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ARTICLE

A newly developed carrier for the vitrification of prepubertal testicular tissue and its comparison with four different carriers

BIOGRAPHY

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KEY MESSAGE

There is ongoing debate around the optimal carrier for vitrification of prepubertal testicular tissue. This study describes the development of a practical and sterilizable carrier, the metal brush, and compared it with others. The results suggest that the metal brush is good at preserving tissue morphology and is a promising carrier.

ABSTRACT

Research question: The cryopreservation of prepubertal testicular tissue is important for children who are to undergo gonadotoxic treatment. There is ongoing debate around the optimal carrier for an inexpensive and rapid vitrification technique. How efficient would a novel, practical and sterilizable metal brush be when compared with previously used carriers?

Design: The testicular tissues of prepubertal rats were vitrified using four different carriers and evaluated by light microscopy and transmission electron microscopy.

Results: Nuclei were easily discriminated in the metal brush, aluminium foil and high-security straw groups, but there was decreased discrimination of structures in the metal wire group. Minimal cytoplasmic degeneration, vacuolization and mild reversible degenerative effects were seen in spermatogonial stem cells and Sertoli cells in the metal brush group. Mild to moderate structural changes were found in the aluminium foil group. Severe pyknosis of the nuclei, a high degree of swelling, expansion of the endoplasmic reticulum, and swelling and blurring of the mitochondria were seen in the metal wire and high-security straw groups. The cell viabilities in the metal brush, aluminium foil, metal wire and high-security straw groups were 91.6 ± 3.85%, 83.0 ± 4.06%, 76.0 ± 3.16% and 68.6 ± 4.93%, respectively.

Conclusions: The metal brush is a promising new carrier for prepubertal testis vitrification.

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KEYWORDS

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Spermatogonial stem cells
Vitrification

INTRODUCTION

The survival rate of children with cancer has increased to more than 80% as a result of new innovations in cancer treatment (*Gatta et al., 2009*; *Magnani et al., 2006*). Although the primary goal for these children is survival, the preservation of fertility is important for quality of life. When oncology treatments are complete and these children reach adulthood, they will be able to produce their own reproductive cells and have children. Cryopreservation can improve quality of life in both psychological and sociological terms. However, no optimized fertility preservation method exists for prepubertal boys undergoing gonadotoxic treatment. *Brinster and Avarbock (1994)* made an important contribution by transplanting spermatogonial stem cells (SSC) from fresh mouse testicular tissue to a recipient and successfully restoring fertility. Successful spermatogenesis (*Avarbock et al., 1996*) and offspring production after the cryopreservation of SSC (*Kanatsu-Shinohara et al., 2003*) have shown that these cells can be preserved for long periods. Recently, *Fayomi et al. (2019)* reported healthy-born primates from frozen prepubertal testicles.

Studies are ongoing to improve preservation methods, as well as how stored prepubertal testicular tissues can be used for fertility preservation. These initial studies used slow freezing (*Avarbock et al., 1996*; *Kanatsu-Shinohara et al., 2003*); however, in recent years, vitrification has been applied to embryo and oocyte cryopreservation (*Chang et al., 2008*; *Desai et al., 2007*) and seems to be the preferred method for prepubertal testis cryopreservation (*Curaba et al., 2011a*; *Zeng et al., 2009*). Vitrification also has practical advantages such as reduced time and cost for the cryopreservation of prepubertal testicular tissue (*Curaba et al., 2011b*; *Gouk et al., 2011*). As a result, vitrification is a good alternative to slow freezing (*Curaba et al., 2011a*, *2011b*; *Poels et al., 2012*).

It is well known that the success of vitrification is dependent on the size of tissue, cooling rate, vitrification media and excess media around the tissue. Another important issue is the simplicity of the method and prevention of cross-contamination in the liquid nitrogen. For these reasons, different carriers have been applied in prepubertal testis tissue vitrification, such as open cryostraws in cooled cryotubes for prepubertal mouse and prepubertal human testicular tissue (Curaba *et al.*, 2011; *Poels et al., 2012*, *2013*) and straw-in-straw (*Gouk et al., 2011*). On the other hand, carrier-free systems like solid surface vitrification (SSV), in which tissue pieces are transferred on floating aluminium foil and partially immersed in liquid nitrogen, or direct cover vitrification (DCV), in which human adult male testicular tissue is placed directly into cryovials, have been used in previous studies (*Baert et al., 2012*, *2013*).

The aim of the current study was to find the optimal carrier for the vitrification of prepubertal testicular tissue. The plan was to increase the cooling rate and accelerate the heat conduction/temperature transmission, initially with a metal wire used as a carrier. A metal brush made of thinner metal wires was subsequently tried, to prevent the merging of tubules. Vitrification was performed on prepubertal testicular tissues in Wistar rats using four different carriers at the same balancing and vitrification times for tissues of equal size with the vitrification medium. A novel metal brush modified as a carrier was used and compared to modified metal wire and other carriers that are already in use, such as high-security straws and aluminium foil, and the effects of these carriers on prepubertal testicular tissues were evaluated.

MATERIALS AND METHODS

Male Wistar rats (5 days old) were sacrificed by decapitation and bilateral abdominal testicles were excised; testicles were 3 × 2 × 1 mm in size. After the tunica albuginea was removed, both testicles were cut into small pieces (1–2 mm) with a scalpel and seminiferous tubules were carefully and gently dissociated using a needle. Five testicular pieces from each rat were used for all control and experimental groups and the experiments were repeated four times. Care was taken to use as few rats as possible and to use the tissue pieces effectively.

The carriers used included a metal brush, a high-security straw (Cryo Bio System, IMV Technologies, L'Aigle, France), aluminium foil and a metal wire. In vitrification, the effects of different carriers on prepubertal testicular tissue were examined and compared under light and transmission electron microscopy (TEM). The effects of cryopreservation on tubular cells were evaluated semi-quantitatively in semi-thin sections under light microscope. Viability tests were conducted.

Vitrification

The vitrification medium was Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM F-12; D8062, Sigma-Aldrich, Inc., St. Louis, MO, USA) supplemented with 2.1 mol/dm^3 dimethylsulphoxide (D2650, Sigma-Aldrich), 2.7 mol/dm^3 ethylene glycol (324558, Sigma-Aldrich), 20% fetal bovine serum (FBS; F6178, Sigma-Aldrich) and 0.5 mol/dm^3 sucrose (840097, Sigma-Aldrich).

The equilibrium medium was a 1:1 dilution of vitrification medium with DMEM F-12. Tissues were incubated in equilibrium medium for 10 min and in vitrification medium for 5 min, then stored in a –196°C liquid nitrogen (MVE XC 47/11-6) tank using the different carriers as a metal J-shaped 1 mm thick copper wire attached to the lid of a 2 ml cryovial (Greiner). The metal brush consisted of roughly 30 pieces of 100 µm thick copper wire wrapped with silicone at one end to form a handle attached to the lid of a 2 mm freezing tube and extended wire hair at the other end (FIGURE 1).

In all groups, samples were transferred to equilibrium and to vitrification media with very fine forceps. Immediately after the vitrification medium, one piece of tissue was loaded to the straw and the open end of the straw closed off using a sealing device. The straw was immersed vertically in liquid nitrogen, moving it for several seconds to prevent the formation of an insulating layer of air. Wire, foil and brush group tissues were transferred to another Petri dish and excess media removed with an absorbent tissue. Tissues were transferred to aluminium foil on the liquid nitrogen for SSV in the aluminium foil groups while seminiferous tubules were gently placed on the metal wire or brush. Care was taken to ensure that the tubules were separated from each other in all the groups. The samples were immersed in liquid nitrogen by moving for a few seconds immediately after loading onto the carriers and transferred to the liquid nitrogen tank after cryovial placement.

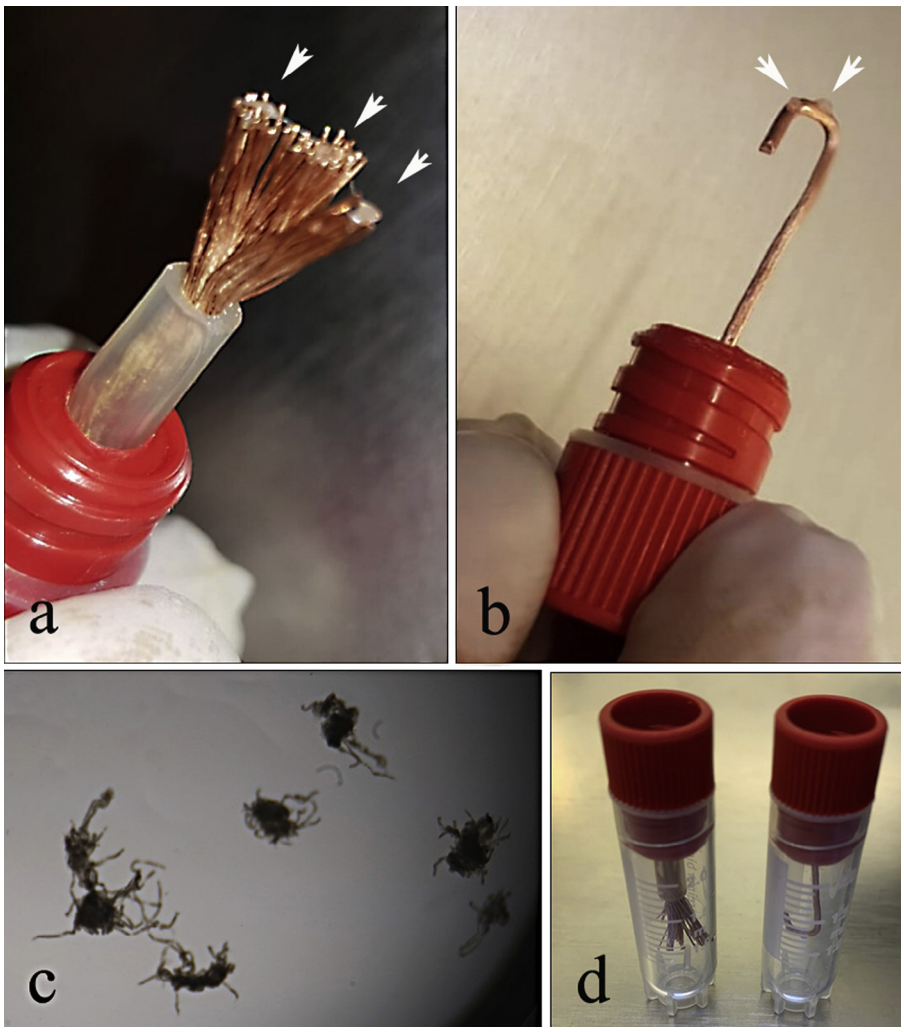

FIGURE 1 (a) The metal brush loaded with testicular tissue pieces (arrow). (b) The metal wire loaded with testicular tissue (arrow). (c) Tissue pieces of equal size. (d) The metal brush and the metal wire in the cryovial.

For warming, tissues were consecutively immersed in 100%, 50% and 25% suspended thawing medium DMEM F-12 supplemented with 1 mol/dm^3 sucrose and 20% FBS for 5 min each at 37°C. The tissues were then incubated in DMEM F-12 supplemented with 10% FBS for 30 min.

Cell suspensions

Half of each thawed prepubertal testicular tissue was enzymatically dissociated first with collagenase type IV and then with trypsin–EDTA. After the trypsin was inactivated with FBS, tissues were washed twice with DMEM F-12 to produce the final cell suspensions.

Viability test (eosin Y dye)

Testicular cell suspensions were stained with 0.5% Eosin Yellow (E0201, Surechem Products, UK) and evaluated using an Olympus IX71 Inverted Hoffman Modulation Contrast HMC Microscope. Two slides were prepared from each sample and a minimum of 200 cells were counted on each slide (*Gouk et al., 2011*).

Semi-thin and thin section preparation

Thawed and control tissues were fixed with glutaraldehyde and processed routinely for TEM (*Contuk et al., 2012*). After embedding tissues in Epon, 1000 nm thick semi-thin sections were cut with an ultramicrotome (Leica Ultracut R Microtome, Germany), stained with toluidine blue and evaluated by light microscope (Olympus BX51, Olympus Corporation, Japan). Thin sections of 60 nm were evaluated using TEM (Jeol 1200 EX II, Japan).

Histological evaluation

The nuclei and cytoplasm of Sertoli cells and SSC were evaluated by light microscope (*Milazzo et al., 2008*, *2010*; *Travers et al., 2011*). Tubular cells were morphologically assessed according to three criteria: (i) Sertoli cells and SSC nuclei discrimination, (ii) pyknotic nuclei (calculation of each pyknotic nucleus in each tubule), and (iii) tubules with cytoplasmic degeneration (vacuolization in intratubular cell cytoplasm and calculation of degenerating and light-coloured heterogenic vacuoles in future tubular lumen) (*Milazzo et al., 2008*, *2010*). The effect of cryopreservation on tubular cells was semi-quantitatively evaluated on a double-blind basis and the following morphological changes on cells of the seminiferous epithelium were analysed: desquamation, nuclear damage, cytoplasmic vacuolization, occurrence of lipid droplets, and swelling and shrinkage of the cell. The morphological changes were semi-quantitatively evaluated according to their intensity/occurrence (no change compared with controls (x);

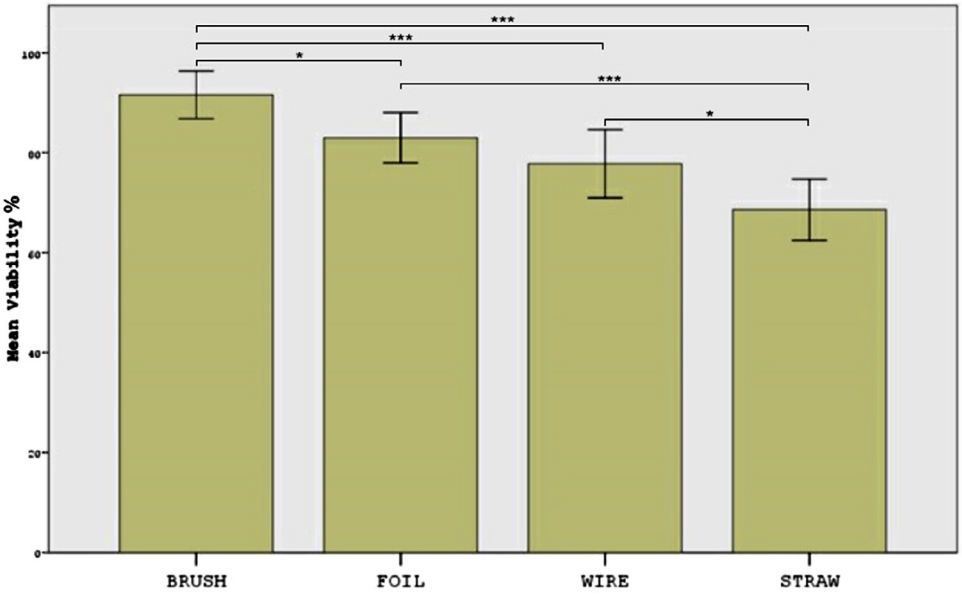

FIGURE 2 Comparison of cell viability among the carrier groups. The brush group demonstrated a significantly higher viability rate than the other groups. Error bars represent SD. *$P < 0.05$; **$P < 0.01$; ***$P = 0.0001$. Brush versus foil, $P = 0.019$; wire versus straw, $P = 0.047$.

low density (+), moderate density (++) and high density (+++) (*Jezek et al., 2001*). Alterations in the nuclei and cytoplasm of Sertoli cells and SSC, the membrane structures, and the lumen of tubules due to cryopreservation were evaluated qualitatively by TEM (*Baert et al., 2012*, *2013*; *Keros et al., 2005*; *Unni et al., 2011*).

Ethical approval

This study was approved by the ethical committee of the Marmara University Animal Research Centre (no. 52.2012. mar, approved 21 June 2012).

Statistical analysis

In this study, statistical analysis was performed using the Number Cruncher Statistical System (NCSS) 2007 Statistical Software (Utah, USA) package and MS Excel 2013. Data are expressed as means ± SEM. One-way analysis of variance and post-hoc Tukey multiple comparison test was used for comparison of groups. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Viability tests

The cell viabilities of samples preserved with the metal brush, aluminium foil, metal wire and high-security straw were 91.6 ± 3.85%, 83.0 ± 4.06%, 76.0 ± 3.16% and 68.6 ± 4.93%, respectively. The differences between groups were statistically significant ($P = 0.0001$). The mean cell viability (MCV) of the brush group was significantly higher than the foil group ($P = 0.019$), wire group ($P = 0.0001$) and straw group ($P = 0.0001$). The MCV of the foil group was significantly higher than the straw group ($P = 0.0001$). The MCV of the wire group was also significantly higher than the straw group ($P = 0.0047$). There was no statistically significant difference between the foil and wire groups ($P = 0.064$) (FIGURE 2).

Light microscopy

The nuclei in the tissues of the metal brush, aluminium foil and high-security straw groups were easily discriminated, but the discrimination of structures decreased in the metal wire group. Severely pyknotic nuclei were observed in the metal wire group and in the high-security straw group. Cytoplasmic degeneration and vacuolization were minimal in the metal brush group but were severe in the high-security straw group (TABLE 1 and FIGURE 3).

Transmission electron microscopy

Mild degenerative indications of reversible changes were observed in SSC and Sertoli cells in the metal brush group. Cell membranes were generally preserved, the endoplasmic reticulum was regular and the crista organization in mitochondria exhibited no degradation other than minimal swelling. Nucleoli were differentiable, and nuclear membranes were normal. Some nuclei displayed a moderate degree of heterochromatin formation (FIGURE 4A). Some cells exhibited a mild to moderate degree of structural change in the aluminium foil and metal brush groups (FIGURE 4B). A high degree of endoplasmic reticulum swelling and expansion and mitochondrial swelling and

TABLE 1 SEMI-QUANTITATIVE MORPHOLOGICAL ANALYSES OF TUBULE-ALIGNING CELLS IN THE CARRIER GROUPS

Group	Sertoli cell–spermatogonial stem cell discrimination	Pyknosis (nuclear condensation)	Degeneration vacuolization
Brush	+++	+	+
Foil	+++	+	++
Wire	++	+++	++
Straw	+++	+++	+++

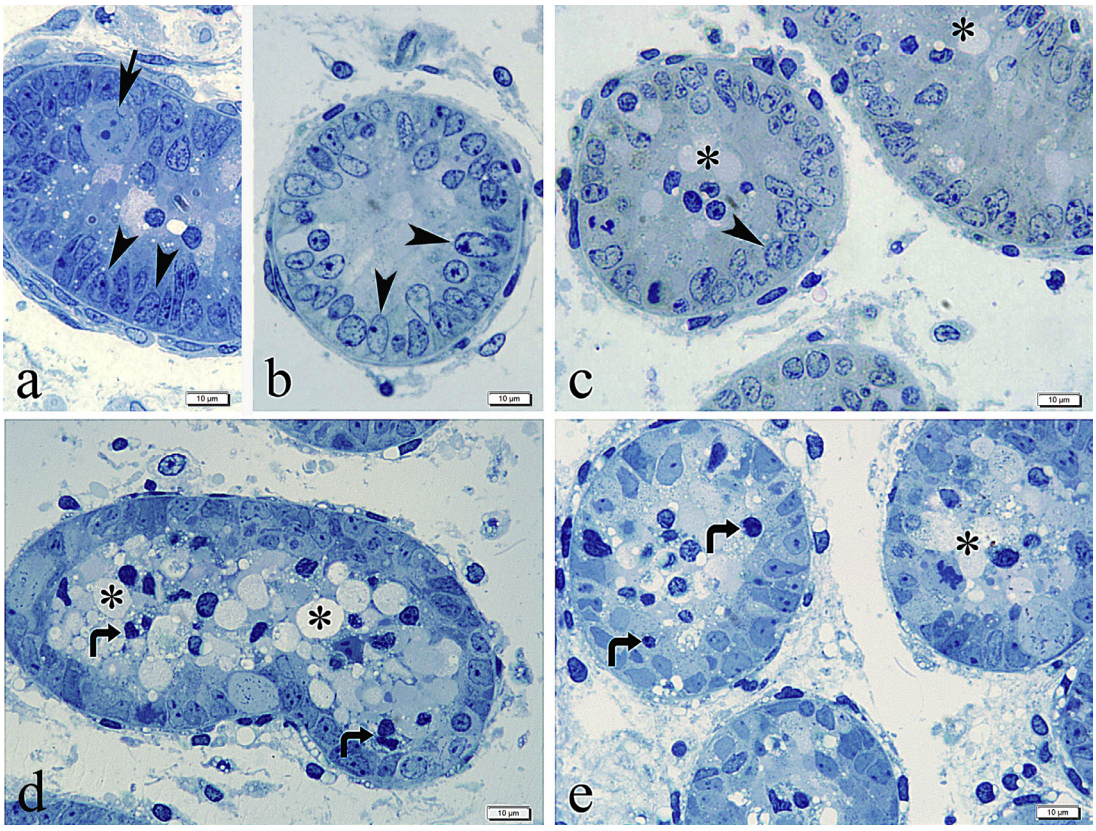

FIGURE 3 Semi-thin sections of rat prepubertal testicular tissue. (a, b) Tubules and intratubular cells were mostly well preserved with the metal brush (arrow indicates spermatogonial stem cells (SSC), arrowhead indicates Sertoli cells). (c) Cells with vacuolization (asterisk) and degenerated cytoplasm (arrowhead) were obvious in some tubule areas in the aluminium foil group. (d) Pyknosis occurred in the metal wire group, and SSC and Sertoli cells were more difficult to discriminate in the metal wire group than in the other groups. (e) The lowest structural preservation was in the high-security straw. The broken arrow indicates pyknosis, and the asterisk indicates cells with vacuolization for (d) and (e). The scale bar represents 10 µm.

blurring were observed in both the metal wire (FIGURE 4C) and the high-security straw (FIGURE 4D) groups.

The cristae of the mitochondria were distorted, and the following were observed in totally degenerated, severely damaged cells: widespread vacuolization of the cytoplasm, swelling and vacuolization of the endoplasmic reticulum, pyknosis of the nuclei and indentation and separation of the nuclear membranes. These irreversible changes were more prominent in damaged cells. Heterogeneous vacuolar structures were observed in future lumen sites. Light-coloured heterogeneous areas in light microscopy were found to be areas of marked degeneration containing swollen cytoplasmic structures. These lacunae consisted of wide vacuoles and intercellular spaces.

DISCUSSION

Cryopreservation studies of prepubertal testicular tissue for fertility preservation are still in the experimental stage. There are a number of laboratory and clinical issues to resolve in the freezing of these tissues (*Andersen et al., 2015*; *Gies et al., 2015*; *Ginsberg et al., 2014*; *Goossens et al., 2013*). This study aimed to modify the vitrification method with different carriers and examine the effects on prepubertal testicular tissue.

In prepubertal testicular tissue vitrification, carriers such as open cryostraws in cooled cryotubes and straw-in-straw have been used; alternatively, tissues have been placed in cryovials and then directly into liquid nitrogen. Open straws effectively preserve the integrity of prepubertal mouse and human testicular tissues (*Curaba et al., 2011a*, *2011b*; *Gouk et al., 2011*; *Poels et al., 2012*, *2013*). Moreover, carrier-free systems, such as in the case of SSV, also exist. The cryopreservation of prepubertal mouse testicular tissue with SSV without a carrier has been performed by placing the tissue onto aluminium foil floating on liquid nitrogen, which successfully preserved testicular tubular morphology and cell ultrastructure. Carrier-free systems have been previously studied for female gonadal tissues to eliminate the negative effects of carriers, such as the reduction in cooling rate (*Amorim et al., 2011a*; *Santos et al., 2007*). When placed into liquid nitrogen, the vitrification solution precipitates around the tissue and affects the cooling rate. It has been reported that carrier-free systems reduce this negative effect. When placed into liquid nitrogen after removing the medium precipitate, vapour coat formation decreases. This is a result of the minimized thermal mass and increased cooling rate, and the cell damage caused by ice crystal formation decreases (*Amorim et al., 2011b*).

SSV is highly successful at maintaining the ultrastructure of prepubertal mouse testicular cells and preserving tubular morphology. However, SSV does not protect human testicular tissue; specifically, the epithelia of seminiferous

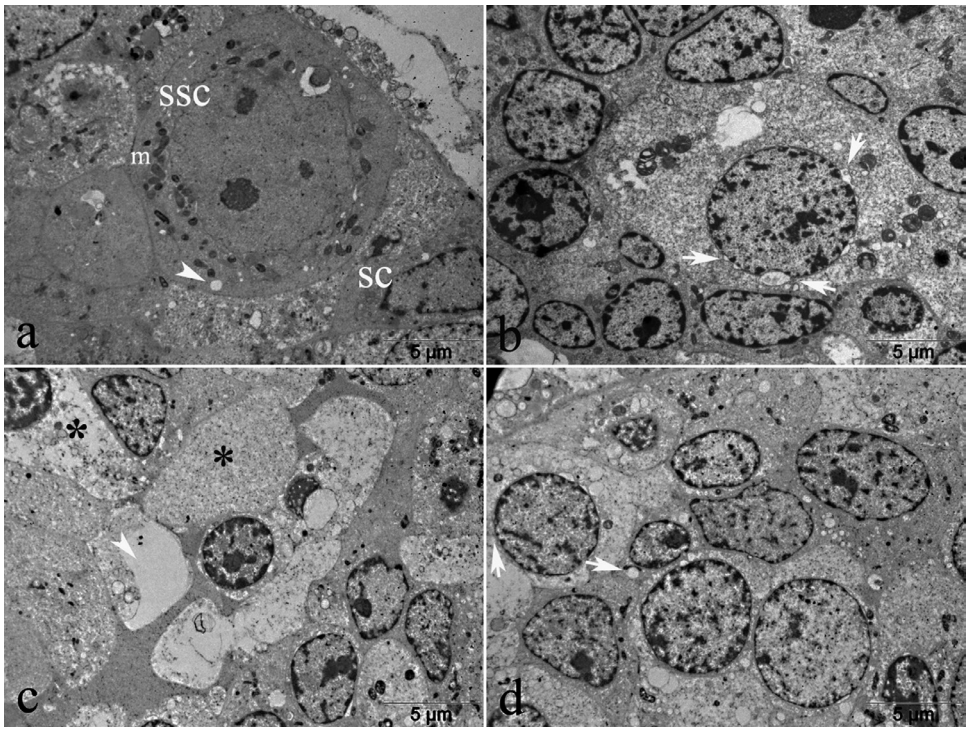

FIGURE 4 Transmission electron micrographs of rat prepubertal testicular tissue. (a) Mild degenerative reversible changes in the metal brush. Spermatogonial stem cells (SSC), Sertoli cells (SC), mitochondria (m) and small vacuoles (arrowhead) can be seen. (b) Mild to moderate degrees of structural changes in the aluminium foil group. Ondulation, notching and dissociation in nuclear membrane (arrow). (c) Cytoplasmic degeneration (black asterisk) in the metal wire group. (d) Structural membrane changes (arrow) in the high-security straw group. The scale bar represents 5 µm.

tubules rupture after cryopreservation (*Baert et al., 2012*, *2013*). The likely reason for this is that the handling is difficult, it is not user-friendly and it is an open system. The high-security straw eliminates the contamination risk, but the volume is a disadvantage as the tubules merge and get larger during vitrification, which decreases the success of the technique.

In this study, more cytoplasmic degeneration was observed in prepubertal testicular tissue with SSV on the aluminium foil than in the metal brush group. This finding could be attributed to the tissue damage caused by the packing and thawing processes with aluminium. The placement of tissue onto a foil floating on liquid nitrogen may also have negatively affected the tissue because of the decrease in cooling rate. The SSV results support the hypothesis that damage occurs because of 'stress on the cells as a result of mechanical forces generated by the extracellular ice', in agreement with the study by *Baert et al. (2013)*. Another disadvantage of SSV and aluminium foil is the possibility of contamination with pathogenic micro-organisms and other cells because the tissue is placed directly into liquid nitrogen with or without a carrier (*Isachenko et al., 2009*, *2010*).

Cell viability is one of the most important criteria when considering the effectiveness of a carrier, and in this study, viability was higher in the metal brush group than in the other groups. The viability of other carriers progressively decreased from aluminium foil to metal wire to plastic high-security straw. The reason for the low viability observed with the high-security straw may be that the straw was made of a non-conductive plastic, which could negatively affect the cooling rate, and the excess cryoprotectant media may have remained around the tissue.

Ultrastructural analysis revealed high nuclear discrimination in SSC and Sertoli cells in the metal brush, aluminium foil and high-security straw groups. These three systems effectively preserved the identities of the cells. The frequency of pyknotic nuclei was low in the metal brush and aluminium foil groups, and the metal brush preserved intratubular cells remarkably better than the other carriers. Collectively, these results indicated that the high-security straw and metal wire negatively affected prepubertal testicular tissue morphology and ultrastructure, possibly because of the amount of precipitated medium around the tissue during freezing. Therefore, prolonged exposure during vitrification could affect tissues negatively and increase tissue damage.

When the metal brush was used, the harmful medium precipitates around the testicular tissue were removed by an absorbent tissue then placed on the carrier, thus leading to tissue distension, which increases the surface area of the tissue when immersed in liquid nitrogen. At the same time, the metal brush acts as a colander because of its brush side. Given these features, the metal brush produced less pyknosis and cytoplasmic degeneration, significantly higher viability and better prepubertal cell preservation than the other carriers. An advantage of the metal brush is its size and it is simpler to use than foil. It can be sterilized and simply put in a sterile cryovial. These features decrease contamination risk in the liquid nitrogen tank. Before the metal brush, the modified metal wire was used. The metal wire was fixed on the lid in the same way as the metal brush, but the tissue was placed on a single wire. Therefore, the carrier provided sterility

and simplicity in use, but it did not have the colander function of the brush, which could have affected the results.

Ethylene glycol and DMSO were used in the vitrification medium, which have previously produced successful results (*Baert et al., 2012*; *Keros et al., 2005*; *Poels et al., 2012*). Vitrification media contain high concentrations of cryoprotectant and these substances have toxic effects on the cell (*David, 2005*). As the media passes through the cell membrane, it affects the structure of the membrane. In cryopreservation, the basement membrane and basal compartment are especially affected. This damage includes the separation of the basal lamina, the separation of the Sertoli cells from the basement membrane and, therefore, the shedding of SSC. On the other hand, cryoprotectant damage in seminiferous tubules decreases from the basement membrane to the lumen (*Jezek et al., 2001*). Morphological deterioration in seminiferous tubule basement membranes caused by cryoprotectant naturally affects the function.

The success of the vitrification method is directly related to the cryoprotectant concentration (*Mazur et al., 1972*). Cleaning of excess medium around the tissue is an important factor in tissue protection, as well as vitrification and equilibration times (*Jezek et al., 2001*). The current authors have developed a metal brush that they believe will remove the excess medium around the tissue by addition to absorbent tissue. Morphological evaluation was used as a criterion in determining the freeze–thaw damage effects. The size of the tissue pieces, together with the cryoprotectant medium and carrier, is another factor in a successful vitrification process. Therefore, all of the tissue pieces used were kept to a similar size by using the bilateral abdominal testicles of 5-day-old rats and dividing each testicle into two or three parts. In order to compare the effects of carriers without being affected by other factors, equilibration and vitrification were applied to tissue pieces of equal size with the same medium for the same time; the only variable that changed was the carrier.

The current study demonstrates that all four carriers are effective for prepubertal testicular tissue vitrification. Among the carriers, the metal brush produced better results and shows great potential for future clinical use.

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