# **ORIGINAL ARTICLE**



# Optimizing the preparation of paraffin sections from stallion testes

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**Summary.** The preparation of paraffin sections is an important experimental technique in animal histological research, and key factors that determine the quality of a section include the dehydration time, waxing time, and drying temperature of the paraffin section. Paraffin sections obtained from testis tissue of adult horses exhibited higher quality with clear tissue structure and complete cell morphology after they underwent gradient dehydration for 6 hours, were immersed in wax for 60 minutes, and were dried in a 75-degree oven for 15 minutes. The detailed, optimized procedures that are developed in the current study may simplify histological experiments and research on equine testes.

**Key words:** Paraffin section, Horse, Testis, Seminiferous tubules

## Introduction

Before the age of two years, stallions are within the prepubertal stage, which is devoid of spermatogenesis (Ing et al., 2004). When stallions reach puberty, Sertoli and Leydig cells mature and begin to support spermatogenesis (Xiao et al., 2014). Meanwhile, the average length and volume of the equine testis increase from 4.83 cm and 182.41 cm<sup>3</sup>, respectively, to 5.65 cm and 264.49 cm<sup>3</sup> (Silva et al., 2021). Until now, studies on the development and functional aspects of equine testes have been scarce. Mature Sertoli cells form the blood-testis barrier in the seminiferous tubules to facilitate the process of spermatogenesis, which

*Corresponding Author:* Gerelchimeg Bou, Inner Mongolia Key Laboratory of Equine Science Research and Technology Innovation, Inner Mongolia Agricultural University, Hohhot, China. e-mail: gerelchimeg@imau.edu.cn www.hh.um.es. DOI: 10.14670/HH-18-775 transports preleptotene spermatocytes from the basal compartment to the apical compartment; then, after reaching this compartment, the preleptotene spermatocytes become round spermatids and spermatids (Li and Cheng, 2016). Leydig cells are found in clusters between the seminiferous tubules (Wen et al., 2018). Leydig cells produce androgens and testosterone, which support the function of Sertoli and germ cells through steroidogenesis (Mok et al., 2013; Trost et al., 2023). By studying the stallion's testicular histology and anatomy, we can gain a better understanding of the spermatogenesis process and related diseases such as orchitis and testicular tumors (Pérez et al., 2015; Yang et al., 2022). Equine species are exposed to various diseases requiring frequent and regular examination (Snider, 2015). Viruses are the most dangerous diseases that infect equines, such as equine herpesvirus-1 and equine arteritis virus, and cause various drawbacks such as abortion in females and persistent infection in stallions (Mahmoud et al., 2022). Before histological or anatomical experiments are performed, most animal cells and tissues are colorless and transparent, and undergo apoptosis shortly after leaving the body; therefore, maintaining the original shape and structure in vitro is difficult (Bergmann and Steller, 2010; Mao, 2013). The most important way to preserve the tissue's morphological and histological characteristics is by performing efficient and rapid preparation of paraffin sections and subsequent staining (Rathi et al., 2006; Li et al., 2020). Today, paraffin sectioning, which includes fixation, dehydration, embedding, sectioning, and drying (Sy and Ang, 2019; Sadeghipour and Babaheidarian, 2019), is used to observe and evaluate the morphological and structural changes of tissues and cells; in addition, the technique is a major approach used in many pathological studies (Wick, 2019; Amin et al., 2020, 2023; Toghan et al., 2022; Ali et al., 2024).

During our preliminary studies, we found that



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creating high-quality sections of equine testis using the conditions described for mice was generally difficult because the testis volume and tissue water content of horses are greater than those of mice. These issues have always limited histological research in equine testicular tissue. Both Rode et al. (2015) and Almeida et al. (2013) reported the sectioning technique to examine the morphology of horse testicular tissue, however, because the description is fairly coarse, the technique cannot be used to guide practice. Therefore, in the present study, nine different paraffin conditions were compared after fresh samples were fixed, and the optimal dehydration time, waxing time, and drying temperature for the paraffin section of horse testis were confirmed. Our optimized paraffin sectioning processes for equine testis provide a ready-to-use reference for application in the histochemical analysis of equine testis.

#### Materials and methods

### Sampling and fixation

All animal experiments were conducted following the regulations of the ethics committee of the Inner Mongolia Agricultural University. Horse testes from the three four-year-old adult Mongolian horses used in this study were obtained from a slaughterhouse in Hohhot, Inner Mongolia, China. The excised testes were immediately brought to the laboratory after being dissected and washed in saline solution. In the sterile laboratory environment, the testes were incised along the longitudinal axis and cut into small pieces of 1 cm×1 cm× 0.5 cm. They were then fixed for 24 hours in Bouin's solution (saturated picric acid, formalin, and glacial acetic acid at 15:5:1 proportions) because our previous research (Asgenbaatar et al., 2020) demon-

Group	Total (h)	Step
1	3	75% ethanol 30 min 75% ethanol 30 min 80% ethanol 30 min 85% ethanol 30 min 95% ethanol 30 mi anhydrous ethanol 30 min
2	6	75% ethanol 30 min 75% ethanol 1.5h 80% ethanol 1h 85% ethanol 1h 95% ethanol 1h anhydrous ethanol 1h
3	12	75% ethanol 1h 75% ethanol 3h 80% ethanol 2h 85% ethanol 2h 95% ethanol 2h anhydrous ethanol 2h

strated that Bouin's solution is a better fixative for horse testicular tissue than 4% PFA.

### Dehydration and infiltration

The following groups of dehydration times were compared: 3h, 6h, and 12h (Table 1). The dehydrated testicles were then placed in a 50 mL centrifuge tube and infiltrated with xylene twice for 15 minutes each.

#### Waxing and embedding

After dehydration, equine testicular tissue was immersed in wax using three different procedures (Table 2). The sample was then placed in paraffin wax for 20 minutes in a 75°C oven. After 2 hours of solidification at room temperature (RT), the testicles were sliced.

#### Microtomy

The embedded tissue wax block was shaped into a cube and then sliced by a rotary microtome (Leica RM2245) with a thickness of 6  $\mu$ m. Floating the tissue section in a 42°C warm water bath will remove wrinkles and make placement on the glass slide easier.

### Drying and sealing

The following drying temperatures were compared for 15 minutes: room temperature, 55°C in a constant temperature oven, and 75°C in a constant temperature oven. The final slice was stored in a 4°C refrigerator.

### Hematoxylin and eosin (HE) staining

Paraffin wax must be removed before tissue staining; thus, deparaffinization was the first step in HE staining. The slides were immersed in xylene twice for 5 minutes each time. The samples were rehydrated by immersing them in ethanol at various concentrations. A 5-minute immersion in 100% ethanol was first performed, followed by 10-minute immersions in 95% ethanol, 85% ethanol, 75% ethanol, and distilled water to wash the xylene. The samples were then stained by

Table 2. Paraffin processing schedules for waxing.

Group	Total (min)	regent
1	30	30% paraffin wax+70% xylene 10 min 50% paraffin+50% xylene 10 min 70% paraffin wax+30% xylene 10 min
2	60	30% paraffin+70% xylene 20 min 50% paraffin+50% xylene 20 min 70% paraffin+30% xylene 20 min
3	120	30% paraffin+70% xylene 40 min 50% paraffin+50% xylene 40 min 70% paraffin+30% xylene 40 min

immersing them in a cuvette that contained hematoxylin for 6 minutes and then carefully washing them with tap water for 10 minutes to remove the excess hematoxylin. Then, the samples were soaked in a differentiating solvent for 1 minute before washing with distilled water for 15 minutes. To stain the remaining cellular structures, the slides were soaked in eosin for 3-5 minutes before being quickly washed for 5 minutes to prevent overstaining. Finally, the samples were dehydrated using different ethanol concentrations, 95% ethanol twice for 1 minute each and then 100% ethanol twice for 1 minute each, before being transferred to a cuvette containing xylene twice for 1 minute each time.

#### Microscope observation

Images were obtained using an inverted microscope (Zeiss Axio Observer D1) with a ZEO Pro software system.

### Results

# The 6h dehydration method is more effective than the 3h and 12h dehydration methods

Three groups of horse testis dehydration times were compared in parallel after the samples were adequately fixed in Bouin's solution at RT for 24h, 3h, 6h, and 12h. The dehydration time significantly affects the dehydration effect. The results showed that the testis tissue blocks embedded in paraffin could not be cut together with paraffin because they were not sufficiently dehydrated after 3 hours. The tissue was too soft and had many wrinkles due to the high residual moisture content (Fig. 1 A,D). After dehydration for 6h, the wax strips and tissue were intact without loss and wrinkles, being suitable for subsequent HE staining experiments (Fig. 1 B,E). After dehydration for 12h, the tissue could easily be minced during the sectioning process, so the sections



Fig. 1. The effect of different dehydration conditions on slicing. A. The waxed tissue was too soft and had many wrinkles on the surface after 3h of dehydration. B. The surface of the waxed tissue was smooth after 6h of dehydration. C. The tissue dries and cracks in the wax block after 12 hours of dehydration. D. The wax strip after dehydration for 3 hours. E. The wax strip after dehydration for 6 hours. F. The wax strip after dehydration for 12 hours.

and paraffin could not be completely cut together. At this point, excessive dehydration was observed (Fig. 1C,F). Therefore, this group of comparative experiments indicates that the optimal total dehydration time for paraffin sections of horse testis tissue is 6 hours.

# The 60 min waxing procedure is more effective than the 30 min and 120 min waxing procedures

The next step was to wax the successfully dehydrated tissue. After 30 min of waxing, the tissue was too soft and did not fit closely to the paraffin, so the tissue could not be sectioned together with the paraffin (Fig. 2A,D). After 60 min, the wax block and the tissue were closely attached, and no air bubbles were observed in the middle. The resulting wax strips and tissue were intact and free of loss and wrinkles (Fig. 2B,E). After 120 minutes, the tissue became hard and the color turned black, as shown in the fragmentation of the slices (Fig. 2C,F). Therefore, the optimal time for waxing the paraffin section of horse testis tissue is 60 min.

# Drying at 75°C for 15 min yields the best HE staining results

The temperature of the drying pronounced affects the postproduction of slices. However, the optimal drying temperature could not be determined from the appearance of slices and by observing the morphology of sliced tissue by HE staining. After the samples were dried at RT, the Leydig cells and vascular cells between the seminiferous tubules fell off, and the germ cells within the seminiferous tubules were present but incomplete (Fig. 3. A,D). After the samples were dried at 55°C, most of the Leydig cells and vascular cells were invisible, leaving only a small portion. The germ cells and Sertoli cells were also incomplete. The effect of overall morphological and HE staining was better than those of the sections dried at RT, however, the cellular structure of the horse testis was still unclear (Fig. 3.



Fig. 2. The effect of different waxing conditions on slicing. A. After 30 minutes of waxing, the block was soft and unevenly colored. B. After 60 minutes of waxing, the block was hard enough, and its color was bright and even. C. After 120 minutes of waxing, the tissue became stiff and turned black. D. After 30 minutes of waxing, testicular tissue could not be sliced into intact wax strips. E. After 60 minutes of waxing, intact wax strips could be obtained. F. After 120 minutes of waxing, testicular tissue could not be sliced into intact wax strips.

B,E). After the samples were dried at 75°C, complete tissue structure and no damage to any cells were observed, which is the best HE staining effect of the paraffin section of horse testis (Fig. 3C,F). Therefore, the optimum temperature for drying the paraffin sections of horse testis tissue is 75°C.

#### Discussion

At present, the conventional method for creating paraffin sections of testicular tissues of model animals such as mice and rats is fixation for 24-48h followed by dehydration in a graded series of ethanol (50, 70, 80, 90, 96, and 100%) for 1h each and incubation in liquid paraffin wax at 56-59°C for 24h (Xiao et al., 2017; Bilinska et al., 2018). Our preliminary study demonstrated that this procedure is not applicable in equine testes. Among the steps, dehydration, infiltration, and drying are crucial in forming paraffin sections of animal tissues (Ramos-Vara, 2017). Therefore, first, in our study, three different dehydration times (3h, 6h, and 12h) were compared in parallel on equine testis tissue after adequate fixation in Bouin's solution at RT for 24 hours. Our results showed that dehydration time plays an important role in paraffin sectioning. With the 3h dehydration strategy, the testis tissue block embedded in paraffin could not be sliced into flat and complete wax strips after they were waxed for 20 min at 75°C and embedded, mostly because the tissue was too soft and contained numerous wrinkles due to a high level of residual moisture (Fig. 1A,D). After 12 hours of dehydration, the tissue became brittle and cracked when sliced (Fig. 1C,F). High-quality wax strips without cracks and wrinkles were only obtained after the 6h dehydration strategy, and these samples were suitable for subsequent HE staining experiments (Fig. 1B,E).

Dehydration aims to replace the residual fixative and cellular water with organic solvents (Liu et al., 2016). As a commonly used dehydrating agent for animal tissue, ethanol can well mix with water at any proportion and simultaneously dehydrate and harden the tissue to facilitate the subsequent embedding (Feldman and Wolfe, 2014). However, because the ethanol solution can rapidly penetrate tissues and contract tissue, the dehydration procedure and time are essential for preparing paraffin section31. With insufficient dehydration times, dehydration is unsuccessful; however, excessive dehydration results in shrunken tissues that are hard and brittle (Qin et al., 2019). Through comparing the sectioning results, we confirmed



**Fig. 3.** HE staining effect obtained after different drying conditions. **A.** Cell morphology before drying at room temperature. **B.** Cell morphology before drying at 55°C. **C.** Cell morphology before drying at 75°C. **D.** HE staining of sections after drying at room temperature for 15 minutes showed that only cells in seminiferous tubules (black arrows) could be detected, and all Leydig cells between seminiferous tubules were lost. **E.** HE staining of sections after drying at room temperature for 15 minutes showed that cells in seminiferous tubules (black arrows) and a small number of Leydig cells (white arrows) can be observed. **F.** HE staining of sections after drying at 75°C for 15 minutes showed that the tissue structure was intact, and all cells in seminiferous tubules (black arrows) and Leydig cells (white arrows) were intact. Scale bar: 200 μm.

that our 6h dehydration strategy is a better dehydration option for horse testis paraffin sections compared with the 3 and 12h strategies.

The second step in the method is to wax tissue sections with paraffin to support the tissue for thin



sectioning. Thus, we also improved the waxing method. Through optimal waxing, tissue and paraffin and tissues can form a close dense entire consecutive wax tape with paraffin and tissues. Hence, exposure time to molten paraffin is vital to the section's quality. As shown in Fig. 2, with a 30-minute waxing procedure, we obtained a soft and uncompacted tissue block (Fig. 2A) due to insufficient waxing, and we could not obtain integrated and continuous wax strips (Fig. 2D); with a 120 min waxing procedure, the tissue became rigid and the color turned black, presumably due to a long time in the hightemperature oven (Fig. 2C), and wax strips were fragile during microtomy (Fig. 2F). It was only with the 60minute waxing procedure (Fig. 2B) that the tissue was thoroughly waxed and free of issues, such as air bubbles, wrinkles, and cracks, allowing it to be sliced into unbroken serial strips (Fig. 2E).

Third, we compared the following drying temperatures: RT, 55°C and 75°C. As shown in Fig. 3 A-C, the state of the tissue under the microscope differs with the three drying conditions. Further HE staining demonstrated that after drying at RT, nearly all Leydig cells and vascular cells within the seminiferous tubules and some germ cells had detached (Fig. 3D). The HE staining is weak after drying at 55°C, and Leydig and vascular cells in the seminiferous tubules, and some germ cells became loose, similar to the previous case (Fig. 3E). However, HE staining results with a clear contrast and structure was obtained only after drying at 75°C (Fig. 3F). The drying process after waxing and slicing enhances adhesion between the tissue and slide. Based on our results, we could observe and study testis morphology and cell development following our optimized drying conditions (Fig. 4A).

It has been confirmed that spermatogonial stem cells undergo a series of mitotic and meiotic divisions to form haploid round sperm cells and then transform into elongated sperm cells, known as spermatozoa (Chojnacka et al., 2016). Afterward, sperm are shed from the spermatogenic epithelium and released into the lumen of the spermatogenic tubules and then enter the epididymis, where they mature and acquire the ability to fertilize an egg (Mruk and Cheng, 2015). In the adult testis, Sertoli cells support germ cell development (Zhang, 2007) by secreting various proteins and other vital biomolecules (Jiang, 2014), such as insulin-like growth factor 1 (Dance et al., 2017), androgen binding protein (Qin, 2012), anti-Mueller hormone (Claes and Ball, 2016), and androgen receptor (Zhang et al., 2020; Yang et al., 2021). Sertoli cells are also involved in the formation of the blood-testis barrier, which protects spermatogenic cells from various harmful factors (Gerber et al., 2016). However, studies on horses are much less detailed than studies on other animal models. As intact structure and cell organization in the testis are important for normal male reproductive physiology, the optimized paraffin sectioning procedure (Fig. 4B) established here will benefit histological, reproductive, and clinical studies on horse testes.

Fig. 4. The obtained high-amplified HE staining result (A) and schematic diagram of the optimized paraffin sectioning procedure (B). Green arrowhead: spermatogonia, black arrowheads: primary spermatocytes, green arrow: secondary spermatocytes, blue arrow: spermatids, red arrow: sperm, red arrowhead: Sertoli cells, and black arrow: Leydig cells. Scale bar: 10 µm.

V 15min

Collection

HE staining

V

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Declaration of competing interest. The researcher claims no conflicts of interest.

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