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RESEARCH ARTICLE

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Persistence of undifferentiated spermatogonia in aged Japanese Black cattle

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Abstract

Aging is a major risk factor for spermatogenesis deterioration. However, the influence of age on spermatogenic stem cells and their progenitors in bulls is largely unknown. Here, we report age-related changes in undifferentiated and differentiating spermatogonia in Japanese Black cattle with nearly constant sperm output, by using spermatogonial markers. The numbers of differentiating spermatogonia and more differentiated spermatogenic cells were significantly decreased in aged bovine testes compared with those in young testes. In contrast, the number of undifferentiated spermatogonia was maintained, and their proliferative activity did not differ significantly between young and aged bovine testes. Although severe calcification was only observed to a small extent in aged testes, fewer Sertoli cells and interstitial fibrosis were observed in noncalcified testicular regions. These results suggest that, even in old bulls with nearly constant sperm output, testicular spermatogenic activity declined whereas undifferentiated spermatogonia numbers were maintained. Thus, we propose that undifferentiated spermatogonia may be resistant to age-related changes in bovine testes. Because undifferentiated spermatogonia may contain stem cell activity, our findings highlight the potential utility of undifferentiated spermatogonia as an agricultural resource to produce spermatozoa beyond the natural bovine lifetime through transplantation and in vitro spermatogenesis in future animal production.

KEYWORDS
aging, bull, PLZF, testis, undifferentiated spermatogonia

1 | INTRODUCTION

In the production of beef cattle, semen ejaculated from a small number of elite bulls is used for artificial insemination. Aging itself is one of the main risk factors for reduced testicular function, and decreased testicular daily sperm production in aged bulls can lead to instability in the beef production chain. The lifespan of a bull (approximately 20 years) is a critical limiting factor for the continuity of spermatogenesis. Some bulls show little to no reduction in seminal sperm output over their lifetime, thus maintaining fertility, whereas others exhibit severe reduction leading to infertility (Bishop, 1970). Although the testes of infertile bulls have been pathologically

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analyzed, changes in the spermatogenesis of aging bulls with nearly constant sperm output have not been elucidated (Bishop, 1970).

Achieving stable and long-term spermatogenesis over the bull's lifespan would be a breakthrough in animal production. In mice, testicular cell transplantation has been suggested to enable recovery from hypospermatogenesis caused by the effects of the surrounding environment (Kanatsu-Shinohara et al., 2020; Ogawa et al., 2000) and to extend the duration of spermatogenesis over the animal's lifespan (Ryu et al., 2006). Thus, the transplantation of spermatogenic stem cells (also called spermatogonial stem cells [SSCs]) in livestock is expected to accelerate the production of genetically modified animals and propagation of eminent genes (Ciccarelli et al., 2020; Savvulidi et al., 2019). Importantly, the transplantation of SSCs has been successfully achieved in bulls, and spermatozoa presumably derived from donor cells were observed in recipient testes (Izadyar et al., 2003; Sharma et al., 2020). If SSCs survive in the testes of aged bulls, consistent production of spermatozoa can be achieved by transplanting SSCs into young bovine testis to ectopically regenerate spermatogenesis. However, little is currently known regarding age-related changes in SSCs in bovine testes.

In mammals, spermatogenesis takes place from the basement membrane to the lumen in convoluted seminiferous tubules in the testis (Russell et al., 1990). Spermatogenic cell number is primarily determined by the population of spermatogonia, which are germ cells in the mitotic stage that can be classified as undifferentiated and differentiating spermatogonia. Importantly, mouse SSCs, which support continuous sperm production by balanced self-renewal and differentiation, have been shown to be present within the population of undifferentiated spermatogonia (Shinohara et al., 1999; Yoshida, 2018).

In bulls, the most primitive type of spermatogonia is morphologically distinguished as type A spermatogonia, while the range of SSCs within this population is unclear. In addition to morphological evaluation, it has been proposed that marker genes may be used to distinguish subpopulations of spermatogonia (Cai et al., 2016). Genetic evidence suggests that promyelocytic leukemia zinc finger (PLZF) is an essential transcriptional factor for SSC maintenance in mice, and its expression is conserved among a variety of mammalian species, including cows (Costoya et al., 2004; Lovelace et al., 2016; Zheng et al., 2014). In bulls, PLZF is reportedly expressed in the type A spermatogonia subpopulation and can be used as an undifferentiated spermatogonial marker (Reding et al., 2010). On the other hand, ubiquitin carboxy-terminal hydrolase L1 (UCHL1) was reportedly expressed in pan-spermatogonia (type A, In, and B spermatogonia) and in the early stages of spermatocytes in bulls (Wrobel et al., 1995). Therefore, PLZF and UCHL1 can be used to distinguish undifferentiated and differentiating spermatogonia; that is, $PLZF^{+}$ spermatogonia are identified as undifferentiated, whereas $PLZF^{-}/UCHL1^{+}$ spermatogonia are identified as differentiating.

In this study, we attempted to clarify age-related changes in the abundance of undifferentiated and differentiating spermatogonia in Japanese Black cattle using spermatogonial markers.

2 | MATERIAL AND METHOD

2.1 | Tissue collection, fixation, and embedding

All experimental procedures conformed to Regulations for Animal Experiments and Related Activities at Tohoku University. The testes and epididymides of healthy and disused Japanese Black cattle were obtained as industrial byproducts from the Livestock Improvement Association of Japan, Inc. (Morioka AI Center) and Miyagi Agricultural Development Corporation, including three aged bulls (10, 17, and 19 years old; $n = 1$, 1, and 1, respectively) and three young bulls (2 years old; $n = 3$), respectively. Young bulls were disused because they were left out of the progeny test, whereas all aged bulls were disused due to drop in their industrial demand. Past records of semen collection frequency, sperm concentration, and semen volume in the aged bulls were used to calculate age-related changes in total sperm output. After orchiectomy under local anesthesia, the testes and epididymides were separated using a scalpel. Then testis size and weight were measured. The testes and epididymides were cut into 1 cm^3, washed in phosphate-buffered saline (PBS), and fixed overnight in 4% (w/v) paraformaldehyde in PBS (Nacalai Tesque, Kyoto, Japan). Tissue samples were dehydrated using a graded ethanol series and permeabilized three times in xylene (Nacalai Tesque) for 2 h each at 4°C. Tissues were preserved in soft paraffin (= 1:1) at 60°C with three paraffin changes for 1.5 h each and finally embedded in paraffin. The paraffin-embedded samples were sliced with a microtome (TU-213; Yamato Kohki, Saitama, Japan) into 4-μm-thick sections and bonded to Matsunami adhesive silane (MAS)-coated glass slides (Matsunami, Osaka, Japan).

2.2 | Periodic acid-Schiff-hematoxylin staining

After deparaffinization, slides were soaked in 0.5% periodic acid aqueous solution for 10 min, washed in tap and distilled water, and soaked in Schiff's reagent (Fujifilm WAKO, Osaka, Japan). They were subsequently soaked three times with sulfurous acid for 3 min each and washed in tap water. The slides were then soaked in Mayer's hematoxylin stain solution (Merck, Darmstadt, Germany) to stain the nuclei. After being washed in tap water for 15 min, the slides were dehydrated, mounted in Entellan New mounting medium (Merck), and covered with glass cover slips (Matsunami). Images of the stained cells were captured using a BX50 light microscope equipped with a DP71 CCD camera (Olympus, Tokyo, Japan).

2.3 | Immunofluorescence staining

After deparaffinization, slides were soaked in the antigen retrieval reagent Hist VTone (Nacalai Tesque) for 30 min at 90°C. Subsequently, they were washed in tap water for 20 min, blocked with blocking buffer (Nacalai Tesque) supplemented with 4% donkey serum for 1 h at room temperature (RT), washed three times in PBS with

0.1% (v/v) Tween20 (PBST) for 10 min each, and incubated with primary antibody in blocking buffer overnight at 4°C. Subsequently, the slides were washed three times in PBST for 10 min each and reacted with the secondary antibody in blocking buffer for 1 h at RT. The slides were then washed three times in PBST for 10 min each, mounted with Slow Fade Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA), and covered with a glass cover slip. Images of the stained cells were captured using a BX63 fluorescent microscope equipped with a DP73 CCD camera (Olympus). The primary antibodies used were anti-VASA/DDX4 (1:400; Abcam, Cambridge, UK), anti-PLZF (1:50; Santa Cruz Biotechnology, Dallas, TX, USA), anti-UCHL1 (1:100; Cell Signaling Technology, Danvers, MA, USA), anti-UCHL1 (1:100; Abcam), anti-Ki67 (1:500; Abcam), and anti-SOX9 (1:500; Merck). For these antibodies, the similarities of the amino acid sequences between antigens for making antibodies and corresponding proteins of cattle were examined using NCBI's blast. All antigens showed more than 80% similarity, predicting the putative cross-reaction of these antibodies for bovine antigens. The secondary antibodies were Alexa-488 conjugated anti-rabbit IgG (1:400; Thermo Fisher Scientific) and Alexa-594 conjugated anti-mouse IgG (1:400; Thermo Fisher Scientific). Hoechst 33342 solution (1:5000; Nacalai Tesque) was used for nuclear staining.

2.4 | Evaluation of sperm density in cauda epididymis

Sperm density evaluation was performed on samples from the young ($n = 3$ epididymis) and aged ($n = 3$ epididymis) bull groups. The number of spermatozoa in the lumen of the cauda epididymis was counted manually from the hematoxylin images of the cauda epididymides. Luminal densities were calculated from the number of spermatozoa/the epididymal luminal area.

2.5 | Evaluation of spermatogenesis progression

Evaluation of spermatogenesis progression was performed on duplicate samples from the young ($n = 3$ testes) and aged ($n = 3$ testes) bull groups. Fifty cross-sections of the seminiferous tubules were randomly selected from the periodic acid-Schiff-hematoxylin (PAS-H) stained images. The integrity of spermatogenesis was evaluated based on the nuclear morphology (Berndston & Desjardins, 1974) using the following grading system: Grade 0, tubule with no germ cells; Grade 1, tubule with single spermatogenic cell layer (only type A spermatogonia); Grade 2, tubule with two spermatogenic cell layers from type A spermatogonia to zygotene spermatocytes; Grade 3, tubule with three layers from type A spermatogonia to pachytene spermatocytes; and Grade 4, tubule with four or five spermatogenic cell layers from type A spermatogonia to elongated spermatids. The stage of the seminiferous epithelium cycle was evaluated by the images of PAS-H staining as described previously (Berndston & Desjardins, 1974).

2.6 | Quantification of undifferentiated/differentiating spermatogonia and Sertoli cells

Cell density analysis was performed on duplicate samples from the young ($n = 3$ testes) and aged ($n = 3$ testes) bull groups. The numbers of undifferentiated spermatogonia ($PLZF^+$ cells on the basement membrane), differentiating spermatogonia ($PLZF^-/UCHL1^+$ cells on the basement membrane), and Sertoli cells were counted in 50 cross-sections of the seminiferous tubules from the immunofluorescence-stained images. $PLZF^+/UCHL1^-$ spermatogonia were not observed (data not shown). To evaluate proliferative activity, the numbers of $Ki67^+$ cells in undifferentiated and differentiating spermatogonia were counted in 25 cross-sections of the seminiferous tubules from the immunofluorescence-stained images. Then the density was calculated from the number of cells for each objective in the seminiferous tubule/the basement membrane length (cell number/μm). For PLZF staining for aged testis samples, small and intense signals were frequently observed near the basement membrane in the stained images of aged bulls. Because these signals could also be observed in section stained only with secondary antibody (anti-mouse IgG antibody) and did not overlap with Hoechst signals, these signals were judged as nonspecific signals.

2.7 | Evaluation of calcified area and interstitial area

Evaluation of the calcified (and severely degenerated) area was determined using ultrasound images of the testes obtained outside the scrotum for the 17- and 19-year-old bulls using an ultrasound system (Xario 200V; Canon, Ohtawara, Japan). The calcified and whole testes areas were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the ratio of the calcified area/whole testes area was calculated. Evaluation of the interstitial area was performed using the PAS-H stained images from duplicate samples of three testes from each of the young and aged bull groups. The seminiferous tubule and interstitial areas in the images were segmented manually and measured using ImageJ software. The ratio of the interstitial area/(interstitial area + seminiferous tubule area) was calculated.

2.8 | Statistical analysis

Statistical analyses of sperm density in the epididymis, testis length (minor axis), the number of differentiating spermatogonia, $Ki67^+$ rate of undifferentiated and differentiating spermatogonia, Sertoli cell density, and ratio of interstitial area were performed using Student's *t*-test with $p < .05$ considered statistically significant. Statistical analyses of the number of undifferentiated spermatogonia, testes length (major axis), and weight were performed using Welch's *t*-test with $p < .05$ considered statistically significant. Data are presented as the mean ± standard error (SEM). Statistical analysis of spermatogenesis progression was performed using the χ^2 test with $p < .05$ considered statistically significant.

3 | RESULTS

3.1 | Sperm productivity in aged bulls

To understand the sperm productivity of aged bulls, we first analyzed past sperm count records in the ejaculated semen of aged bulls (10, 17, and 19 years old) without obvious disease. Although a simple comparison might be difficult because of fluctuations in the frequency of daily and yearly semen collection largely due to industrial demand, which should affect sperm output, the estimated total sperm count per ejaculation indicated that spermatozoa were not depleted in the aged bulls (Figure 1a). This was also supported by the presence of a certain number of spermatozoa distributed in the lumen of the cauda epididymal tube in aged bull testes, although spermatozoa density was decreased from that in young bull testes ($p = .06$) (Figure 1b,c). These data suggested that sperm production persisted in aged bulls.

3.2 | Testis size and ultrasonographic evaluation in aged bulls

We then focused on testis size, which may roughly represent spermatogenic activity. The major and minor lengths and weights of testes did not differ significantly between young and aged bulls (Figure 2a,b). It has been reported that ultrasonography can identify areas of severe testicular lesions including calcification and severe fibrosis (Kastelic & Brito, 2012; Lim et al., 2015). Ultrasonography revealed small patches of intensely white regions in the testicular parenchyma near the rete testes of the aged bulls (Figure 2c), most of which were nearly round in shape with varying diameters (approximately 2.5 mm on average) (Figure 2c). Nevertheless, 91% of the testis parenchyma was not clearly evident on ultrasound even in the testes of the 17- and 19-year-old bulls, suggesting that most parenchymal regions in their testes were not calcified (Figure 2d).

3.3 | Age-related changes in spermatogenic cell layers in aged testes

We then examined whether spermatogenesis occurred properly in the seminiferous tubules in noncalcified regions of aged bull testes. The results of PAS-H staining revealed that the majority of the seminiferous tubules in aged bull testes had four or five layers of spermatogenic cells, as observed in young testes (Figure 3a,b). However, the densities of spermatocytes and spermatids, visualized as strongly VASA-positive stained cells, appeared to decrease in the seminiferous

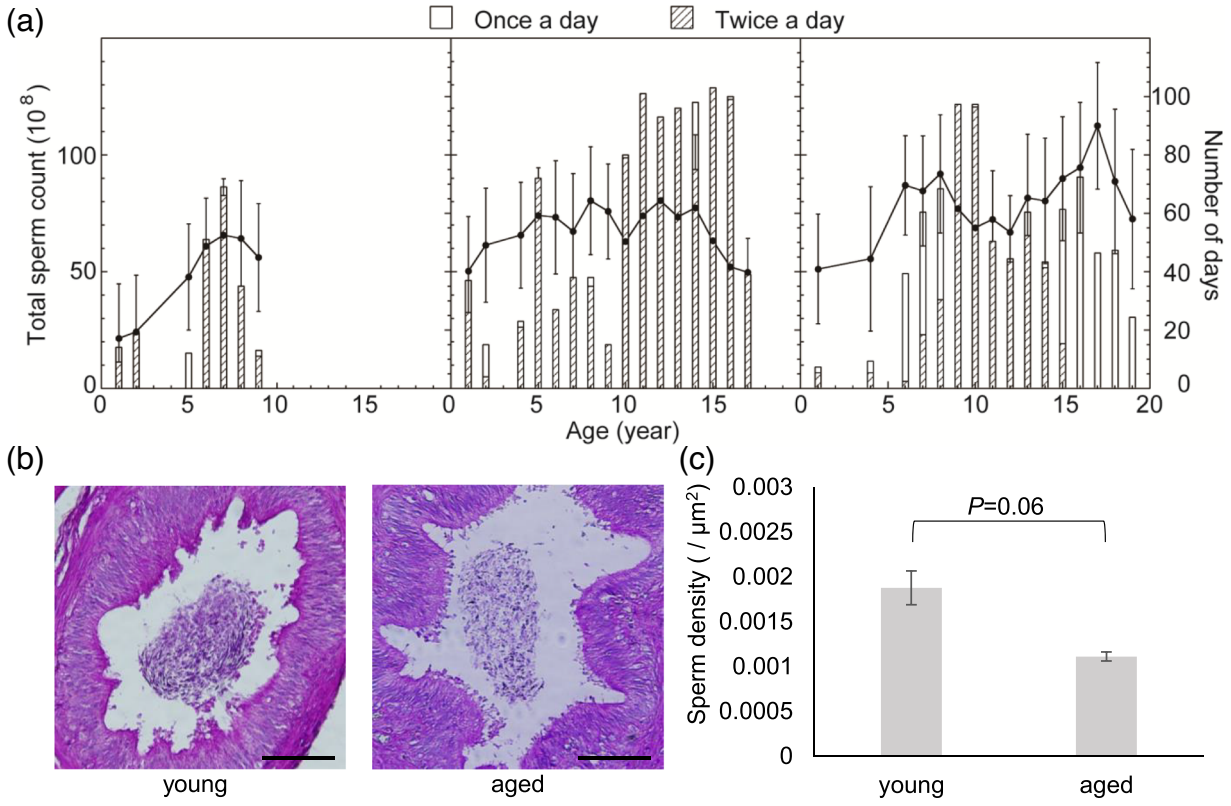

FIGURE 1 Sperm output of aged bulls. (a) Past records of age-related changes of estimated total amount of sperm in ejaculated semen of 10- (left), 17- (middle), and 19-year-old bulls (right). Lines indicate the average total sperm output. White bars indicate the total days of daily semen collection, whereas striped bars indicate total days of twice daily semen collection. (b) PAS-H stained images of cauda epididymis of young (left) and aged (17 years old, right) bulls. Representative images of sperm volume in the caudal parts of the epididymides are shown for young and aged bulls. Scale bars indicate 100 μm. (c) Intraluminal sperm densities in cauda epididymides of young and aged bull epididymides (young, n = 3; aged, n = 3). Data are expressed as mean ± SEM. p-value is the result of Student's t-test between young and aged adult groups

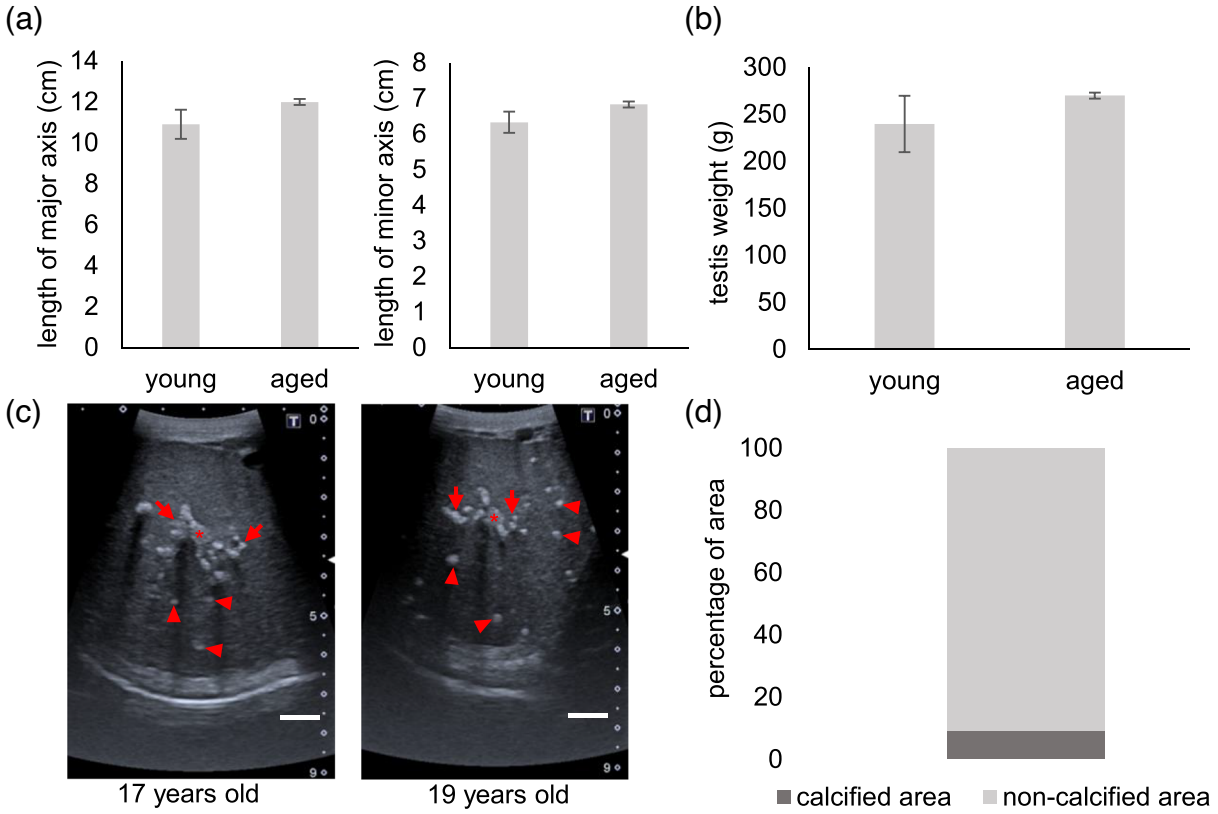

FIGURE 2 Testis size and ultrasonographic analysis for aged bovine testes. (a) Comparison of testis length (major axis, left; minor axis, right) between young and aged bulls. Data for each bull are average value of left and right testes and expressed as mean ± SEM (young, n = 3; aged, n = 3). (b) Comparison of testis weight between young and aged bulls. Data for each bull are average value of left and right testes and expressed as mean ± SEM (young, n = 3; aged, n = 3). Data are expressed as mean ± SEM. (c) Representative transversal ultrasonographic images of testes from 17- and 19-year-old bulls. Asterisks represent the rete testis, arrows represent the cluster of calcification area near the rete testis, and arrow heads represent the calcification areas with various sizes. Scale bars indicate 10 mm. (d) Average ratio of calcified (light gray) and noncalcified area (dark gray) in total testicular parenchymal area from 17- and 19-year-old bulls

epithelia of aged bull testes compared with that in young bull testes (Figure 3c). Moreover, the ratio of seminiferous tubules with a decreased number of spermatogenic cell layers to the total number of seminiferous tubules was significantly increased in the testes of aged bulls compared with that in young bulls ($p < .001$) (Figure 3a,d). Taken together, these results suggested that the abundance of spermatogenic cells was decreased in the seminiferous tubules of aged bull testes.

3.4 | Age-related changes in spermatogonia in aged testes

Because spermatogenic cell number is primarily determined by spermatogonial behavior, we analyzed spermatogonial density and proliferative activity in the testes of aged bulls. Undifferentiated spermatogonia can be identified by using PLZF as a marker. The density of $PLZF^+$ undifferentiated spermatogonia did not differ between young and aged bull testes (Figure 4a,b). Nuclear localizations of PLZF in spermatogonia were observed in both young and aged testes, suggesting that roles of PLZF as nuclear transcriptional factor in undifferentiated spermatogonia were persisted from young to aged bulls (Figure 4a). In addition, the fraction of $Ki67^+$ cells out of the total $PLZF^+$ spermatogonia did not differ significantly between young and aged bull testes (Figure 4c). In contrast, the density of differentiating spermatogonia ($PLZF^-/UCHL1^+$ cells attached to the basement membrane) in the testes of aged bulls was significantly decreased compared with that in young bulls ($p = .022$) (Figure 4d,e). The fraction of $Ki67^+$ cells out of the total differentiating spermatogonia did not differ significantly between young and aged bull testes, indicating that the decline in differentiating spermatogonia was not caused by their lowered proliferative activity (Figure 4f). These results suggested that whereas the number of undifferentiated spermatogonia was maintained, differentiation of spermatogonia was decreased, which may have contributed to the decline in germ cell number in subsequent stages of spermatogenesis in the testes of aged bulls.

3.5 | Age-related changes in Sertoli cells and interstitium in aged testes

Finally, to understand the influence of age on the surrounding environment for spermatogenesis, we analyzed age-related changes in Sertoli

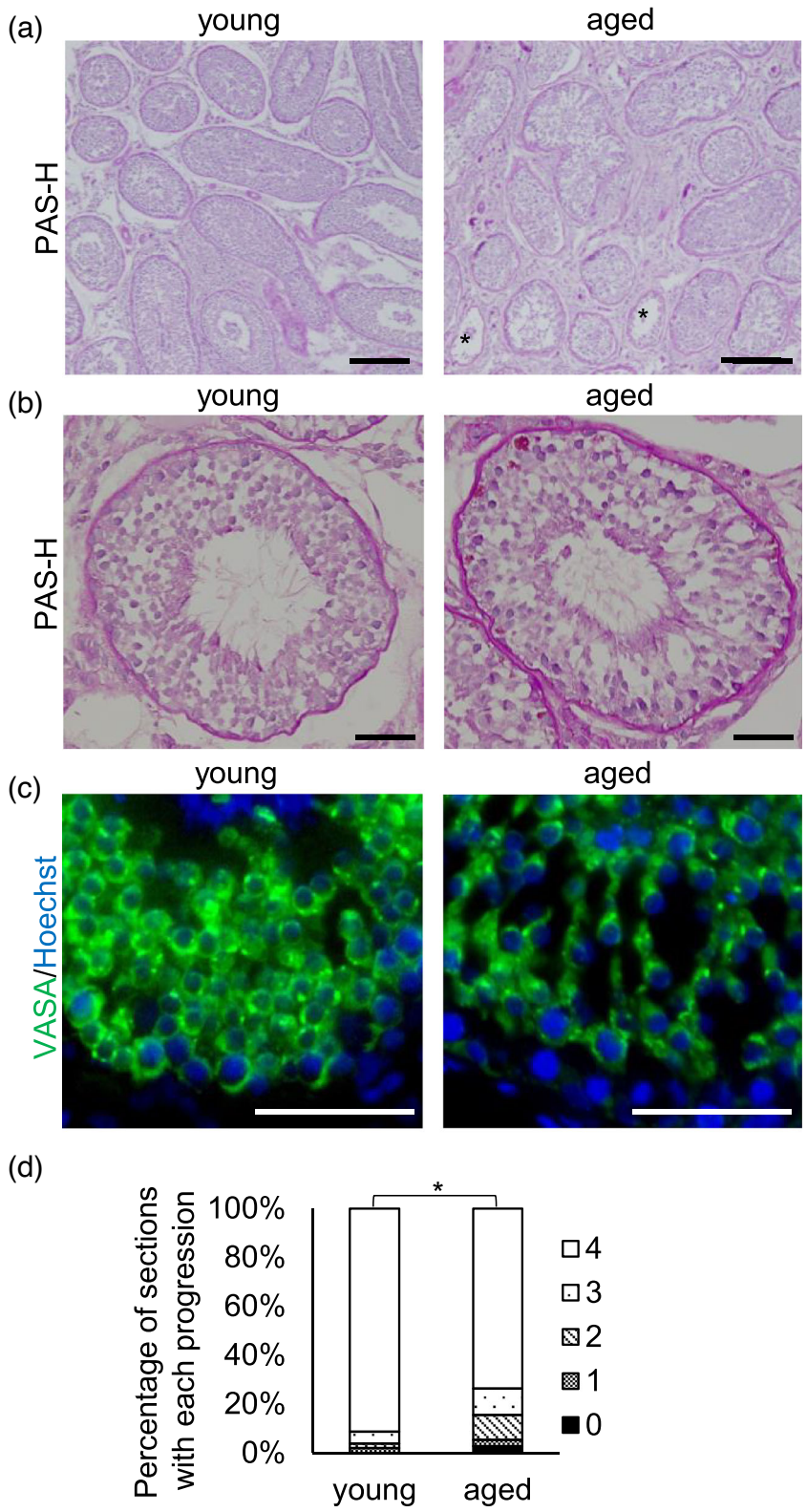

FIGURE 3 Age-related changes in spermatogenesis in bovine testes. (a) PAS-H images of testes in young (2 years old, left) and aged (19 years old, right) testes. Asterisks represent seminiferous tubules classified as Grade 0 or 1 (see Section 2 and (d)). (b) Higher magnification of PAS-H images of normal seminiferous tubules in young (left) and aged (17 years old, right) testes. (c) Seminiferous epithelia in young (left) and aged (10 years old, right) testes. The upper images are representative of PAS-H staining, and the lower images are representative of immunofluorescence staining for VASA (green) with Hoechst 33342 (blue). The stages of seminiferous epithelium cycle in each image are stageIV (upper left), stageV (upper right), stageIV (lower left), and stageVII-VII (lower right). (d) Degree of spermatogenesis in each seminiferous tubule evaluated by spermatogenesis progression (see Section 2). The percentages of seminiferous tubules with each grade are shown (young, n = 3; aged, n = 3). Scale bars indicate 200 μm (a) and 50 μm (b and c). Asterisks represent significant difference ($p < .05$) between young and aged groups (χ^2 test)

cells and the interstitium. Within the seminiferous tubules, $SOX9^+$ Sertoli cell density was decreased in the testes of aged bulls ($p = .019$) (Figure 5a). Further, in noncalcified parenchyma, the ratio of the interstitial region to the total parenchyma increased significantly in the testes of aged bulls compared with that in young bulls ($p = .045$) (Figure 5b,c). In the interstitial region, fibrotic regions were more apparent in aged bull testes and even more pronounced in the testes of the 19-year-old bull (Figure 5b). These results suggested manifestations of age-related changes in the somatic environment, including fewer Sertoli cells and fibrosis-associated expansion in the interstitia.

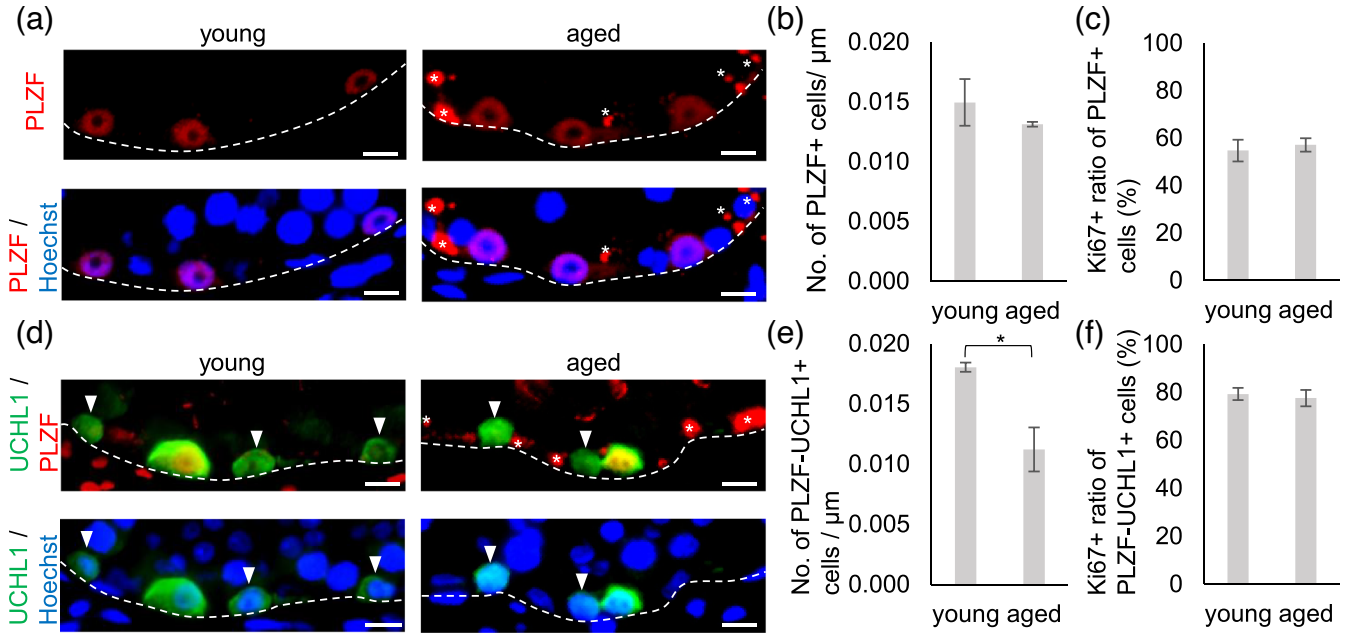

FIGURE 4 Density and proliferative activity of undifferentiated spermatogonia in aged bovine testes. (a) Representative images of basal compartment of seminiferous epithelia to show undifferentiated spermatogonia visualized by immunohistochemical staining for PLZF (upper) and Hoechst 33342 (lower) in young (left) and aged (19 years old, right) bovine testes. (b) Densities of $PLZF^+$ spermatogonia in young and aged testes. (c) Ratio of $Ki67^+$ cells out of total $PLZF^+$ cells for young and aged testes. (d) Representative images of basal compartment of seminiferous epithelia to show differentiating spermatogonia by immunohistochemical staining for UCHL1 and PLZF (upper) and UCHL1 and Hoechst 33342 (lower) in young (left) and aged (19 years old, right) bovine testes. White arrowheads indicate $UCHL1^+$ and $PLZF^-$ spermatogonia. (e) $PLZF^-/UCHL1^+$ cell densities in the basement membrane in young and aged testes. (f) Ratio of $Ki67^+$ cells out of total $UCHL1^+$ spermatogonia in young and aged testes. Average $PLZF^-/UCHL1^+/Ki67^+$ cell densities are shown for each age group. Throughout, scale bars indicate 10 µm. Dashed lines indicate basement membrane. White asterisks indicate autofluorescence signal. Data are expressed as mean ± SEM (young, n = 3; aged, n = 3). Asterisks represent significant differences ($p < .05$) between young and aged bulls (Student's t-test)

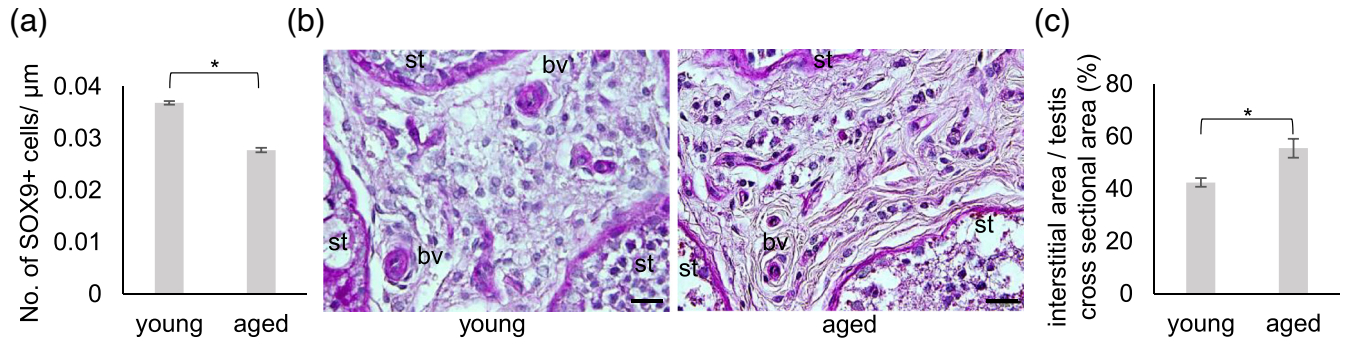

FIGURE 5 Sertoli cell number and progression of interstitial fibrosis in aged bovine testes. (a) Average densities of $SOX9^+$ cells in seminiferous tubules in young and aged bulls. (b) Representative images of interstitial tissues of young (left) and aged (19 years old, right) testes by PAS-H staining. Bars indicate 20 µm. bv, blood vessel; st, seminiferous tubule. (c) Percentages of interstitial area to the total testis parenchyma in young and aged bulls. Data are expressed as mean ± SEM (young, n = 3; aged, n = 3). Asterisks represent significant differences ($p < .05$) between young and aged bulls (Student's t-test)

4 | DISCUSSION

In this study, we analyzed spermatogonial density and proliferative activity in the testes of Japanese Black cattle. Although testicular size and weight did not differ significantly between young and aged bulls, the overall number of spermatogenic cells decreased in association with a decline in Sertoli cell number and expansion of fibrotic interstitium. However, whereas the abundance of differentiating spermatogonia and more differentiated cells decreased, that of undifferentiated spermatogonia and their proliferative activity was

maintained in the testes of aged bulls. Thus, we propose that undifferentiated spermatogonia may be more resistant to age-related changes, which mainly affects more differentiated types of spermatogenic cells. Because of limited age and number of bulls used in this study, it will be important to analyze more testis samples from bulls with variable genetic backgrounds and different ages for the deeper understandings of age-related changes in spermatogonial population over bovine life time.

4.1 | High resistance to aging of undifferentiated spermatogonia in bulls

Our study findings demonstrated that undifferentiated spermatogonia were robustly maintained in aged bovine testes, in stark contrast to decreased number of differentiating spermatogonia. This finding indicates that the undifferentiated state is more resistant to age-related changes than the differentiating state within the spermatogonial pool in normal bull testes. Maintenance of transplantable SSC number in normal seminiferous tubules has been reported in the testes of aged mice without atrophy (Ryu et al., 2006; Zhang et al., 2006), suggesting that high resistance of SSCs to aging may be conserved in mammals. Although age-related changes in the actual SSC number within undifferentiated spermatogonia remain to be elucidated in bovine spermatogenesis, the expression and nuclear localization of PLZF, and retained proliferative activity support the possibility of SSC self-renewal activity even in aged bovine testes. The stem cell function of undifferentiated spermatogonia in bulls will be need to be further evaluated by transplantation or lineage-tracing assays in vivo or in vitro (Brinster & Zimmermann, 1994; Ciccarelli et al., 2020; Hara et al., 2014; Izadyar et al., 2003; Sharma et al., 2020).

4.2 | Age-related defects in testicular germ cells in bulls

In the current study, aged bulls demonstrated a slightly decreased number of spermatogenic cells even in the noncalcified parenchymal region, except for very minor undifferentiated spermatogonia, whereas the ejaculated seminal sperm number did not decrease remarkably with age. Regardless of decreased numbers of spermatogenic cells, spermatozoa abundance in the cauda epididymal lumen and ejaculated semen of aged bulls was comparable with that in young bulls. This inconsistency might be caused by the sperm storage function in aged bovine epididymis. Decreased abundance of spermatogenic cells in aged bull testes has also been reported for other dairy breeds (Humphrey & Ladds, 1975). Moreover, age-associated decreases in spermatogenic cell numbers have been reported in other mammalian species, including rodents (mice, rats, and hamsters), canines, and humans (Bernal-Mañas et al., 2014; Bhanmeechao et al., 2018; Gosden et al., 1982; Paniagua et al., 1991). Therefore, the decreased number of spermatogenic cells in the testes of aged Japanese Black cattle without severe atrophy might be a conserved phenomenon in mammalian species, rather than due to inbreeding degeneration. However, these changes may indicate early hypospermatogenesis; therefore, the results of this study highlight the importance of evaluating intratesticular changes via testicular biopsy as the most reliable and accurate indicator of testicular sperm productivity during breeding improvements (Rohländer et al., 2020).

4.3 | Decrease of differentiating spermatogonia may contribute to germ cell loss

Because spermatogenic cell number is primarily determined by the differentiation stage of the spermatogonial population, we analyzed the density and proliferative activity of differentiating spermatogonia. The decreased number of differentiating spermatogonia in aged bovine testes suggests that the partial loss of differentiating spermatogonia may contribute to the low density of spermatogenic cells. In hamsters, the decreased abundance of male germ cells associated with aging was reportedly caused by a decline in the proliferation of differentiating spermatogonia (Bernal-Mañas et al., 2014). In the current study, the consistent ratio of Ki67 positive cells to the total number of differentiating spermatogonia suggests that the proliferative activity of differentiating spermatogonia does not change significantly in aged testes. Thus, in aged bovine testes, differentiating spermatogonia may be decreased by other mechanisms, probably through lowered production of differentiating spermatogonia from undifferentiated spermatogonial population or lowered cell survival of differentiating spermatogonia.

4.4 | Age-related defects in testicular somatic cells in bulls

Testicular severe lesion (probably being associated with calcification) detected as intense white signals by ultrasonography was only observed in 9% of the testicular parenchyma in the testes of the 17- and 19-year-old Japanese Black cattle. Because the diameter of bovine seminiferous tubules was approximately 200 μm, the larger size of the calcified area suggests that calcification spread concentrically over multiple seminiferous tubules and associated interstitial spaces in aged testes, rather than expanding along the length of the seminiferous tubules (Morales et al., 2007). Even in noncalcified regions, some other age-related changes were apparent in aged testes, such as the decline in Sertoli cell number and presence of interstitial fibrosis. Importantly, Sertoli cell number has been correlated with the number of male germ cells in mice and humans (Jiang et al., 2014; Paniagua et al., 1991; Rebourcet et al., 2017; Wang et al., 1993). Thus, there is a possibility that bovine differentiating spermatogonia, spermatocytes, and spermatids may be affected by the decline in Sertoli cell number. On the other hand, interstitial fibrosis can interfere with the diffusivity of intratesticular molecules

supplied from blood or lymphatic vessels, such as follicular stimulating hormone. Thus, fibrosis of the interstitial tissue might have a negative effect on the proliferation and differentiation of male germ cells. To understand the factors influencing germ cell loss, further studies are warranted to analyze the causal relationship between age-related changes in the spermatogenesis-supporting environment and hypospermatogenesis.

4.5 | Potential utility and limitations of undifferentiated spermatogonia for livestock production

Our study highlights the potential utility of undifferentiated spermatogonia from the testes of aged bulls. If bovine undifferentiated spermatogonia can be manipulated to differentiate into spermatozoa by testis transplantation or in vitro spermatogenesis, as was successfully achieved in mice (Brinster & Zimmermann, 1994; Sato et al., 2011), spermatozoa with high industrial value can be used longer than the original lifespan of the bull. However, technical barriers must be overcome to implement these technologies in cattle. Particularly, a method of identifying donor cells is needed for transplantation, and a repeatable culture method has not been established for in vitro spermatogenesis until now. Nonetheless, one needs to consider the effect of paternal age as a potential risk factor in the long-term use of SSCs for animal production. Named the paternal age effect (PAE), paternal age has been reportedly associated with increased probability of disorders in offspring, partly caused by age-associated accumulation of mutations in SSCs (Goriely et al., 2003, 2005; Goriely & Wilkie, 2012). Because the PAE has not yet been evaluated in bulls, judicial long-term use of undifferentiated spermatogonia of aged bulls is necessary to establish a safe and sustainable technology for food production.

In conclusion, our study provides direct evidence showing the persistence of undifferentiated spermatogonia in testes of aged Japanese Black cattle. This finding will provide a biological basis for robust, long-term sperm production using SSCs through genetic manipulation, transplantation, and in vitro culture.

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CONFLICT OF INTEREST

The authors declare no conflict of interests for this article.

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REFERENCES

Bernal-Mañas, C. M., Cortes, S., Morales, E., Horn, R., Seco-Rovira, V., Beltran-Frutos, E., Ferrer, C., Canteras, M., & Pastor, L. M. (2014). Influence of histological degree of seminiferous tubular degeneration and stage of seminiferous cycle on the proliferation of spermatogonia in aged Syrian hamster (*Mesocricetus auratus*). *Andrologia*, *46*, 672–679. https://doi.org/10.1111/and.12134

Berndston, W. E., & Desjardins, C. (1974). The cycle of the seminiferous epithelium and spermatogenesis in the bovine testis. *American Journal of Anatomy*, *140*, 167–179. https://doi.org/10.1002/aja.1001400204

Bhanmeechao, C., Srisuwatanasagul, S., & Ponglowhapan, S. (2018). Age-related changes in interstitial fibrosis and germ cell degeneration of the canine testis. *Reproduction in Domestic Animals*, *53*(Suppl 3), 37–43. https://doi.org/10.1111/rda.13354

Bishop, M. W. (1970). Ageing and reproduction in the male. *Journal of Reproduction and Fertility. Supplement*, *12*, 65–87.

Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 11298–11302. https://doi.org/10.1073/pnas.91.24.11298

Cai, H., Wu, J. Y., An, X. L., Zhao, X. X., Wang, Z. Z., Tang, B., Yue, Z. P., Li, Z. Y., & Zhang, X. M. (2016). Enrichment and culture of spermatogonia from cryopreserved adult bovine testis tissue. *Animal Reproduction Science*, *166*, 109–115. https://doi.org/10.1016/j.anireprosci.2016.01.009

Ciccarelli, M., Giassetti, M. I., Miao, D., Oatley, M. J., Robbins, C., Lopez-Biladeau, B., Waqas, M. S., Tibary, A., Whitelaw, B., Lillico, S., Park, C. H., Park, K. E., Telugu, B., Fan, Z., Liu, Y., Regouski, M., Polejaeva, I. A., & Oatley, J. M. (2020). Donor-derived spermatogenesis following stem cell transplantation in sterile *NANOS2* knockout males. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 24195–24204. https://doi.org/10.1073/pnas.2010102117

Costoya, J. A., Hobbs, R. M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K. E., Wolgemuth, D. J., & Pandolfi, P. P. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature Genetics*, *36*, 653–659. https://doi.org/10.1038/ng1367

Goriely, A., McVean, G. A., Röjmyr, M., Ingemarsson, B., & Wilkie, A. O. (2003). Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. *Science*, *301*, 643–646. https://doi.org/10.1126/science.1085710

Goriely, A., McVean, G. A., van Pelt, A. M., O'Rourke, A. W., Wall, S. A., de Rooij, D. G., & Wilkie, A. O. (2005). Gain-of-function amino acid substitutions drive positive selection of FGFR2 mutations in human spermatogonia. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 6051–6056. https://doi.org/10.1073/pnas.0500267102

Goriely, A., & Wilkie, A. O. (2012). Paternal age effect mutations and selfish spermatogonial selection: Causes and consequences for human disease. *The American Journal of Human Genetics*, *90*, 175–200. https://doi.org/10.1016/j.ajhg.2011.12.017

Gosden, R. G., Richardson, D. W., Brown, N., & Davidson, D. W. (1982). Structure and gametogenic potential of seminiferous tubules in ageing mice. *Journal of Reproduction and Fertility*, *64*, 127–133. https://doi.org/10.1530/jrf.0.0640127

Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., Simons, B. D., & Yoshida, S. (2014). Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell*, *14*, 658–672. https://doi.org/10.1016/j.stem.2014.01.019

Humphrey, J. D., & Ladds, P. W. (1975). A quantitative histological study of changes in the bovine testis and epididymis associated with age. *Research in Veterinary Science*, *19*, 135–141. https://doi.org/10.1016/S0034-5288(18)33523-9

Izadyar, F., Den Ouden, K., Stout, T. A., Stout, J., Coret, J., Lankveld, D. P., Spoormakers, T. J., Colenbrander, B., Oldenbroek, J. K., Van der Ploeg, K. D., Woelders, H., Kal, H. B., & De Rooij, D. G. (2003). Autologous and homologous transplantation of bovine spermatogonial stem cells. *Reproduction*, *126*, 765–774. https://doi.org/10.1530/rep.0.1260765

Jiang, H., Zhu, W. J., Li, J., Chen, Q. J., Liang, W. B., & Gu, Y. Q. (2014). Quantitative histological analysis and ultrastructure of the aging human testis. *International Urology and Nephrology*, *46*, 879–885. https://doi.org/10.1007/s11255-013-0610-0

Kanatsu-Shinohara, M., Ogonuki, N., Matoba, S., Ogura, A., & Shinohara, T. (2020). Autologous transplantation of spermatogonial stem cells restores fertility in congenitally infertile mice. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 7837–7844. https://doi.org/10.1073/pnas.1914963117

Kastelic, J. P., & Brito, L. F. (2012). Ultrasonography for monitoring reproductive function in the bull. *Reproduction in Domestic Animals*, *47*(Suppl 3), 45–51. https://doi.org/10.1111/j.1439-0531.2012.02042.x

Lim, B., Song, S. H., Song, G., & Kim, K. S. (2015). Changes of calcific density in pediatric patients with testicular microlithiasis. *Korean Journal of Urology*, *56*, 318–323. https://doi.org/10.4111/kju.2015.56.4.318

Lovelace, D. L., Gao, Z., Mutoji, K., Song, Y. C., Ruan, J., & Hermann, B. P. (2016). The regulatory repertoire of PLZF and SALL4 in undifferentiated spermatogonia. *Development*, *143*, 1893–1906. https://doi.org/10.1242/dev.132761

Morales, E., Ferrer, C., Zuasti, A., Garcia-Borron, J. C., Canteras, M., & Pastor, L. M. (2007). Apoptosis and molecular pathways in the seminiferous epithelium of aged and photoinhibited Syrian hamsters (*Mesocricetus auratus*). *Journal of Andrology*, *28*, 123–135. https://doi.org/10.2164/jandrol.106.000778

Ogawa, T., Dobrinski, I., Avarbock, M. R., & Brinster, R. L. (2000). Transplantation of male germ line stem cells restores fertility in infertile mice. *Nature Medicine*, *6*, 29–34. https://doi.org/10.1038/71496

Paniagua, R., Nistal, M., Sáez, F. J., & Fraile, B. (1991). Ultrastructure of the aging human testis. *Journal of Electron Microscopy Technique*, *19*, 241–260. https://doi.org/10.1002/jemt.1060190209

Rebourcet, D., Darbey, A., Monteiro, A., Soffientini, U., Tsai, Y. T., Handel, I., Pitetti, J. L., Nef, S., Smith, L. B., & O'Shaughnessy, P. J. (2017). Sertoli cell number defines and predicts germ and Leydig cell population sizes in the adult mouse testis. *Endocrinology*, *158*, 2955–2969. https://doi.org/10.1210/en.2017-00196

Reding, S. C., Stepnoski, A. L., Cloninger, E. W., & Oatley, J. M. (2010). THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. *Reproduction*, *139*, 893–903. https://doi.org/10.1530/REP-09-0513

Rohländer, M., Otzen, H., Rode, K., Jung, K., Schmicke, M., Harborth, T., Langeheine, M., Brehm, R., & Bajcsy, Á. (2020). Histological comparison of testicular needle biopsy and en bloc samples in abattoir calves. *Animals (Basel)*, *10*, 918. https://doi.org/10.3390/ani10050918

Russell, L., Ettlin, R., Sinha Hikim, A., & Clegg, E. (1990). *Histological and histopathological evaluation of the testis*. Cache River Press.

Ryu, B. Y., Orwig, K. E., Oatley, J. M., Avarbock, M. R., & Brinster, R. L. (2006). Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. *Stem Cells*, *24*, 1505–1511. https://doi.org/10.1634/stemcells.2005-0580

Sato, T., Katagiri, K., Gohbara, A., Inoue, K., Ogonuki, N., Ogura, A., Kubota, Y., & Ogawa, T. (2011). In vitro production of functional sperm in cultured neonatal mouse testes. *Nature*, *471*, 504–507. https://doi.org/10.1038/nature09850

Savvulidi, F., Ptacek, M., Savvulidi Vargova, K., & Stadnik, L. (2019). Manipulation of spermatogonial stem cells in livestock species. *Journal of Animal Science and Biotechnology*, *10*, 46. https://doi.org/10.1186/s40104-019-0355-4

Sharma, A., Kumaresan, A., Mehta, P., Nala, N., Singh, M. K., Palta, P., Singla, S. K., Manik, R. S., & Chauhan, M. S. (2020). Successful transplantation of transfected enriched buffalo (*Bubalus bubalis*) spermatogonial stem cells to homologous recipients. *Theriogenology*, *142*, 441–449. https://doi.org/10.1016/j.theriogenology.2019.10.019

Shinohara, T., Avarbock, M. R., & Brinster, R. L. (1999). β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 5504–5509. https://doi.org/10.1073/pnas.96.10.5504

Wang, C., Leung, A., & Sinha-Hikim, A. P. (1993). Reproductive aging in the male brown-Norway rat: A model for the human. *Endocrinology*, *133*, 2773–2781. https://doi.org/10.1210/endo.133.6.8243304

Wrobel, K. H., Bickel, D., Kujat, R., & Schimmel, M. (1995). Configuration and distribution of bovine spermatogonia. *Cell and Tissue Research*, *279*, 277–289. https://doi.org/10.1007/BF00318484

Yoshida, S. (2018). Open niche regulation of mouse spermatogenic stem cells. *Development, Growth & Differentiation*, *60*, 542–552. https://doi.org/10.1111/dgd.12574

Zhang, X., Ebata, K. T., Robaire, B., & Nagano, M. C. (2006). Aging of male germ line stem cells in mice. *Biology of Reproduction*, *74*, 119–124. https://doi.org/10.1095/biolreprod.105.045591

Zheng, Y., Zhang, Y., Qu, R., He, Y., Tian, X., & Zeng, W. (2014). Spermatogonial stem cells from domestic animals: Progress and prospects. *Reproduction*, *147*, R65–R74. https://doi.org/10.1530/REP-13-0466

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