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Evaluation of *CHD5*, *H3K9me3*, and *H4K12ac* in Human Testes with Spermatogenic Maturation Arrest: A Cross-Sectional Study

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Abstract

Background: Spermatogenic maturation arrest is thought to be caused by epigenetic defects, specifically in chromatin remodeling and histone modification. This study evaluated the status of chromatin remodeling chromodomain helicase DNA binding protein 5 (*CHD5*) and histone modifications histone 4 lys-12 acetylation (*H4K12ac*) and histone 3 lys-9 trimethylation (*H3K9me3*) in human testicular biopsies, based on maturation arrest type.

Materials and Methods: The cross-sectional study utilized 18 Bouin-fixed paraffin-embedded (BFPE) specimens prepared from residual tissue from routine laboratory tests of infertile patients. The expression of CHD5, H4K12ac, and H3K9me3 was examined through immunohistochemistry (IHC). The intensity was measured using ImageJ with IHC Profiler and StarDist plugins. Statistical analysis was performed using Python with Scipy.Stats module. The data were tested with Shapiro–Wilk for normality and Levene test for homogeneity. The differences in the intensity of spermatogenic cells were assessed using Kruskal-Wallis and Mann-Whitney tests. A difference was considered statistically significant if $P<0.05$.

Results: We found three types of maturation arrest, including Sertoli cell only (n=5), spermatocyte arrest (n=4), and spermatid arrest (n=9). *CHD5* was positive in spermatogonia and round spermatids but absent in spermatocytes. The mean grey value (MGV) of *CHD5* in spermatogonia was generally weak in spermatocyte arrest (157.4 ± 16.6) and spermatid arrest (155.3 ± 16.8), and there was no significant difference between them [$P=0.49$, 95% confidence interval (CI): (-4.3, 6), effect size (r): 0.02]. Although there was a significant difference in the expression of *H3K9me3* and *H4K12ac* ($P<0.001$), both histone modifications were found in all observed spermatogenic cells.

Conclusion: The expressions of *CHD5*, *H3K9me3*, and *H4K12ac* in different spermatogenic cell types produce similar results, indicating that they cannot be used as markers to determine the type of spermatogenic maturation arrest in humans. The significant finding in this research is the expression of *CHD5* in human spermatogonia cells, which requires further study for elaboration.

Keywords: Azoospermia, CHD5, H3K9me3, H4K12ac, Spermatogenic Arrest

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Introduction

Male infertility accounts for approximately 20% of infertility cases (1). Azoospermia is one of the causes of male infertility (2). This condition can be further categorized into obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). OA occurs when there is a blockage in the ejaculatory ducts, resulting in the absence of sperm in the ejaculate. On the other hand, NOA is characterized by disturbances in spermatogenesis (3). Spermatogenic maturation arrest refers to the cessation of spermatogenesis before the formation of spermatozoa without any damage to Sertoli cells and Leydig cells. It encompasses several types, such as spermatogonia maturation arrest, spermatocyte maturation arrest, and spermatid maturation arrest (4). In addition to maturation arrest, there was another condition known as Sertoli cell only, wherein no spermatogenic cells were present, only Sertoli cells.

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Various factors, including genetics and epigenetics, can cause infertility. In recent years, epigenetics has been extensively studied, including its relationship with spermatogenesis. A critical aspect of epigenetics studied is its role in regulating spermatogenesis through chromatin remodeling and histone modifications. *CHD5* is one of the important proteins involved in chromatin remodeling and is considered a master regulator. It belongs to the chromodomain helicase DNA-binding group, which consists of nine members and regulates multiple molecular processes, including hyperacetylation of histone 4, expression of histone variants, nucleosome eviction, and core protein expression. Studies conducted on mice with the deliberate removal of the *CHD5* gene demonstrated various molecular disruptions, such as a decrease in H4 acetylation and an abnormal increase in protamine 1 levels, ultimately leading to infertility (5). In humans, based on whole-exome sequencing, there is strong evidence suggesting that *CHD5* plays a crucial role in cases of NOA (6). Furthermore, due to its significant involvement in spermatogenesis, *CHD5* can be a valuable marker for NOA cases (7).

CHD5 is known to interact with *H3K9me3* and *H4K12ac*. Previous studies have reported an indirect correlation between *CHD5* and *H3K9me3*. For instance, Li et al. (5) identified concurrent colocalization of *CHD5* and *H3K9me3* during steps 7-8 of mouse spermiogenesis. Knockdown of the *SETDB1* gene, which affects *H3K9me3* levels, resulted in disturbances and meiotic arrest in mice before pachytene, leading to spermatocyte apoptosis (8). Similarly, the knockdown of the *Suv39h* gene responsible for methylating *H3K9me3* in mice caused spermatogenic failure due to the association of nonhomologous chromosomes (9). *CHD5* also plays a role in H4 acetylation, including *H4K12ac*, during the early stages of spermiogenesis (5). Knockout of the Sirtuin 1 (*SIRT1*) deacetylation enzyme, responsible for deacetylating *H4K12* in mice, resulted in impaired chromatin condensation and the compromised transition of histones to protamine (10).

Studies conducted on mice demonstrated spermatogenesis cessation due to knockout of the *CHD5* chromatin remodeler, as well as the writer and eraser genes responsible for *H3K9me3* and *H4K12ac*, as mentioned earlier (5, 8, 10). In humans, it is believed that impaired maturation arrest can also occur due to interference with *CHD5* or genes regulating *H3K9me3* and *H4K12ac*. Therefore, this study aimed to evaluate the status of chromatin remodeling protein *CHD5* and the histone modifications *H4K12ac* and *H3K9me3* in human testicular biopsies with different types of maturation arrest. To accomplish this evaluation, we employed the IHC profiler with the deep learning-based StarDist plugin for spermatogenic cell segmentation and intensity measurement of each cell type.

Materials and Methods

The cross-sectional study was conducted between August 2021 and November 2021. A total of 18 Bouin-fixed paraffin-embedded (BFPE) specimens were prepared from residual tissue from routine laboratory tests of infertile patients. The use of residual tissue has been approved by the Health Research Ethics Committee. The type of maturation arrest was determined in the Andrology Laboratory at the Department of Medical Biology, Faculty of Medicine, Universitas Indonesia (FMUI). The categories were established based on the types of spermatogenic cells that had ceased their development (4). Testes samples from three adult mice (*Mus musculus*) used as a positive internal control for the IHC experiment were obtained. The mice were 7-8 weeks old and weighed 20-25 g.

Histopathology staining

The type of maturation arrest was identified through a histopathology examination, wherein Hematoxylin Eosin (HE) staining was applied for the analysis. Initially, BFPE specimens were cut to a 5-6 µm thickness. The tissue was placed on a slide and stained with hematoxylin-eosin. It was immersed in 100, 95, 80, and 70% alcohol for about 3-5 minutes and then rinsed with distilled water for 1 minute. The tissue was then immersed in a solution of hematoxylin for 5 minutes, rinsed under running water, and placed in a solution of HCl. After that, the tissue was immersed in eosin Y solution, followed by immersion in 70, 95, and 100% alcohol for 1-2 minutes and in xylol solution for 5 minutes. The slides were examined under a microscope at 400x magnification. The results were grouped according to the type of maturation arrest, including spermatogonia maturation arrest, spermatocyte maturation arrest, spermatid maturation arrest, and Sertoli cell only.

Immunohistochemistry staining

We performed immunohistochemistry staining with anti-CHD5 (Cell Signaling, 44829S), anti-*H3K9me3* (Abcam, ab8898), and *H4K12ac* (Abcam, ab46983) antibodies on testicular biopsy from azoospermic patients. Immunohistochemistry was performed according to the Starr Trek Universal HRP Detection System Protocol (Biocare Medical, US). Sections as thick as 3 µm were deparaffinized in xylene, hydrated through a graded ethanol series, and blocked with Endogen Peroxide 3%. After that, sections were pretreated with Tris EDTA pH9 and incubated in the decloaking chamber at 96°C for about 20 minutes. Sections were incubated with blocking Background Sniper for about 30 minutes and then incubated with the primary antibody (1:1000). Slides were then incubated with Trekie Universal Link, TrekAvidin-HRP, and 3,3'-Diaminobenzidine (DAB), respectively. Finally, we stained slides with hematoxylin, set them in lithium carbonate, dehydrated them in a graded series of alcohol, cleared them in xylol and mounted them in deck glass. The slides were then examined under a microscope with 400x magnification.

Digitalization and image processing

Digitalization aimed to obtain digital files from microscopic images of the seminiferous tubules, while image processing performed the processing and interpretation of these digital

files. The steps involved taking pictures, conducting IHC DAB image deconvolution, segmenting and grouping each type of spermatogenic cell, and interpreting the results for each cell.

HE and IHC slides were digitized using an Olympus CX23 light microscope (Olympus, Japan) and an Indomicro digital camera equipped with IndomicroView 3.7 software (CV Indomicro, Indonesia). Images of the seminiferous tubules were taken at 400x magnification from 3 different fields of view. Image files with a resolution of 1920×1080 pixels were stored in tif format. HE images were used to determine the type of maturation arrest, while IHC images were further processed to measure the expression and location of *CHD5*, *H3K9me3*, and *H4K12ac*.

IHC image analysis was performed using ImageJ software version 1.53t. The IHC Profiler plugin was utilized for color deconvolution to obtain an image that would be used to measure the intensity of antigen expression. The resulting image consisted of Color 1, representing Hematoxylin (H Channel) staining, and Color 2, representing DAB (DAB Channel) staining. Color 3 was not required for analysis. The original IHC image was used to determine the segmentation position. Based on the segmentation results, Color 2 images were utilized to assess the expression levels of *CHD5*, *H3K9me3*, and *H4K12ac*.

Segmentation was done using the StarDist plugin (11). Before segmentation with StarDist, the original IHC image was converted to an 8-bit grayscale type, and then the image was inverted. Subsequently, segmentation was performed using StarDist. Once the region of interest from the segmentation results was obtained, the segmentation area was reduced by 3 pixels inward. Following that, visual labeling of spermatogenic cells was conducted. Spermatogonia cells were labeled with code 1, spermatocytes with code 2, round spermatids with code 3, elongating spermatids with code 4, and Sertoli cells with code 5.

The labeled region of interest, corresponding to the type of spermatogenic cell, was used to calculate the DAB color intensity. The parameter analyzed was MGV, representing the intensity of DAB staining. The gray value ranged from 0 to 255, where 0 represented the darkest color, and 255 represented the lightest color. The examination results were classified based on the classification provided by the IHC Profiler. MGV values were classified as strong if they fell between 0-60, moderate if they fell between 61-120, weak if they fell between 121-180, and negative if they exceeded 180 (12). For each sample, the analysis was conducted on three seminiferous tubules, and the results were subsequently combined. The classification outcomes for each type of spermatogenic cell were further analyzed.

Statistical analysis

All the statistical analyses were conducted using Python with Scipy. Stats module, version 1.11.1 (SciPy Project Team, US). Quantitative data were presented as the number of cells (mean intensity ± standard deviation). The data were assessed for normality using the Shapiro-Wilk and homogeneity using the Levene test. The differences between the intensities in Sertoli cells were examined with the Kruskal-Wallis. The effect size of the Kruskal-Wallis was computed using eta-squared, while the 95% confidence interval was determined utilizing the bootstrapping method. The differences in intensities between spermatocytes and spermatids were analyzed using the Mann-Whitney. The effect size in the Mann-Whitney was computed by dividing the Z value by the square root of the dataset's size. The 95% confidence interval was calculated based on the difference between the medians of the two groups. In all cases, $P \leq 0.05$ was considered statistically significant.

Ethical considerations

The study was approved by the FMUI-Cipto Mangunkusumo Hospital (RSCM) Health Research Ethics Committee (KET-610/UN2.F1/ETIK/ PPM.00.02/2021).

Results

In this study, we collected 18 BFPE specimens from the testes of patients diagnosed with infertility. By interpreting HE samples based on the type of maturation arrest, we discovered that 5 samples were of the Sertoli cell only type, 4 were of the spermatocyte maturation arrest type, and 9 were of the spermatid maturation arrest type. None of the samples were identified as spermatogonia maturation arrest.

CHD5 expression in various types of maturation arrest was observed and recorded in Table 1, while an example of histology can be seen in Figure 1. *CHD5* expression in the mice testes used as positive controls was observed in Supplementary Information (Fig.S1, See Supplementary Online Information at www.ijfs.ir). In the Sertoli cell only, no *CHD5* expression was found. In the case of spermatocyte maturation arrest, spermatogonia cells were identified as *CHD5* positive, but spermatocytes were negative. Furthermore, in spermatid arrest conditions, spermatogonia and round spermatids were found to be *CHD5* positive, while spermatocytes and elongating spermatids were negative.

The *CHD5* intensity in spermatogonia was positive but generally weak (157.4 ± 16.6 in spermatocyte arrest and 155.3 ± 16.8 in spermatid arrest). We found that the percentage of spermatogonia with negative *CHD5* in spermatocyte arrest was 46% (n=240), and in spermatid arrest, it was 48% (n=691). Additionally, the percentage of spermatids with negative *CHD5* in spermatid arrest was 51% (n=1086, Table 1). There was no significant difference between the intensity of the spermatogonia in the two maturation arrests [P=0.49, 95% confidence interval (CI): (-4.3, 6), effect size (r): 0.02].

H3K9me3 and *H4K12ac* were expressed in all spermatogenic cells in spermatocyte and spermatid maturation arrest. Similarly, these two modified histones were also found in Sertoli cells at different types of maturation arrest. The histological picture of *H3K9me3* and *H4K12ac* expression in the types of maturation arrest can be seen in Figure 2.

The intensity of *H3K9me3* and *H4K12ac* from spermatogonia cells to round spermatids was mainly in the moderate category (MGV 61-120). Only in elongating spermatids, the intensity of *H4K12ac* was most commonly found in the weak category (50% with MGV 146.9 ± 19.5). There were significant differences in the intensity of all spermatogenic cells in the three types of maturation arrest (P<0.001, Table 2).

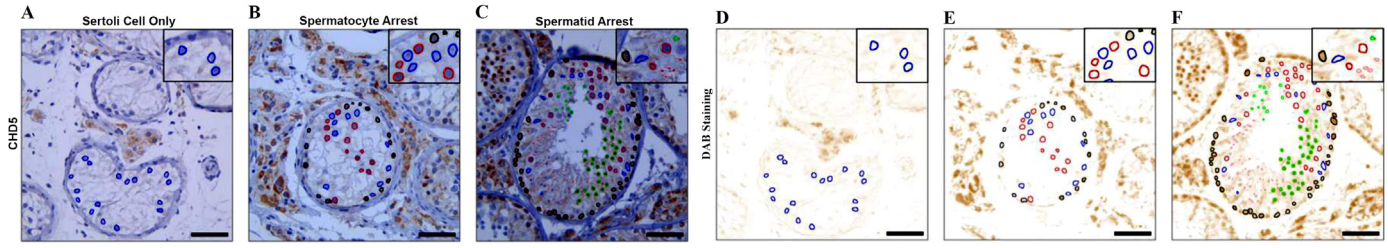

Fig.1: Immunohistochemistry of human testicular tissue stained with anti-CHD5. **A-C.** Original image and **D-F.** DAB channel image, in various types of spermatogenic maturation arrest, that is, **A, D.** Sertoli cell only, **B, E.** Spermatocyte maturation arrest, and **C, F.** Spermatid maturation arrest (scale bar 50 µm). Blue; Sertoli cell, Black; Spermatogonia, Red; Spermatocyte, Green; Round spermatid, and Pink; Elongating spermatid.

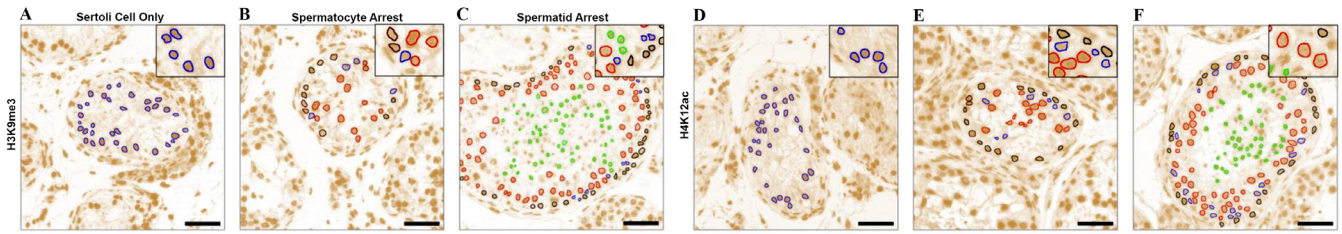

Fig.2: DAB channel image. Immunohistochemistry with **A-C.** Anti-*H3K9me3* and **D-F.** Anti-*H4K12ac*, in various types of spermatogenic maturation arrest, that is, **A, D.** Sertoli cell only, **B, E.** Spermatocyte maturation arrest, and **C, F.** Spermatid maturation arrest (scale bar 50 µm). Blue; Sertoli cell, Black; Spermatogonia, Red; Spermatocyte, Green; Round spermatid, and Pink; Elongating spermatid.

Table 1: IHC staining with anti-CHD5 on human testicular spermatogenic cells

Sertoli cell only (n=5)	Maturation arrest type		Statistical result
	Spermatocyte arrest (n=4)	Spermatid arrest(n=9)	
Sertoli - N:245 (241.2 ± 18.4) -W: 0 -M: 0 -S: 0	Sertoli - N: 145 (239 ± 17.3) - W: 0 - M: 0 - S: 0	Sertoli - N: 426 (244.4 ± 12.2) - W: 0 - M: 0 -S: 0	P=0.08[a] 95% CI: [0.05, 7.3] η^2: 0.004
	Spermatogonia - N: 111 (207.4 ± 19.8) - W: 120 (157.4 ± 16.6) - M: 9 (109.3 ± 8.5) - S: 0	Spermatogonia - N: 332 (210 ± 19.7) - W: 270 (155.3 ± 16.8) - M: 89 (104.9 ± 10.9) - S: 0	P=0.49[b] 95% CI: [-4.3, 6] r: 0.02
	Spermatocyte - N: 414 (238.4 ± 17.4) - W: 0 - M:0 - S:0	Spermatocyte - N: 1008 (247.2 ± 11.8) - W: 0 - M: 0 - S: 0	P<0.001[b] 95% CI: [5.9, 9.6] r: 0.28
		Round Spermatid - N: 551 (224.6 ± 25.9) - W: 447 (152.8 ± 16) - M: 88 (104.7 ± 10.6) - S: 0	
		Elongating Spermatid - N: 356 (243.9 ± 11.4) - W: 0 - M: 0 - S: 0	

Data are presented as a number of cells (mean of intensity ± SD). Assessed by [a]; Kruskal Wallis and [b]; Mann Whitney. N; Negative (>180), W; Weak (121-180), M; Moderate (61-120), S; Strong (0-60), n; Number of slides, CI; Confidence interval, η2; Eta squared, and r; Effect size.

Table 2: IHC staining with anti-*H3K9me3* and anti-*H4K12ac* on human testicular spermatogenic cells with various maturation arrest types

Maturation arrest type			Statistical result
Sertoli cell only (n=5)	**Spermatocyte arrest (n=4)**	**Spermatid arrest (n=9)**	
Sertoli	Sertoli	Sertoli	
H3K9me3 - W: 18 (131 ± 7.6)\ - M: 321 (86.8 ± 13.2)	*H3K9me3* - W: 24 (135.1 ± 13.5) - M: 88 (94.1 ± 15.9) - S: 3 (58.8 ± 0.8)	*H3K9me3* - W: 150 (136.1 ± 12.2) - M: 290 (94.7 ± 14.8)	P<0.001[a] 95% CI: [0.05, 6.6] η^2: 0.15
H4K12ac - W: 62 (143.6 ± 9.9) - M: 217 (85.9 ± 13.9) - S: 6 (59.1 ± 1)	*H4K12ac* - W: 27 (135.4 ± 10.1) - M: 82 (93.3 ± 16)	*H4K12ac* - W: 196 (142.3 ± 14.1) - M: 258 (98.6 ± 12.5)	P<0.001[a] 95% CI: [0.05, 7.3] η^2: 0.12
	Spermatogonia	Spermatogonia	
	H3K9me3 - W: 21 (131.4 ± 8.4) - M: 133 (91.8 ± 14) - S: 3 (57.6 ± 3.3)	*H3K9me3* - N: 3 (187.8 ± 3.9) - W: 242 (141.9 ± 15.2) - M: 317 (96.7 ± 13.8)	P<0.001[b] 95% CI: [16.9, 24.5] r: 0.3
	H4K12ac - M: 160 (83.1 ± 13) - S: 7 (58.4 ± 1.8)	*H4K12ac* - W: 86 (137.1 ± 13.2) - M: 404 (88.7 ± 13.8) - S: 13 (57.6 ± 2.9)	P<0.001[b] 95% CI: [7.7, 13.4] r: 0.27
	Spermatocyte	Spermatocyte	
	H3K9me3 - W: 36 (130 ± 7.6) - M: 404 (88.9 ± 13.7) - S: 14 (57.5 ± 2.8)	*H3K9me3* - N: 12 (193.1 ± 8.8) - W: 349 (141.6 ± 15.1) - M: 769 (94.6 ± 14.7) - S: 2 (58.7 ± 1.9)	P<0.001[b] 95% CI: [15.3, 19.9] r: 0.32
	H4K12ac - W: 5 (125.3 ± 3.9) - M: 214 (89.2 ± 14.2) - S: 6 (59.3 ± 0.7)	*H4K12ac* - W: 301 (139.5 ± 13.2) - M: 563 (92.7 ± 14.9) - S: 18 (56.8 ± 3)	P<0.001[b] 95% CI: [11.6, 17] r: 0.28
		Round Spermatid *H3K9me3* - N: 4 (190.9 ± 11.9) - W: 313 (142.7 ± 15.2) - M: 325 (99.6 ± 12.7) *H4K12ac* - W: 250 (137.2 ± 13.1) - M: 475 (95.6 ± 14.5) - S: 4 (54.6 ± 4.1)	
		Elongating Spermatid *H3K9me3* - N: 30 (200.7 ± 12.6) - W: 21 (151.8 ± 18.1) - M: 28 (104.5 ± 9.3) *H4K12ac* - N: 11 (199.4 ± 16.6) - W: 37 (146.9 ± 19.5) - M: 26 (99.7 ± 15.2)	

Data are presented as a number of cells (mean of intensity ± SD). Assessed by [a]; Kruskal Wallis and [b]; Mann Whitney. N; Negative (>180), W; Weak (121-180), M; Moderate (61-120), S; Strong (0-60), n; Number of slides, CI; Confidence interval, η2; Eta squared, and r; Effect size.

Discussion

The expression of the chromatin remodeling protein *CHD5* and the histone modifications *H4K12ac* and *H3K9me3* in human testicular biopsies showed similar results for each type of maturation arrest. We observed *CHD5* expression in spermatogonia in both spermatocyte and spermatid maturation arrest. Furthermore, *CHD5* expression was also detected in round spermatids from spermatid maturation arrest. As for *H3K9me3* and *H4K12ac*, these two histone modifications were present in all spermatogenic cells (spermatogonia, spermatocytes, round spermatids, and elongating spermatids) across all types of maturation arrest.

We found no significant difference in *CHD5* expression in spermatogonia between spermatocyte and spermatid arrest. Meanwhile, spermatocyte and elongating spermatid cells did not express *CHD5*. Therefore, it was possible that the type of maturation arrest was not affected by the role of *CHD5* in spermatogonia cells. *CHD5* expression in round spermatids between different types of maturation arrest could not be compared because these cells were only found in spermatid maturation arrest. Another finding was that not all spermatogonia and round spermatids expressed *CHD5*. The percentage of spermatogonia with negative *CHD5* in spermatocyte and spermatid maturation arrest was 46 and 48%, respectively. Additionally, the percentage of round spermatids with negative *CHD5* in

spermatid arrest was 51%. This suggested that *CHD5* was only involved in a few steps of all stages of spermatogonia and round spermatid cell development.

Previous reports stated that *CHD5* in the mice testes was only found in round spermatids and not in other spermatogenic cells such as spermatogonia, spermatocytes, and elongating spermatids (5, 13). *CHD5* expression in these reports was the same as that found in the testes of mice, which were used as a positive control. *CHD5* was positive in the heterochromatic chromocenter area at step 4-10 mice round spermatids and was thought to play an important role in the histone-to-protamine transition process (5). The role of *CHD5* in human round spermatids was considered to be similar to that in mice, including helping chromatin condensation when histone-to-protamine transitions occurred.

There were several reasons why *CHD5* in humans was positive for two types of spermatogenic cells (round spermatids and spermatogonia), while mice were only positive for one type of spermatogenic cells (round spermatids) (5). Firstly, spermatogonia differentiation in mouse testes slightly differed from differentiation in human testes. In mice, undifferentiated spermatogonia (A single, A paired, A aligned) underwent six amplifying divisions (A1-A4, intermediate, B) before turning into spermatocytes. In humans, undifferentiated spermatogonia (A pale and A dark) underwent only one amplifying division (B) before becoming spermatocytes. Secondly, the genes involved in the differentiation process differed between the two species. The role of *CHD5* in human testicular spermatogonia was unknown. Spermatogonia cells, which were progenitor cells, underwent two different processes, including proliferation or self-renewal to maintain stem cell supplies and differentiation into spermatozoa (14). It was assumed that *CHD5* was expressed due to spermatogonia proliferation and differentiation. This assumption was based on the similarity of the process of spermatogenesis in the testes with neurogenesis in the adult brain. These two organs had many similarities; they both had the highest *CHD5* expression, underwent stem cell proliferation and differentiation processes, and had many protein expression similarities in the proteomic analysis (15). The findings of *CHD5* expression in human spermatogonia impacted our understanding of the process of spermatogenesis, where this protein was involved in the mitosis of primordial germ cells. Previously, it had been that *CHD5* was only involved in spermiogenesis, the final stage of spermatogenesis (16). In addition, these findings also explained why the expression of *CHD5* in the testes and brain was higher compared to other organs (5, 17).

In the present study, we found that *H3K9me3* and *H4K12ac* were expressed in all spermatogenic cells, but the intensity of these histone modifications differed significantly among cell types for different arrests. To determine whether these differences correlated with the type of maturation arrest, it would be necessary to conduct further studies using more advanced detection methods, such as immunofluorescence. Previous studies have reported that *H3K9me3* in normal human testes was detected in spermatogonia, spermatocytes, and spermatids. *H3K9me3* was primarily concentrated in the nucleus, particularly in spermatocytes and spermatids (18). Previous research also found that *H4K12ac* was detected in human spermatogonia, spermatocytes, round spermatids, and elongated spermatids (19). In Sertoli cells, we have found that in Sertoli cell-only cases and other types of maturation arrest, *CHD5* expression was negative, while *H3K9me3* and *H4K12ac* were positive. It has previously been reported that *H4K12ac* was highly expressed in the nucleus of Sertoli cells. Meanwhile, *CHD5* was negative in Sertoli cells (13, 19).

In experimental animals such as mice, manipulation of *CHD5*, *H3K9me3*, and *H4K12ac* was shown to induce infertility (5, 8, 10). However, in azoospermic patients where infertility occurred naturally, it was believed that disruptions in spermatogenesis were not caused by impaired expression of *CHD5*, *H3K9me3*, or *H4K12ac*. This notion was supported by the findings of this study, where the expression patterns of *CHD5*, *H3K9me3*, and *H4K12ac* were similar across different types of maturation arrest. It seemed that other factors were at play in the infertility of azoospermic patients.

This study had some limitations. First, the study sample size was only 18 BFPE specimens. A larger sample size was expected to increase the statistical power and the generalization of the findings. Second, we only examined spermatogenesis cells from spermatogonia to elongating spermatids, specifically focusing on samples with spermatid maturation arrest. We did not analyze normal testicular samples encompassing the complete range of spermatogenic cells, from spermatogonia to spermatozoa. To our knowledge, this study represents the first investigation of *CHD5* expression in human spermatogenic maturation arrest. Furthermore, additional research is necessary to investigate the role of *CHD5* in the proliferation and differentiation of human spermatogonia and uncover the underlying molecular processes involved.

Conclusion

The expressions of *CHD5*, *H3K9me3*, and *H4K12ac* at various levels of maturation arrest, when examined in terms of the types of spermatogenic cells involved, produce similar results. Therefore, all three cannot be used as markers to determine the type of spermatogenic maturation arrest in humans. The expression of *CHD5* in human spermatogonia cells becomes an important finding in this research, and further studies are needed to elaborate on it.

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Authors' Contributions

P.P.; Conducted examinations, Ensured accurate data collection, Analyzed, Presented the results, and Drafted manuscript. D.A.P.; Supervised the histopathological examination, Assisted in interpreting, and Presenting data. K.K.; Supervised the immunohistochemistry examination and Participated in statistical analysis. P.B.; Involved in research design, Sample collection processes, and Provided clinical advice. A.; Contributed to conception, Design, Administrative support, and Manuscript revision. All authors read and approved the final version of the manuscript.

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