A Study Of Ibuprofen-Induced Renal Papillary Necrosis In Neonatal Mice Of The BALB/c db Strain

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A STUDY OF IBUPROFEN-INDUCED RENAL PAPILLARY NECROSIS IN NEONATAL MICE OF THE BALB/c db STRAIN

Submitted in Partial fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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March 28, 1988
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March 28, 1988
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I would like to express my gratitude to Dr. John Addis, my director, for his encouragement and invaluable assistance in organizing and carrying out this research. I would also like to thank Fr. Eugene Peoples and Fr. Joseph Harrington for their time and effort in reading this paper. A special thanks goes to Scott W. Nye for his long hours spent on the computer. I'd like to thank Dr. Jack Stumphling at the McLaughlin Institute, Great Falls, for donating the mice used in this experiment and Dr. Roger Landers at St. Peter's Hospital, Helena, for his expertise in examining the slides. Finally, I would like to express my appreciation to my parents, without whom this experience would not have been possible.
ABSTRACT

It is now well established that renal papillary necrosis may be induced by a number of nonsteroidal anti-inflammatory agents. In this experiment, pregnant mice of the BALB/c db strain were intravenously injected with 0.1 ml of buffered Ibuprofen or buffer alone on days six, eleven, and sixteen of their gestational period. The control group was given 0.1 M sodium phosphate solution. The experimental group was given 69.73 mg/kg of Ibuprofen in 0.1 M sodium phosphate solution. Within twenty-four hours after birth, the pups were sacrificed and the left kidneys were removed. The kidneys were fixed, sectioned, and stained with hematoxylin and eosin. The slides were examined histologically. Comparative histological examination of representative sections of the kidneys revealed no significant difference between the tubules in the papillae of the two groups.
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INTRODUCTION

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID), administered to relieve aches, pains, menstrual cramps, and to reduce inflammation (1,2). It was patented in England in 1964 and has been available there as a nonprescription drug for several years (2). In the United States, the Federal Drug Administration approved the nonprescription sale of Ibuprofen in 1984 (2). It is presently being sold over-the-counter under several brand names including Advil, Nuprin, Trendar, and Pamprin (3,4).

It has become evident that some NSAID's can occasionally cause serious damage to the kidneys (5,6,7). Although the kidneys form only 0.4% of the total body weight, they receive approximately 25% of the cardiac output and are therefore extremely vulnerable to drug toxicity (6). There are other factors contributing to their cytotoxic susceptibility including a large glomerular epithelial area, the capacity to develop extremely high concentrations of a drug within lumen and tubule cells, the medullary concentration of potential nephrotoxins by the countercurrent system, and the accumulation of drugs and their metabolites due to tubular obstruction or nephron loss (6).

Renal papillary necrosis is the hallmark of analgesic-induced kidney disease (8). Although this has been known for over 25 years and extensive research of the condition has been conducted, the cause of renal papillary necrosis is unknown. One of the more popular theses to explain the disease is that the NSAID's injure the kidneys in large part through their ability to diminish prostaglandin synthesis (9).

The purpose of this study is to examine the ability of Ibuprofen to cause renal papillary necrosis in neonatal mice of the BALB/c db strain when injected into the mother during pregnancy. Studies show that renal papillary necrosis can be induced by Ibuprofen (5,7,17). Since Ibuprofen crosses the placental barrier (4,12), it seems likely that it could cause renal
papillary necrosis in the embryo. Histological examinations were employed in the interpretation of the results.
LITERATURE REVIEW

Chemical Properties of Ibuprofen

Ibuprofen (C_{13}H_{18}O_{2}) is a phenylpropionic acid derivative (10,11). Other NSAID's derived from phenylpropionic acid are Fenoprofen and Naproxen (9). The chemical structures of these drugs are shown in Fig. 1.

Ibuprofen is a white to off-white crystalline stable solid with a melting point of 75°-77°C (10). It is relatively insoluble in water but readily soluble in most organic solver (10). The apparent pKₐ is 5.2. The LD₅₀ in male mice is 495 mg/kg intraperitoneal injection and 1255 mg/kg orally (10). The molecular weight of Ibuprofen is 206.27 (10).

![Fenoprofen](image1)

(Fenoprofen (\(\alpha\)-methyl-1-3-phenoxybenzeneacetic acid))

![Naproxen](image2)

(Naproxen (6-Methoxy-\(\alpha\)-methyl-1-2-napthalene-acetic acid))
**Metabolism and Physiological Effects of Ibuprofen**

Ibuprofen possesses analgesic and antipyretic activities (12,13). As is the case with other NSAID's the exact mechanism of action of the drug is not known (4,12). The anti-inflammatory action of Ibuprofen may be due to the inhibition of synthesis and release of prostaglandins (2,5,6,7,9,14). Ibuprofen probably produces antipyresis by acting on the hypothalamus with a heat dissipation increase resulting from vasodilation and increased peripheral flow (4). As indicated by animal studies Ibuprofen is a peripherally acting analgesic (4).

Animal studies also indicate that the distribution of Ibuprofen in the body varies according to species (4). Approximately 90-99% of a dose is bound to plasma proteins (4). Ibuprofen and its metabolites cross the placental barrier in rats, mice, and rabbits. It has not been detected in the milk of lactating mothers (4,12).

Although animal reproduction studies have not yet demonstrated any teratogenic effects, the safe use of Ibuprofen during pregnancy has not been established (2,3,4). Ibuprofen may interfere with labor and delay parturition due to its ability to inhibit prostaglandin synthesis and release (3,4). Because of this, the use of Ibuprofen by pregnant women, especially during the third trimester, is not recommended (2,3,4).

**Renal Medullary Structure**

The human kidney has a multilobar structure not seen in other primates. Common
laboratory animals such as mice, rats, and rabbits have a unilobar kidney with a structure that is essentially the same as one lobe of a human kidney (5). The number of papillae in a human kidney may range from nine to twenty (5). Although the shape of the papillae is primarily pyrimidal, it may be short, asymmetric, or broad based (5).

Detailed studies have been conducted on the structure of the rat kidney which is essentially the same as that of the mouse (19,25). These studies show that collecting ducts in the inner medulla are surrounded by thin ascending limbs of Henle, a capillary plexus, and interstitial cells lying in a relatively abundant matrix rich in mucopolysaccharides (5). The ascending vasa recta and the descending vasa recta lie in close proximity to the descending loops of Henle (5,19). In the outer medulla, the structure is similar. The ascending limb of Henle is thick-walled, but the capillary plexus is much more dense (5). Interstitial tissue is less abundant in the outer medulla (5).

In subcortical zone venous recta diverge from the vascular bundles to rise with collecting ducts toward their junction with arcuate and interlobular veins (5). Anastomoses between pelvic and medullary vessels have been observed in animals (5).

In studies by Burry et al. (1977), differences in the rat have been demonstrated between cells of the ascending and descending limbs of Henle. In the long descending limbs, the epithelium in the outer medulla and outer half of the inner medulla is complex with shallow occluding junctions and numerous lateral interdigitations between cells. In the remainder of the descending limb to near the loop, the epithelium is relatively simple without interdigitations between cells and with deep tight junctions. The epithelial cells within the loop have broad, less complex interdigitations, and a narrow occluding junction extends to the junction with thick ascending limb epithelium. Multilayering of the basement membrane is typical of the ascending limb (5).

Function of Renal Papilla

The detail of the structure of the renal papilla is important for its function, which is the concentration of urine (6). Quite evident is the importance of the anatomic arrangement of the vascular and tubular elements in countercurrent loops, but no less significant are the details
of the submicroscopic design of the membranes of the cells lining these structures. It is this ultrastructure which confers upon the tubular elements the differential permeability properties that enable the medullary countercurrent loops to function as a concentrating system (5).

It is experimentally evident that a medullary concentration gradient is present. A model for this gradient is described by Burry et al. (1977). The loops of Henle run deep into the medulla in close proximity to the collecting ducts. Sodium chloride is actively pumped out of the ascending limb and accumulates in the tightly restricted space of the medullary interstitium. The resulting high concentration of interstitial sodium provides an osmotic force which extracts water from the collecting ducts leading to concentration of the final urine. The countercurrent arrangement of the vasa recta ensures that the sodium is not washed out of the medullary interstitium (5).

In this model, water is separated from solute by a process of solute reabsorption from a water impermeable segment of the renal tubule. Sodium and chloride, moving from the thick ascending loop, leave the fluid in the tubule hypotonic to blood plasma at this point, which is referred to as the diluting segment. If the tubule distal to this segment remains impermeable to water, a dilute urine develops. The kidney is able to switch to a concentrating state through antidiuretic hormone (ADH), which increases the permeability of the distal tubule to water. Dilute fluid leaving the diluting segment comes into osmotic equilibrium with the peritubular capillary blood in the cortical distal tubule and is subsequently concentrated by removal of water from the collecting duct. Most of the water extracted in the elaboration of a concentrated urine is removed in the cortical distal nephron in the process of rendering tubular fluid isotonic. Therefore, the amount of water that is extracted from the collecting duct to concentrate the urine is too small to significantly dilute the sodium concentration of the medullary interstitium (5).

Analgesic Nephropathy

Analgesic nephropathy is the expression of a pattern of disease which results from the unique structure and function of the renal medulla (5,6). It was first recognized in Switzerland by Spuhler and Zollinger in 1953 when pathologists encountered cases of
chronic renal failure (15). The kidney in these cases displayed widespread cortical atrophy with a nonspecific histologic appearance of chronic interstitial nephritis. In a proportion of the cases, papillary necrosis was seen. As more cases were reported, it became evident that papillary necrosis was an integral and constant component of the pathological findings (5). It was suspected that there was an association between this new papillary necrosis and heavy analgesic consumption (7). Most of the analgesics taken were mixtures containing phenacetin and the disease was therefore referred to as "phenacetin nephritis" (5,7,16). However, more cases were reported with the same pathological pattern in which phenacetin had not been consumed. The disease is now generally titled "analgesic nephropathy" (5,7,16).

**Microscopic Pathology of Analgesic Nephropathy**

The earliest recognizable changes have been necrosis of the limbs of Henle, capillaries, and interstitial cells surrounding groups of collecting ducts (5). The process is a slow progressive necrosis commencing at the tip of the papilla and extending toward the cortex. Calcification may be seen within the cells of the limb of Henle which retain pyknotic nuclei, and between cell cytoplasm and basement membrane. Calcium granules may also be present in the interstitium concentrated around the limbs of Henle (16). The tubules and vessels in the necrotic area show a lamellation of the basement membrane and may be permeated by empty vacuoles (5,16,17).

As the disease progresses, necrosis extends to the vasa recta and related limbs of Henle (5,16). Only the collecting ducts and some vasa recta remain well preserved. Within the limbs of Henle and in the necrotic matrix, calcium deposition may be heavy. In the outer medulla there is atrophy and sclerosis around groups of collecting ducts (5,16,17).

Fragments of necrotic papilla may detach or remain with a thin connecting stalk. It is not uncommon for the entire papilla to become detached (5). Where an entire papilla is detached the line of sequestration becomes coated by pelvic transitional epithelium. The medulla above the line of sequestration is never normal (5).

There is an increase of connective tissue in the interstitium embedded in an amorphous osmiophilic matrix which is also permeated by lipid vacuoles (17). There are few interstitial
cells most of which do not contain lipid droplets. Scattered in the interstitium are lymphocytes and histiocytes (5,17).

The damage of analgesics affects all structures of the kidney - tubules, capillaries, larger vessels, collecting ducts, and interstitium. The collecting ducts and interstitium seem to be fairly resistant to the damage, while the loops of Henle and the capillaries are the most susceptible (17).

It appears there are two patterns of development in complete necrosis (5). One pattern is a superficial necrosis covering the papilla extending toward the cortex in a crescent shape. The second is a centropapillary necrosis extending up the center of the papilla. In both cases, if the disease progresses, the end result is total papillary necrosis (5,7,17).

Prostaglandins

The primary mechanism of the phenomenon of analgesic nephropathy has been ascribed to inhibition of renal prostaglandin synthesis and release (6). Prostaglandins are a group of fatty acids that affect a wide variety of physiological processes (18). Within the kidney prostaglandins exert significant hemodynamic and tubular effects. Analgesics suppress prostaglandin synthesis leading to complex physiologic responses and compensatory feedback mechanisms (6).

There are several prostaglandins presently recognized (Fig. 2, p.9). Prostaglandin products made by the kidneys, PGI\(_2\), PGE\(_2\), PGD\(_2\), PGF\(_{2a}\), and thromboxane \(A_2\), are shown at the bottom of Fig. 2. All of these classic prostaglandins are derived from the cyclic endoperoxides PGG\(_2\) and PGH\(_2\) (9,18). The lipoxygenase pathway also uses arachidonic acid as a substrate but the products are leukotrienes. Arachidonic acid is made available for prostaglandin synthesis by a phospholipase which frees it from membrane lipids (18). Steroid hormones can inhibit this step (9). Free arachidonic acid, made available within the cell, will either go through the lipoxygenase pathway to the leukotrienes or through the cycloxygenase pathway to the classic prostaglandins (9,18). The latter step is inhibited by a variety of nonsteroidal anti-inflammatory agents (9,18). Endogenous renal
prostaglandins are diminished with this inhibition, which is popularly believed to play a role in the renal dysfunction associated with nonsteroidal anti-inflammatory drug administration (9).

\[ \text{Phospholipids} \xrightarrow{\text{Phospholipase}} \text{Arachidonic acid} \xrightarrow{\text{Lipoxigenase}} \text{Hydroxy fatty acids} \]

\[ 2 \text{O}_2 \xrightarrow{\text{Cyclo-oxygenase}} \text{PGG}_2 \xrightarrow{\text{NSAID's}} \text{Thromboxane A}_2 \]

\[ \text{Prostaglandin (PG1 or PGX)} \]

\[ \text{6-keto-PGF}_1 \alpha, \text{PG}_{2 \alpha}, \text{PGD}_2 \]

\[ \text{Leukotrienes} \]

Fig. 2. Chemical structures of the various prostaglandins and the pathways involved in their production. NSAID's inhibit the production of PGG2 as indicated.
The arachidonic acid combines with oxygen to yield the cyclic endoperoxide PGG$_2$ (9,18). This step is believed to be mediated by cyclooxygenase and therefore sensitive to inhibition by nonsteroidal anti-inflammatory drugs (9,18). This enzyme is permanently inactivated by acetylsalicylate (aspirin) which is believed to acetylate this enzyme directly, whereas other NSAID's are reversible inhibitors of cyclooxygenase (9,18). Within the clinical range that nonsteroidal anti-inflammatory drugs are used, it is likely that the level of cyclooxygenase activity is reduced relative to previous levels rather than blocked completely (9). Thus, following cyclooxygenase inhibition, small but measurable amounts of prostaglandins are frequently found in the renal tissue (9).

Role of Prostaglandins

The prostaglandins are found in virtually all organs and are products of virtually all cells (20). There are various possible roles and mechanisms of action (9,18,19). One of the popular themes at present suggests that there is a balance between thromboxane A$_2$ production by the platelet (which would tend to promote platelet aggregation, clot formation, and vascular contraction) and PGI$_2$, produced by the endothelium (which would tend to inhibit platelet aggregation and relax blood vessels) (9,19,20). This is believed to go on in all vessels of the body including the kidney. Under normal conditions it is believed that there is a balance between these two opposing arachidonic acid products, maintaining the normal circulation and lack of hypercoagulable state (9).

Renal Effects of Prostaglandins

The effect of prostaglandins have several major consequences at the level of the kidney. Renal hemodynamics - renal blood and glomerular filtration rate - are affected by prostaglandins (9,18,20). Prostaglandins also affect sodium and water excretion (9,19). Prostaglandins appear to antagonize the action of antidiuretic hormone on the collecting duct. A prostaglandin antagonist such as Ibuprofen, therefore tends to enhance the effects of antidiuretic hormone (9).
Effect of Nonsteroidal Anti-inflammatory Agents

There have been several studies to determine the effect of inhibitors of prostaglandin synthesis (1,5,6,7,8,9,14,15). Under most circumstances when normal animals have been treated with NSAID's, there is a transient decrease in sodium excretion or no change in sodium excretion (5,6,7). There are a few studies in which small increases in sodium excretion were found (9). However, in conditions in which the kidney is avidly retaining sodium, the administration of prostaglandin synthesis inhibitors such as NSAID's seems to further increase sodium reabsorption (6,7).

In a variety of experiments it has been found that PGF$_2$ has a relatively direct inhibiting effect on the ability of ADH to increase the collecting tubule's permeability to water. Under conditions in which the urine is relatively dilute to begin with, NSAID administration increases urine osmolality (9).

There are believed to be at least two mechanisms that may play a role in the ability of prostaglandin inhibition to increase the osmolality of the urine. One is that the lower amounts of endogenous renal prostaglandins tend to enhance the effect of antidiuretic hormone on the collecting system itself (9). The second possibility, also supported by experimental data, is the relatively direct effect of the nonsteroidal anti-inflammatory drugs to increase papillary interstitial osmolality. This creates a greater gradient for water to move from the collecting system into the interstitium of the kidney and out of the urine (9).
MATERIALS AND METHODS

Experiments were conducted using twenty-eight BALB/c db female mice, approximately six to seven months of age, supplied by the McLaughlin Institute in Great Falls, Montana. The mice were weighed and divided into two groups of fourteen. The control group received injections of 0.1 M sodium phosphate saline solution (see appendix I), while the experimental group received injections of Ibuprofen dissolved in 0.1M sodium phosphate saline solution (see appendix I). Each of these groups were subdivided into four groups of three mice and one group of two mice. The ten groups of female mice were placed in separate cages. After three days one male of strain BALB/c was placed into each of the ten cages for breeding purposes. The experiment proceeded for twenty-five days. The mice were provided Purina Laboratory Rodent Chow and water ad libitum. The room in which they were kept was on a twelve hour light cycle.

Five days following the introduction of the male, the female mice were given their first injection. The mice of the control group (B) were given one 0.1 ml intravenous injection of sodium phosphate saline solution. The mice of the experimental group (I) received one 0.1 ml intravenous injection of the Ibuprofen solution. The concentration of the Ibuprofen was 69.73 mg/kg. The mice were given one injection every five days for a total of three injections throughout their gestational period. The male breeders were not injected.

Only the pups of the mice that gave birth exactly twenty-one days after the males were introduced were used for the experiment. The mothers of these mice had received injections on days six, eleven, and sixteen of their gestational period.

Within twenty-four hours following birth the pups were removed from their mother and sacrificed by use of a chloroform chamber. An abdominal incision was made to allow for exposure of the kidneys to the fixative: 3.7% formalin buffered with sodium phosphate.
(see appendix I). After two weeks in the fixative the left kidney was removed from each of the pups.

Five representative kidneys were randomly selected from the control group and the experimental group. The kidneys were fixed and embedded in paraffin (see appendix II). Sections were cut at a thickness of seven micrometers and mounted on microscopic slides. Three slides of each kidney were selected for staining with hematoxylin and eosin and covered with a coverslip (see appendix II).

The histological examinations were made by Dr. Roger Landers, a registered pathologist at St. Peter's Hospital in Helena, Montana. The slides were viewed at 400X using an American Optical light microscope.

Photomicrographs were taken of the kidney sections using a Nikon microscope equipped with an AFX-II photomicrographic attachment. Kodak Panatomic X film (A.S.A. 32) was used. Photomicrographs were taken at 100 and 200 power with a green interference filter.
RESULTS

Histological and Pathocytological

Comparative histological examination of representative kidney sections taken from the B (control) and I (69.73 mg/kg Ibuprofen) groups revealed no significant differences between the two groups of kidneys. Both kidneys show focal tubular necrosis in the cortex and intact tubules in the papilla. Fig. 3 is a photomicrograph of a representative kidney section of the I group showing the papilla and part of the cortex. Fig. 4 is a photomicrograph of a representative section through the kidney of the B group also showing the papilla and part of the cortex. Both of these photomicrographs show areas of necrotic tubules in the cortex and healthy tubules in the papilla. The focal tubular necrosis is characterized by areas of tubules with cells lacking a nucleus. The shape of these cells is irregular and the cytoplasm is relatively pale. The intact tubules are lined with cuboidal cells with a distinct nucleus. Fig. 5 is a photomicrograph of a representative kidney section through the cortex of the B group showing focal tubular necrosis. Fig. 6 is a photomicrograph of a representative section through the kidney of the B group.
Fig. 3. Photomicrograph of mouse kidney from I (69.73 mg/kg Ibuprofen) group. Papilla (P) and cortex (C) are shown. Necrotic tubules (Nt) as well as healthy tubules (T) are present. H & E stain, x 200.
Fig. 4. Photomicrograph of mouse kidney from B (control) group. Papilla (P) and cortex (C) are shown. Necrotic tubules (Nt) as well as healthy tubules (T) are present. H & E stain, x200.
Fig. 5. Photomicrograph of mouse kidney from B (control) group. The cortex (C) is shown with numerous necrotic tubules (Nt). H & E stain, x200.
Fig. 6. Photomicrograph of mouse kidney from the B (control) group. Cortex (C) and papilla (P) are present. Also shown are necrotic tubules (Nt) and healthy tubules (T). H & E stain, x100.
DISCUSSION

The purpose of this study was to examine the ability of Ibuprofen to induce renal papillary necrosis in neonatal mice of the BALB/c db strain when injected into the mother during pregnancy.

Comparative histological examination of kidneys from the control group and experimental group revealed no significant difference between the tubules of the papillae of the two groups. The tubules in the papilla of each group showed no necrosis. This examination also revealed areas of necrotic tubules in the cortex of the kidneys from both the control and experimental groups. This is known as focal tubular necrosis (23). This is most often caused by one of two factors: toxicity or hypoxia (24,25). The most probable explanation is that the necrosis is due to hypoxia (26). The hypoxic condition probably occurred while the mice were in the chloroform chamber at the time of sacrifice (26).

Several improvements of this experiment would allow for more definite results in determining whether Ibuprofen is able to induce renal papillary necrosis in neonatal mice when injected into the mother.

First, in order to determine if hypoxia rather than toxicity was the cause of the focal tubular necrosis in the cortex, it would be necessary to employ an alternative form of sacrifice. The most likely method to eliminate the effects of hypoxia is the use of a guillotine.

A second problem encountered in this experiment was the method of administering the Ibuprofen. The mice were injected intravenously through a tail vein. This proved to be very difficult because of the small size of the vein. Ibuprofen is an ulcerative drug and many of the tails developed ulcerations. Therefore, the mice were only able to be injected a total of three times. Analgesic nephritis is caused by analgesic abuse (5,7,16). Since the mice were only able to be injected with the Ibuprofen three times, they may not have received a high enough
amount of the drug. This may have been a reason why the mice did not develop renal papillary necrosis. A more efficient method of administration would have been the use of a cannulation tube.

There have been no published studies on the embryological renal effects of Ibuprofen during pregnancy. It seems possible that Ibuprofen could potentially harm the kidney, because it crosses the placental barrier (4,12) and is known to cause papillary necrosis in adults taking high doses of the drug (5,6,7). A more detailed experiment with the improvements mentioned above is necessary to further test this hypothesis.
APPENDIX I

0.1 M Saline Solution:
2.76 g NaH$_2$PO$_4$
200 ml H$_2$O

Ibuprofen Solution (formula supplied by the Upjohn Company):
400 mg Ibuprofen
15 ml H$_2$O
3.0 ml NaOH (1.0 N)
57.5 mg NaH$_2$PO$_4$

1. Mix until the solute is completely dissolved
2. Add 1.0 N HCl (drops) to bring pH to 7.2-7.8
3. Add H$_2$O to bring total volume to 20 ml
4. Dilute with 1.5 ml 0.1 M saline solution (above)
   (concentration is now 1.86 Ibuprofen/0.1 ml solution)
5. Filter solution with a 0.45 μm Acrodisc into 10 ml vacutainer

Fixative:
100 ml saline solution (above)
80 ml H$_2$O
20 ml Formalin
APPENDIX II

Fixing (21) and Staining (22) Techniques

Fixing:
Bouin's fluid:
Saturated picric acid (12g) to 1 liter in distilled water  
Formalin  
Glacial acetic acid

Procedure:
1. Bouin's 15 minutes
2. Bouin's 30 minutes
3. 80% alcohol 15 minutes
4. 80% alcohol 15 minutes
5. 95% alcohol 15 minutes
6. 95% alcohol 15 minutes
7. Absolute alcohol 15 minutes
8. Absolute alcohol 30 minutes
9. Chloroform 15 minutes
10. Chloroform 15 minutes
11. Paraffin 15 minutes
12. Paraffin 1 hour

Staining:
Delafield's Hematoxylin:
Dissolve 4 gm hematoxylin in 25 ml absolute ethyl alcohol. Mix gradually into 400 ml ammonia alum, Al₂(SO₄)₃(NH₄)SO₄·24H₂O, saturated aqueous (approximately 1 part alum to 11 parts distilled water). Leave exposed to light in a flask with a cotton plug for 3-5 days. Filter. Add to filtrate, 100 ml methyl alcohol.

Counterstain (Eosin):

1.0 g eosin Y C.I. 45380
1.0 l 70% ethyl alcohol
5.0 ml glacial acetic acid

Dilute with equal volume of 70% alcohol for use and add 2-3 drops acetic acid.

Scott's Solution:

2.0 g sodium bicarbonate
20.0 g magnesium sulfate
100.0 ml distilled water

Add a pinch of thymol to retard molds.

Procedure:
"Running down" slides to water:
1. xylene (2 changes) 2-3 minutes
2. absolute alcohol 2-3 minutes
3. 95% alcohol 2-3 minutes
4. 70% alcohol 2-3 minutes
Staining:
5. running water
6. hematoxylin, Delafield's
7. running water
8. Scott's solution
9. running water
10. counterstain, Eosin
"Running up" slides:
11. 70% alcohol
12. 95% alcohol
13. absolute alcohol
14. absolute alcohol
15. absolute alcohol-xylene
16. xylene
17. xylene
18. mounting medium; keep sections moist with xylene during this process. They must not dry.
Add coverglass.
REFERENCES


