

Research Article

# Development of Testes and Expression of $\beta$ -catenin in Testes Tissue of Mice Born from Vitrified/Cryopreserved Embryos

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## Abstract

**Objective:** To evaluate the impact of vitrifying embryo from mice on the development of testes of offspring.

**Materials and methods:** This study included 60 male offspring mice, 30 male mice from transferred vitrified embryos and 30 male mice from transferred fresh embryos. We tested the morphology and  $\beta$ -catenin gene expression of testes of difference time after they born.

**Results:** The HE staining results of the testes of the vitrification group and the fresh group showed dark blue staining in the nuclei and varying degrees of red staining in the cytoplasm and fibrous tissue. And there were no significant differences in the morphology of the testis tissues at the different developmental stages between the two groups. With the use of reverse transcription polymerase chain reaction and Western blotting, the data showed that there were no significant differences in the expression of  $\beta$ -catenin in the testes between the two groups of mice at different developmental stages.

**Conclusion:** These data suggest that vitrifying pre-implantation embryos may have no effects on morphology and  $\beta$ -catenin gene expression of the testes of offspring.

**Keywords:** Vitrifying; Testes; Offspring; B-Catenin; Development

## Introduction

In recent years, with the development of reproductive medicine, embryo vitrification/cryopreservation methods have become an important derivative technology in assisted reproduction techniques (ARTs) [1,2] Embryo vitrification/cryopreservation has been utilized for the birth of many babies in China and in other countries. However, during the process of vitrification/cryopreservation, the exposure of the embryo to a high concentration of cryoprotectant may be toxic for the embryo. Furthermore, the cryopreservation process itself may also cause significant intracellular changes, resulting in genomic chromosomal changes in the embryo [3,4] therefore the relative safety of vitrification/cryopreservation methods has been of wide concern.

Clinically, the impact of vitrification/cryopreservation on the development of offspring has drawn more attention, including whether the method causes congenital malformations, chromosomal abnormalities, and acquired developmental abnormalities in neonates. However, to date, no agreements on these issues have been reached. Studies have demonstrated that males born using ART showed manifestations of hypospadias, reduced serum testosterone levels and low semen quality [5]. A few studies have suggested that the steps involved in intra-cytoplasmic sperm injection (ICSI), i.e., superovulation, *in vitro* culture, and mechanical stimulation, cumulatively impact the expression of reproduction-related genes [6]. However, most of these published studies lacked sufficient clinical controls and utilized small sample sizes, which included cases in pregnancy or with short post-natal periods. The observations of congenital fetal malformations do not fully explain the impact of vitrification/cryopreservation on neonates. At the same time, because vitrification/cryopreservation technology has only been relatively recently clinically applied, data are lacking regarding long-term follow-ups after birth. Furthermore, safety studies on long-term effects of vitrification/cryopreservation on organ development have rarely been reported. No studies on whether vitrification/cryopreservation causes morphological changes in testicular development or differences in reproduction-related genes have been reported.

The Wnt/ $\beta$ -catenin signal pathway is a classic signal transduction pathway of Wnt, which participates in the proliferation and differentiation of normal cells and stem cells and is closely related to the development of many types of tumors [7]. As a bifunctional protein,  $\beta$ -catenin simultaneously affects cell adhesion and mediates the classical cell signal transduction of Wnt/ $\beta$ -catenin.  $\beta$ -catenin is significantly expressed in testicular supportive cells and germ cells after meiosis, suggesting that  $\beta$ -catenin plays a role in spermatogenesis. In male gonads, when using a protamine promoter-driven Cre transgene (PRM-CRE), the conditional inactivation of the  $\beta$ -catenin gene (CTNNB1) has been reported to cause partial infertility, reduced sperm count, and abnormal spermatogenesis [8].

In this study, we investigated the development of testes and the expression of  $\beta$ -catenin in the testis tissue of mice born after vitrification/cryopreservation. This study is expected to provide effective laboratory data for the safe development of vitrification/cryopreservation in clinical practice.

## Materials and Methods

### Main materials

**Experimental animals:** In this study, a total of 200 healthy white female Kunming mice, 6-7 weeks old, clean grade, weighing 25  $\pm$  3 g

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and 60 healthy white Male Kunming mice, 8-10 weeks old, clean grade, weighing  $32 \pm 5$  g were purchased from the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology, China. The mice were reared with freely accessible pellet feed and drinking water. The room temperature was  $24 \pm 2^\circ\text{C}$ , and the humidity was 40%-60%, with a 12 h-12 h light-dark cycle and regular ventilation.

**Main reagents:** The main reagents used in this study included pregnant mare serum gonadotropin (PMSG; Ningbo Hormone Product Co., Ltd., China), human chorionic gonadotropin (HCG; Ningbo Hormone Products Co., Ltd.), *in vitro* fertilization (IVF) culture medium 30 (Gibco, USA), and F12 culture medium (Gibco, USA). Fetal bovine serum and 0.25% trypsin were purchased from Gibco; mouse anti-mouse  $\beta$ -catenin monoclonal antibody, FITC-goat anti-mouse fluorescent secondary antibody, and FITC-goat anti-rabbit fluorescent secondary antibody were purchased from Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., China. Rabbit anti-mouse C-myc polyclonal antibody, RNA extraction kits, and RT-PCR kits were purchased from Wuhan Boster Biological Technology Co., Ltd., China.

## Methods

**Preparation of embryos with vitrification/cryopreservation:** According to method reported by Murphy and Martinuk [9], murine embryos were obtained. Based on the grading standard reported by Steer et al. [10] the embryos in the grade I-II cleavage stage were selected for subsequent experiments (with more than six cells, showing similar cell sizes, and having a debris volume  $\leq 50\%$  of the total volume of the embryo). An embryo vitrification/cryopreservation method reported by Linlin et al [11] was applied, with resuscitation after 2 weeks. High quality, fresh embryos and vitrified/cryopreserved embryos after resuscitation were selected and cultured for 1-3 hand then transferred to either side of the uterus in the female mice showing false pregnancy for 63-67 h, with 8 embryos on each side. After 17-20 day pregnancy, natural birth occurred.

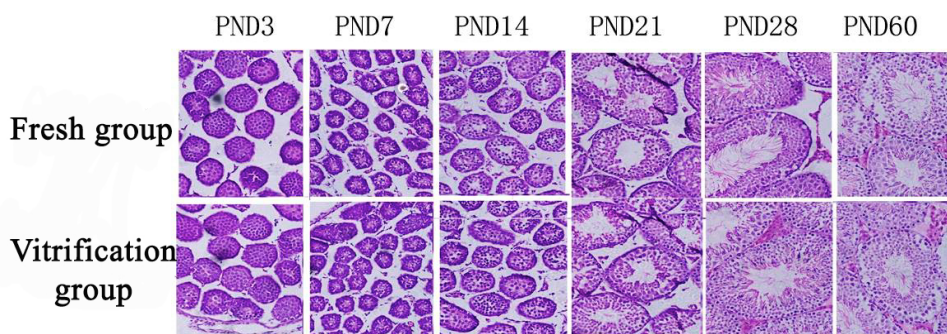
**Specimen processing and grouping:** Male offspring mice born after the transfer of the fresh embryos (fresh group) and the vitrified/cryopreserved embryos (vitrification group) were sacrificed on post-natal days 3, 7, 14, 21, 28 and 60 by cervical dislocation. Under sterile conditions, the testes were collected via laparotomy, and six mice were used at each time point for each group. At the same time, the left testis tissues of three offspring mice in each group were fixed with 4% paraformaldehyde at  $4^\circ\text{C}$  overnight, followed by paraffin-embedded sectioning and hematoxylin and eosin (HE) staining for morphological observation. The remaining sections were prepared for immune histochemical staining. The right testis tissues were stored at  $-70^\circ\text{C}$  for detection by fluorescence quantitative reverse transcription (RT) PCR. The testis tissues of the remaining three offspring mice of each group were stored at  $-70^\circ\text{C}$  for Western blot analysis.

**Immuno histochemical staining for  $\beta$ -catenin:** Testis tissues were fixed with 4% paraformaldehyde at room temperature followed by paraffin-embedded sectioning. Immunohistochemical staining was performed according to the instructions of the SP immunohistochemistry kit and the diaminobenzidine (DAB) staining kit (purchased from Beijing Zhong Shan Biological Technology Co., Ltd.). The samples were washed with PBS for 10 min (x3) and blocked with goat serum for 30 min.  $\beta$ -catenin primary antibody (1:300) was added, and the sections were incubated in a humidified chamber at  $4^\circ\text{C}$  overnight, followed by phosphate buffer (PBS) washes for 10 min (x3). Subsequently, the samples were incubated with 30 mm biotin-labeled goat anti-rabbit IgG secondary antibody working solution(1:200)

at  $37^\circ\text{C}$  in the dark for 1 h, followed by PBS washes for 10 min (x3). Horseradish enzyme-labeled streptavidin working solution was used for counter stain. After mounting, the results were observed using an inverted phase contrast microscope. The primary antibody was replaced with PBS as a negative control. The localization and expression of  $\beta$ -catenin in the testis tissues of the two groups of offspring mice were detected. The positive expression of  $\beta$ -catenin was reflected as brown particles in the cell membrane and cytoplasm.

**The expression of  $\beta$ -catenin detected by RT-PCR:** Total RNA was extracted from 0.05-0.10 g of the testis tissues of the two groups of mice using TRIzol. The RNA was dissolved in 50  $\mu\text{L}$  of diethyl pyrocarbonate (DEPC)-treated water. RNA purity and concentration were estimated by measuring the OD260 and OD280 after appropriate dilutions. The integrity of the total RNA was identified by formaldehyde denaturing agarose gel electrophoresis. An RT reaction with 1  $\mu\text{L}$  of total RNA was performed to synthesize cDNA from each sample, and phosphoglycerate dehydrogenase (GAPDH) was used as an internal reference. The mRNA sequence of the  $\beta$ -catenin gene was obtained from the GenBank database. Primers were designed using GeneRunner software and synthesized by Beijing AuGCT Biotechnology Co., China. The sequences of the primers were as follows:  $\beta$ -catenin, sense: 5'-TAC TGT TCT ACG CCA TCA CGA C-3', anti-sense: 5'-CCT TCC ATC CCTTCC TGCTTA-3', with an amplified fragment length of 448 bp; and GAPDH, sense: 5'-TGG AGT CTA CTG GCG TCT TC-3', anti-sense: 5'-ITC ACA CCC ATC ACA AAC ATG -3', with an amplified fragment length of 121 bp. The reaction volume was 25  $\mu\text{L}$  (10  $\mu\text{M}$  upstream primer, 1  $\mu\text{L}$ ; 10  $\mu\text{M}$  downstream primer, 1  $\mu\text{L}$ ; 2.5 mM dNTPs, 2  $\mu\text{L}$ ; 10x Taq buffer with  $\text{MgCl}_2$ , 2.5  $\mu\text{L}$ ;  $\text{ddH}_2\text{O}$ , 14.5  $\mu\text{L}$ ; cDNA template, 2  $\mu\text{L}$ ; and Taq DNA polymerase, 2  $\mu\text{L}$ ). The reaction conditions were as follows: pre-denaturation at  $94^\circ\text{C}$  for 4 min; denaturation at  $94^\circ\text{C}$  for 35 s, annealing at  $56^\circ\text{C}$  for 45 s and extension at  $72^\circ\text{C}$  for 60s, for 32 cycles; and a final extension at  $72^\circ\text{C}$  for 10 min. The PCR products were electrophoresed on a 2% agarose gel (80 V, 40 mA, 30 min). Gel images were obtained with a gel imaging system.

**The expression of  $\beta$ -catenin protein detected by western blot:** The testes collected on post-natal days 3, 7, 14, 21, 28 and 60 were weighed and 100 g/L protein lysate (with 1:100 protease inhibitors) was homogenized on ice After centrifugation at  $4^\circ\text{C}$ , 13,000 r/min  $\times$  20 min, the supernatants were collected and the protein contents were measured using an ultraviolet spectrophotometer. After the protein concentrations were determined using a nucleic acid protein analyzer, equivalent amounts of protein (20  $\mu\text{g}$ ) were subjected to SDS-PAGE. Proteins were separated via electrophoresis (1 h, 20 mA) through a 3.5% stacking gel and 10% separating gel, followed by Coomassie brilliant blue (R-250) staining overnight. Protein separation was observed. After ensuring that the proteins were well separated, they were transferred onto a nitrocellulose membrane, followed by Ponceau red staining for 1-2 min. After rinsing briefly, the sections with the marker and standards were cut off, and the remaining membrane sections were blocked with 1% bovine serum albumin (BSA; 1 h,  $37^\circ\text{C}$ ) and incubated with  $\beta$ -catenin antibody (1:800) overnight at  $4^\circ\text{C}$ . After washing with PBST (w/v 0.05% Tween 20 in PBS buffer) three times and drying, biotin-labeled secondary antibody was added. The membrane was incubated at  $37^\circ\text{C}$  for 30 min and subsequently, rinsed as before. Horseradish peroxidase (HRP)-labeled streptavidin was drop wise added, and the membranes were incubated at  $37^\circ\text{C}$  for 30 min. After rinsing with PBST three times, a DAB coloring reaction was performed and was terminated with tap water. Images of the samples were captured. The optical density (OD) values of the target bands in the Western blot were obtained by scanning with a Bio-Rad image analysis system. Quantity One software was used for analysis. A UVP gel imaging analysis system was used to analyze the



**Figure 1:** Histological pictures of postnatal testis tissues development in the mouse in vitrification group and fresh group. There was no major malformation from PND3 to PND60.(HE  $\times 400$ )

OD value of each target band; this value reflected the relative expression level of the corresponding protein. A large OD value indicated strong expression of the target protein and a small OD value indicated weak expression of the target protein.

### Statistical methods

The SPSS 13.0 software package was used for statistical analyses. The measurement data are represented as  $\bar{x} \pm s$ . One-way analysis of variance (ANOVA) was used to compare differences between continuous variables. The chi-square test was used to compare categorical variables. Differences with  $P < 0.05$  were considered significant.

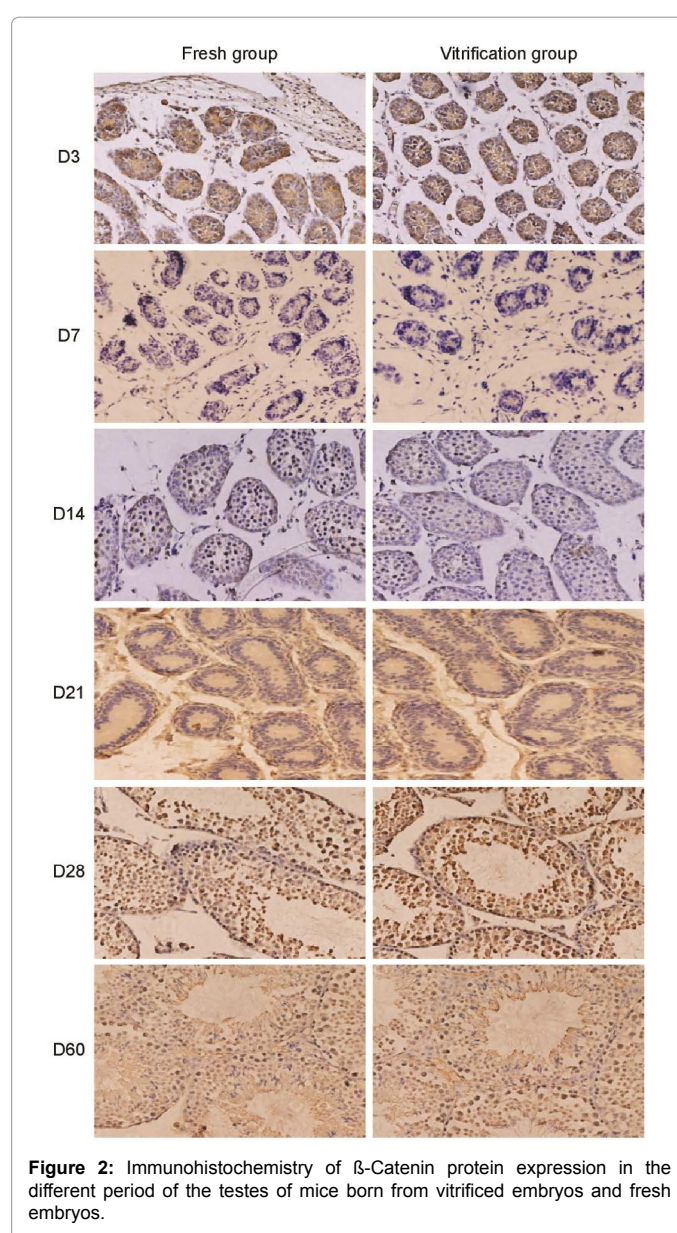
### Results

#### Morphological observation of the testes of the two groups of offspring mice

The HE staining results of the testes of the vitrification group and the fresh group showed dark blue staining in the nuclei and varying degrees of red staining in the cytoplasm and fibrous tissue. There were no significant differences in the morphology of the testis tissues at the different developmental stages between the two groups. As shown in Figure 1, at post-natal day 3 (PND3), the spermatogenic tubal lumen was not visible, parietal cells were arranged in a monolayer, only spermatogonia and naive supportive cells were observed and were all attached to the basement membrane, and testicular interstitial cells were not visible. At post-natal day 7 (PND7), the spermatogenic tubal lumen was small, cells were arranged in 2-3 layers, spermatogonia proliferated and differentiated, and the supportive cells and testicular stroma began to develop. At post-natal day 14 (PND14), the spermatogenic tubal lumen continued to enlarge and spermatogonia proliferated and differentiated, showing interstitial cells. At post-natal day 21 (PND21), the number of cell layers increased, spermatogenic cells were observed at all levels, proliferated progeny cells were distributed near the surface of the lumen (showing intercellular bridges and developing sperm) and the supportive cells and interstitial cells were mature. At post-natal day 28 (PND28), the spermatogenic tubal lumen was enlarged, showing nearly matured sperm and testicular development was nearly completed. At post-natal day 60 (PND60), a large number of mature sperm was observed in the spermatogenic tubal lumen, indicating no significant aberrant testicular development or delayed development after vitrification/cryopreservation (Figure 1).

#### The expression and localization of $\beta$ -catenin in the testis tissue of the two groups of mice

Six mice were selected from both the fresh group and the vitrification group at PND7, PND14, PND21, PND28 and PND60. As shown in Figure 2,  $\beta$ -catenin expression in the testes of the two



**Figure 2:** Immunohistochemistry of  $\beta$ -Catenin protein expression in the different period of the testes of mice born from vitrified embryos and fresh embryos.

groups was compared by immunohistochemistry, and the impact of vitrification/cryopreservation on the testicular development of the offspring was further investigated.  $\beta$ -catenin was expressed in the

epithelium and stroma, with no significant differences between the two groups at different developmental stages ( $P > 0.05$ ) (Figure 2).

Representative images were showed on postnatal days (PNDs) 3, 7, 14, 21, 28 and 60. The average  $\beta$ -Catenin immune reactivity scores in each group were:  $4.4 \pm 0.9$ ,  $5.5 \pm 0.6$ ,  $6.7 \pm 0.8$ ,  $7.8 \pm 0.7$  and  $7.9 \pm 0.5$  in vitrified group and  $4.2 \pm 0.3$ ,  $5.7 \pm 0.8$ ,  $6.3 \pm 0.2$ ,  $7.5 \pm 0.5$  and  $7.8 \pm 0.8$  in fresh group, respectively. There was no significant difference in testes sections between vitrified group and fresh group ( $P > 0.05$ ).

### The expression of $\beta$ -catenin mRNA in the testes of the two groups of mice

As shown in Figure 3, there were no significant differences in the expression of  $\beta$ -catenin mRNA in the testes between the two groups of mice at different developmental stages ( $P > 0.05$ ) (Figure 3).

RT-PCR was repeated at least three times.  $\beta$ -Catenin expression was analyzed by densitometry and normalized to GAPDH. Average  $\beta$ -Catenin/GADPH ratio in each group was:  $0.12 \pm 0.05$ ,  $0.23 \pm 0.02$ ,  $0.25 \pm 0.04$ ,  $0.26 \pm 0.01$ ,  $0.32 \pm 0.02$  and  $0.33 \pm 0.13$  in vitrified group, and  $0.13 \pm 0.08$ ,  $0.24 \pm 0.03$ ,  $0.26 \pm 0.01$ ,  $0.27 \pm 0.05$ ,  $0.32 \pm 0.05$  and  $0.34 \pm 0.16$  in fresh group, respectively. There was no significant difference between vitrified group and fresh group ( $P > 0.05$ ).

### The expression of $\beta$ -catenin protein in the testes of the two groups of mice

As shown in Figure 4, there were no significant differences in the expression of  $\beta$ -catenin protein in the testes between the two groups of mice at different developmental stages ( $P > 0.05$ ) (Figure 4).

Western analysis performed on testes in vitrified group and

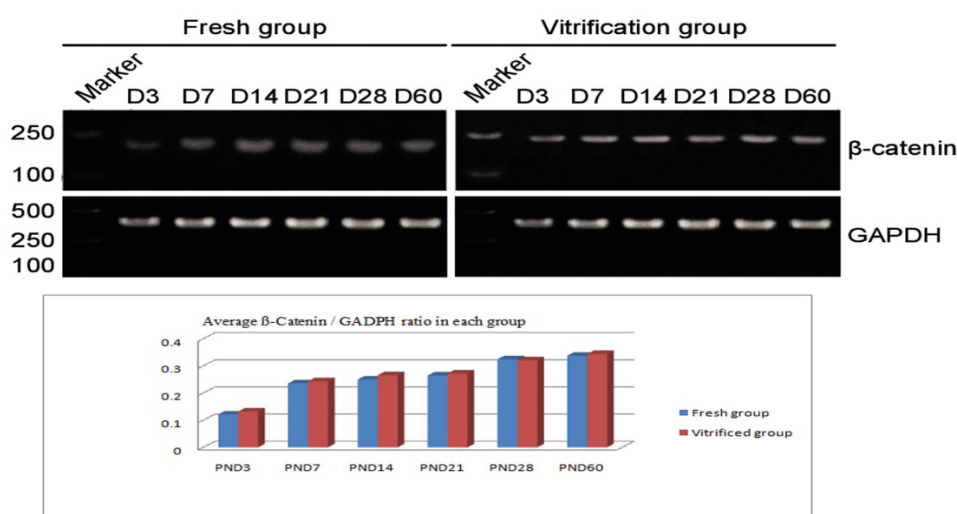


Figure 3: RT-PCR analysis of  $\beta$ -catenin mRNA in the testes of the two groups of offspring mice.

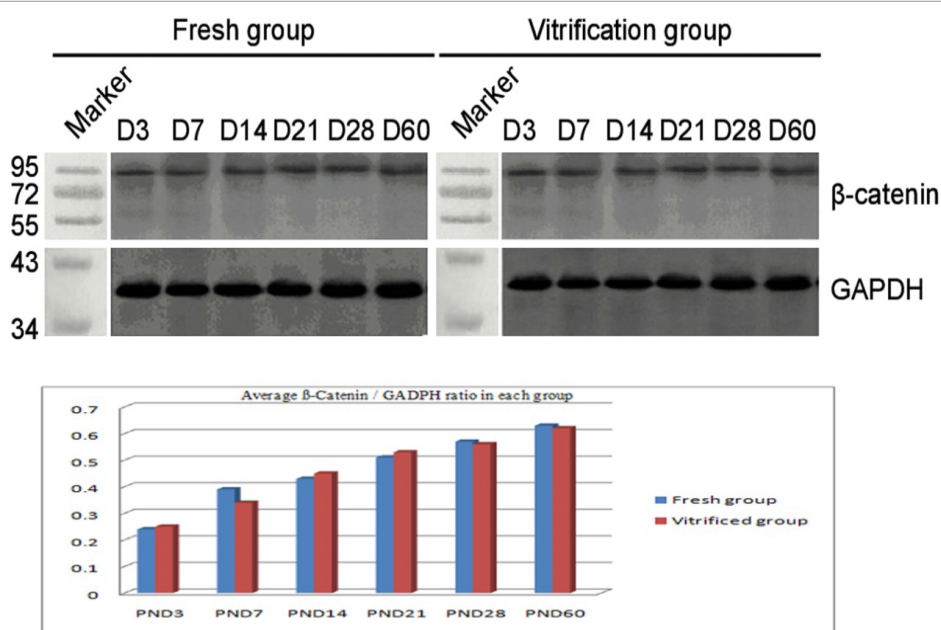


Figure 4: The expression level of  $\beta$ -catenin protein in the testes of the two groups of offspring mice.

fresh group (five animals in each postnatal stage in each group). A representative western blot image is shown  $\beta$ -catenin protein expression was analyzed by densitometry and normalized to GAPDH values. Western blot analysis and quantification were repeated at least three times. There was no significant difference between two groups ( $P > 0.05$ ).

## Discussion

Since vitrification/cryopreservation technology was first reported by Rall and Fahy in 1985 [12] the technology has been widely applied in clinical practice. Due to reports that vitrification/cryopreservation technology causes damage in embryos [3,13,14] the safety of vitrification/cryopreservation for the cryopreservation of human embryos has received extensive attention [15] in this study, we first observed the morphology of testicular development for male mice born from embryos after vitrification/cryopreservation. At PND1-PND3, spermatocytes were located on the medial side of supporting cells and the central part of spermatogenic tubules. The supportive cells were near the basement membrane. At PND3-PND4, a small number of spermatocytes were attached to the basement membrane, showing the development of primary A-shaped spermatogonia and adjacent cells were trapped in one or more supportive cells. However, most of the spermatogenic cells remained located in the center of the seminiferous tubules. At PND5-PND7, almost all of the spermatogenic cells were attached to the basement membrane and had proliferated and developed to undifferentiated spermatogonial stem cells (SSCs) that were smaller than the spermatogenic cells. After PND5, a large number of the testicular stromal cells were degraded and died due to the lack of stimulation of the gonadotropin produced in the placenta. At the same time, because the supportive cells were relatively immature, the secretions of cell viability factors and cell nutrients involved in the generation of sperm were lacking. Thus, the spermatogonial stem cells were not induced to differentiate. After PND10, stromal cells began to develop after being stimulated by a certain amount of interstitial cell-stimulating hormone (ICSH) produced in the pituitary. This was accompanied with the synthesis of small amounts of androgens, which could bind to androgen receptors on the supportive cell membrane and thereby induce the proliferation and differentiation of A-type spermatogonial stem cells. The number of spermatogenic cells, including B-type spermatogonia and primary spermatocytes, appearing on the section surface increased. The functions of two types of non-spermatogenic cells were not yet mature, resulting in a lower production of inducing hormones and cell nutrients. Thus, the differentiated cells could not develop into mature sperm and, subsequently, showed apoptotic tendencies at different stages of development. After PND20, with the development of the hypothalamus-pituitary-gonad axis and the maturation of stromal cells and supportive cells in structure and function, the spermatogenic cells proliferated and developed at all levels, showing various cell combinations and intercellular bridges. The same results were observed in the two groups of mice at different developmental stages. The results demonstrated that the testicular development processes of the two groups of mice were essentially the same, indicating that the vitrification/cryopreservation process had no obvious impact on the morphological development of the testes.

To further study the impact of embryo vitrification/cryopreservation on testicular development, the expression of  $\beta$ -catenin during testicular development in the two groups of offspring mice was investigated.  $\beta$ -catenin is primarily located in various cell membranes and in the cytoplasm. The  $\beta$ -catenin located in cell membranes mediates cell adhesion; the  $\beta$ -catenin in the cytoplasm is involved in the expression of genes, leading to cell proliferation, differentiation and apoptosis [16]

In the process of embryonic development, most downstream target genes of the Wnt/ $\beta$ -catenin signaling pathway are important genes involved in cell cycle regulation, i.e., regulating generation, growth and development [17] The continuous activation of the Wnt/ $\beta$ -catenin signaling pathway in the Sertoli cells of post-natal mice could result in infertility in male mice, which is manifested as degenerated seminiferous tubules, loss of spermatogenic cells, suppressed maturation of Sertoli cells and proliferation of Sertoli cells after adulthood [18] However, the inactivation of the  $\beta$ -catenin gene (CTNNB1) could also lead to partial infertility, reduced sperm count and abnormal spermatogenesis [8] Thus, the expression and level of  $\beta$ -catenin play important roles in the generation of sperm.

In the present study, real-time PCR and Western blot analysis were used to detect the expression levels of  $\beta$ -catenin mRNA and protein at post-natal days 3, 7, 14, 21, 28 and 60 in the testis tissues of offspring mice in the vitrification group and the fresh group. The results of the comparison showed no significant differences between the two groups. Thus, the vitrification/cryopreservation process for the embryos did not seem to affect the expression of  $\beta$ -catenin in the testis tissues of the offspring.

## Conclusion

Our study showed that the vitrification/cryopreservation process likely did not affect testicular development or function in the offspring mice. However, the indicators used in this study are useful for limited assessments of potential abnormalities in late developmental stages caused by mutagenic or toxic effects during early embryonic development. A comprehensive assessment of the safety of vitrification/cryopreservation process for embryos and even a comprehensive assessment of the safety of vitrification/cryopreservation technology for human embryos requires more detailed investigations and long-term follow-up studies.

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