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Fixation of Testes and Eyes Using a Modified Davidson's Fluid: Comparison with Bouin's Fluid and Conventional Davidson's Fluid

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ABSTRACT

Most recent revisions of regulatory guidelines for testing effects of chemicals on reproduction recommend Bouin's fluid (BF) or a "comparable fixative" instead of formalin to preserve the morphologic detail of testes for histopathological evaluation. However, picric acid in BF is a health and safety hazard, as well as a laboratory waste disposal problem. Furthermore, use of BF is labor intensive, requiring multiple alcohol rinses to remove picric acid for optimum preservation and immunohistochemical (IHC) detection of testicular antigens that may potentially be used to identify and quantify cells and functional proteins with critical roles in spermatogenesis. Recently a modified Davidson's fluid (mDF) has been reported as an alternative to BF to fix testes for routine histopathological examination. This study compared the overall histomorphologic clarity and the immuno- and histochemical staining of testicular specimens fixed in BF and mDF. Additionally, because conventional Davidson's fixative (DF) is used routinely for optimum fixation of eyes, preservation of ocular histomorphology by DF and mDF was compared. mDF resulted in noticeably less shrinkage of the seminiferous tubules and superior overall morphologic detail compared to BF. Unlike DF, the mDF also supported excellent staining of acrosomes with periodic acid-Schiff (PAS) reagent when staging of spermatogenesis was required. IHC detection of androgen receptor and PCNA (to directly and indirectly identify Sertoli cells) as well as protein gene product 9.5 (to label spermatogonia) was superior in mDF compared to BF-fixed specimens. For histopathological examination of the eye, apposition and preservation of rods and cones, and nuclear layers of the retina were slightly inferior with mDF compared to DF. This paper has demonstrated that mDF provides comparable, and in many respects superior preservation of the testes to that of BF, both for IHC staining and for detailed histopathological examination. It also provides an acceptable fixative for eyes, although the quality of cellular preservation is inferior to that of DF.

Keywords. Immunohistochemistry; histochemistry; fixation; histomorphology; methods, spermatogonia; Sertoli cell antigenic markers; Davidson's fixative; Bouin's fixative.

INTRODUCTION

Most of the recently revised regulatory guidelines for reproductive toxicity tests have recommended that the testes should be fixed in Bouin's fluid (BF) or in a "comparable fixative." Implicit in this recommendation is that formalin should not be used as the fixative of choice. These recommendations have arisen from a general drive by the regulatory authorities to improve the sensitivity and quality of histopathological evaluation of the male reproductive system. The new guidelines now provide recommendations on tissue sampling, fixation, and staining (Table 1) and also provide criteria for evaluation of toxicological changes. The general move to using BF as a routine fixative for the testis has certainly improved the general quality of cellular preservation and the resolution of cellular detail that can be achieved, but it presents a number of problems of its own. The presence of picric acid in the fixative results in safety hazards and disposal problems, as well as bright yellow staining of working surfaces and anything that comes in contact with it. For optimum fixation and immunohistochemical detection of antigens, BF-fixation requires numerous alcohol rinses to remove picric acid in a timely manner.

When there is a continuously heavy volume of specimens with a specified relatively short and consistent fixation interval, the processing logistics can become burdensome and difficult to control. This can result in inconsistent detection of antigens in BF-fixed testis. In addition, the fixative causes significant, differential shrinkage of the tubules away from the interstitial tissue. This poses a particular problem for evaluation of interstitial edema, detection of tubular contraction or dilatation, and for carrying out any quantitative measurements of tubular diameter.

Zenker's and Helly's fixatives, which contain potassium dichromate and mercuric chloride have also traditionally been used for testis fixation, but they also suffer from significant safety and disposal problems. There is an urgent need for an alternative fixative that provides similar penetration and preservation properties, but which is more convenient and safer to handle and dispose of. Recently, a modified Davidson's fluid (mDF), has been reported as an alternative to BF to fix testes for routine histopathologic evaluation (3). The primary purpose of this study was to compare the

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TABLE 1.—Recommendations from recently revised regulatory guidelines for reproductive studies relating to sampling and fixation of male reproductive tissues.

Guideline	Tissues to be weighed	Tissues to be preserved	Tissues to be examined
OECD 416: 2-Generation reproduction toxicity study (January 2001) (12)	Testes Epididymides (total and cauda) Seminal vesicles with coagulating glands and their fluids and prostate (as one unit)	One testis* (preserved in Bouin's or comparable fixative) One epididymis* Seminal vesicles Coagulating glands Prostate	Testis Epididymis Seminal vesicles Coagulating gland Prostate
OECD 421: Reproduction/developmental toxicity Screening test (July 1995) (11)	Testes Epididymides	Testes (preserved in Bouin's or comparable fixative) Epididymides Seminal vesicles Coagulating glands Prostate	Testes Epididymides
OPPTS 870.3800: Reproduction and fertility Effects (August 1998) (17)	Testes Epididymides (total and cauda) Seminal vesicles with coagulating glands and their fluids Prostate	Right testis* (preserved in Bouin's or comparable fixative) Right epididymis* Seminal vesicles Coagulating glands Prostate	Testis Epididymis Seminal vesicles Coagulating gland Prostate
ICH S5A (1994) S5B (1996): Detection of toxicity to reproduction for medicinal products (including addendum on male fertility studies). (6)	None	Testes (preserved in Bouin's or comparable fixative) Epididymides	Testes and epididymides if indicated by altered fertility indices

*Remaining testis/epididymis is retained for assessment of sperm parameters.

Abbreviations: OECD: Organization for Economic Co-operation and Development; OPPTS: Office of Prevention, Pesticides and Toxic Substances; ICH: International Conference on Harmonization.

immuno- and histochemical staining characteristics and overall histomorphological clarity of testicular specimens fixed in Bouin's and Davidson's fluids.

Although routine histopathological evaluation of the testes is carried out in hematoxylin and eosin (H&E) or periodic acid-Schiff-hematoxylin (PASH)-stained sections, immunohistochemical techniques can be extremely useful for investigational studies. Utilization of specific antigenic markers for Sertoli cells, Leydig cells, or the various populations of germ cells within the testis can monitor and compare functional activity or be used to identify and quantify the individual cell populations. Mechanistic studies in reproductive toxicology or extrapolation of dose-response data from animals for human risk assessment sometimes requires this type of approach. In this study we evaluated the distribution and expression of a number of antigenic markers for their ability to distinguish between different cell types within the seminiferous epithelium. We also compared the quality of immunostaining between the 2 different fixatives. Protein gene product (PGP) 9.5 is an ubiquitin carboxyl-terminal hydrolase expressed in spermatogonia, but not the other generations of testicular germ cells (16, 18). Anti-PGP 9.5 selectively labels spermatogonia using immunohistochemistry (IHC), distinguishing these germ cells from spermatocytes and round spermatids. Likewise, anti-androgen receptor (AR) and antiproliferating cell nuclear antigen (PCNA) will, respectively, detect Sertoli cells directly (13, 14) and indirectly ie, negatively (personal observation). Moreover, depending on the stage of spermatogenesis, AR may be upregulated or downregulated in Sertoli cells in accordance with physiologic requirements for testosterone (14, 15). The use of IHC can be useful for mechanistic studies in reproductive physiology and toxicology to visualize and potentially measure modulation of certain structural and functional proteins, for example AR, that are important in sperm production in association with the different stages of spermatogenesis. These IHC methods have been evaluated using BF and mDF-fixed rat testicular sections. We optimally fixed testes in either BF or mDF. Then we prepared specimens stained with H&E and PASH to compare morphology, IHC to detect spermatogonia and Sertoli cells, and IHC/PASH to demonstrate the utility of dual staining to detect important structural and functional proteins in different stages of the spermatogenic cycle.

Conventional Davidson's fixative (DF) is used routinely in many laboratories to optimize the fixation of eyes. To address the possibility that mDF could be used to fix both eyes and testes with 1 fixative, we carried out a comparative evaluation of the structural preservation of eyes using a conventional and the modified recipe.

MATERIALS AND METHODS

Animals

mDF Versus BF for Testes in Rats. To compare mDF and BF for histomorphologic qualities and immunohistochemistry techniques, both testes were removed from 10 sexually mature untreated Fisher-344 rats that had been euthanized humanely with carbon dioxide.

mDF for Testes in Large Animals. The testes from purpose-bred, untreated mature New Zealand White rabbits, beagle dogs, and cynomologus monkeys that were euthanized for other purposes were also fixed in mDF to evaluate the utility of this fixative for large as well as small laboratory animal species. These testes were examined for histomorphologic quality only.

mDF Versus DF for Testes and Eyes. Comparison of mDF with DF for the fixation of eyes and testes was carried out on tissues taken from mature Sprague–Dawley (Crl:CD IGS BR) rats being euthanized for other purposes. In addition the eyes from the New Zealand White rabbits and cynomologus monkeys (see before) were used to compare the 2 fixatives.

Tissue Handling and Fixation: Testes

Table 2 summarizes the fixatives and procedures used for the various comparative investigations. The fixatives were

TABLE 2.—Fixation procedures for comparison of different fixatives for eyes and testes.

Species	Tissue	Fixative	Fixation time	Post fixation storage fluid	Morphologic/IHC examination
F344 rat	Testes	mDF, BF	24 h	70% alcohol	Morphology IHC
SD rat	Testes	mDF, DF	48 h	NBF	Morphology
NZW rabbit	Testes	mDF	48 h	NBF	Morphology
Beagle dog					
Cynomologous monkey					
SD rat	Testes	mDF, DF	48 h	NBF	Morphology
SD rat	Eyes	mDF, DF	48 h	NBF	Morphology
NZW rabbit					
Cynomologous monkey					

Abbreviations: mDF: modified Davidson's fluid; DF: Davidson's fluid; BF: Bouin's fluid; NBF: neutral buffered formalin; IHC: immunohistochemistry.

prepared fresh from the recipes for mDF (30% of a 37–40% solution of formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled H_2O) and DF (2% of a 37–40% solution of formaldehyde, 35% ethanol, 10% glacial acetic acid, and 53% distilled H_2O). BF was purchased from Poly Scientific R&D (Bay Shore, NY). Testes were removed taking care to handle specimens gently to minimize trauma to the delicate seminiferous tubules. Prior to placement of each testicle into fixative, the tunica albuginea was shallowly pierced at each pole 5 times with a 21-gauge needle to aid in the penetration of the fixative. For IHC and morphological comparison of mDF and BF procedures on F344 rat testes, fixation time was limited to 24 hours and tissues were transferred to 70% ethyl alcohol (ETOH) prior to trimming. For all other comparisons, tissues were fixed for 48 hours, then briefly washed in tap water before being transferred to 10% neutral buffered formalin for storage prior to trimming and processing (Table 2).

Postfixation Procedure for Bouin's Fluid. After fixation in BF, the specimens were transferred to 70% ETOH. The ETOH was changed 3 times daily for 2 days before transferring the specimens to a saturated solution of 70% ETOH and lithium carbonate to neutralize the picric acid in BF. The ETOH-lithium carbonate solution was changed 3 or more times until the yellow color of BF was almost completely depleted from the tissue. The testes were stored in 70% ETOH until they were trimmed and further processed.

Sectioning and Staining: Testes. Slices 3–4 mm thick were cut transversely from the middle portion of each of 10 pairs of testes, 1 testis fixed in BF and the other in mDF. These were processed through graded alcohols and cleared in xylene before being embedded, one specimen from each fixative in the same paraffin block. Four-micron sections were cut and stained with hematoxylin and eosin (H&E) and with a modification of the McManus periodic acid-Schiff reaction (PASH) (8). The modification increased the interval in Schiff reagent from 15 to 30 minutes (to enhance acrosomal staining) and decreased the time in Mayer's hematoxylin counterstain from 2 minutes to 8 seconds (to increase contrast between the nuclear membrane and acrosome).

Immunohistochemical (IHC) methods were carried out on additional sets of the F344 rat testes (serially sectioned) to detect androgen receptor (AR), proliferating-cell nuclear antigen (PCNA), and protein gene product (PGP) 9.5 (Table 3). One additional set was dual stained, first immunohistochemically to detect AR followed by PASH to visualize the acrosome.

TABLE 3.—Antibodies used for immunohistochemical detection.

Primary antibody[a]	Ig class[b]	Working concentration	Vendor	Vendor's address
AR	Rabbit IgG	1:200 for 60 min	Santa Cruz Biotechnology	Santa Cruz, CA
PCNA	Mouse mc	1:4,000 for 60 min	Dako corporation	Carpinteria, CA
PGP 9.5	Mouse mc	1:50 overnight at 4°C	Vector laboratories, Inc	Burlingame, CA

[a] AR = androgen receptor; PCNA = proliferating cell nuclear antigen; PGP = protein gene product.
[b] Ig = immunoglobulin; mc = monoclonal.

Briefly, for the IHC methods, after rehydration, the specimens were placed in phosphate buffered saline. Endogenous peroxidase was quenched with 3% H_2O_2 containing 0.1% sodium azide for 10 minutes. The sections were placed in 40 ml of antigen-retrieval solution consisting of either 0.01 M citrate buffer (AR and PGP 9.5) or 1% zinc sulfate in deionized water (PCNA) and heated for 7.5 minutes in a 700-watt microwave oven on full power. A routine streptavidin procedure was performed, beginning with application of 0.5% aqueous casein (Sigma, St. Louis, MO) to block nonspecific binding of subsequent antibody and sequential incubations of sections in primary antibody (Table 3) followed by the appropriate biotinylated link antibody, and streptavidin-conjugated horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA). Each antibody was titrated for optimum concentration (Table 3) for detection of each of the three antigens (AR, PCNA, PGP 9.5) in formalin-fixed specimens, as opposed to specimens preserved in either of the fixatives to be tested. The immunoreactive cells were visualized by incubating the sections in 3,3′-diaminobenzidene hydrochloride (DAB) chromogen followed by a Mayer hematoxylin (Poly Scientific R&D (Bay Shore, NY) counterstain at full strength for 10 seconds.

The dual-staining procedure to detect AR and the spermatid acrosome was accomplished by performing the IHC first. After visualization of the AR-positive cells using DAB, slides were rinsed in deionized water and further stained with PASH to delineate the acrosome and nucleus.

Comparison of the Fixatives: Testes. Endpoints used in the comparison of mDF and BF for rat testes included overall clarity of the morphologic detail, shrinkage of the seminiferous tubules, cytoplasmic shrinkage of the seminiferous epithelium, cytoplasmic "graininess," nuclear chromatin aggregation, sharpness of acrosomal staining, and IHC staining intensity. Peripheral and central regions were compared for those endpoints where comparisons seemed meaningful or distinct differences were observed. Each evaluated parameter was graded subjectively between 1 and 4, with 4 being the most severe. If a particular effect was not observed, it was listed as negative. The grading evaluation was carried out by 1 pathologist and reviewed by 2 other pathologists, with concurrence of results.

Tissue Handling, Preparation Procedures and Microscopic Evaluation of Eyes

Both eyes were carefully removed from each animal taking care to minimize trauma. Excess tissue was trimmed from

the globe and 1 eye from each animal was placed into mDF and the other into DF for 48 hours. They were then rinsed briefly in tap water before being transferred into 10% neutral buffered formalin for storage.

The rabbit and monkey eyes were trimmed to provide a midsagittal slice of 4–5 mm thickness. The rat eyes had a thin (1–2 mm) slice trimmed from the lateral aspect of the globe and were placed trim side down in a cassette. All specimens were further processed through graded alcohols and cleared in xylene before embedding in paraffin. Midsagittal, 4-micron sections were cut and stained with H&E.

Endpoints used in the comparison of the 2 fixatives included retinal attachment, histomorphologic detail of the nuclei and sensory endings of retinal layers, and general corneal and lens morphology.

RESULTS

Comparison of Bouin's and Modified Davidson's Fluid for Testis Morphology and Immunohistochemical Staining

A summary of morphological differences in testicular histology observed comparing mDF and BF is given in Table 4. The overall clarity of morphologic detail was slightly superior for mDF-fixed specimens stained with H&E, IHC, PASH, and dual IHC/PASH (Figures 2–8). Shrinkage artifact of the seminiferous tubules and germ cells was present mostly in the central area of the specimen and not the periphery. Central tubular shrinkage was appreciably more pronounced in BF-fixed testis (Figure 1). Broad interstitial spaces accompanied by small diameter tubules with numerous tubules lacking patent lumens characterized the shrinkage artifact. In contrast, cytoplasmic shrinkage of the seminiferous epithelium was not observed in BF-fixed testes, but was minimally present in mDF-fixed specimens (Figure 2). This artifact resulted in small clefts between cells that should have been abutting, but was not considered to be enough to compromise evaluation. There was slightly less chromatin aggregation of the nuclei and less "graininess" of the cytoplasm in mDF-fixed specimens (Figure 3a) compared to BF-fixed testes (Figure 3b).

Table 5 summarizes the differences in immunoreactive staining intensity labeling AR, PCNA, and PGP 9.5 antigens in testicular specimens fixed in mDF or BF. For both fixatives detection was better in the peripheral tubules compared to the central tubules. AR labeling was least effective in BF-fixed testes where detection was weak in the peripheral tubules (Figure 4a) and negative in those in the center (Figure 4b). In contrast, AR detection in mDF-fixed specimens was exceptional in the peripheral tubules (Figure 5a), but approximately the same intensity in central tubules (Figure 5b) as was present in the peripheral tubules in specimens fixed in BF (Figure 4a). For both PCNA (Figure 6a, 6b) and PGP 9.5 (Figure 7a, 7b), labeling after fixation in mDF was superior to that observed in BF-fixed testes in either the peripheral or central tubules.

TABLE 4.—Summary of morphological effects seen comparing modified Davidson's fluid and Bouin's fluid fixation of testis.

Effect	Fixation method	
	mDF	BF
Shrinkage of seminiferous tubule		
Peripheral tubules	+	+
Central tubules	++	++++
Cytoplasmic shrinkage of seminiferous epithelium		
Peripheral tubules	–	–
Central tubules	+	–
Cytoplasmic graininess	+++	++++
Chromatin aggregation	+	++
Overall clarity of morphologic detail	++++	+++

Finding severity: + = minimal, ++ = slight, +++ = moderate, ++++ = marked, – = absent.
Abbreviations: mDF: modified Davidson's fluid; BF: Bouin's fluid.

TABLE 5.—Summary of IHC staining intensity seen comparing modified Davidson's fluid and Bouin's fluid fixation of testis.

Effect	Fixation method					
	mDF			BF		
	AR	PC	PGP	AR	PC	PGP
Staining intensity						
Peripheral tubules	++++	++++	++++	++	+++	+++
Central tubules	++	++++	+++	–	+	++

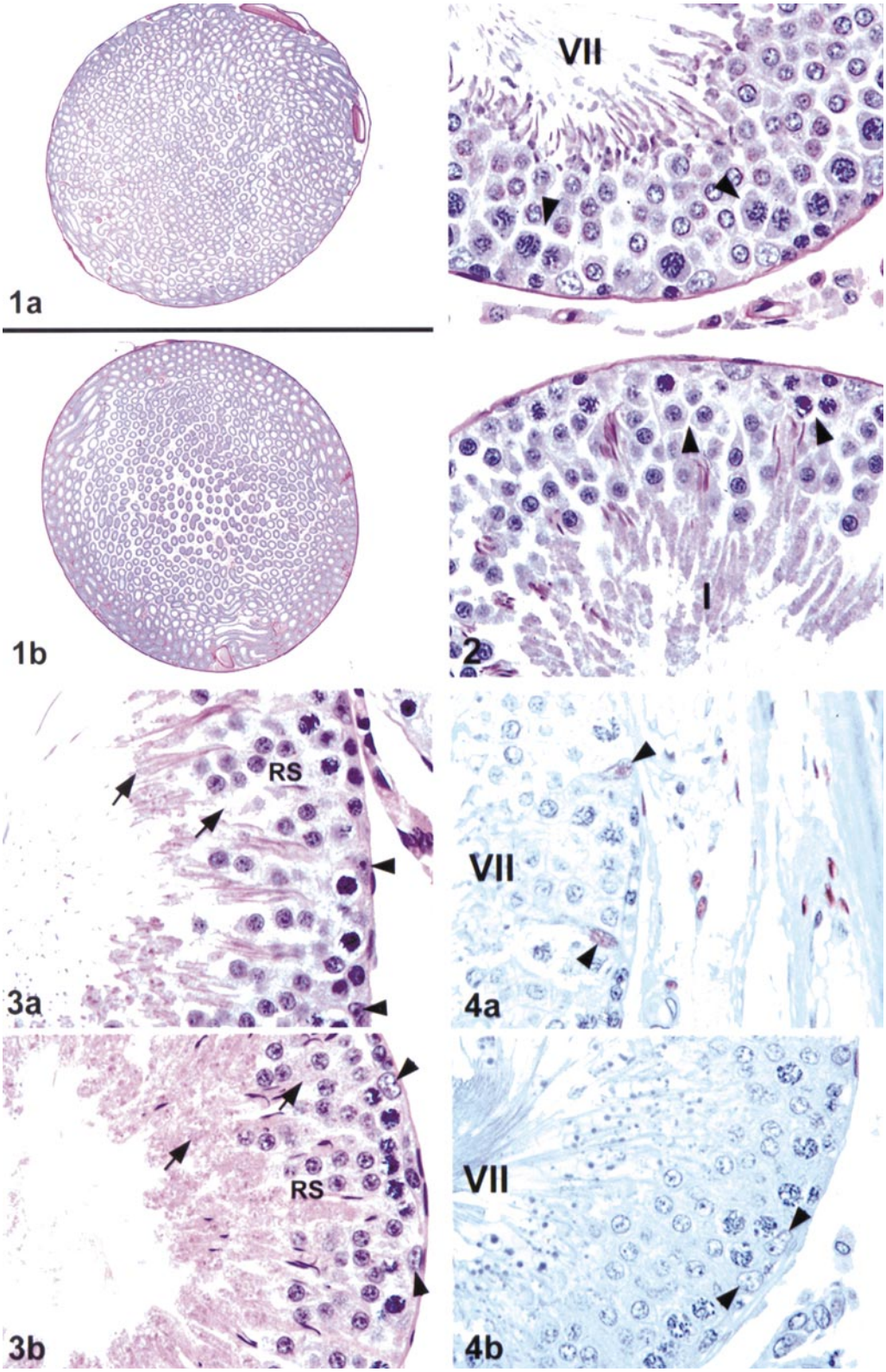
AR = androgen receptor; PC = proliferating cell nuclear antigen; PGP = protein gene product 9.5.
Intensity of staining: + = minimal, ++ = slight, +++ = moderate, ++++ = marked, – = absent.

Detection of the acrosome using PAS-staining was satisfactory alone or in combination with IHC (Figure 8a, 8b) for both fixatives in the peripheral tubules (Table 6). However, it stained slightly less distinctly in the center of the specimens fixed in BF compared to mDF with PASH alone or in combination with IHC to detect AR. In contrast, immunoreactivity for AR using dual staining was excellent in the peripheral tubules fixed in mDF (Figure 8a), but less satisfactory in peripheral tubules in BF-fixed testes (Figure 8b) and central tubules in specimens preserved by both fixatives.

Fixation of Rabbit, Dog, and Monkey Testes with Modified Davidson's Fixative. The overall quality of testis fixation was similar in the rabbit, dog, and monkey to that in the rat (Figure 9). Tubular shrinkage was minimal and the cytoplasmic "graininess" seen with BF, was less pronounced with mDF. Slight shrinkage of the germ cells away from one another was evident, but as with the rat, the degree of shrinkage did not compromise cytological evaluation. Acrosomal staining is not a relevant parameter for assessment in these species, because this structure does not stain adequately with PASH.

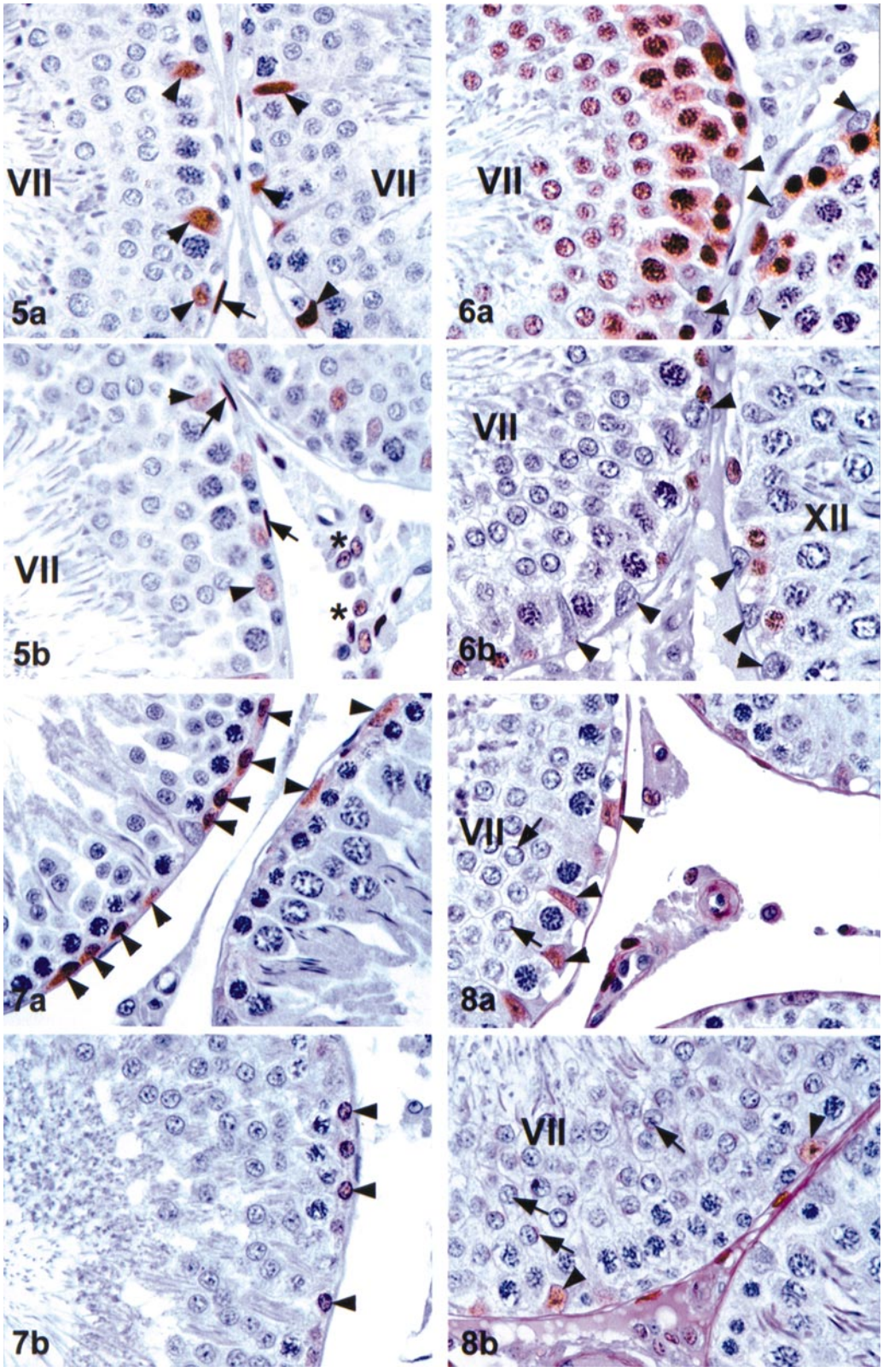
Comparison of Conventional Davidson's with Modified Davidson's for Fixation of the Eye and Testes

Fixation of the rat testis in DF resulted in prominent chromatin clumping of all nuclei as well as prominent "graininess" of the cytoplasm. Tubular and cytoplasmic shrinkage were minimal but its major disadvantage was the very poor staining of the acrosomic cap and acrosomic granule, making it impossible to confidently identify the stage of the spermatogenic cycle. Fixation of the rat testis in mDF for 48 hours with transfer to neutral buffered formalin for storage, gave similar results to those seen in the previous trial where testes were fixed for 24 hours and transferred to 70% alcohol. Flexibility in tissue fixation time and the ability to transfer tissue into formalin rather than alcohol provides for better logistics in a busy routine histology laboratory. The longer fixation time is also probably preferable for large animal testes (see previous discussion) to allow additional time for fixative penetration.



Figures 1–4

FIGURE 1.—In the central region, BF (b) compared to mDF (a) caused significantly more shrinkage of seminiferous tubules resulting in widened interstitium between tubules and tubules lacking patent lumens. PASH. Final magnification ×4.5. 2.—Cytoplasmic shrinkage artifact of germ cells in stages I and VII of testis fixed in mDF (arrowheads). PASH. Final magnification ×425. 3.—Both cytoplasmic graininess (arrows) and chromatin clumping (arrowheads) are less with mDF (a) compared to BF fixation (b). RS = layers of round spermatids. H&E. Final magnification ×425. 4.—IHC detection of AR in Sertoli cell nuclei (arrowheads) is weak in peripheral testis (a) and negative centrally (b) in BF-fixed testes. Hematoxylin counterstain. Final magnification ×425.



Figures 5–8

FIGURE 5.—IHC detection of AR in Sertoli cell nuclei (arrowheads) is strong in peripheral testis (a) and good centrally (b) in mDF-fixed testis. Peritubular myoid (arrows) and Leydig (∗) cells are also positive for AR. Hematoxylin counterstain. Final magnification ×425. 6.—PCNA immunoreactivity of the seminiferous epithelium in the peripheral testis fixed in mDF (a) and BF (b). Along the basal lamina, only Sertoli cell nuclei do not stain, making them readily detectable. Hematoxylin counterstain. Final magnification ×425. 7.—Protein gene product 9.5 immunoreactivity of spermatogonia in testis fixed in mDF (a) and BF (b). Final magnification ×425. 8.—Dual staining to detect AR in Sertoli cells (arrowheads) and the acrosome in round spermatids (arrows) in stage VII tubules fixed mDF (a) and BF (b). IHC and PASH. Final magnification ×425.

TABLE 6.—Dual staining to detect receptor expression in different stages of spermatogenesis following modified Davidson's fluid and Bouin's fluid fixation.

Effect	Fixation method			
	mDF		BF	
	PASH	AR-PASH	PASH	AR-PASH
Sharpness of acrosomal staining				
Peripheral tubules	++++	+++	++++	+++
Central tubules	++++	+++	+++	++
IHC staining intensity				
Peripheral tubules	NA	++++	NA	+
Central tubules	NA	+	NA	–

Note: Periodic acid-Schiff-hematoxylin (PASH) alone was included for comparison; AR = Androgen receptor labeled by immunohistochemistry.

Intensity of staining: + = minimal, ++ = slight, +++ = moderate, ++++ = marked, – = absent.

NA = Not applicable.

Compared to the modified version described here, conventional Davidson's fixative (DF) has a much higher alcohol and acetic acid concentration and a much lower formalin concentration. It is an excellent fixative for the eye, maintaining retinal attachment during fixation and processing and providing excellent preservation of the retinal nuclear layers and of the sensory specializations of the rods and cones (Figure 10a). However, as with most fixatives, lenticular fixation is relatively poor, resulting in varying degrees of shattering and inconsistent staining of the central layers. Use of the mDF for eyes resulted in detachment of the retina and reduced quality of preservation of the sensory endings of the rods and cones (Figure 10b). There was also a difference in the compactness and staining intensity of the nuclear layers (Figure 10a,10b). This was true of all species examined, although with rabbit eyes slight retinal detachment was also seen with DF. With mDF the collagen of the corneal substantia propria appeared shrunken and laminated with clefts between the collagen layers whereas with DF it was more homogeneous and lacked clefts (Figure 11a, 11b). This was true in rat and rabbit eyes, but for cynomologous monkey eyes, the same degree of separation of the corneal layers occurred whether fixed in mDF or DF The degree of lenticular disruption for all species was similar with both fixatives.

DISCUSSION

Fixation of the testis and eye presents a number of problems. Most tissues are trimmed to a size that allows rapid

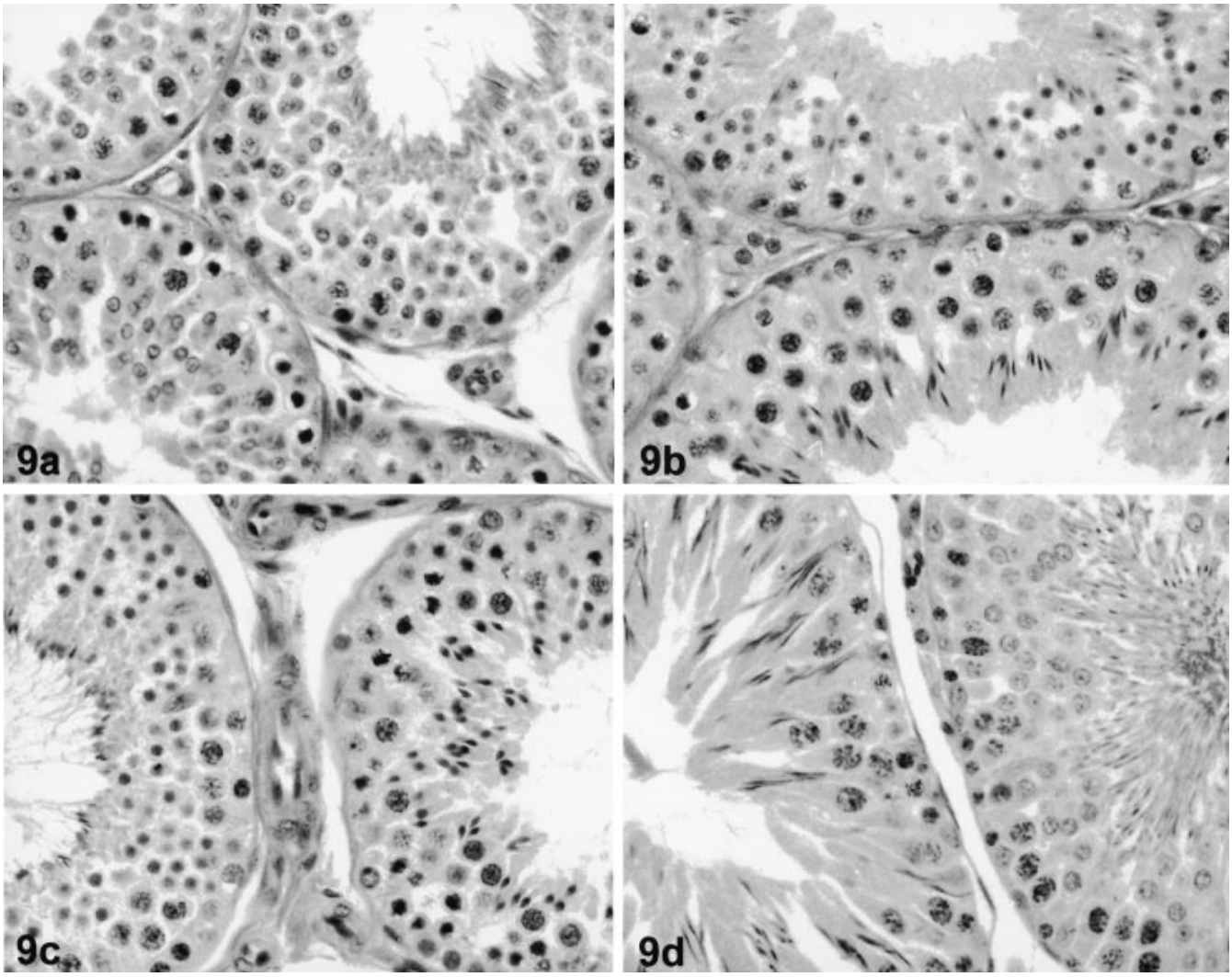


FIGURE 9.—Testis fixed with mDF: a) rabbit, b) dog, c) monkey, and d) rat. H&E. Final magnification ×288.

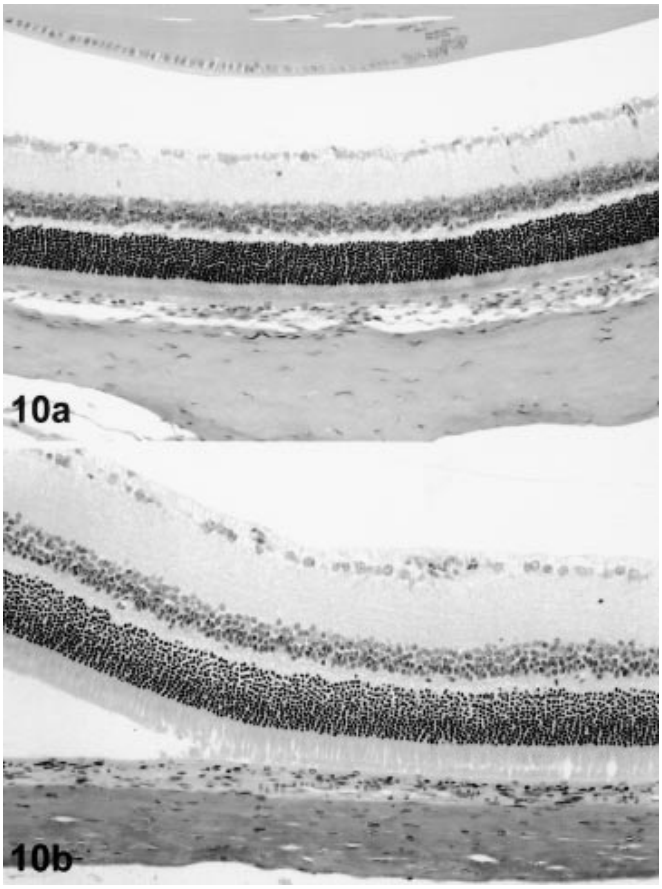


FIGURE 10.—Rat eye fixed in DF (a) has compact retinal layers attached to underlying choroid while the mDF-fixed retina (b) shows detachment of sensory rods and cones and less compact nuclear layers. H&E. Final magnification ×267.

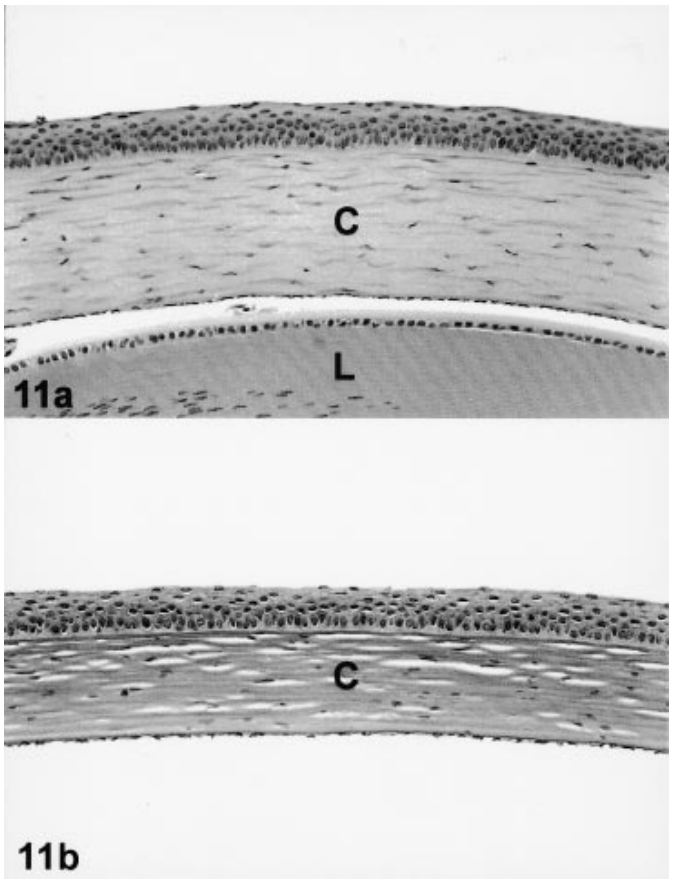


FIGURE 11.—DF-fixed rat eye shows an intact nonlaminated cornea (a) while the mDF-fixed corneal substantia propria has laminating clefts (b). Cornea (C), lens (L). H&E. Final magnification ×126.

penetration of fixative but the these 2 tissues need to be fixed whole to maintain the delicate intra- and intertubular cellular relationships in the testis and the integrity of retinal and intraocular structures in the eye. This makes the penetration characteristics of the fixative of prime importance. The poor morphological appearance of the testis using formalin fixation followed by paraffin embedding has been described previously (1, 5). The severe shrinkage of the germ cells and Sertoli cells within the tubules, results in clefts and vacuoles and makes identification of degenerative cellular changes extremely difficult. In comparison, BF maintains good intercellular contact between tubular cells and also provides good nuclear and cytoplasmic features for the Sertoli and germ cells. Its main disadvantage is in the excessive shrinkage of tubules from one another, which is most marked in the center of the testis. These shrinkage artifacts are substantially alleviated if the testis is embedded in glycol methacrylate (GMA) instead of paraffin, suggesting that the shrinkage occurs during processing or embedding into paraffin (1, 5). Chapin et al (1) have suggested that the distortion is probably caused by the need for total dehydration of the tissue during processing into paraffin. Because GMA is water-soluble and contains about 5% water, tissue processing does not involve total elimination of water.

Although formaldehyde is a rapidly penetrating fixative, it cross-links proteins relatively slowly and, even with specimens trimmed to ideal thickness (<5 mm thickness), it takes at least a week to achieve full stabilization of histological structure (7). Because testes are fixed whole, it is probable that structural stability is never complete and therefore susceptible to postfixation distortion during subsequent processing into paraffin.

BF is a mixture of different chemically active ingredients: picric acid, acetic acid, and formaldehyde with each component having a specific function. Picric acid is a slowly penetrating fixative that precipitates proteins by forming salts (picrates) with basic proteins. It is particularly good for glycogen preservation but causes excessive shrinkage of tissue (7). Acetic acid does not fix proteins but it coagulates nucleic acids. It is often included in fixative mixtures to preserve chromosomes and precipitate the chromatin of interphase nuclei (7), so it is particularly beneficial in testis fixation for visualizing the developing meiotic chromosomes in the spermatocytes. Another important property of acetic acid is its rapid penetration and production of swelling, which partially counteracts the shrinkage from the picric acid. Formaldehyde is a noncoagulative fixative that cross-links proteins, but does so relatively slowly compared with most fixatives (7). At neutral pH it is rapidly penetrating but under the acidic conditions of BF, it shows reduced penetration (4).

DF is an acetic acid-alcohol-formalin-based fixative that has been advocated and widely used for the preservation of

eye, bone marrow, breast, testis, and other tissues for histologic examination (www.histosearch.com, Histonet archives, Davidson's fixative). Davidson's (also called Hartmann's) fluid is reportedly a rapid fixative that for small samples, should be limited to 24 hours before further processing or transferring specimens to alcohol or formalin for storage ⟨http://members.aol.com/RSRICHMOND/histology.html⟩. According to Richmond, it was named after William McKay Davidson, a British hematologist, who apparently never published the formulation, but it was published by Moore et al (9, 10), who realized its attributes for preserving nuclear detail in cytologic specimens. The ingredients of DF and BF are similar, except that alcohol is substituted for the 'undesirable' picric acid. Alcohol denatures proteins by breaking hydrogen bonds and disturbing their tertiary structure. Unlike picric acid, it is rapidly penetrating but it has the same disadvantage of causing excessive shrinkage of tissues. As with BF, the presence of acetic acid, counteracts this shrinkage and the presence of formaldehyde adds an additional rapidly penetrating, general fixative. The major disadvantage with fixation of testes with DF is the poor staining of the spermatid acrosome with PAS. To alleviate this problem the relative proportions of the alcohol, acetic acid, and formalin components were modified until acceptable acrosome staining was achieved (3). Testes fixed with this mDF had the excellent nuclear resolution of BF, and the germ cells also had improved cytoplasmic preservation, lacking the "graininess" caused by precipitation of cytoplasmic proteins that is seen with BF. In comparison with Bouin's (and DF), mDF fixation was associated with some shrinkage of the germ cells away from each other, but the extent was slight and did not compromise evaluation of cellular detail. Unlike formalin-induced shrinkage, where nuclei and cell cytoplasm are severely condensed and cytological detail is severely compromised, the contour of the individual cells and cell membranes could be visualized and the cytoplasmic and nuclear detail were not altered. This was true of the large animal testes as well as the rat. Fixation of large animal testes is more difficult by nature of the size and the need to be fixed whole. Even with BF fixation, there is some intercellular shrinkage, but the degree seen, as with mDF, did not interfere with cytological detail.

Shrinkage of individual tubules away from each other with closure of the tubular lumen is a major disadvantage of BF-fixation in the center of the testis. The empty interstitial space is frequently filled with lightly staining fluid, making it difficult to distinguish artifact from edema. Fixation-induced tubular shrinkage also presents a problem for the detection of tubular dilatation, which can be a subtle but important toxicant-induced lesion (2). Testes fixed with mDF showed significantly less shrinkage of the central tubules and an absence of stained fluid in the interstitial space.

The advantages of eliminating the use of picric acid in favor of ethanol are numerous. Picric acid is potentially explosive, a severe irritant and allergen, and a mutagen ⟨http://www-ehs.ucdavis.edu/sftynet/sn-104.html⟩, ⟨http://www.cdc.gov/niosh/rtecs/tj7829b8.html#Q⟩ presenting significant safety and disposal problems to laboratory personnel. Following BF fixation, tissues should be washed in several changes of 50–70% alcohol to remove excess picrates, otherwise staining is compromised. Storage in 70% alcohol is also recommended. This special handling is labor and time consuming for routine laboratories processing a high throughput of tissues. In comparison, the alcohol-based fixative provides no additional safety hazards or disposal problems than routine formalin solutions, and after 24–48 hours of fixation, tissues can be transferred to 10% buffered formalin for storage.

The IHC results from this study demonstrate the utility of mDF as a general fixative for the testis. In most respects, the results obtained with mDF were superior to those of BF, which until now has been the fixative of choice for such studies. The selection of antigenic markers used also provided a useful staining method for distinguishing between different cell types within the seminiferous epithelium. Quantification of cells within the seminiferous epithelium by conventional histopathological methods of manual counting is laborious and time-consuming. In addition, distinguishing Sertoli cells from early spermatogonia, or late spermatogonia from early spermatocytes can often be difficult. Differential staining of these cell populations by IHC provides a tool for improving identification as well as potential use with image-analysis technology for automated quantification. The ability to carry out dual staining for IHC parameters and acrosomal structure with PASH also provides the opportunity to monitor cell populations or cell function (eg, AR expression) in a stage-specific manner. This is important because most physiologic, metabolic, and regulatory functions of the seminiferous epithelium occur in a stage-specific pattern.

DF is a recommended fixative for preserving eyes. This is another tissue that benefits from being fixed whole, but conventional fixation in 10% formalin causes artifactual cellular shrinkage and poor cellular and nuclear resolution of the retina. In comparison with the Davidson's recipe used to fix the testes, the mix of components for ideal retinal preservation requires approximately double the alcohol and acetic acid concentration and 1/17th the concentration of formalin. Fixation of the testes with the mixture recommended for eyes caused significant chromatin clumping and cytoplasmic graininess of the Sertoli and germ cells and also prevented any appreciable staining of the spermatid acrosome with PASH. Fixation of the eyes in the testis fixative resulted in varying degrees of retinal detachment and reduced resolution of the nuclear layers and the sensory photoreceptor endings. Although the overall morphology was acceptable and markedly superior to neutral buffered formalin, it was inferior to the DF recommended for eyes. It is possible that modification of the relative proportions of the components could produce a single fixative ideal for fixing both eye and testis, but this was not attempted.

Although most of the regulatory guidelines specifically mention use of BF fixative for the testes, they also add "or a comparable fixative." Implicit in this recommendation [and actually stated in OECD 421(11)] is that formalin should not be used. Our work has demonstrated that a mDF fixative using alcohol, acetic acid, and formalin in different proportions from the conventional Davidson's recipe, provides comparable, and in many respects superior, preservation to that of BF, both for IHC staining and for detailed histopathological examination. It also provides an acceptable fixative for eyes, although the quality of cellular preservation is inferior to that of DF.

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