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Melatonin promotes development of haploid germ cells from early developing spermatogenic cells of *Suffolk* sheep under in vitro condition

Abstract: Promotion of spermatogonial stem cell (SSC) differentiation into functional sperms under in vitro conditions is a great challenge for reproductive physiologists. In this study, we observed that melatonin (10^{-7} M) supplementation significantly enhanced the cultured SSCs differentiation into haploid germ cells. This was confirmed by the expression of sperm special protein, acrosin. The rate of SSCs differentiation into sperm with melatonin supplementation was 11.85 ± 0.93% which was twofold higher than that in the control. The level of testosterone, the transcriptions of luteinizing hormone receptor (LHR), and the steroidogenic acute regulatory protein (StAR) were upregulated with melatonin treatment. At the early stage of SSCs culture, melatonin suppressed the level of cAMP, while at the later stage, it promoted cAMP production. The similar pattern was observed in testosterone content. Expressions for marker genes of meiosis anaphase, Dnmt3a, and Bcl-2 were upregulated by melatonin. In contrast, Bax expression was downregulated. Importantly, the in vitro-generated sperms were functional and they were capable to fertilize oocytes. These fertilized oocytes have successfully developed to the blastula stage.

Shou-Long Deng[1], Su-Ren Chen[1], Zhi-Peng Wang[1], Yan Zhang[1], Ji-Xin Tang[1], Jian Li[1], Xiu-Xia Wang[1], Jin-Mei Cheng[1], Cheng Jin[1], Xiao-Yu Li[1], Bao-Lu Zhang[2], Kun Yu[2], Zheng-Xing Lian[3], Guo-Shi Liu[3] and Yi-Xun Liu[1]

[1]State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China; [2]National key Lab of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, P.R. China; [3]Laboratory of Animal Genetics and Breeding, College of Animal Science and Technology, China Agricultural University, Beijing, P.R. China

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Address reprint requests to Guo-Shi Liu, Laboratory of Animal Genetics and Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, P.R. China.
E-mail: gshliu@cau.edu.cn and Yi-Xun Liu, State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China.
E-mail: liuyx@ioz.ac.cn

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Introduction

Spermatogonial stem cells (SSCs) are a class of primordial germ cells (PGCs) located in mammalian testis. These cells are characterized by self-renewal or proliferation, generation of differentiated progeny cells. Spermatogenesis is a multiple process through mitotic phase of stem cell renewal, differentiation, and meiotic and postmeiotic phases of spermatogenesis. Genetically modified SSCs could be used for the treatment of genetic diseases and transgenic animal production after in vitro differentiation [1]. An induction of spermatogenesis in vitro may also provide a therapeutic strategy for male infertility [2]. Spermatogenesis at early stage occurs in testis at a unique environment (niche), surrounded mainly by Sertoli cells and other somatic cells outside of the blood–testis barrier (BTB). Key time points regarding appearance of particular germ cell types are well defined during the first spermatogenic cycle. During spermatogenesis, Sertoli cells and reproductive hormones, such as testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), play important roles. Meanwhile, it has been reported that several cytokines, including epidermal growth factor (EGF), stem cell factor (SCF), glial derived neurotrophic factor (GDNF), and insulin-like growth factor (IGF), were also crucial for spermatogenesis [3].

Evidence have shown that spermatogenesis could take place in in vitro conditions by a variety of culture systems, such as three-dimensional culture [4], tissues culture [5, 6], and testis mix cells culture [7]. These methods could successfully generate haploid germ cells, through microinjection, and these haploid sperm cells were functional and able to fertilize oocytes and generate offspring [8, 9]. Currently, an effective method is co-culture of SSCs with Sertoli cells, and this method probably does not fully simulate the in vivo microenvironment, but the cytokines secreted by Sertoli cells might support SSCs self-renewal and differentiation [10]. A more effective method probably is to add differentiation-induced components in the culture system to promote SSCs differentiation. For example, retinoic

acid (RA) was frequently used to initiate the meiotic process by the action on the retinoic acid gene 8 (Stra8) to promote spermatogenesis [11]. Testosterone is essential for spermatogenesis because lack of testosterone could lead to male infertility due to spermatogenesis disorder [12]. Luteinizing hormone receptor (LHR) activation by LH could increase cellular cAMP synthesis. In addition, LH binding with 3β-HSD, 17α-hydroxylase, and steroidogenic acute regulatory proteins (StAR) could increase testosterone production [13].

Melatonin, a major secretory product of the pineal gland, possesses both lipophilic and hydrophilic properties, and it passes through blood–testis barrier and enters testis cells [14]. It is also synthesized by the testes [15]. Melatonin has been known as a pleiotropic molecule. It is a potent free radical scavenger and antioxidant [16, 17], and it also regulates cell proliferation and differentiation [18–21]. Both melatonin membrane receptors 1 (MT1) and 2 (MT2) are G protein-coupled receptors and regulate the activation of adenylate cyclase (AC) and phospholipase C (PLC) [22]. It is one of the important regulators being responsible for seasonal reproduction in photoperiodic animals. Melatonin exhibits different effects on the long-day and the short-day breeding animals. A constant supplementation of melatonin to the short-day breeding animals stimulated the gonad function [23]. In rams, exogenous melatonin supplementation promoted their LH and FSH secretions, subsequently activation of the testis function and enhancement of blood testosterone concentrations [24]. These changes eventually led to the germ cell maturation [25, 26]. It was observed that pinealectomized rams lost their LH impulse; however, their LH secretory rhythm was recovered after 70 days of melatonin implantation [27]. Also in rams, melatonin stimulated the activity of acrosin in spermatozoa and this action was not related to the changes of testosterone parameters [28].

Melatonin could modify the morphological characteristics of spermatogenic cells and influence the synthesis of cAMP in testicular cells [29]. Some of these actions of melatonin were mediated by receptors on testis, for example, melatonin's actions on gonadal hormone synthesis and secretion [30, 31]. Based on these observations, we speculated that melatonin might effectively regulate spermatogenesis in photoperiodic animals. To test this hypothesis, a variety of concentrations of melatonin were added into the SSCs culture system, and the efficiency of spermatogenesis at various stages was examined. In addition, the molecular mechanisms of the melatonin-signaling pathway on spermatogenesis were also examined. This study might provide a new method for in vitro haploid sperm generation from SSCs and that might be used to further improve the transgenic animal production.

Materials and methods

Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Ethics statement

Sheep surgical biopsy was performed at the experimental station of the China Agricultural University, and the whole procedure was carried out in strict accordance with the protocol approved by the Animal Welfare Committee of China Agricultural University.

Isolation of testicular tissues from the sheep

Testicular tissues were obtained from 2-month-old sheep and transported to the laboratory within 3 h contained in normal physiological saline at 4°C. The testicular tissues were washed thrice with phosphate-buffered solution (PBS; Ca^{2+} and Mg^{2+} free) supplemented with penicillin 100 IU/mL and streptomycin 100 mg/mL. After decapsulation, dissociated cells and tubule fragments were collected. The testicular tissues were dissociated by modified one-step enzymatic digestion [32]. Briefly, the short seminiferous tubules were incubated with tenfold (w/v) enzyme cocktail containing collagenase type IV 1 mg/mL, hyaluronidase 1 mg/mL, and DNase I 500 μg/mL at 37°C for 20 min, followed by neutralization with DMEM containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). Dissociated cells and seminiferous tubules fragments were filter by 40-mesh filter.

In vitro differentiation of sheep SSCs

Dissociated cells and seminiferous tubular fragments were seeded onto 60-mm culture dishes and maintained at 33°C in 5% CO_2 incubator. The cells/tissue segments were cultured in the medium (containing DMEM, 3% FBS, 1% nonessential amino acids, SCF 10 ng/mL, bFGF 10 ng/mL (Peprotech 100-18B), EGF 25 ng/mL, GDNF 40 ng/mL (Peprotech 450-10), FSH 500 ng/mL, LH 500 ng/mL, insulin 1 μg/mL, L-glutamine 4 M, transferrin 5 mg/mL, and 1% penicillin–streptomycin (Gibico) for 3 days, and then, various concentrations of melatonin (0, 10^{-9}, 10^{-7}, 10^{-5}, and 10^{-3} M, respectively) were added into culture system. A half of the culture medium was changed every 3 days [33]. In part of the cultured cells, the medium was added melatonin (10^{-7} M) with either luzindole (10^{-7} M) (a nonselective MT1/MT2 inhibitor) or 4-P-PDOT 10^{-7} M (MT2-specific inhibitor), respectively. The cells were monitored periodically, and the images were captured.

After 30 days of culture, the cells were transfected. For the sperms, the exogenous DNA is relatively easy to be integrated into host DNA due to the nature of histone/DNA relationship [34, 35]. After replacement of the histones by protamine, they bind tightly with host DNA to avoid the foreign DNA insertion. The pIRES2-GFP vector was transfected into the culture cells utilizing Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA, USA). Genomic DNA of the transfected culture cells was extracted (Invitrogen) and amplified for the detection of GFP and transition protein 1 (Tnp1) fragment. Primers used for real-time PCR are listed in Table 1.

Table 1. The primer sequences

Gene (Accession no.)	Primer sequence	Product size (bp)
MT1 (NM001009725.1)	5′ CCTCGCCTCCATCCTCATC 3′ 5′ GCTCCACCCATTGTTAACTATAGAC 3′	196
MT2 (NM001130938.1)	5′ GGCAACCTCCTGGTCATCC 3′ 5′ GGCGGTGATGTTGAAGACG 3′	231
RORα (XM015096689.1)	5′ AGCAGCGAGACCACCAACA 3′ 5′ ATGTAGTTGCTGAGGTCATCGTG 3′	109
FSHR (NM001009289.1)	5′ CCCCAACATCACATCCTCTTC 3′ 5′ CAGGACCAGGAGGATCTTTGA 3′	166
LHR (JX471148.1)	5′ GGGGCTCTACCTGCTACTCATT 3′ 5′ ACTGTGAGGGTGTAGACGGAGA 3′	153
StAR (AF290202.1)	5′ GGGACGAGGTGCTGAGTAAAG 3′ 5′ GGTTCCACTCGCCCATAGC 3′	132
c-kit (AM293661.1)	5′ ATCCCAAACCTGAACACCGA 3′ 5′ CGTGAAATGTGTAAGTGCCTCC 3′	162
Star8 (XM015095396.1)	5′ GGCAAGTTTTGAATAAGGCGA 3′ 5′ CCGATGAACTTTATTTGACACCA 3′	185
Tnp1 (XM0040 04917.2)	5′ AAAGAGTCAGGGCACGAGG 3′ 5′ TCACAAGTGGGAGCGGAAA 3′	148
Tnp2 (XM004020771.2)	5′ GCCACAGATACACCATGCACTC 3′ 5′ CCTGCTTGCTCCTCTTGACC 3′	132
Prm1 (FJ900270.1)	5′ GAAGATGTCGCAGACGAAGGA 3′ 5′ TGGAGAACCAGAGGGCAAGA 3′	149
c-Jun (AF257464.1)	5′ GCCAAGAACTCCGACCTCCT 3′ 5′ ATATGCCCGTTGCTGGACTG 3′	101
c-Fos (U94719.1)	5′ GAGCCAGTCAAGAGCATCAGC 3′ 5′ AAGGAATTGCTGTGCAGAGGC 3′	173
H19 (AY091484.1)	5′ GAGAATGGGAGAGAAGGCAGC 3′ 5′ CTCCCTGCTGCGGGTATCT 3′	105
Dnmt3a (HQ202740.1)	5′ GAAGAGGAGAAGAACCCCTACAAA 3′ 5′ TGACCTTAGGCTTCTCCGTTG 3′	127
CDYL (XM012101338.2)	5′ TCATTCAGTTCAAGAAACCTATTATCG 3′ 5′ TCCTGGGGAACATCACGGT 3′	177
Bax (AF163774.1)	5′ GACGGCAACTTCAACTGGG 3′ 5′ ACAAAGATGGTCACGGTCTGC 3′	227
Bcl-2 (DQ152929.1)	5′ GCCCTGTGGATGACCGAGTA 3′ 5′ GACAGCCAGGAGAAATCAAACA 3′	128
GFP	5′ TCAAGGACGACGGCAACT 3′ 5′ GGTGTTCTGCTGGTAGTGGTC 3′	260
β-Actin (JN033788.1)	5′ TCCGTGACATCAAGGAGAAGC 3′ 5′ CAGGAAGGAAGGCTGGAAGA 3′	176

Immunohistochemistry

Testis samples from 2-month-old sheep were fixed with 4% paraformaldehyde. The samples were cryo-embedded in OCT compound and then cut into 7-μm-thick sections. H&E strain was employed to observe seminiferous tubules. MT1 distributions were observed by immunohistochemistry. Briefly, after washing three times with PBS,the slides were incubated for 1 hr in PBS containing 1% bovine serum albumin (BSA) at room temperature. Antibody of MT1 (Santa sc-13180, final concentration 1:100) was added to the solution, after 4 hr of incubation, and the secondary antibody was applied for another 1-hr incubation. Staining was visualized using a DAB substrate kit.

The cultured cells were collected for immunofluorescence analysis. Briefly, after fixed in 70% alcohol for 2 hr, the cells were washed twice with PBS, and then, the samples were rinsed with PBS and permeabilized with 0.5% Triton-X100 for 1 hr and blocked with 1% BSA for 1 hr at room temperature.

After 7 days of culture, the dissociated cells and seminiferous tubules fragments were staining with the sheep spermatogonia-specific marker vasa (Santa sc-67185, final concentration 1:100) and Plzf (Santa sc-22839, the final concentration 1:100) [36].

After 30 days of culture, the cells were used to identify whether they expressed acrosin, a marker for the differentiated spermatozoon [37]. Antibody of acrosin (Bioss bs-5151R, final concentration 1:200) was added to the solution for 4 hr, and then, the secondary antibody was applied for another 1 hr. Following washing as mentioned above, the nuclei were stained with DAPI and visualized with microscope.

Western blot

The proteins were isolated from 30-day culture cells. Prm1 (Santa sc-30174), Tnp1 (Santa sc-169653), and c-Fos (Santa sc-253) were examined with Western blot analysis. β-Actin served as a control. The proteins were electrophoresed

under reducing conditions in 12% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were blocked in 5% BSA and incubated overnight at 4°C with the primary antibody, followed by incubation with the secondary antibody for 1 hr at room temperature. The protein bands were visualized using enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China) and X-OMAT BT film (Eastman Kodak Co., Rochester, NY, USA).

Flow cytometric analysis

After 7 days of culture, the cells were used to identify their expressions of PGP9.5 and vasa, markers for the germ cells, by the method of flow cytometry. Briefly, after fixed in 70% alcohol for 2 hr, the cells were washed twice with PBS and then resuspended in PBS with BSA over an hour. Anti-PGP9.5 antibody (Bioss, bs-3806R, final concentration 1:200) and antivasa antibody (final concentration 1:200) were added to the solution for another hour. After washing three times with PBS, the cells were collected by centrifugation at 500 × g for 5 min, and then the secondary antibody was applied to the cells with 45-min incubation. Finally, the cells were washed thrice with PBS and resuspended in 0.5 mL PBS. The cells were then analyzed by flow cytometry.

The ploidy of the individual pre- and postcultured cells, as well as the adult sperms, was also analyzed by the flow cytometry. Briefly, the cells and sperms were fixed in 70% ethanol for over 4 hr, respectively. After washing thrice with PBS, the cells and the sperms were incubated at 37°C for 10 min in PBS plus RNase I 200 μg/mL and propidium iodide (PI) 20 μg/mL. Finally, the DNA content of the cells was detected by flow cytometry analysis.

Quantitative real-time PCR

MT1 and MT2 expressions in the testis were analyzed. Testicular tissues from 1-, 2-, 3-, 4- and 5-month-old sheep were collected and stored in liquid nitrogen. Primordial germ cells, Sertoli cells, and Leydig cells were isolated from sheep testis using Percoll discontinuous gradient centrifugation. MT1 expressions of primordial germ cells, Sertoli cells, and Leydig cells were detected. cDNA from ram sperms were used as the control in evaluation of the methylation and histone acetylation. Dissociated cells and seminiferous tubular fragments (0, 5, 10, 20, 30, and 40 days, respectively) were collected for specific genes expression study. These included hormone receptors (MT1, MT2, RORα, LHR, and FSHR), proto-oncogenes (c-Fos, c-Jun), apoptosis-related genes (Bax, Bcl-2), spermatogenesis-related genes (c-kit, Stra8, Tnp1, Tnp2, and Prm1), DNA methylation genes (H19, Dnmt3a), histone acetyl transferase gene (CDYL), and testosterone synthesis rate-limiting enzyme (StRA), and they were studied by RT-PCR. The primers sequences were listed in Table 1. Total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's protocol. Revers transcription–PCR was performed by cDNA synthesis kit (Promega, Madison, WI, USA) using 2 μL of total RNA according to the manufacturer's protocol. Real-time PCRs were carried out with a Real Master Mix SYBR Green Kit (Tiangen, China) using MX300P (Stratagene; Agilent Technologies Inc., Santa Clara, CA, USA). Fold change of the gene expression was calculated with 2^{-ddct} method as a ratio of expression levels of treated groups to the expression level of the control group.

Elisa and NO concentration

Suspension of testicular cells was obtained from 1-, 2- and 5-month-old sheep, respectively, following the instructions of the protocol. Enzyme-linked immunosorbent assay (Elisa) kits were used to detect levels of melatonin (Hermes Criterion Biotechnology, Vancouver, Canada) in accordance with the manufacturer's instructions. Cultured cells at different stages were collected for cAMP and testosterone detection following the instructions of the manufacturer's protocol of the ELISA kits (Hermes Criterion Biotechnology) and nitric oxide (NO) concentrations (Nanjing Jiancheng Bioengineering Institute) were determined by spectrophotometry according to the manufacturer's instructions.

Intracytoplasmic microinjection

Ovine ovaries from slaughterhouse were collected and immediately stored in the physiological saline at 30°C and transported to the laboratory within 3 hr. Cumulus and cumulus–oocytes complexes (COCs) were selected and cultured into in vitro maturation (IVM) medium including 10% FBS TCM-199, EGF 10 ng/mL, FSH 5 μg/mL, LH 5 μg/mL, 17β-estradiol 1 μg/mL, and glutamine 2 mM at 38.5°C in a humidified atmosphere of 5% CO_2 for 19 hr. After that, COCs were transferred into TCM-199 medium containing 0.1% hyaluronidase to make oocytes free from cumulus cells. Oocytes were selected for haploid sperm cell injection.

In vitro-generated haploid sperm cells were collected as the donor cells from the culture dishes. The mature sperms are round shape and about 10 μm in diameter with flagellum. In this study, only those sperms with round shape and single tail were selected [38], and they were injected into the matured oocytes using a micromanipulator. These couplets were transferred into IVM medium for recovery at 38°C, 5% CO_2 for 30 min, and then, recovered couplets were cultured in vitro development culture (IVC) medium including modified synthetic oviduct fluid with amino acids, glutamine 0.2 mM, BSA 6 mg/mL, 3% essential amino acids, 1% nonessential amino acids, and inositol 0.5 mg/mL at 38°C, 5% CO_2 for 30 min; subsequently, these couplets were activated with ionomycin 5 μM for 5 min, followed by addition of 6-dimethylaminopyridine 2 mM for another 4-hr incubation. After activation, the reconstructed embryos were cultured at 38°C, 5% CO_2 for their development. The cleavages of reconstructed embryos were observed after 120 hr incubation.

Statistical analyses

All experiments were repeated, at least, three times. One-way ANOVA was used to determine statistical significance following by the Duncan's test to determine the statistical significance between the relative groups. Statistical analysis

was conducted using Statistical Analysis System software (SAS Institute, Cary, NC, USA). All data were expressed as mean ± S.E.M. Differences were considered to be significant when $P < 0.05$.

Results

MT expression in sheep testis

Real-time PCR analysis identified that both MT1 and MT2 were expressed in the sheep testis. The both expressions reached to the peak level at 2-month development; thereafter, they decreased with the increased ages of the sheep (Fig. 1A,B). MT1 expression kept in a relatively high level during the first 4 months. While at the 2 months of age, MT2 expression was lower than that of it at 1 month. In addition, melatonin was detectable in the sheep testis and its levels were reduced with increased age of the sheep (Fig. 1C). Histological study on 2-month-old sheep shows that spermatogenic cells in the seminiferous tubules remained in an undifferentiating state (Fig. 1D). The positive staining of MT1 in the 2-month-old sheep testis was shown with yellowish or dark brown color. The MT1 was localized mainly inside of the cytomembrane of primordial germ cells, and it was also detected in the seminiferous tubules and leydig cells with less intensity. Real-time PCR results indicated that the expression of MT1 in Leydig cells was dramatically higher than that of in primordial germ cells and Sertoli cells ($P < 0.05$) (Fig. 1E).

SSCs differentiation

Testicular somatic cells began to attach the wall of the plate after 12 hr of culture, while the SSCs initiated a process to attach the somatic cells after 24 hr of culture. The fastest growing cells were Sertoli cells. They almost confluence on the 7 day of culture. Meanwhile, spermatogonia adhered to the surface of the single layer of Sertoli cells which were around the seminiferous tubules. The paired or short-chain-shaped cytoplasmic bridges were found between spermatogonia. Spermatogonium showed a typical phenomenon of homologous (Fig. 2A,B). Both immunohistochemical analysis and flow cytometry study failed to find undifferentiated SSCs as shown by the specific antibody on 7th day of culture (Fig. 2D,E).

On the 10th day of culture, small clusters of the spermatogonium were observed (Fig. 2C) and the seminiferous tubules began to flatten. Spermatocyte-like cells were identified on the 20th day of culture. After 30 days of culture, both round sperm-like cells and fusiformis elongated sperm-like cells were observed either in the culture medium or on the bottom of the plate. The cell clusters were only observed floating in the medium. Most of the differentiated round-head cells developed apoptosis, and only few of them continued to grow a long flagellum and these cells with long flagella finally differentiated into sperm-like cells with a tail (Fig. 3Ab). The time schedule for SSCs differentiation into sperm-like cells in vitro was

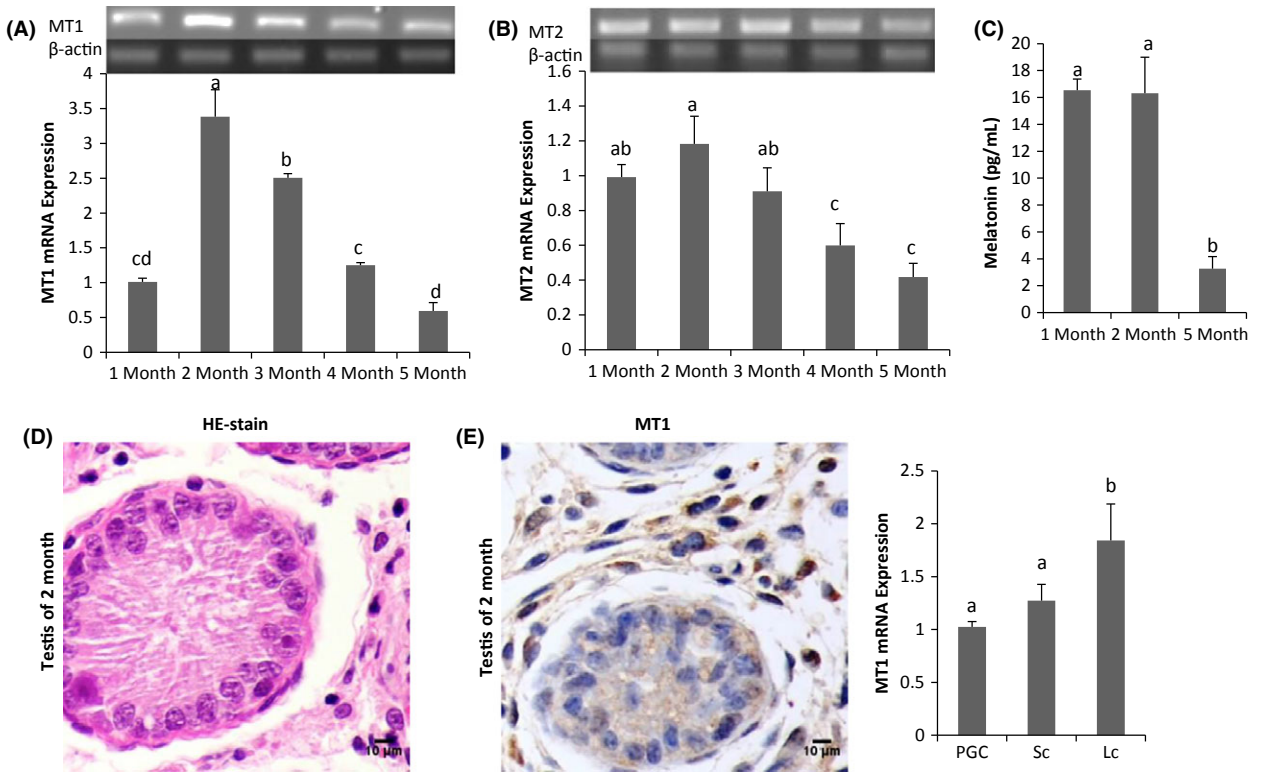

Fig. 1. MT expression and localization in sheep testis. (A, B) real-time PCR detection for MT1 and MT2 at various times in the testes. (C) Melatonin content in sheep testis. (D) H&E staining in 2-month-old sheep testis. (E) Immunohistochemical study of MT1 in 2-month-old sheep testis, real-time PCR detection of MT1 expression in primordial germ cells (PGC), Sertoli cells (Sc), and Leydig cells (Lc) from sheep testis. The superscript different letters (a–d) represent a significant difference of these columns ($P < 0.05$).

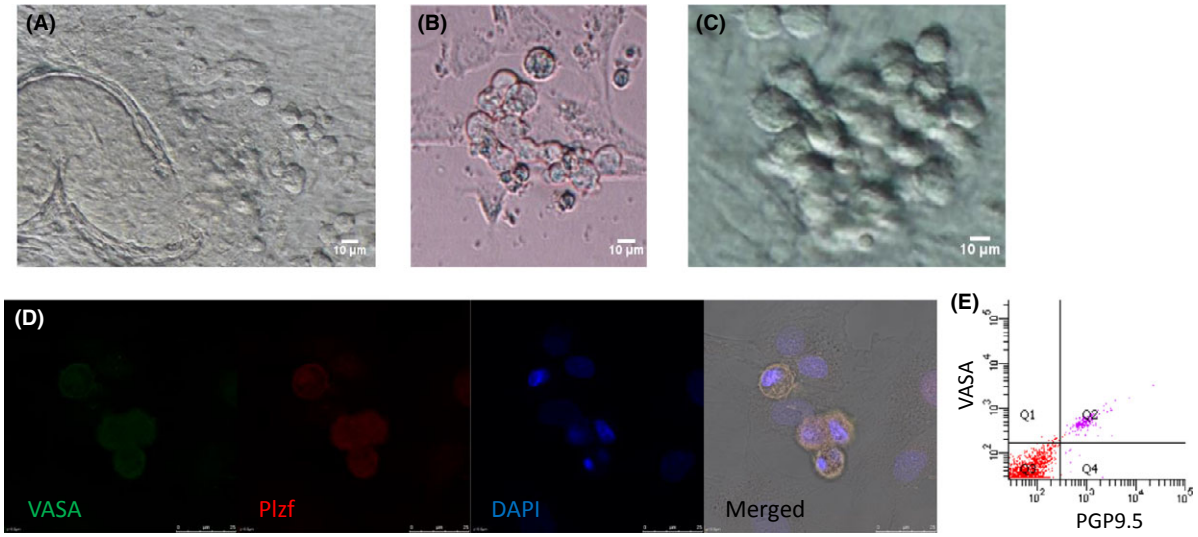

Fig. 2. Germ cell morphologies in differentiation culture system. (A, B) germ cells aggregated into clumps with cytoplasmic bridges between cells. (C) Small cluster of SSCs. (D) Images of vasa and Plzf on 7th day of cultured cells. (E) Flow cytometric graph of vasa and PGP9.5 double-labeled germ cells.

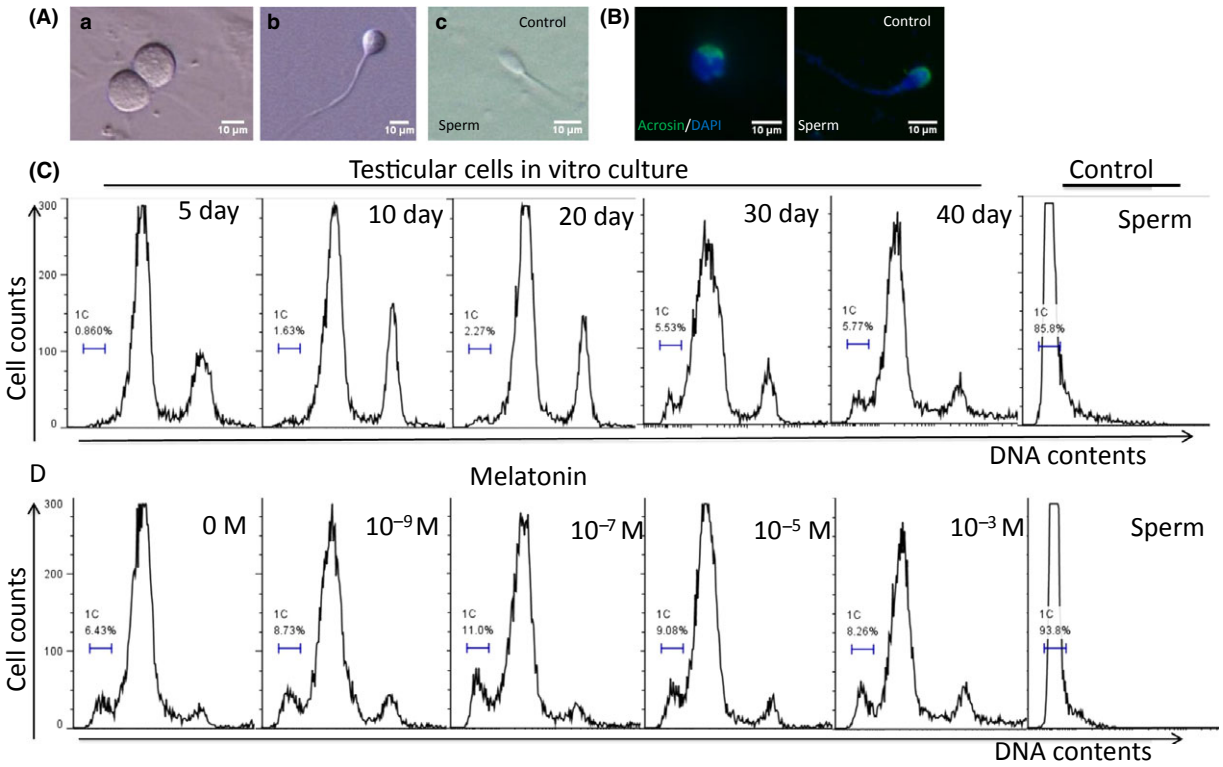

Fig. 3. Effects of melatonin on sheep haploid spermatozoa in vitro culture. (A) Morphological changes in sheep spermatogonial stem cells during culture. (a) 7th day of culture: SSCs, (b) 30th day of culture: sperm-like cells, and (c) adult sheep sperm as a control. (B) The haploid cell expressed the mature sperm protein acrosin (green), nuclei of the cells were stained by DAPI, and ram sperms used as control. (C) DNA content of the floating cells with flow cytometry analysis at 5, 10, 20, 30, and 40 days of culture, respectively, adult ram sperm cells as a positive control, peaks of 1C haploids. (D) Cells were cultured in different concentrations of melatonin (0, 10^{-9}, 10^{-7}, 10^{-5}, and 10^{-3} M, respectively) for 30 days, the DNA content of the floating cells.

earlier than that of these cells did in vivo (Movie S1). Haploid cells expressed the mature sperm protein acrosin (Fig. 3B).

The DNA content of the sperm-like cells showed three peaks (including a haploid, a prominent diploid and a tetraploid peaks) (Fig. 3C). It was calculated that around

5.77 ± 1.05% of the cells differentiated into haploid cell population during 40 days of incubation. Melatonin supplementation at the concentration of 10^{-7} M not only shortened the time schedule of the sperm formation (30 days of incubation versus 40 days) but also significantly increased the haploid cell population to 11.85 ± 0.93% which was twofolds higher than that in control group ($P < 0.05$). However, the higher concentrations of melatonin than those of 10^{-7} M appeared to decrease the efficiency as to promote the sperm formation from SSCs (Fig. 3D and Table 2).

Function test of the haploid sperm cell

Positive haploid cells were identified by detecting the expression of GFP (Fig. 4A). The results from the multiplex PCR confirmed that haploid cell expressed the sperm-specific gene Tnp1 and the exogenous GFP gene was integrated into its chromosome (Fig. 4B). Haploid cells with a tail and GFP were microinjected into the oocytes (Fig. 4C,D). More blastocysts were harvested from melatonin-treated group (14.04 ± 1.53%), compared with the control group (10.26 ± 1.27%) ($P < 0.05$) (Table 3). The results indicated that the haploid cells were biologically functional to fertilize the oocytes for the further development. Results from the studies of melatonin membrane receptor antagonists showed that melatonin could affect SSCs differentiation through its receptors. There were no significant differences between the groups of luzindole and 4-P-PDOT regarding the differentiation efficiencies ($P > 0.05$). In addition, the haploid efficiency under the combination of these two receptor inhibitors was still higher than that in the control group ($P < 0.05$) (Fig. 4E).

Exogenous melatonin in the in vitro SSCs culture system upregulated cAMP and testosterone contents

MT1, MT2, and RORα expression was identified by real-time PCR in cultured cells on 30th day of culture. Results showed that MT1, MT2, and RORα expressions were enhanced by melatonin supplementation with all concentrations tested compared with the control group (Fig. 5A–C) ($P < 0.05$). However, melatonin at the concentration of 10^{-7} M was the most effective compared with other groups ($P < 0.05$). A similar pattern was found with the changes in testosterone levels. The highest content of testosterone was detected in the melatonin 10^{-7} M group on 30th day of culture (Fig. 5D). Furthermore, the levels of testosterone were monitored during the entire period of SSCs differentiation in the melatonin 10^{-7} M group. Results showed that testosterone content in culture medium was

Table 2. Ratio of sperm-like cells to total suspending cells in 30 days after SSCs cultured with different melatonin concentrations

Melatonin	0 M	10^{-9} M	10^{-7} M	10^{-5} M	10^{-3} M
Haploid	5.57 ± 1.05%^{d}	7.53 ± 1.44%^{c}	11.85 ± 0.93%^{a}	9.18 ± 0.87%^{ab}	8.35 ± 0.76%^{bc}

Different superscript letters indicate significantly different values between groups ($P < 0.05$).

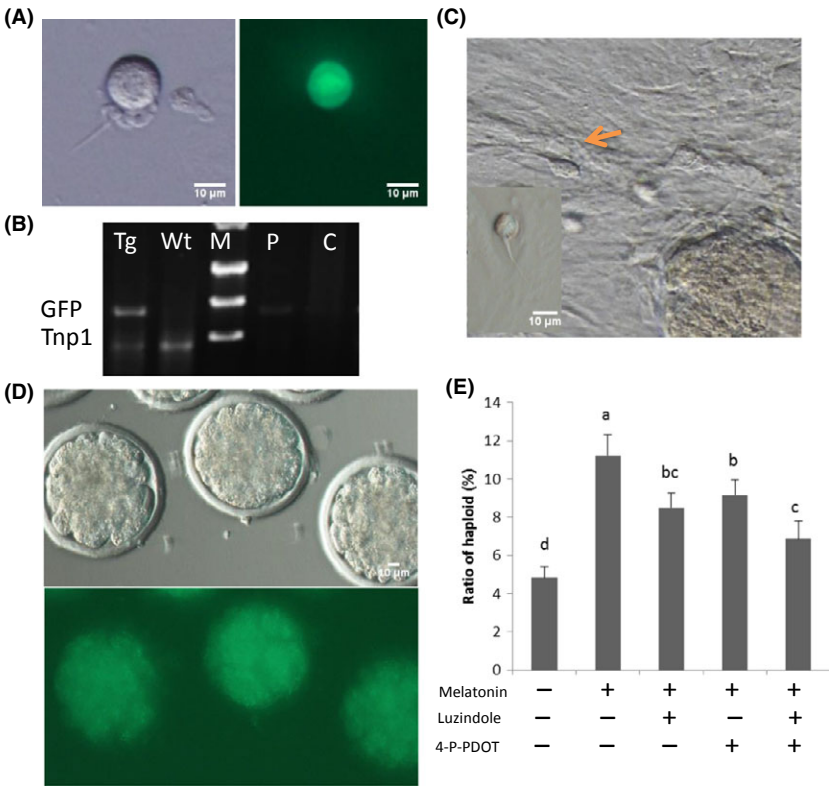

Fig. 4. Images of haploid spermatozoa from in vitro culture. (A) GFP in haploid cell. (B) Positive haploid cell detected using polymerase chain reaction, and the floating cells expressed GFP and Tnp1. Tg: expression GFP and Tnp1; lane Wt: expression Tnp1; lane P: pIRES2-GFP vector; C: negative control without template; lane M: marker DNAs. (C) SSCs differentiated sperm-like cell with single tail. (D) Production of GFP-haploid reconstruction morulae. (E) The ratio of in vitro differentiated haploids with luzindole, 4-P-PDOT, and both of them, respectively. Different superscript letters (a–d) in each column represent statistical significant differences ($P < 0.05$).

Table 3. The outcomes of the embryonic development of sheep oocytes after intracytoplasmic spermatozoa injection (ICSI) or round spermatid injection (ROSI)

	COCs-IVM	IVC	2-cell (%)	Morulae (%)
ROSI	63	39	12 (23.08 ± 3.11)[b]	4 (10.26 ± 1.27)[c]
ROSI (Melatonin 10^{-7}M)	90	57	17 (29.82 ± 2.43)[b]	8 (14.04 ± 1.53)[b]
ICSI	60	29	21 (72.74 ± 5.88)[a]	15 (51.72 ± 4.57)[a]

Different superscript letters indicate significantly different values between groups ($P < 0.05$). ROSI, these sperm-like cells were obtained from the in vitro culture. ICSI, the sperms were obtained from the adult rams.

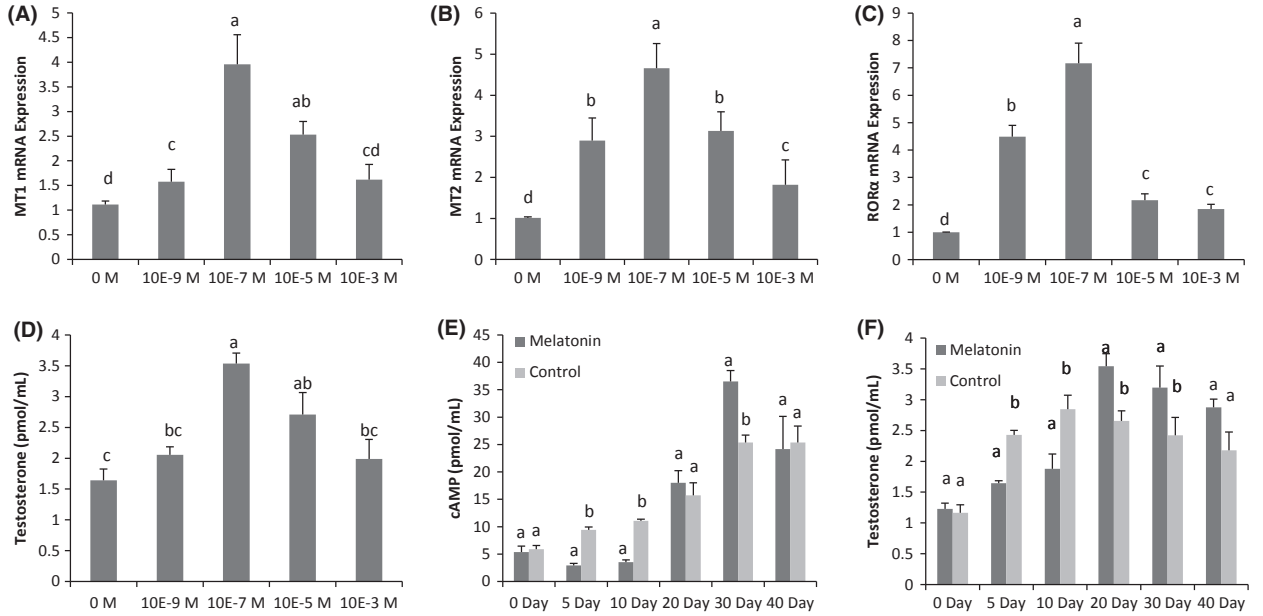

Fig. 5. Effects of long term of melatonin treatment on cAMP and testosterone secretion. (A) MT1, (B) MT2, and (C) RORα expression patterns in cultured cells with various melatonin concentrations. (D) Testosterone content on the 30th day of culture. (E, F) cAMP and testosterone concentrations in 10^{-7} M melatonin-treated group at indicated time. Data are expressed as means ± S.E.M.; different superscript letters (a–d) in each column represent statistical significant differences ($P < 0.05$).

lower in melatonin 10^{-7} M group than that in control group at the early stage of cell culture. However, at the stages of 20th and 30th day of the cell culture, melatonin (10^{-7} M) significantly promoted the medium testosterone content as compared to the control group ($P < 0.05$). The data suggest that the influence of melatonin on testosterone production in the SSCs culture system was time and dose sensitive manner. The similar influence of melatonin on cAMP production was observed in the cell culture (Fig. 5E,F). This observation implied that melatonin may promoted the in vitro spermatogenesis in a time- and dose-dependent manner through regulating both cAMP and testosterone production.

Exogenous melatonin stimulated expressions of LHR, and postmeiotic-related genes

At a 10^{-7} M concentration, melatonin significantly upregulated LHR expression ($P < 0.05$) and had no significant impact on FSHR expressions ($P > 0.05$) (Fig 6A). In addition, melatonin upregulated the expressions of both testosterone synthesis rate-limiting enzyme gene (StAR), SSCs differentiation genes (Stra8, c-kit), and oncogene (c-Fos and c-Jun) (Fig. 6B,C). The postmeiotic genes (Tnp1, Prm1, and Tnp2) and c-Fos were also upregulated by melatonin in the differentiated haploid sperm-like cells as compared with control cells (Fig. 6D,E). In addition, melatonin did not influence H19 and CDYL, but Dnmt3a was dramatically upregulated by melatonin ($P < 0.05$) (Fig. 6F).

Antioxidant and anti-apoptotic effects of melatonin on SSCs differentiation

NO content in melatonin (10^{-7} M)-treated group was significantly lower than that in control group ($P < 0.05$) (Fig. 7A). This tendency was maintained during entire SSCs differentiation process in the melatonin-treated group. In addition, melatonin upregulated Bcl-2 expression and downregulated Bax expression and this phenomenon was more obvious in the late stage of the cell culture than that in the early stages (Fig. 7B,C).

Discussion

Testicular microenvironments provide a variety of hormones and cytokines which support and promote SSCs differentiation. Without these microenvironments, the

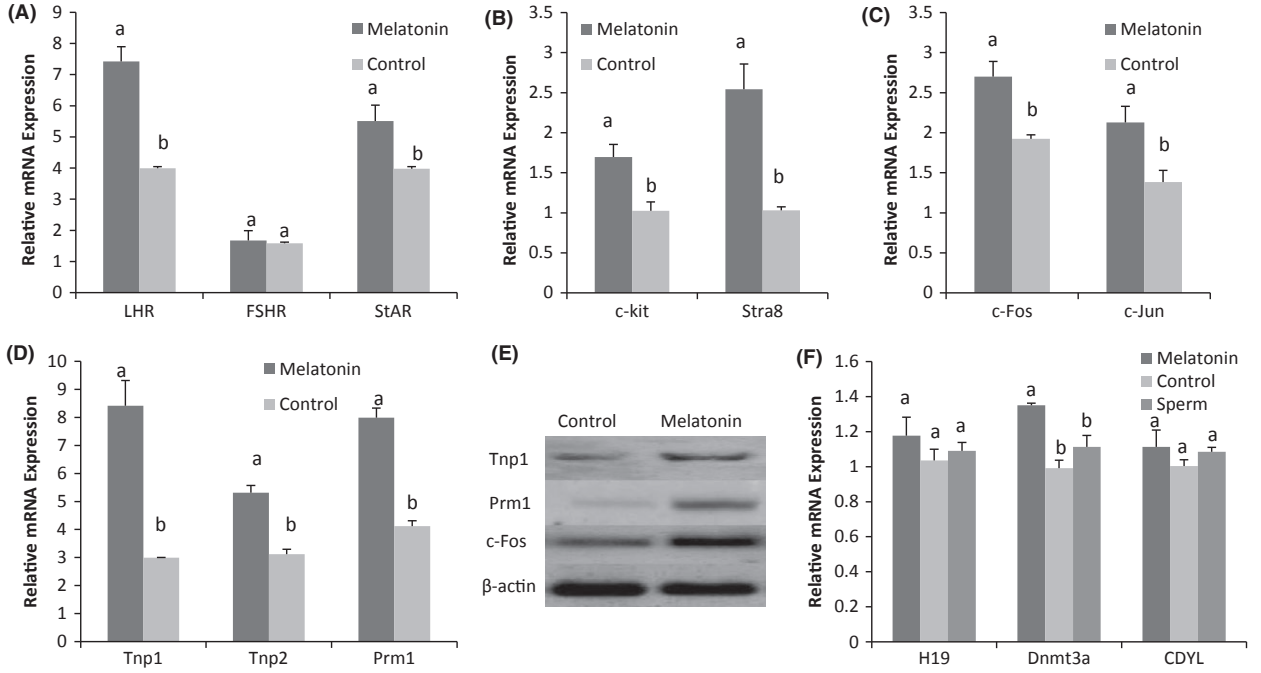

Fig. 6. Effects of melatonin 10^{-7} M on the expressions of hormone receptor genes, oncogenes, and postmeiotic genes. (A) mRNA levels of hormone receptor genes. (B) Expression patterns of SSCs differentiation genes (Stra8, c-kit). (C) Expression patterns of oncogenes (c-Fos and c-Jun). (D) Expression patterns of postmeiotic genes (Tnp1, Tnp2, and Prm1). (E) Prm1, Tnp1, and c-Fos in cultured cell detected with Western blotting method. (F) Expression patterns of DNA methylation genes (H19, Dnmt3a) and histone acetyl transferase genes (CDYL). Data are expressed as means ± S.E.M.; the letters (a and b) in each column represent statistically significant differences ($P < 0.05$).

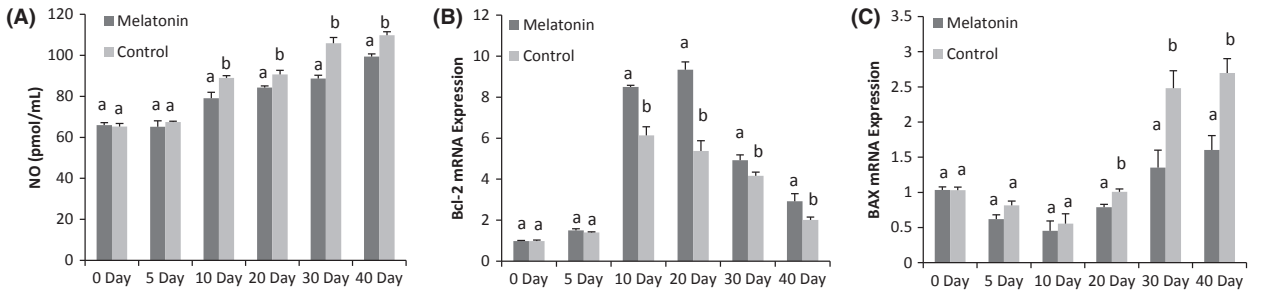

Fig. 7. Effects of melatonin at 10^{-7} M on NO level, expressions of anti-apoptosis (Bcl-2), and pro-apoptotic gene (Bax). (A) The levels of NO. (B) Bcl-2, and (C) Bax transcription in culture cells. Data are expressed as means ± S.E.M.; different superscript letters (a and b) in each column identify statistical significant differences ($P < 0.05$).

development of the SSCs would be impeded. Therefore, many efforts have been made to simulate the testicular microenvironments and to promote SSCs differentiation into functional sperms under in vitro conditions [39]. Testicular cells have the capacity to reconstitute seminiferous tubule ectopically which provides possibility for reconstitution of testicular microenvironments in vitro; thus, it will provide the supporting factors for SSCs differentiation [40, 41]. Among many factors, retinoic acid (RA) is a powerful cytokine for cell differentiation and antioxidation [42]. It was reported that RA deficiency caused spermatogonial cells degeneration and with RA supplementation the spermatogenesis was fully recovered [43]. RA binding with retinoic acid receptor gamma (RARγ) induces type A SSCs differentiation into type B spermatogonia [44]. RA supplementation at a concentration of 10^{-7} M induced bovine mGCs differentiation into haploid sperm cell in vitro [45]. Stra8 is the target gene of RA-signaling pathway [46]. Bone morphogenetic protein-4 (Bmp-4)/Smad pathway not only involves in neural stem cell proliferation and differentiation [47] but also participates in spermatogonia differentiation by upregulate c-kit expression [48].

Melatonin is a ubiquitous molecule. It is present in almost all tissues and cells of organisms including neural cells [49] and testicular cells [50]. It was reported that melatonin promoted neural stem cell differentiation [51]. The potential mechanism is that melatonin, similar to the RA mentioned above, binds to retinoic acid receptor

(RAR) subfamily, RZR/RORα, which has homology with MT1 [52]. In the subfamily of these two ligands, there are RZRα1, RZRβ, and RORα which probably mediate the melatonin-signaling pathway. RZRβ mainly exists in the nervous system. RZRα is expressed in testis [53]. Aromatase could be activated by RORα and, as a result, conversion of androgen into estrogen was promoted [54]. MT1 and MT2 are found in the testes of many species, where they mediate the functions of melatonin on testes. Furthermore, testicular somatic cells (Sertoli cells and Leydig cell) were also regulated by melatonin via its MT1- and MT2-signaling pathways in time- and dose-dependent manners [55]. In the current study, there is no differentiated germ cell being found in the seminiferous tubules of the 2-month-old sheep testis. While melatonin receptors were found in sheep testis, melatonin 10^{-7} M supplementation upregulated MT1, MT2, and RORα expressions [56]. The key genes (Stra8, c-kit) which are related to SSCs differentiation were upregulated by melatonin. The melatonin supplementation promoted SSCs differentiation into functional haploid sperm-like cells under in vitro conditions. In addition to the melatonin membrane receptors, its nuclear binding site, RZRα, is assumed, at least partially, to mediate melatonin-signaling pathway to active SSCs differentiation.

The differentiated round spermatid has a complete haploid chromosome and has the capacity to fertilize metaphase-II stage oocyte. However, the rate of successful fertilization and the birth rate after fertilization from the round spermatid injection (ROSI) are quite low [57]. One of the reasons may be the lack of oocyte activation factors, which result in deficiency in the activation of oocyte. In this study, when the single flagellum spermatid generated by melatonin supplementation was injected into oocyte, relatively high level of spermatid reconstructed embryonic blastocyst rate was achieved. This indicated that the haploid sperm cells generated from the melatonin treatment exhibited some potentials to activate the oocyte and then to promote its further development.

Mechanistic study indicated that the effects of melatonin on spermatogenesis might partially involve c-Fos and c-Jun that are important cytokines for primordial germ cell differentiation. c-Fos, expressing in type B spermatogonia, involves regulation of gene transcription during meiosis. The results from the GT1-7 cell line study indicated that, by initiation of PKC and MAPK pathway, melatonin activated c-Fos and JunB [58]. Other studies showed that activation of melatonin membrane receptors also increased the activity of c-Jun-N-terminal kinase (JNK) [59]. c-Fos and c-Jun were proposed to mediate the long-term responses of Leydig cells to some hormones in vivo [60]. These genes mentioned above have recently been linked to the molecular events involved in the transcription of the StAR gene [61]. Our results revealed that melatonin upregulated expressions of c-Fos, c-Jun, and StAR genes in sheep testis under in vitro differentiation system.

Other import factors on SSCs differentiation and spermatogenesis are reproductive hormones. Effects of melatonin on the levels of reproductive hormones are variable and depend on species and physiological conditions of animals [62]. Melatonin significantly upregulated LHR expression and enhanced the progesterone secretion in ovary granule cells; however, it downregulated the FSHR expression [63, 64]. In current study, the similar results were observed in testicular cells. Melatonin stimulated testosterone secretion in human prostate epithelial cells [65]. In rams, prolonged melatonin supplementation significantly enhanced their testosterone and FSH levels and resulted in their advanced sperm generation [66]. In contrast, short-term melatonin injection had an opposite effect in rams [67]. This indicated that to promote LH secretion, a proper concentration and/or long term of melatonin administration was required. Our results showed that melatonin application suppressed testosterone secretion in the early stage of cell culture and promoted its production at later stage as well as upregulated the LHR expression. These may also result in the promotion of spermatogenesis with melatonin application.

Follicle-stimulating hormone, luteinizing hormone, and their receptors expressions were impacted by cAMP [68]. It was found that melatonin accelerated the accumulation of cAMP in SHY-SY5Y cell (a human neuroblastoma cell line) via calcium/calmodulin-dependent pathway rather than via G protein mechanism [69]. Results from PT cell study (cell line from sheep anterior pituitary of pituitary gland) confirmed this observation. One of MTs functions was to rapidly inhibit cAMP synthesis [70]. In melatonin prolonged-treated PT cells, adenylate cyclase is kept in a sensitizing state, and cAMP basic level was enhanced [71]. In this study, cAMP level was reduced by melatonin at early stage of the cell culture, so did the testosterone synthesis. In prolonged incubation with melatonin, the cAMP synthesis and secretion were significantly increased. As a result, the expressions of postmeiosis-related genes (Prm1, Tnp1, and Tnp2) were upregulated. These may relate to different melatonin-signaling pathways mediated by subtypes of melatonin receptors.

Establishing a mature epigenetic status in the genome is also considered as the spermatogenesis. DNA methylation in CpG sites is one of the epigenetic modifications that regulate gene expression. H19 and Dnmt3a are the main genes to regulate methylation status in the sperm [72]. Activation of histone acetyl transferase (HAT) is important for histone acetylating. HAT is encoded by chromodomain protein Y-like (CDYL). In the round sperms, histone acetyltransferase is selectively expressed [73]. Melatonin has been reported to involve in epigenetic regulation including the modification of the genome methylation status [74]. In consistent with other's report, we observed that Dnmt3a expression was significantly upregulated in melatonin-treated SSCs culture system. This suggests that melatonin regulates epigenetic status during spermiogenesis.

In the study, we observed that apoptosis caused the low yield of the differentiated sperm cells. Both Bcl-2 family and Fas/Fas ligands were involved in the cellular apoptosis and spermatogenesis. Overexpressed Bax induced apoptosis in spermatogenic cells, and Bcl-2 overexpression inhibited apoptosis [75]. Melatonin (1 mg/kg) was given to high fat-fed mice for 12 wk significantly increased their spermatogenic cells and reduced their germ cell apoptosis, especially on their spermatocyte and round sperm cells

[76]. Melatonin treatment significantly enhanced Bcl-2 expression and decreased pro-apoptotic gene Bax expression in cultured bovine embryo [77]. Similar results were observed in the current study, that is, melatonin downregulated the pro-apoptotic gene of Bax, while upregulating anti-apoptotic gene Bcl-2 in culture system. These effects of melatonin may lead to less cellular apoptosis and more cells differentiate into sperms as we observed in the current study.

Melatonin is a wide-spectrum antioxidant, and it scavenges varieties of reactive oxygen and nitrogen species (ROS and RNS) including superoxide anion, hydroxyl radical, hydrogen peroxide, and NO [78]. Melatonin can also stimulate the synthesis of variety of antioxidant enzymes [79]. The impacts of ROS or RNS on spermatogenesis depend on their levels. Low levels of these species are necessary for spermatogenesis [80]. However, high levels of these species result in lipid peroxidation and DNA damage of the sperms [81]. Melatonin application effectively protected spermatozoa from lipid peroxidation and DNA-oxidative damage in the samples of humans and rams [82, 83]. Melatonin was also used to improved ram semen quality [84].

Based on observations mentioned above, we reported for the first time that melatonin at a concentration of 10^{-7} M significantly promoted the primitive spermatogonium to differentiation into functional haploid sperm cell under in vitro culture conditions. The mechanisms are multifold. These include melatonin nuclear and membrane receptor mediated events, upregulations of gene expression related to spermatogenesis, alterations of the reproductive hormones and cytokines as well as antioxidant activities of melatonin. Most importantly, the in vitro differentiated functional haploid sperm cells promoted by melatonin are functional. When the haploid sperm cell was microinjected into the oocyte, the embryo, at least, can develop into blastula. Melatonin supplementation provides an effective alternative to improve the sperm quality and production in the in vitro sperm differentiation system. To further improve the efficiency of melatonin on the in vitro spermatogenesis and to clarify its detailed signaling pathways are our future research projects.

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Competing financial interests

The authors declare that they have no competing interests.

Author contributions

Shou-Long Deng, Zheng-Xing Lian, and Yi-Xun Liu conceived and designed the experiments. Shou-Long Deng, Zhi-Peng Wang, Guo-Shi Liu, and Bao-Lu Zhang performed the experiments. Shou-Long Deng and Guo-Shi Liu analyzed the data. Su-Ren Chen, Ji-Xin Tang, Jian Li, Xiu-Xia Wang, Jin-Mei Cheng, Xiao-Yu Li, and Cheng Jin contributed reagents/materials/analysis tools. Shou-Long Deng, Kun Yu, Guo-Shi Liu, and Yi-Xun Liu wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Movie S1. On the 35th day of culture, sperm-like cells with single tail was observed.