

Original Article

Effects of 4% paraformaldehyde and modified Davidson's fluid on the morphology and immunohistochemistry of Xiang pig testes

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Abstract: Modified Davidson's fluid (mDF) is a good fixative for morphological and antigen preservation. However, recent studies have shown that 4% paraformaldehyde (PFA) can better preserve the actin structure in rodent testes. It remains controversial which of these fixatives is best for testicular tissue. This study investigated the effects of both mDF and 4% PFA on the morphology and antigen preservation of Xiang pig testes using hematoxylin-eosin (HE) staining and immunohistochemistry (IHC). The stronger testis penetration of mDF compared with that of 4% PFA was primarily manifested as testicular color change and decrease in tissue weight loss. Testes fixed with 4% PFA displayed a severe shrinkage of both the tubular and interstitial compartments and the seminiferous tubule area decreased by 12.02% compared with that in mDF-fixed tissues. In contrast, IHC results showed that 4% PFA fixation achieved better IHC-positive performance than mDF fixation for antigens specifically expressed in germ cells, Leydig cells and Sertoli cells. Due to this improved antigen preservation by 4% PFA fixation, the relative immunoreactions intensity significantly increased by 39.8%, 27.8%, and 76.4%, respectively, compared with that in mDF fixation. In summary, fixation of Xiang pig testes with mDF was suitable for HE staining, while fixation with 4% PFA was more suitable for IHC. (DOI: 10.1293/tox.2019-0072; J Toxicol Pathol 2020; 33: 97–104)

Key words: fixative, testis, morphology, hematoxylin-eosin staining, antigen preservation, immunohistochemistry

Introduction

The evaluation of testicular histomorphology is an important part of investigations of reproductive pathology, growth, and development of laboratory animals. The testis morphology directly reflects reproductive diseases such as testicular cancer¹, Sertoli cell-only syndrome², and spermatogenesis failure³. Furthermore, specific antigen markers in Sertoli, Leydig, and germ cells can be used to both identify and evaluate the developmental process of animals and cellular functions⁴. Prior to testicular evaluation, testes should be fixed appropriately.

Testes are more difficult to fix than other organs and tissues due to their lower protein content and less linked structure in the seminiferous epithelium⁵. Based on previous studies, researchers typically chose 4% paraformaldehyde (PFA), modified Davidson's fluid (mDF), and Bouin's fluid (BF) to fix mammalian testes^{6, 7}, since histomorphological

details can be better preserved, and tissue processing is also easy. Recently, 4% PFA has been favored by researchers due to its convenience and satisfactory antigen preservation^{8–10}. A further related study showed that 4% PFA-fixed nucleoprotein antigens offered remarkable immunohistochemistry (IHC) performance compared with 10% neutral buffered formalin (NBF)-fixed antigens¹¹. However, several studies have reported that 4% PFA-fixed tissue samples showed tissue shrinkage and mislocalization of target proteins^{12–14}. For testes, the main morphological differences were the shrinkage of both seminiferous epithelium and interstitial compartment after fixation¹³. Cell structures and morphological details of testes can be better preserved with BF^{15, 16}. However, the main ingredient of BF (the residual picric acid) is assumed to be a health and safety hazard after fixation, causes laboratory waste disposal problem, and needs to be removed by multiple alcohol rinses^{15, 17}. Consequently, in 2002, the Society of Toxicologic Pathology recommended the use of mDF to fix animal testes¹⁵. Later, researchers found that mDF-fixed testes showed slight shrinkage of morphological details compared with BF-fixed testes¹⁵. IHC is a technique for the study of localization and quantification of proteins; however, its sensitivity is easily affected by tissue fixatives, antigen retrieval methods, and detection systems (especially fixatives)^{18–20}. For example, IHC based detection of mDF- and BF-fixed mouse testes have shown that specific antigen

Received: 15 September 2019, Accepted: 11 December 2019

Published online in J-STAGE: 18 February 2020

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expression is more easily detected with mDF fixation than with BF fixation¹⁵.

Fixatives used in common laboratory animal tissues have considerably matured. However, the use of fixatives and reproductive toxicology models remains unassessed in domestic animals such as pigs. The Xiang pig is a rare breed of Chinese miniature pig, which is renowned for its small size, early sexual maturity, and favorable meat quality as well as its similarities to human physiology. The Xiang pig is widely bred in the border area of Guizhou and Guangxi in China, and has been used for domestic animal breeding and reproduction, in addition to clinical medical research²¹. Several molecular mechanisms of precocious puberty have been reported before in Xiang pigs²². However, the morphological characteristics and protein function have not been reported. To select an optimal fixative for Xiang pig testes and establish a stable system for morphological examination and protein expression, the present study evaluated the performance of two fixatives with respect to morphological and antigen preservation. Hematoxylin-eosin (HE) staining of cross-sections and IHC of specific marker proteins were conducted using Xiang pig testes that had been fixed with either mDF or 4% PFA. The results of this study will be useful for the comparative anatomical study on the male reproductive organs of domestic animals and thus, will provide useful information to improve Xiang pig breeding soundness and fertility potential assessment.

Materials and Methods

Animals and tissue preparations

Six health male Xiang pigs (weighing 10.53 ± 1.38 kg at 4 months of age) were chosen for this study. The pigs were obtained from Guiyang Lvshengyuan Animal Husbandry Technology Development Co., Ltd. (SCXK: 20160007, SYXK (Qian) 2018-0010, Guiyang, China). Testes samples were collected via orchietomy after the animals were anesthetized by 0.04mg/kg atropine sulfate salt monohydrate (A0257; Sigma-Aldrich, St. Louis, MO, USA). According to the experimental design, testes were divided into four parts, and trimmed to $5 \times 5 \times 3$ mm³. Two parts were fixed in mDF for HE staining and IHC, and the remainder was fixed with 4% PFA for the same assays. The ingredients of mDF and 4% PFA are listed in Table 1. All procedures were performed by a veterinarian and strictly followed the approved guidelines of the laboratory animal ethics committee of Guizhou University, China (Grant No. 1801227).

HE staining

Tissue samples measuring $5 \times 5 \times 3$ mm³ were collected from the center of testes and were routinely fixed, embedded, and sectioned as described by Dutta *et al.*²³. To analyze testes morphology, testicular cross-sections were stained using a commercial HE staining kit (G1120; Solarbio Co., Ltd., Beijing, China), and photographed using a Nikon ECLIPSE-Ni+DS-Ri2 (Nikon Instruments Inc., Tokyo, Japan) with NIS-Elements BR analysis software ver-

sion 5.01 (Nikon Instruments Inc.). The area of 100 round seminiferous tubules at stage I–II of the seminiferous epithelial cycle²⁴ was quantified using NIS-Elements BR and the relative shrinkage rate was calculated according to the following formula:

$$\text{Relative shrinkage rate (\%)} = \frac{\text{Area of seminiferous tubules fixed by mDF} - \text{Area of seminiferous tubules fixed by 4\%PFA}}{\text{Area of seminiferous tubules fixed by mDF}} \times 100\%$$

Immunohistochemistry

Germ cells, Leydig cells, and Sertoli cells in the testis were, respectively, identified using the following antibodies: DEAD-box helicase 4 (DDx4; ab13840, diluted 1:200; Abcam Inc., Cambridge, UK), 3beta-hydroxysteroid dehydrogenase/Delta5–Delta4 isomerase (β -HSD; sc-515120, diluted 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and transcription factor GATA-binding factor 4 (GATA4; sc-1237, diluted 1:100; Santa Cruz Biotechnology)²⁵. These were used in reference to the previously reported IHC protocol^{26, 27} to compare the antigen preservation abilities in both fixatives. Specifically, 5 μ m testicular cross sections were dewaxed with xylene, hydrated by a gradient ethanol series, immersed in sodium citrate (pH=6.0), and heated in a microwave oven at about 100°C for 5 min for antigen retrieval. Then, endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 1 h at room temperature (about 23°C) and nonspecific activity was blocked using 5% bovine serum albumin (BSA; SW3015, Solarbio Co., Ltd.) for 30 min at 37°C. Thereafter, sections were treated overnight with primary antibodies (DDx4, β -HSD, and GATA4) at 4°C, respectively. After primary antibody treatment, these specific antigens were detected using HRP-labeled Goat Anti-Rabbit IgG or HRP-labeled Goat Anti-Mouse IgG-SABC kits (SA2001, Booster Biological Technology Co., Ltd., Wuhan, China) and the results were visualized with 3,3'-Diaminobenzidine kit (D6190, Sigma-Aldrich). Subsequently, all sections were counterstained with hematoxylin for 50 s and embedded in neutral resin. In negative control groups, the sections were treated with phosphate buffered saline (PBS; P1031, Solarbio Co., Ltd.) instead of primary antibodies. All images were captured using Nikon ECLIPSE-Ni+DS-Ri2 and qualitatively analyzed with NIS-Elements BR software. To quantify the intensity of the immune reaction, the average intensity was used to represent the positive IHC rate as previously described²⁸. Forty positive immunohistochemical digital images (DDx4: stage III; β -HSD: stage V; GATA4: stage V; n=10 per group) were used to calculate the integrated optical densities (IOD) and the area of interest (AOI) using Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The following formula was used to calculate the average intensity:

$$\text{Average intensity} = \frac{\text{IOD}}{\text{AOI}}$$

Table 1. Ingredients (for 1,000 mL) and Fixing Conditions of mDF and 4% PFA

Fixing fluid	Composition					Fixing condition	
	Formaldehyde	Paraformaldehyde	Alcohol	Acetic acid	Water	Temperature	Time
mDF	30%	-	15%	5%	50%	4 °C	24 h
4% PFA	-	40 g	-	-	0.1 M PBS	4 °C	24 h

mDF, modified Davidson's fluid; PFA, paraformaldehyde; PBS, phosphate buffered saline.

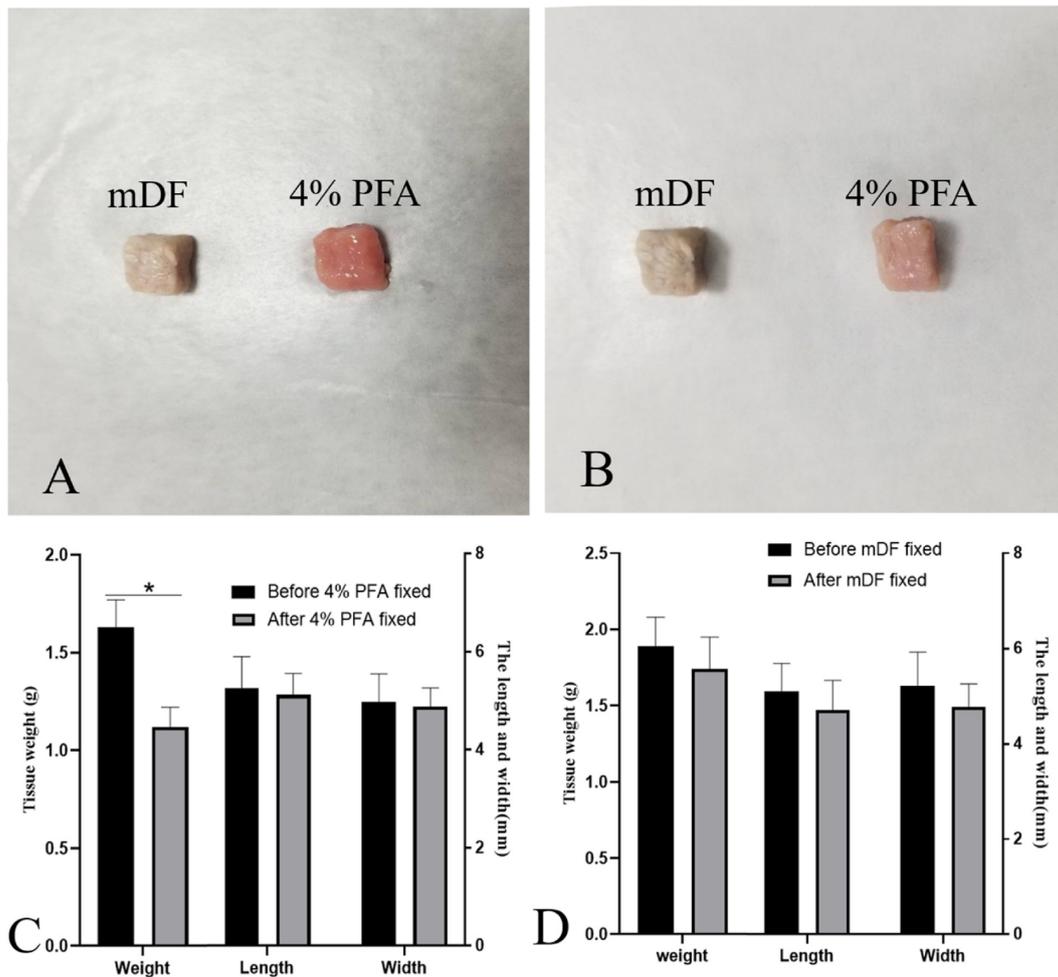


Fig. 1. Effects of testicular cross-section processing. Testis color change in response to modified Davidson's fluid (mDF) and 4% paraformaldehyde (PFA) after fixation for (A) 1 min and (B) 24 h. Testicular weight, length, and width change before and after (C) 4% PFA and (D) mDF fixation for 24 h (n=6 in each group). The testis weight decreased significantly in response to 4% PFA fixation compared with that in response to mDF fixation (*, T-test; $P < 0.05$).

Statistical analysis

All results were expressed as means \pm standard deviation (SD); GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA) were used to visualize the results and T-tests were used to assess the seminiferous tubule area and testicular weight (left-hand vertical axis), length, and width (right-hand vertical axis). Differences were considered significant at $P < 0.05$ and different letters (a and b) in the same column indicate significant differences.

Results

Changes in testis tissue processing for paraffin embedding

The color of testes immediately began fading after placing in mDF solution, while the color of 4% PFA fixed-testes did not change (Fig. 1A). After fixing for 24 h, the 4% PFA-fixed testes were darker in color than the mDF-fixed testes (Fig. 1B). After 4% PFA fixation, the testes weight decreased by 68.71% independent of the testis length and

Table 2. Changes of Testicular Weight in 4% PFA and mDF-fixed Testes after Dehydration

Fixed fluid	Testis number	Before dehydration (g)	After dehydration (g)	Loss (%)
mDF	n=6	0.67 ± 0.03 ^a	0.49 ± 0.02 ^b	26.87
4% PFA	n=6	0.69 ± 0.03 ^a	0.38 ± 0.03 ^b	44.93

mDF, modified Davidson's fluid; PFA, paraformaldehyde. Different letters (a and b) in the same line indicate significant differences ($P < 0.05$) by T test.

Table 3. Area of Seminiferous Tubules Fixed with 4% PFA and mDF

Fixing fluid	Testis number	Seminiferous tubules	Age (d)	Area (μm^2)
4% PFA	n=6	100	120	35829.40 ± 2534.86 ^a
mDF	n=6	100	120	40722.26 ± 2933.88 ^b

mDF, modified Davidson's fluid, PFA, paraformaldehyde.

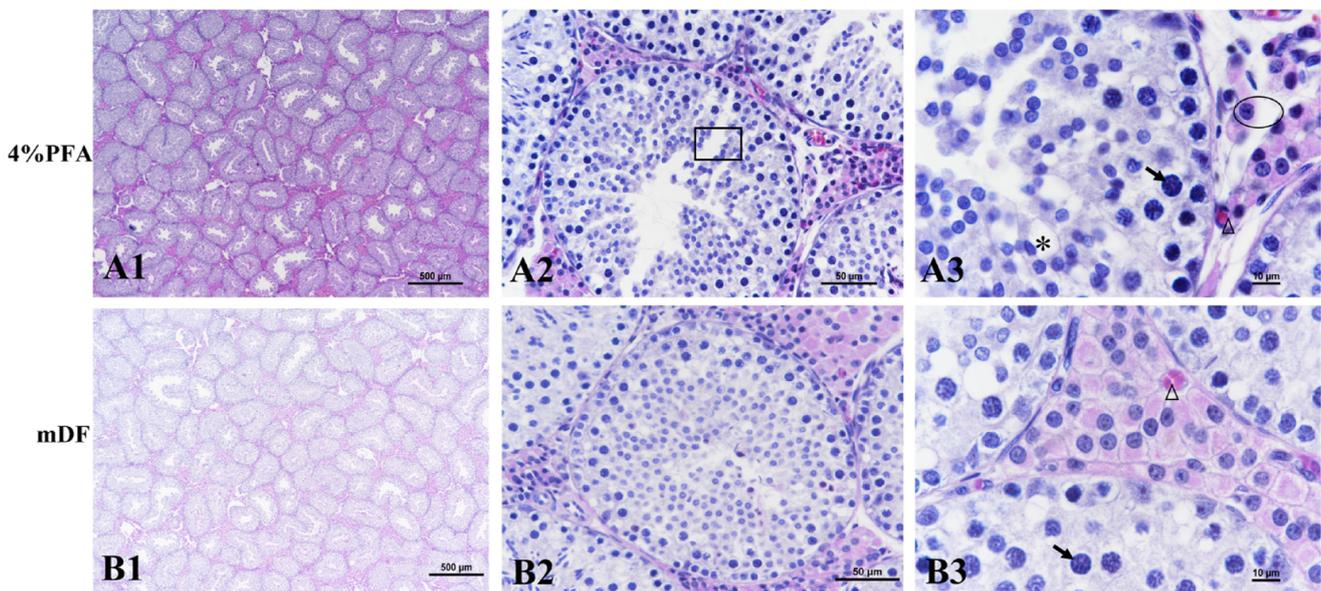


Fig. 2. Effects of modified Davidson's fluid (mDF) and 4% paraformaldehyde (PFA) on Xiang pig testis morphology. The testes were removed from 120-day-Xiang pigs and fixed with mDF and 4% PFA at 4°C for 24 h. A1, A2, and A3 show testes fixed with 4% PFA, while B1, B2, and B3 show testes fixed with mDF. Shrinkage of the spermatogenic epithelium (marked with a "square box" in A2); shrinkage of Leydig cells (marked with an "oval box" in A3); cytoplasmic vacuolization (marked with an "asterisk" in A3); nuclear staining of the primary spermatocyte in interphase (marked with an "arrow" in A3 and B3); erythrolysis (mark with a "triangle" in A3 and B3); bars = 500 μm (A1 and B1), 50 μm (A2 and B2), and 10 μm (A3 and B3).

width (Fig. 1C) Tissue sizes were not statistically different (including testis weight, length, and width) after mDF fixation for 24 h (Fig. 1D). Table 2 shows a testis weight comparison after dehydration via alcohol gradient for mDF- and 4% PFA-fixed testes. A decrease in testes weight loss was found for mDF-fixed tissues compared with that for 4% PFA-fixed tissues, which may be because mDF contains alcohol and dehydrated in advance.

Morphology of Xiang pig testis preserved in two different fixatives

The histomorphology of testes fixed with either 4% PFA or mDF were assessed by HE staining. The results showed

that the area of seminiferous tubules fixed with 4% PFA was clearly smaller than that fixed with mDF (see Table 3). For morphological details, the shrinkage of seminiferous tubules (i.e., intercellular clefts) and interstitial compartments (i.e., intercellular spaces and cytoplasmic vacuolation) was more pronounced in 4% PFA-fixed testes than in mDF-fixed tested (Fig. 2). Specifically, a severe seminiferous epithelial cleft was present in 4% PFA-fixed testes due to the loosening of connections between germ cells, especially late spermatogenic cells (marked with a "square box" in Fig. 2A2). Large intercellular spaces were found between Leydig cells (marked with an "oval box" in Fig. 2A3). With regard to cell structure, cytoplasmic vacuolization was observed in

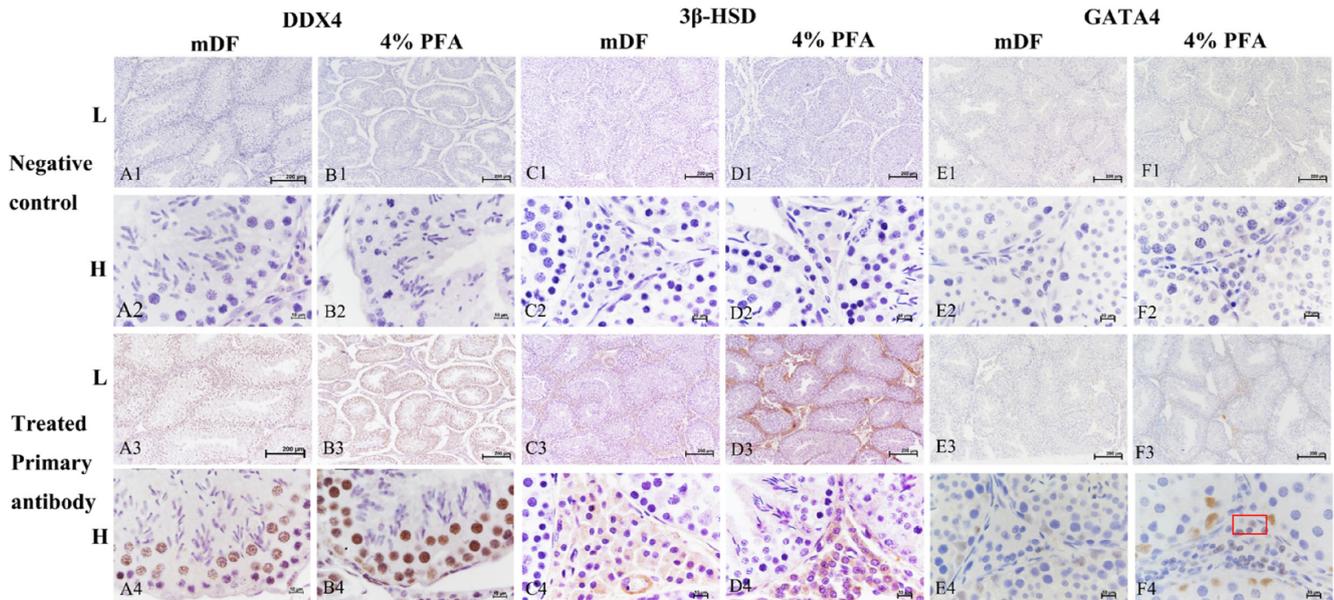


Fig. 3. Effects of modified Davidson's fluid (mDF) and 4% paraformaldehyde (PFA) on antigen preservation in Xiang pig testes. L and H represent low and high magnification, respectively. Testes were removed from 120-day Xiang pigs ($n=6$) and fixed with mDF and 4% PFA at 4°C for 24 h. A1–A4, C1–C4, and E1–E4 represent images of mDF-fixed testes, while B1–B4, D1–D4, and F1–F4 represent images of 4% PFA-fixed testes. Testicular cross-sections were immunostained with primary antibodies for DDX4 (A3–A4 and B3–B4), 3 β -HSD (C3–C4 and D3–D4), and GATA-binding factor 4 (GATA4) (E3–E4 and F3–F4). Negative controls used phosphate buffered saline (PBS) for incubation (A1–A2, B1–B2, C1–C2, D1–D2, E1–E2, and F1–F2). Bars = 200 μ m (A1–F1) and (A3–F3) and 10 μ m (A2–F2) and (A4–F4). Positive immunostaining is shown in brown color after counterstaining with hematoxylin. DDX4 and 3 β -HSD expressions were evident in germ and Leydig cells of 4% PFA-fixed testes but not in mDF-fixed testes. GATA4 was clearly expressed in 4% PFA-fixed Sertoli cells and weakly in Leydig cells (F4, Rectangle mark), but mDF-fixed testes showed inconspicuous immunostaining in these cells.

testes fixed with both fixatives and the difference was not significant (marked with an “asterisk” in Fig. 2A3). Furthermore, in 4% PFA-fixed testes, the tissue was slightly darker, similar to erythrolysis (marked with a “triangle” in Fig. 2A3 and B3) and nuclear staining of primary spermatocyte in the interphase (marked with an “arrow” in Fig. 2A3 and B3), than in mDF-fixed testes. This affected the observation of nucleus chromosomes of germ cells. In conclusion, mDF-fixed testes showed better morphological details both in tubular and interstitial compartments of testis than 4% PFA-fixed testes.

IHC differences between 4% PFA- and mDF-fixed testes

To analyze the effects of the two fixatives on antigen preservation, IHC was conducted in the present study. The results showed that regardless of whether mDF or 4% PFA was used to fix Xiang pig testes, germ cells (Fig. 3A1–B4), Leydig cells (Fig. 3C1–D4), and Sertoli cells (Fig. 3E1–F4) stained positive for DDX4, 3 β -HSD, and GATA4, respectively. However, due to the effect of the fixatives on antigen preservation, immunoreactions with the DDX4 antibody were decreased (by about 39.8%) at the nucleus of germ cells fixed with mDF compared with those in cells fixed with 4% PFA (Fig. 3A1 and B4). The strong 3 β -HSD antibody-specific staining in the cytoplasm of Leydig cells in 4% PFA-testes was significantly deeper (about 27.8%) than

in mDF-fixed testes (Fig. 3C4 and B4). Accordingly, GATA4 antibody-specific staining was apparent in 4% PFA-fixed Sertoli cells and its IOD was about 76.4% higher than that of mDF-fixed Sertoli cells (Fig. 4). Furthermore, GATA4 was also weakly positively expressed in 4% PFA-fixed Leydig cells, but not in mDF-fixed (Fig. 3E4 and F4). In summary, 4% PFA is the better fixative of testis for IHC analysis.

Discussion

IHC and histopathology can be used for comparative pathology and translational studies. When tissue samples are separated from the blood supply, they will undergo autolysis. Consequently, tissue must be fixed to preserve its morphology and antigenicity or RNA structure. Poor fixation can cause poor morphology and reduce the specificity of IHC or in situ hybridization (ISH). However, over-fixation may result in antigen masking or strong non-specific background staining.

Testes, eyes, and fat tissues are difficult to fix due to their specific structure. Inappropriate fixatives are considered to be susceptible to loss of glycogen, nucleic acid, and tissue protein, which will affect morphological observations and protein expression studies²⁹. As an important component of the male reproductive system, appropriately fixed testes contribute to histopathological examination, reproductive toxicology, and developmental biology studies in animals.

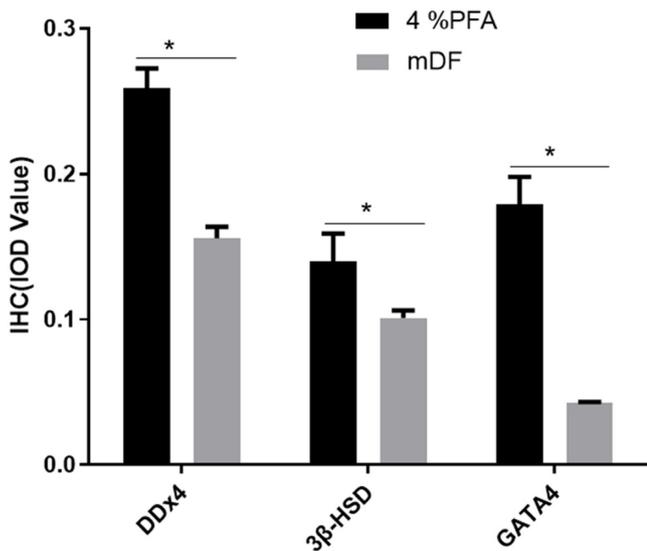


Fig. 4. Comparison of DDx4, 3 β -HSD, and GATA-binding factor 4 (GATA4) expressions in modified Davidson's fluid (mDF)- and 4% paraformaldehyde (PFA)-fixed testes. Immunohistochemistry (IHC) testicular cross-sections (n=6) of DDx4, 3 β -HSD and GATA4 were used for the integrated optical densities (IOD) analysis. Testes fixed with 4% PFA showed significantly higher IOD of IHC compared with mDF-fixed testes for all three proteins (*, T-test; $P < 0.05$).

The Xiang pig is a rare Chinese miniature breed with small size and favorable meat. In previous studies, Xiang pigs were frequently used as an experimental animal model for clinical research due to their similar anatomical and physiological characteristics to those of humans. Moreover, due to their small size, the sample size can be increased and this breed is more manageable than larger animals^{21, 22}. Consequently, a reproductive toxicology model of the Xiang pig and their histological features, as well as the interactions of testis-related proteins are worth investigating. This study showed that mDF works as a better fixative than 4% PFA for HE staining of testes, while 4% PFA fixation is preferable for IHC. For many animals, it has been reported that different fixatives show distinct morphological details of testes. For example, formalin-fixed mouse testes showed severe shrinkage of both, germ cells and Sertoli cells, as well as wide gaps and vacuoles in the spermatogenic epithelium³⁰. The results of another study showed that testicular cross-sections were difficult to fix and suffered from seminiferous epithelium shrinkage after 10% NBF fixation¹⁵. For BF fixation, even if the morphological evaluation was not affected by the shrinkage of intra-tubule and inter-tubule compartments of mouse and human testes, it is not recommended due to the existence of picric acid^{31, 32}. Instead, mDF, modified from traditional Davidson's Fluid (DF), was found to achieve less shrinkage of the seminiferous tubules and superior overall morphologic details of testes for histopathological evaluation. The underlying reason for this is

the rapidly penetrating dehydrating effect of the coagulative fixative ethanol and acetic acid present in this fixative. However, the drawback is that the seminiferous epithelium at stages I and VII shrunk in mDF-fixed mouse testes, while they were perfect in BF-fixed mouse testes^{13, 15, 31}. Consistently, mDF was found to be suitable for HE staining as indicated by the satisfactory morphological details of Xiang pig testes in the present study. The change of testis color and weight was likely related to strong mDF penetration. In contrast, as a single-component fixative, 4% PFA-fixed testes were flesh-red-like and soft and showed altered testis weight and area of seminiferous tubules after fixation. The weak penetration of formaldehyde in 4% PFA likely caused tissue debris in the fixative, which may be an acceptable explanation. Generally, shrinkage of spermatogenic epithelium and interstitium is considered the main challenge in morphological evaluation of 4% PFA-fixed testes. Several studies have reported that this unsatisfactory performance is due to weak fixative penetration. These results suggest that 4% PFA may not be as good as mDF for the testicular morphological analysis of Xiang pigs.

IHC staining of GATA4, 5-bromo-2-deoxy Uridine (BrdU), and proliferating cell nuclear antigen (PCNA) by using mDF fixative have been reported in many studies^{33, 34}; however, which of these are the best fixatives for testes remains controversial. For example, mDF-fixed testes provided better IHC stain intensity than BF-fixed testes for PCNA and neuron cytoplasmic protein 9.5 (PGP 9.5) antigens¹⁵. In addition, androgen receptor (AR) expression was detected in the peripheral and central seminiferous tubules in mDF-fixed testes, but was observed only in peripheral seminiferous tubules in BF-fixed testes¹⁵. Moreover, it was reported that mDF-fixed testes showed more satisfactory effects of vimentin-IHC than did 10% NBF-fixed testes³⁰. However, another study argued that the actin of *Drosophila melanogaster* and reproductive and respiratory syndrome virus (PRRSV) of porcine were better preserved than organic solvents, since the free amino acids can chemically cross-link with 4% PFA^{9, 10}. Although the comparison of IHC differences between 4% PFA- and mDF-fixed mouse testes was reported via qualitative description in recent studies^{33, 35}, quantitative evidence is not yet available. In Xiang pigs, GATA4, 3 β -HSD, and DDx4 expressions were detected in Sertoli cells, Leydig cells, and germ cells, respectively, in both mDF- and 4% PFA-fixed testes; however, IHC staining achieved the better results in 4% PFA-fixed testes than in mDF-fixed testes in the present study. When Xiang pig testes were fixed with mDF, specific antibodies (GATA4, 3 β -HSD, and DDx4) showed a weak positive signal compared with that in 4% PFA-fixed testes. In fact, previous studies have reported that cytoskeletal proteins can be destroyed by the absolute ethanol and acetic acid present in mDF, resulting in antigen dispersion and loss²⁰. In addition, GATA4, a specific marker antigen of Sertoli cells involved in testicular development and regulation of biological function^{36, 37}. The weakly positive GATA4 expression

in Leydig cells was only detected in 4% PFA-fixed testes. Overall, although mDF fixation provides a considerable advantage over the morphological preservation of Xiang pig testes, it cannot match the result of 4% PFA fixation in IHC detection. In summary, mDF fixation better preserves the morphological details of Xiang pig testes than 4% PFA fixation. Although 4% PFA-fixed testes showed morphological shrinkage, IHC-positive staining for testicular proteins was superior than that in mDF-fixed testes.

Disclosure of Potential Conflicts of Interest: The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions: WYW analyzed the data for this study and drafted the manuscript; TG and YY designed the study and revised the manuscript; LJM performed the immunohistochemistry and hematoxylin–eosin staining; YJX prepared the tissue and testicular cross-sections.

Acknowledgements: The authors appreciate the use of instruments and platform of the Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, Guizhou University. This study was supported by the National Natural Science Foundation of China (31702117), the Science and Technology project of Guizhou Province (QKHJC-2018-1404), the Scientific Research Cooperation Project with America and Oceania Region, Ministry of Education (JWSM-2016-2191), the project for the Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education (Guizhou University) (GYSD-K-2018-05), and the Construction Program of Biology First-class Discipline in Guizhou (GNYL[2017]009), Graduate Education Innovation Project of Guizhou Province (QJH-YJSCXJH-2018-042) and the introduced talent project of Guizhou University (GDRJHZ [2016]72).

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