A Novel Approach of Tracing in Vivo Bioluminescence Imaging Expression of Vitrified Immature Testicular Tissue Grafts Until Adulthood: A Translational Transgenic Mouse Model

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Research Article

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Abstract

Background: The optimal method for cryopreserving immature testicular tissue (ITT) remains unknown and there is no standardized protocol. Controlled slow freezing remains the mainstream method of choice in human prepubertal male fertility preservation. Currently, the outcomes for ITT vitrification are conflicting, and most data are limited to in vitro animal studies.

Methods: A total of 12 pairs of donor and recipient mice were included in our experiments. The donors were immature transgenic mice, and the recipients were wild-type male mice. In the vitrification group, ITT was vitrified and thawed before transplantation. In the control group, ITT was transplanted to the recipients immediately. After thawing, we measured the expression of apoptosis-related mRNA caspase-3. More importantly, we monitored to adulthood all the transplanted grafts in vivo using noninvasive bioluminescence imaging (BLI) technology. On day 31, we removed the grafts for evaluation via hematoxylin and eosin staining and immunohistochemistry (IHC).

Results: We traced the survival of the grafts by in vivo BLI on days 1, 2, 5, 7, and 31 after transplantation. In both the vitrification and the control groups, bioluminescence decreased between days 2 and 5. Subsequently, the bioluminescence showed an upward trend until day 31. Compared with day 1, the bioluminescence was significantly stronger on day 31 after transplantation ($P = 0.009$). The differences between the two groups were constantly insignificant after analysis. These results indicate that both fresh and frozen–thawed testicular tissues can survive for at least 31 days after transplantation. Moreover, the vitrification group showed BLI signals comparable with those of fresh tissues. Compared with the control group, expression of the caspase-3 gene was significantly increased after vitrification ($P = 0.04$). Histology and IHC showed that both tissue structure and protein expression were intact in both groups.

Conclusions: Transplanted vitrified ITT grafts could survive till adulthood with BLI intensity comparable to that of the fresh control. Intact cells and structures for spermatogenesis in vitrified ITT grafts were as well-preserved as those in the control group. This translational model of self-repairing vitrified ITT grafts in vivo, lends weight to the role of vitrification in prepubertal male fertility preservation.

Background

According to cancer statistics in 2020, the incidence of childhood and adolescent cancer has increased slightly, while overall mortality has been greatly reduced, declining by 68% in children and 63% in adolescents [1]. As many cancer therapies are considered gonadotoxic, fertility preservation has become an important issue for these patients [2].

According to the guidelines published by the American Society of Clinical Oncology in 2018, for prepubertal children, the only fertility preservation options are ovarian and testicular cryopreservation using methods that are currently investigational [3]. The first and only live birth after autograft of ovarian tissue cryopreserved during childhood was reported in 2015 [4, 5]. For males, previous studies in rhesus

monkeys demonstrated that autologous grafting of cryopreserved prepubertal testis could produce sperm and healthy offspring [6]. However, to our knowledge, there has been no live birth following cryopreserved immature testicular tissue (ITT) transplantation in humans.

The optimal method for ITT cryopreservation remains unknown and there is no standardized protocol [2]. There are two methods for ITT cryopreservation: slow freezing and vitrification. Currently, controlled slow freezing (CSF) is the most commonly used freezing protocol globally [7]. Vitrification is a faster and cheaper alternative and it may avoid ice crystal formation and the ensuing injury to the tissues [8]. Since 2010, many studies have compared the feasibility of these two methods in both animal and human models, and the results are conflicting. Most of the studies evaluated the tissue by in vitro morphology and immunohistochemistry (IHC) assessment instead of the function of the tissue transplanted in vivo. Two studies comparing CSF and vitrification for human ITT cryopreservation have demonstrated that vitrification is able to maintain the proliferation capacity of spermatogonial cells and may be a feasible alternative [8, 9]. However, more data are necessary before vitrification could be widely used in the real world.

The development of noninvasive bioluminescence imaging (BLI) makes it possible to follow tissue-specific luciferase expression in transgenic mice and monitor the biological processes such as signaling or protein interactions of transplanted tissues in vivo [10]. As described in our previous study, we used FVB/N-Tg (*Polll-luc*) Ltc transgenic mice and showed that BLI is a viable tool for assessing the efficacy of germ cell transplantation in vivo in a transgenic male mouse model [11]. Our team has used this technology to track the survival of mouse ovarian iso- and allografts [12], the effects of immunosuppressant treatment after allotransplantation of ovarian grafts [13], and the fate of cryopreserved murine ovarian grafts [14]. These previous studies mainly focused on ovarian grafts since the BLI technology is rarely used in experiments for fertility preservation of ITT.

While testicular cryopreservation is the only option available for fertility preservation in prepubertal boys, this method is still considered experimental. More studies are necessary not only to optimize the protocol, but also to make this technology more user friendly. The objective of this study was to investigate the feasibility of vitrification for ITT cryopreservation. We studied the apoptosis of thawed ITT, and the histology after transplantation for 31 days. More importantly, we used BLI to monitor the in vivo fate of cryopreserved testicular grafts longitudinally to determine their biological activity in real time.

Material And Methods

Mice

The donors were 3-week-old immature male FVB/N-Tg (*Polll-luc*) Ltc transgenic mice with an H-2 haplotype (H_2^q). These mice were created by the transgenic service of Level Biotechnology (New Taipei City, Taiwan), and the generation process was described in our previous studies [11, 13, 14]. In brief, after pronuclear microinjection of the *Polll-Luc* transgene into the FVB/N embryos, they could encode a 712-bp

mouse RNA polymerase II promoter (*PolII*) and a modified firefly luciferase cDNA (Promega pGL-2). The animals were hemizygotes, and could express the transgene for luciferase (*luc*) and transmit this gene to their offspring. The recipients were 3-week-old immature FVB/NJNarl wild-type male mice with an H2q. We obtained these mice from the National Laboratory Animal Center (Taipei City, Taiwan).

A total of 12 pairs of donor and recipient mice were included in our experiments. They were all bred in the animal house of Taipei Medical University at a temperature of 22–24 °C and 12/12 h light/dark regimen. All procedures were reviewed and approved by the Animal Experimental Committee at the Taipei Medical University, in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Experimental design

The study was divided into three parts (Figure 1). First, we removed the 3-week-old donors' gonads, and a total of 24 donor mice were divided into two groups. In the vitrification group, the testis from the donor mice were vitrified and thawed before transplantation. For the other 12 mice in the control group, the ITT was transplanted to the recipients immediately. After thawing, we measured the level of expression of caspase-3 by real-time reverse transcription polymerase chain reaction (RT-PCR). Each transplanted testicular graft was measured volume 10 μg. After washing in Dulbecco's phosphate buffered saline (DPBS; Gibco®; Thermo Fisher Scientific, USA), the grafts were implanted in the scrotum of each recipient. We monitored all the transplanted grafts in vivo using BLI technology for 31 days until adulthood. On day 31, we removed the grafts and evaluated the tissue by hematoxylin and eosin (H&E) staining and IHC.

Preparation of donor ITT

The immature male FVB/N-Tg (*PolII-luc*) Ltc transgenic donor mice were euthanized by cervical dislocation under isoflurane anesthesia. Their testes was isolated, and the tunica albuginea was immediately removed into medium and maintained at 4 °C. The seminiferous tubules were isolated and cut into pieces less than 0.5 mm. Tissues were transferred to a 1.5 mL microfuge tube and washed three times with DPBS before cryopreservation or transplantation.

Cryopreservation protocol

The vitrification protocol was based on previous studies [15–17]. The vitrification solution used consisted of 1.5 M dimethyl sulfoxide (DMSO, D2650, Sigma, USA), 0.1 mol/L sucrose (S1888, Sigma, USA), 10% fetal bovine serum (Biological Industries, Israel), 1% penicillin streptomycin in L-15 medium (20183-027, Gibco®; Thermo Fisher Scientific). To achieve equilibration, 1 mL of cryoprotectant was gently introduced every minute with orbital shaking for 10 min. The tissues were incubated for 5 min at room temperature until precipitation of the ITT. The tissues were divided into 30 mL for each centrifuge and transferred to a foil (CoolRack®; Corning Inc., New York, USA) at 4 °C. Finally, the foil was immersed in liquid nitrogen and

the ITT was transferred to a cryovial (Biomate, Taiwan) for 1 month. The warming procedure involved removing the ITT from the liquid nitrogen and quickly plunging it into a prewarmed 37 °C solution containing sucrose (1 M). The ITT was placed on an orbital shaker and DPBS was introduced at the rate of 1 mL per minute to lower the concentration of DMSO to 0.15 M. Before further assessment, the ITT was washed twice with DPBS.

Assessment of thawed ITT recovery

After general anesthesia with Zoletil (20–40 mg/kg, Virbac) and Xylazine (5–10 mg/kg, Bayer), we proceeded to the transplantation procedure and the skin was shaved around the testicular region. We performed unilateral orchiectomy and refilled the space with 10 mL of fresh or thawed seminiferous tubules. Finally, we closed the wound and a dose of Carprofen (5 mg/kg, Selleck Chemicals, USA) was given.

Real-time polymerase chain reaction

Total RNA was extracted with the RNA extraction kit (Qiagen, USA) and synthesis of the cDNA was done with the SuperScript III synthesis kit (Invitrogen, USA). All experimental steps were performed according to the manufacturer instructions. Caspase-3 mRNA expression was determined using StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific, USA). The relative mRNA was normalized with b-actin. All experiments were repeated three times [18].

Transplantation and in vivo BLI imaging assessment

Bioluminescence imaging was obtained using the In Vivo Imaging System 200 (Xenogen Corp., USA). The recipients were injected with D-luciferin intraperitoneally (150 mg/kg, BSAL-8220; Biosynth Carbosynth®, USA) 10 min before imaging, anesthetized (1%–3% isoflurane, Abbott, USA), and placed into a light-tight camera box on the stage of the imaging chamber. An overlay image (black-and-white picture) was taken with the aid of a light inside the imaging chamber. Luminescence was quantified using Living Image software. Luminescence was quantified by summing pixel intensities within the region of interest, as described [10].

H&E staining

For H&E staining, the testes of mice were fixed in Bouin’s solution (HT10132, Sigma-Aldrich). Then, the tissues were embedded in paraffin and the sections were cut at 5 mm. Histological and morphological changes of the testicular structures were examined under light microscopy [19].

Immunohistochemistry

Testes were collected and the tissue sections were incubated in 0.01 M citrate buffer (pH 6.0) at 95 °C for 30 min. Then, the sections were washed three times in PBS. Endogenous peroxidase was blocked with H_2O_2 for 10 min. Next, unspecific staining was blocked with 0.5% non-fat milk in PBS for 1 h. We used the following antibody probes: proliferating cell nuclear antigen (PCNA antibody 10205-2-AP; Proteintech), anti-sperm acrosome associated 1 (Spaca1 antibody 12829-1-AP; Proteintech), luciferase (ab181640; Abcam), sex determining region Y-box 9 (Sox 9 A19710; ABclonal), 3b-hydroxysteroid dehydrogenase (3b-HSD A1823; ABclonal), and DEAD (Asp–Glu–Ala–Asp)-box polypeptide-4 (DDX4, 51042-1-AP; Proteintech). The sections were placed in a humidified chamber overnight at 4 °C and then incubated for 15 min with rabbit/mouse horseradish peroxidase-labeling secondary antibody. After washing with PBS three times, the sections were mounted on glass slides and analyzed by light microscopy.

Statistical analysis

We performed all the statistical analysis using Statistical Package for the Social Sciences version 25.0 (SPSS; IBM, USA). The changes of the photons after transplantation were measured by the following equation: (measurement on a certain day – measurement at baseline) / measurement at baseline. Differences between the vitrification group and control group were compared using Student's t-test, and a P value < 0.05 was considered statistically significant.

Results

Effect of the vitrification intervention on caspase-3 mRNA expression within ITT

We detected the expression of the apoptosis-related caspase-3 gene in ITT immediately after vitrification [20]. As compared with the control group, the expression of this gene was significantly increased after vitrification (P = 0.04) (Figure 2).

In vivo BLI of mouse testicular grafts in fresh and frozen–thawed testicular transplant groups on days 1, 2, 5, 7, and 31 after transplantation

After we removed one of the recipients' testes, we replaced it with fresh or frozen–thawed ITT from the FVB/N-Tg (PolII-luc) Ltc transgenic mice. We traced the survival of the graft by in vivo BLI on days 1, 2, 5, 7, and 31 after transplantation (Figure 2A). For both the vitrification and the control groups, the bioluminescence decreased between day 2 and day 5. After day 5, the bioluminescence showed an upward trend until day 31. Compared with day 1, the bioluminescence was significantly stronger at day

31 after transplantation (9.3 ´ 105 photons/s versus 15.4 ´ 105 photons/s, P = 0.009). The bioluminescence of the vitrification group was initially stronger than that of the control group. However, the signals were lower than the control group after day 7 (Figure 2B). The differences between the two groups failed to reach significance (Figure 2C). These results indicate that both fresh and frozen–thawed testicular tissues can survive for at least 31 days after transplantation. Moreover, the vitrification group revealed bioluminescence signal intensity that was comparable with that of the fresh tissues.

Histology and IHC staining analysis of ITT grafts on day 31 after transplantation

Since the BLI signals revealed that both fresh and frozen–thawed testicular tissues could survive for at least 31 days after transplantation, we determined their structure and function by histology and IHC, 31 days after transplantation. Seminiferous tubules could be observed in both groups, but the structure was more intact in the control group (Figure 3A).

Proliferating cell nuclear antigen (PCNA) is a useful marker for mitotically proliferating spermatogonia, but not for spermatocytes that have just entered meiosis [21]. Sperm acrosome membrane-associated protein 1 (SPACA1) is a membrane protein with a function in sperm–egg fusion [22]. The expression of luciferase indicated that the cells originated from the transgenic ITT. Sox-9, 3b-HSD, and DDX4 represent Sertoli cells, Leydig cells, and germ cells, respectively [23–25]. The expression of the above antigens were all detectable in both the vitrification and control groups. We assumed that the ITT integrities were preserved during the process of vitrification, consistent with the result of BLI imaging. However, compared with the control group, the protein expression was decreased in the vitrification group (Figure 3B).

Discussion

Many studies have investigated the feasibility of vitrification for ITT cryopreservation. To the best of our knowledge, this is the first study to report the survival of ITT in vivo by adopting the BLI imaging system. Apoptosis of the vitrified ITT was noted before transplantation. During longitudinal observation of the bioluminescence in vivo for 31 days, we confirmed that the tissues were viable after transplantation in both the vitrification and the control groups with comparable bioluminescence intensity. The intensity was significantly stronger on day 31 compared with the first day, indicating the possibility of tissue self-repair after transplantation. On day 31, the histology and IHC demonstrated that the vitrified tissues had preserved the structures and cells for spermatogenesis.

The aim of our study was to adopt the translational model of BLI signals in transgenic mice to track the survival of the grafts in vivo. In our previous study, this animal model was shown to be useful for quantifying germ cells in vitro and assessing the efficacy of germ cell transplantation in vivo [11]. In the present study, our results indicated that at follow-up on days 1, 2, 5, 7, and 31 after transplantation, bioluminescence signals were similar between the vitrification group and the control group. This finding

suggests that ITT can recover from the vitrification–thawing process and establish revascularization in a manner similar to fresh tissues from the beginning of transplantation. This observation is comparable with earlier in vitro studies that reported similar outcomes with vitrification after CSF or with fresh controls [9,26, 27]. Unlike in ITT, ovarian cryopreservation by slow freezing may compromise ovarian reserve through cryoinjury and ischemia. We found the BLI signals persisted lower in the slow freezing group on days 1, 3, 5, 7, and 10 than the fresh controls after transplantation [14]. The process of revascularization takes 2 to 7 days to complete, depending on the size of the implant [28].
The bioluminescence intensity decreased between day 2 and day 5, and then increased linearly. The BLI signals were significantly higher on day 31 than on day 1, indicating the presence of revascularization after day 5, and the subsequent restoration of function of the ITT grafts.

According to previous reviews, eight studies have compared vitrification to slow freezing for ITT cryo-storage [2, 7]. In 2010, Abrishami et al. reported that ITT vitrification could maintain cell viability and restore spermatogenesis after xenograft [29]. They found that exposure to DMSO for 5 min yielded numerically higher cell numbers than in grafts exposed for 15 or 30 min. Accordingly, we limited DMSO exposure of our grafts to less than 10 min. Curaba et al. compared vitrification and slow freezing in terms of IHC and tissue integrity. They confirmed that vitrification was a promising approach, but additional studies should be conducted in vivo to assess completion of spermatogenesis [16]. The same authors also published a case report assessing vitrification of human ITT, and revealed that the histology characteristics of spermatogonia and Sertoli cells were preserved [8]. Gouk et al. focused on harvesting spermatogonial stem cells (SCCs) for fertility preservation. They found that vitrification maintained post-warming cell viability and function significantly better than conventional slow and rapid freezing protocols [15]. Baert et al. and Poels et al. both agreed that vitrification is an effective strategy to maintain the proliferative capacity of SCCs and integrity of ITT [9, 17]. Poels et al. was the first group to adopt a human ITT xenotransplantation model. They observed spermatogonia proliferation 6 months after transplantation, but then there was a blockage at the pachytene stage. Time-consuming CSF protocols are commonly used in human testicular tissue banking. Baert et al. have investigated the alternatives to conventional CSF using testicular tissues from 14 adult patients. In the vitrification group, they found increased numbers of seminiferous tubules displaying a ruptured epithelium and considered this method to have a negative impact on spermatogonial number [30]. On the other hand, Dumont et al. reported the superiority of the vitrification protocol in terms of testicular structure maintenance, tubular morphology, and tissue function [31]. In a recent review, these authors concluded that the results of comparison between vitrification and slow freezing for ITT were still conflicting, and most of them were limited to in vitro animal studies [2].

In vitro cell culture is an interesting method to avoid re-introduction of malignant cells to cancer patients, especially with hematological malignancies [32]. Sato and Ogawa were the first to show complete spermatogenesis from cryopreserved ITT after it was thawed and cultured in vitro [33]. Furthermore, Yokonishi et al. suggested that tissues undergoing vitrification showed spermatogenesis similar to that of unfrozen control tissues [26]. The cryopreservation method is not as critical as culture conditions, such as type of culture medium or temperature. Dumont et al. also confirmed that in vitro spermatogenesis

maturation strongly modified apoptosis and autophagy-related protein levels, and the impact of cryopreservation was minimal at the end of the culture [27].

The limitation of this study is that the BLI signals could represent tissue viability only, and not its ability for spermatogenesis. Despite the fact that the vitrification group revealed substantial BLI signals similar to the control group, we could not detect spermatids during the morphology evaluation on day 31 after transplantation in either group. Histology and IHC confirmed the presence of some important cells and structures for spermatogenesis. Others have reported live spermatogenic cells recovered at 2 months after transplantation and produced the first live birth of rabbit offspring [34]. Recently, the same group retrieved post-meiotic spermatids 8 to 12 months after transplantation, and successfully produced offspring from autologous grafting of cryopreserved prepubertal rhesus monkeys [6]. The lack of spermatogenesis in our study may simply reflect insufficient observation time.

Conclusion

Apoptosis was present in vitrified ITT before transplantation. Transplanted vitrified ITT grafts could survive until adulthood with comparable BLI intensity to the fresh control while stained vitrified ITT grafts indicated intact cells and structures preserved for spermatogenesis. This translational model of self-repairing vitrified ITT grafts in vivo lends weight to a role for vitrification in prepubertal male fertility preservation.

Declarations

Ethics approval and consent to participate

The fore mentioned procedures were approved by the Animal Experimental Committee at the Taipei Medical University (LAC-2016-0354) and adhered to the Guide for the Care and Use of Laboratory Animals (National Institute of Health).

Consent for publication

All authors approve the manuscript and give their consent for submission and publication.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Chen CH designed the study and performed the study process. Lu BJ contributed the analysis of digital of BLI and manuscript editing. Liu YL and Lin BZ supported the data analysis and interpreted data related to HE staining and IHC. All authors reviewed the manuscript and approved the final version of the manuscript.

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Figures

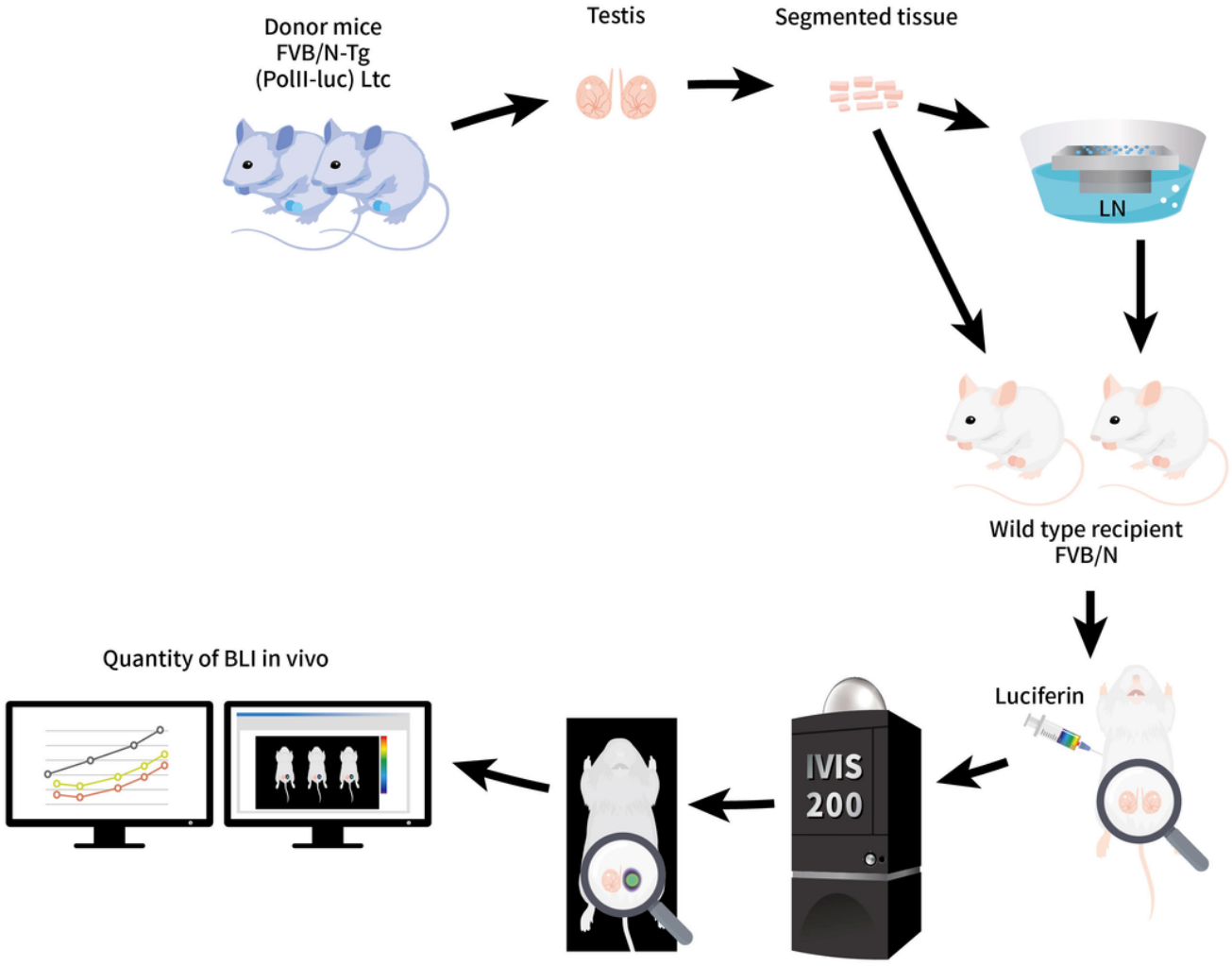


Figure 1

Study design flowchart. The donor mice were 3-week-old immature male FVB/N-Tg (*PolII-luc*) Ltc transgenic mice with an H-2 haplotype (H_2^q). Their immature testicular tissues (ITT) were removed and fragmented. Then, the ITT were either vitrified and thawed before transplantation or transplanted directly to wild-type recipient mice. After anesthesia and D-luciferin injection, we used the In Vivo Imaging System

200 (IVIS 200) to monitor the bioluminescence (BLI) signal intensity in vivo. Finally, the quantified BLI signal data were exported to the computer for further longitudinal research.

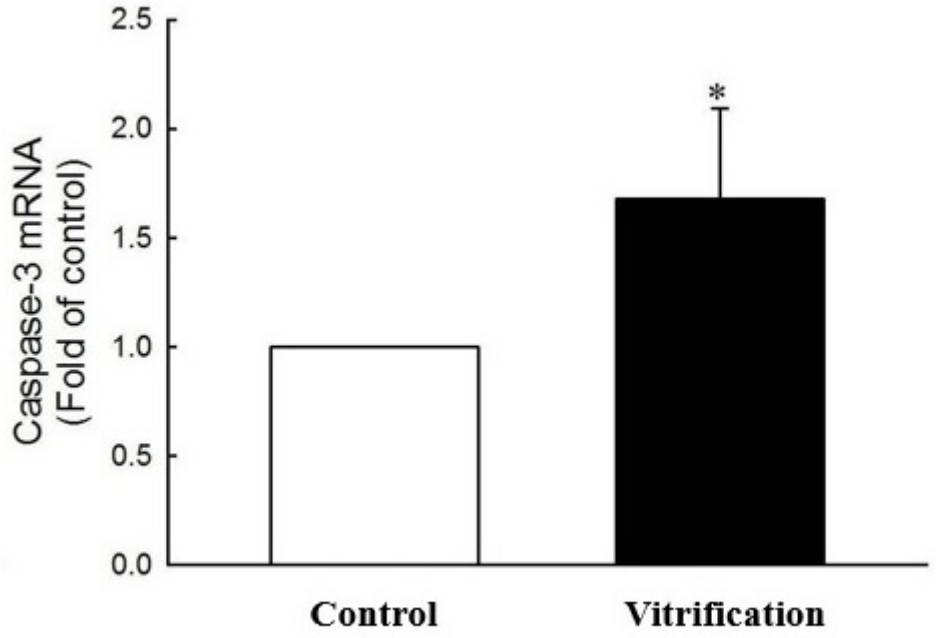
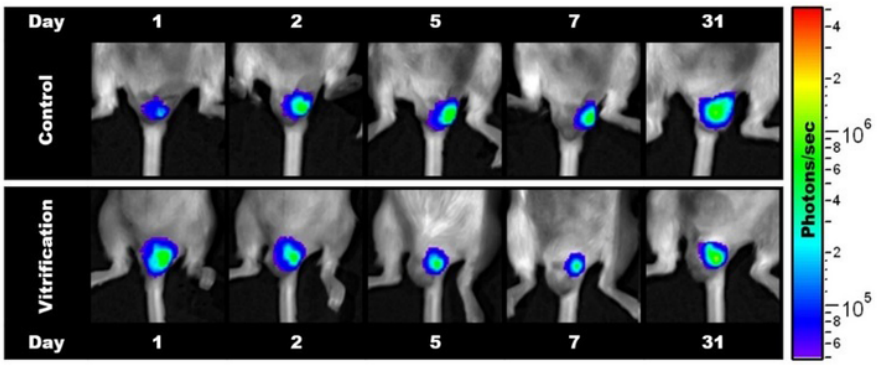


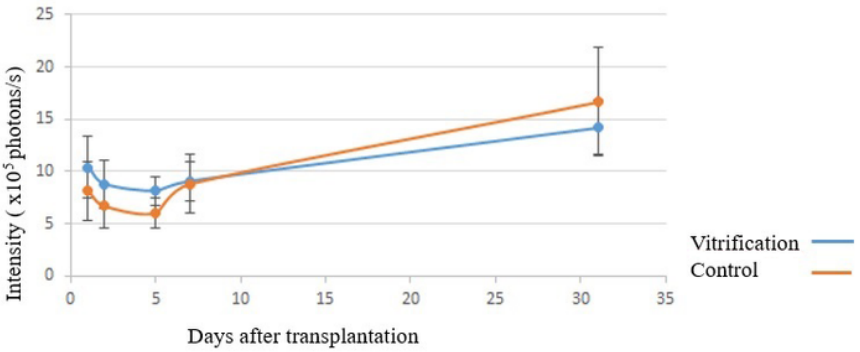
Figure 2

Caspase-3 mRNA expression was determined by real-time qPCR. Its expression was significantly higher in the vitrification group ($P = 0.04$).

A. In-vivo bioluminescence imaging



B. Bioluminescence intensity



C. Comparison of bioluminescence intensity by student's t-test from day 1 to day 31 after transplantation

Days after transplant	Vitrification (x 10^5 photons/s)	Control (x 10^5 photons/s)	p-value
1	10.41 (±2.94)	8.14 (±2.79)	0.58
2	8.77 (±2.28)	6.68 (±2.11)	0.51
5	8.12 (±1.43)	6.03 (±1.41)	0.31
7	9.07 (±1.89)	8.83 (±2.79)	0.95
31	14.23 (±2.60)	16.67 (±5.17)	0.68

Figure 3

(A) We traced the survival of testicular graft in vivo by bioluminescence imaging (BLI) on days 1, 2, 5, 7, and 31 after transplantation. The upper column shows the mice from the control group, and the lower column shows the mice from the vitrification group. (B) Curves show the digitized photon intensity with standard deviation for the vitrification (blue) and the control (orange) group, respectively. (C) Analysis of the bioluminescence intensity by Student's t-test from days 1 to 31 after transplantation. The differences were all not significant between the two groups.

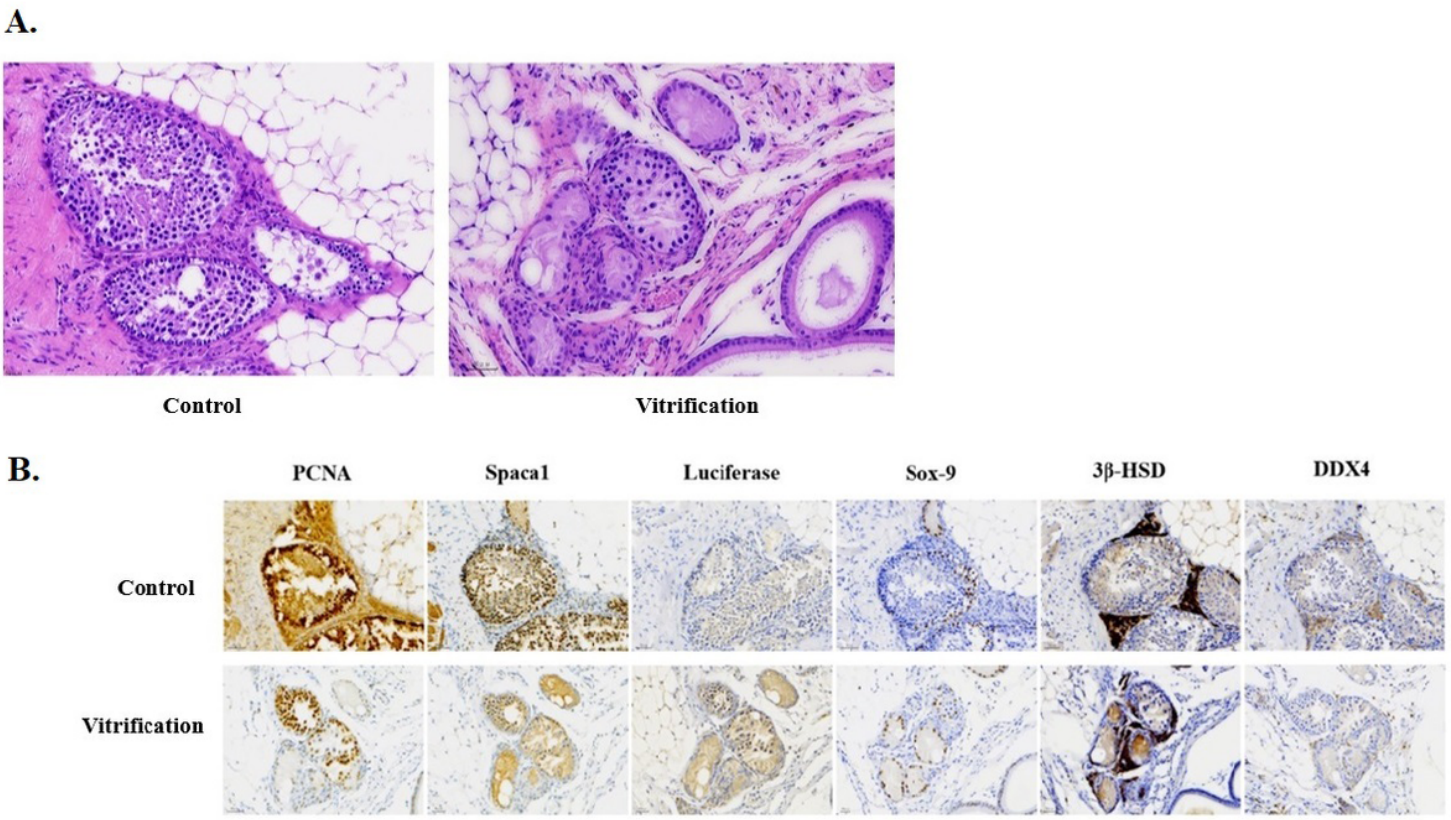


Figure 4

We observed the survival of ITT grafts 30 days after transplantation for both groups. (A) Hematoxylin and eosin (H&E) staining: the structure of the seminiferous tubules was more intact in the control group. (B) Immunohistochemistry (IHC) staining: the expression of antigens including PCNA, Spaca1, Luciferase, Sox-9, 3b-HSD, and DDX4 was stronger in the control group. The results indicate that ITT grafts were damaged during the vitrification procedure.