Short-term hypothermic preservation of human testicular tissue: the effect of storage medium and storage period

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Objective: To optimize the storage medium and period during short-term preservation of human testicular tissue.
Design: First, human testicular tissue fragments from five patients were kept at 4°C for 3 days in different media (Dulbecco's modified Eagle's medium [DMEM]/F12, DMEM/F12 + 20% human serum albumin [HSA], DMEM/F12 + 50% HSA, and HSA). Secondly, fragments from four patients were kept in DMEM/F12 for 3, 5, or 8 days at 4°C.
Setting: Laboratory research environment.
Patient(s): Adult human testicular tissue.
Intervention(s): Biopsy and short-term storage of human testicular tissue at different conditions.
Main Outcome Measure(s): Viability, general tissue morphology, Sertoli cell morphology, number of spermatogonia, and apoptosis. The experimental conditions were compared with fresh control samples.
Result(s): Storing human testicular tissue in DMEM/F12 did not alter any of the investigated parameters. In most conditions containing HSA, tissue morphology was altered, and in all of them the Sertoli cell morphology was affected. The number of spermatogonia was only affected when tissue was stored in 100% HSA. In the second part of the study, tissue morphology deteriorated significantly as of 5 days of hypothermic storage, and Sertoli cell morphology after 8 days.
Conclusion(s): Human testicular tissue can be preserved for 3 days at 4°C in DMEM/F12 without altering tissue morphology, Sertoli cell morphology, number of spermatogonia, or number of apoptotic cells. (Fertil Steril® 2016;105:1162–9. ©2016 by American Society for Reproductive Medicine.)
Key Words: Human, fertility, preservation, short-term, testis

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Since spermatogonial stem cells (SSCs) are the backbone of spermatogenesis, damage to these cells causes infertility. Damage can be initiated genetically (e.g., Klinefelter syndrome) or can occur after treatment with high doses of radio- or chemotherapy (1). Whereas semen samples can be obtained and stored for adult patients, this preservation method is not possible in prepubertal patients, owing to the lack of spermatogenesis. Therefore, two preservation methods have been described to help this group of patients: SSC transplantation (2) and testicular tissue grafting (3). Until the time that these techniques become clinically available, testicular tissue is being cryopreserved (4).

Because specific knowledge is required to cryopreserve testicular tissue samples and manage the testicular tissue bank, this activity is available in only a few hospitals worldwide. If testicular tissue is biopsied in a distant hospital, correct transportation is therefore needed to guarantee the quality of the biopsied tissue. To guard that the tissue is preserved in the best manner possible, the transport conditions and conditions for short-term storage of testicular tissue should be determined. However, to date this research question has been the subject of very few investigations in animal models and, as far as we know, has not been addressed for human testicular tissue.

During ex vivo storage the goal is to inhibit the metabolism of the cells and to significantly retard the chemical

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and biochemical processes responsible for tissue degradation (5). When cells or tissues are stored at a temperature between 4°C and 10°C, this is referred to as *hypothermic storage*. This temperature is often used to transport many different tissue types between tissue donation sites and end users, and for short-term storage. Because during hypothermic preservation the cellular metabolism is slowed down and the oxygen and intracellular energy consumption is minimized, it may lead to prolonged cell viability (6).

Zeng et al. (7) have shown that immature porcine testis fragments still showed high viability rates after 48 hours of preservation at 4°C in Dulbecco's phosphate-buffered saline. Another group tested different media for storing immature porcine testicular tissue and concluded that the tissue could be best maintained in HypoThermosol solution–FRS (HTS-FRS). Cell survival was not affected during 3 days at 4°C, and tissue morphology was even not affected after 6 days at 4°C (8). For immature, nonhuman primates, storage of testicular tissue pieces in ice-cold Leibovitz L15 (L15) medium during 24 hours did not cause morphologic changes (9). Even more, ectopic xenografting to nude mice did not affect the ability to initiate spermatogenesis (9) and could even lead to full spermatogenesis (7).

Fetal bovine serum is commonly used in cell cultures to provide growth factors and as protein source. It is also used in cryopreservation media because it stabilizes cell membranes, is a key element in regulating the osmotic pressure, and has a proven antioxidant role (10, 11). This protective role was also demonstrated during organ preservation (12).

To our knowledge, no data are available on the short-term storage of human testicular tissue. Therefore, in this study, we address two parameters, storage medium and storage period, which could affect the viability and morphology of the testicular biopsy during hypothermic short-term storage or transport. Because the use of fetal bovine serum is not feasible in clinic, owing to batch variations and the associated health concerns (13), this should be replaced by a clinically approved replacement like human serum albumin (HSA). In this study we opted to test Dulbecco's modified Eagle's medium (DMEM)/F12 solely and with increasing percentages of HSA, analogous to a study performed on porcine testicular cells (6) to evaluate whether increasing the HSA concentrations results in a higher viability. Because the preserved tissue will most likely be cryopreserved afterward, and both DMEM/F12 and HSA are also present in the cryopreservation medium (14), using these solutions avoids extra manipulation (washing steps) of the tissue.

MATERIALS AND METHODS

Tissue Source

All human samples were obtained from patients undergoing vasectomy reversal after providing written, informed consent. All patients had a normal tubular structure as proven by histology. Immediately after biopsy, the tissue was transported on ice to the laboratory. The experiments have been approved by the ethics committee of the UZ Brussel (2013/397).

Short-term Storage

In a first part of the study, tissue of five patients (biological replicates) was used to determine the best storage medium. After receiving the testicular biopsy, the tissue was washed in DMEM/F12 (31330-095; Invitrogen) containing L-glutamine and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and cut in fragments of ± 6 mm^3, which corresponds to the size of fragments that is cryopreserved (14). Per patient, two fragments were used as day-0 controls (D0), of which one was digested to a cell suspension to measure the cell viability by TALI Image cytometer (Life Technologies) (according to the manufacturer's guidelines), and the other one was fixed in Acidified Formal Alcohol fixative (10056710; Labonord) for histology and immunohistochemistry. Four different media were tested: DMEM/F12, DMEM/F12 supplemented with 20% HSA (20 mg/mL; 10064, Vitrolife), DMEM/F12 supplemented with 50% HSA (50 mg/mL), and pure HSA (100 mg/mL) medium. The osmolarity of the solutions was measured and is summarized in Supplemental Table 1 (available online). The fragments from each patient were evenly distributed among the different experimental conditions. Of each patient, four fragments were stored per experimental condition (technical replicates). The fragments were kept at refrigerator temperature (4°C) for 3 days. At the end of the experiment, two fragments of each patient were digested for cell viability measurement, as described earlier (15), and two were fixed for further evaluation by histology and immunohistochemistry. In total, 20 tissue fragments were investigated per experimental condition, of which 10 for cell viability and 10 for histology and immunohistochemistry.

In a second part of the study, tissue from four patients (biological replicates) was used to define the maximal storage period in which no important morphologic or functional alterations occurred. Therefore, fragments were kept for 3, 5, or 8 days at refrigerator temperature in the best medium found in the first part of this study. The fragments from each patient were evenly distributed among the different experimental conditions. In total, 16 tissue fragments were investigated per experimental condition, of which 8 for cell viability and 8 for histology and immunohistochemistry.

An overview of the experimental design is given in Supplemental Figure 1.

Histology and Immunohistochemistry

After fixation, the samples were embedded in paraffin and cut into 5-μm-thick serial sections.

For histology, per fragment (one for D0; two for the experimental conditions) two sections, made at different depths, were investigated for their morphologic appearance by hematoxylin-eosin staining. Per section, 10 tubules were evaluated for four parameters: structure of tubules, ruptures of the basement membrane, swelling of tubular cells, and tubular cell loss. Each parameter was scored as indicated in Supplemental Table 2. Maximal score is 3, and the minimal is 0.

For immunohistochemistry, per fragment (one for D0; two for the experimental conditions) two sections, made at different depths, were investigated per fragment. Per section, 10 tubules were investigated.

To evaluate the appearance of the supporting Sertoli cells, vimentin (M0725, Dako) staining was performed. On the consecutive serial section, the number of spermatogonia per area (mm^2) was evaluated by staining the sections for melanoma-associated antigen 4 (MAGE-A4; provided by Dr. Giulio Spagnoli, University of Basel, Switzerland) as described previously (16). Evaluation was performed on an inverted light microscope (Olympus IX81). Analysis of the vimentin staining was executed in the same manner as hematoxylin-eosin evaluations, but this time the Sertoli cell morphology was taken into account by looking at the shape, the detachment from the basement membrane, or cell loss (Supplemental Table 3).

Because MAGE-A4 is known to stain both the spermatogonia and the primary spermatocytes, only positive cells lying at the basement membrane were counted, using the ImageJ software program (version 1.48). Per condition, the average number of MAGE-A4–positive cells per area (mm^2) was calculated. Human testicular tissue was used as positive and negative control. For the negative control, the first antibody was replaced by a mouse IgG isotype control (SC2025; Santa Cruz Biotechnology).

To evaluate the amount of apoptosis, DeadEnd colorimetric TUNEL (G7130, Promega) was performed according to the manufacturer's protocol. The positive control was induced by incubating the section with DNase I. A negative control was taken along by incubating the section with a TdT reaction mix without the rTdT enzyme. The sections were evaluated on an inverted light microscope by counting the TUNEL-positive cells in the tubules.

All evaluations were performed blindly.

Statistics

The data are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with post hoc Bonferroni correction (SPSS Statistics version 19, IBM). Statistical significance was set at $P<.05$.

RESULTS

Different Storage Media

The viability data did not reveal a significant difference between the fresh control (87% ± 13%) and any of the experimental conditions (84% ± 13%, 88% ± 7%, 86% ± 8%, and 87% ± 5% for DMEM/F12, 20% HSA, 50% HSA, and HSA, respectively) (Fig. 1C).

Hematoxylin-eosin staining revealed that the structure deteriorates with increasing HSA concentration. Average scores were 2.75 ± 0.19, 2.62 ± 0.19, 2.35 ± 0.32, 2.16 ± 0.12, and 1.51 ± 0.18 for D0, DMEM/F12, 20% HSA, 50% HSA, and HSA, respectively. Although no significant difference was seen between D0, DMEM/F12 and 20% HSA, the two other media differed significantly from the control (50% HSA: $P=.002$; 100% HSA: $P=.001$) (Fig. 1A and D).

The supporting Sertoli cells also underwent morphologic changes when the amount of HSA was increased (Fig. 1B). No significant difference was seen between D0 (2.69 ± 0.40) and the DMEM/F12 condition (2.34 ± 0.37), whereas all other conditions (20% HSA: 1.91 ± 0.28; 50% HSA: 1.49 ± 0.25; HSA: 1.01 ± 0.13) differed significantly from the control (20% HSA: $P=.006$; 50% HSA: $P<.001$; 100% HSA: $P<.001$) (Fig. 1E).

According to the MAGE-A4 staining, the average number of spermatogonia per mm^2 was 374.55 ± 68.51, 372.40 ± 99.01, 327.74 ± 70.20, 249.92 ± 87.53, and 133.08 ± 73.92 for D0, DMEM/F12, 20% HSA, 50% HSA, and HSA, respectively. The number of spermatogonia only dropped significantly in the tissue fragments stored in HSA ($P=.001$) (Fig. 2A and B). High standard deviations are due to large interpatient differences on D0.

Analysis of the TUNEL staining only highlighted very little percentages of apoptotic cells (D0: 0.01 ± 0.01; DMEM/F12: 0.10 ± 0.12; 20% HSA: 0.20 ± 0.32; 50% HSA: 0.16 ± 0.14; HSA: 0.06 ± 0.04). No significant difference was observed between the control and the different storage media used (Fig. 2C, Supplemental Fig. 2A and B).

On the basis of all the findings mentioned above, DMEM/F12 was selected as the medium to take forward to test how long the samples could be preserved in hypothermic conditions.

Storage Period

The average percentage of viability on the different evaluation time points was not altered significantly compared with D0 (D0: 82% ±9%; day 3: 82% ± 6%; day 5: 85% ± 7%; day 8: 85% ± 10%) (Fig. 3C).

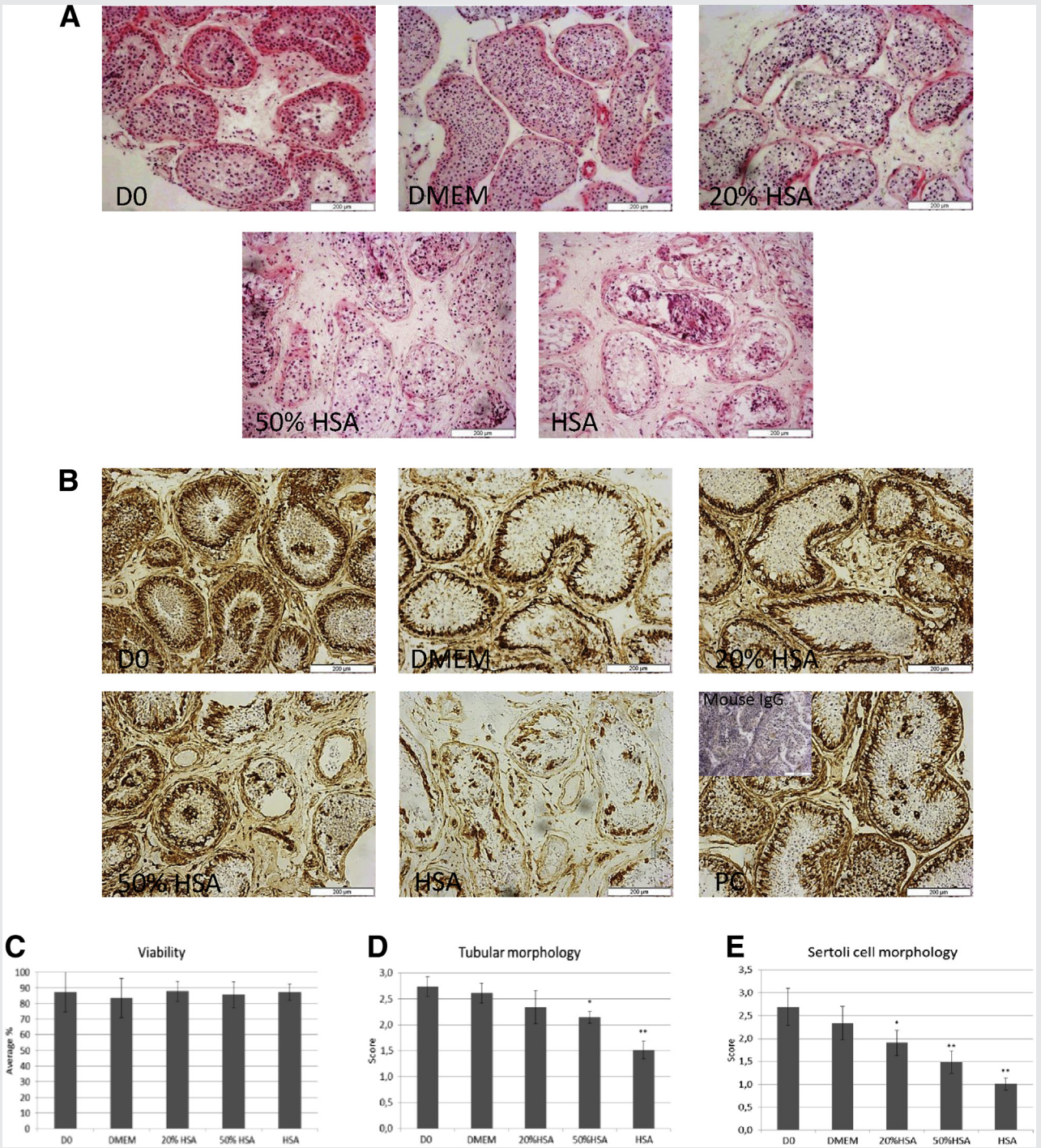
Evaluation of the hematoxylin-eosin staining has revealed that the tissue morphology deteriorated significantly with increasing hypothermic storage period. Between D0 (2.65 ± 0.16) and 3 days (2.32 ± 0.38) of hypothermic storage no significant difference was noticed, but after 5 (1.82 ± 0.31; $P=.007$) and 8 days (1.47 ± 0.18; $P<.001$) of hypothermic storage, significant alterations were seen (Fig. 3A and D).

Evaluation of the vimentin staining led to the following average scores for Sertoli cell integrity for D0 up to day 8, respectively: 2.58 ± 0.59, 2.13 ± 0.40, 1.62 ± 0.54, and 1.19 ± 0.17. Significant difference was only observed between the control and after 8 days of storage ($P=.005$) (Fig. 3B and E).

No difference was seen in the average number of spermatogonia per mm^2 between D0 (337.36 ± 237.17) and any of the storage periods (day 3: 232.83 ± 59.95; day 5: 220.13 ± 74.01; and day 8: 163.86 ± 77.66) (Fig. 4A and B). High standard deviations on D0 are due to large interpatient differences.

Analysis of the TUNEL staining revealed only very little percentages of apoptotic cells in all conditions. Average percentages of TUNEL-positive cells per tubule were 0.02 ± 0.02, 0.08 ± 0.09, 0.19 ± 0.18, and 0.21 ± 0.19 for D0, day 3, day 5, and day 8 of hypothermic storage, respectively. No significant difference was obtained for the different storage periods (Fig. 4C, Supplemental Fig. 2C and D).

FIGURE 1



(**A**) Tubular morphology after hypothermic preservation in different storage media. Normal tissue morphology is seen in the fresh condition (D0), DMEM, and DMEM + 20% HSA. As the concentration of HSA increases, the tissue morphology deteriorates and normal spermatogenic structure is lost. (**B**) Comparison of Sertoli cell morphology between D0 and the different storage media. Normal Sertoli cell morphology is seen in the fresh control (D0) and in the DMEM condition. In DMEM + 20% HSA, some Sertoli cells start to lose their typical columnar shape. More round and detached Sertoli cells appear as the concentration of HSA in the medium increases. Mouse IgG isotype control as negative control (human adult testicular tissue); PC = positive control (human adult testicular tissue). (**C**) Percentage of viability (n = 5). Error bars show the standard deviation. (**D**) Scoring for the tissue morphology based on hematoxylin-eosin data (n = 5). Data shown as mean ± SD. Statistical analysis was performed according to one-way ANOVA followed by post hoc Bonferroni correction and compared with D0: **P*=.002, ***P*<.001. (**E**) Scoring for Sertoli cell morphology by vimentin staining. Data shown as mean ± SD. Statistical analysis was performed according to one-way ANOVA followed by post hoc Bonferroni correction and compared with D0: **P*=.006, ***P*<.001.

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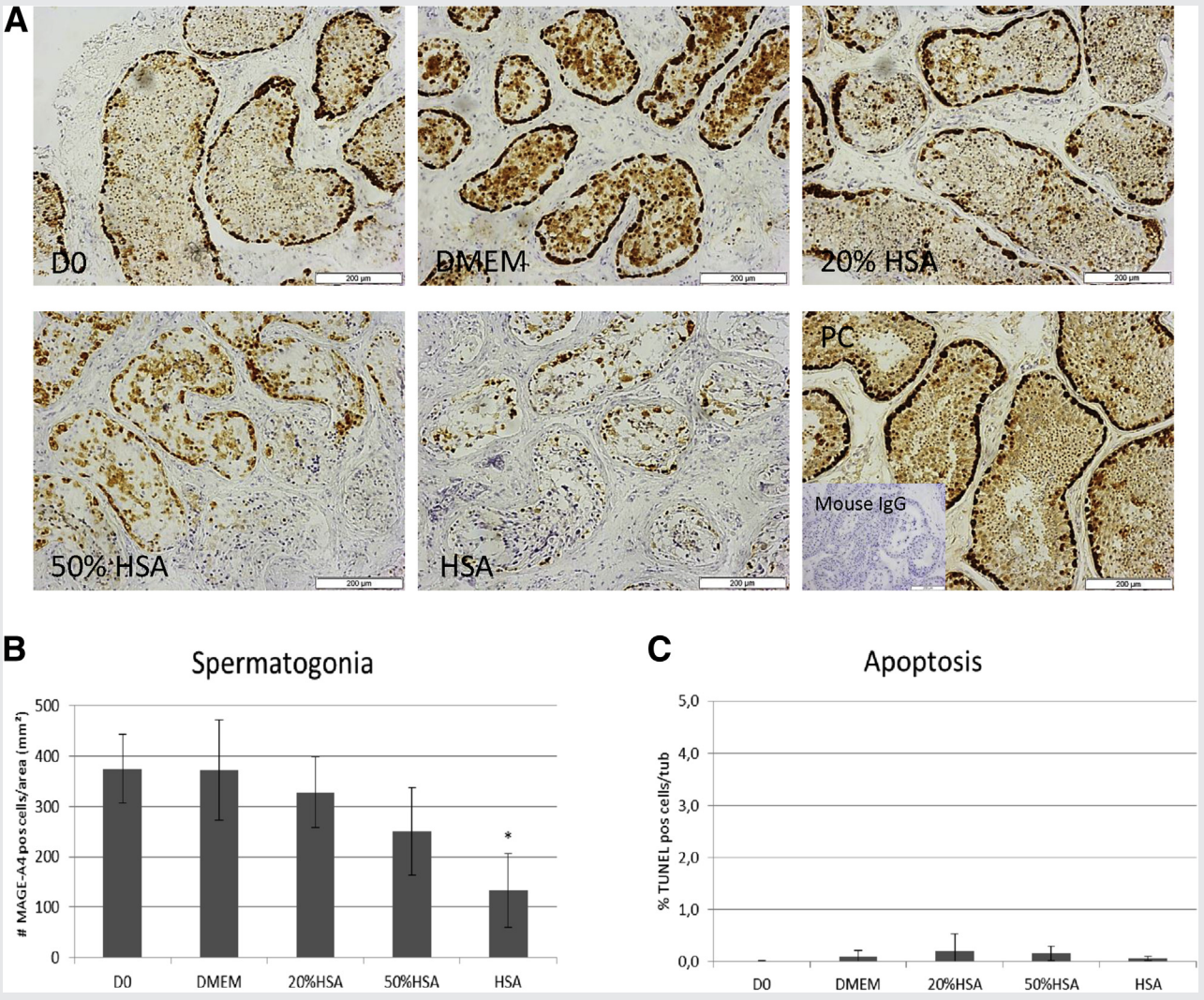
FIGURE 2



(**A**) Comparison of MAGE-A4 positivity (*brown* cells) between D0 and the different storage media. Mouse IgG isotype control as negative control (human adult testicular tissue); PC = positive control (human adult testicular tissue). (**B**) Number of spermatogonia per mm^2 according to MAGE-A4 immunostaining. Data shown as mean ± SD. The number of spermatogonia per mm^2 differed significantly between D0 and 100% HSA. Statistical analysis was performed according to one-way ANOVA followed by post hoc Bonferroni correction and compared with D0: **P*=.001. (**C**) Overview of the TUNEL evaluations of D0 and the different storage media. Error bars show the standard deviation.

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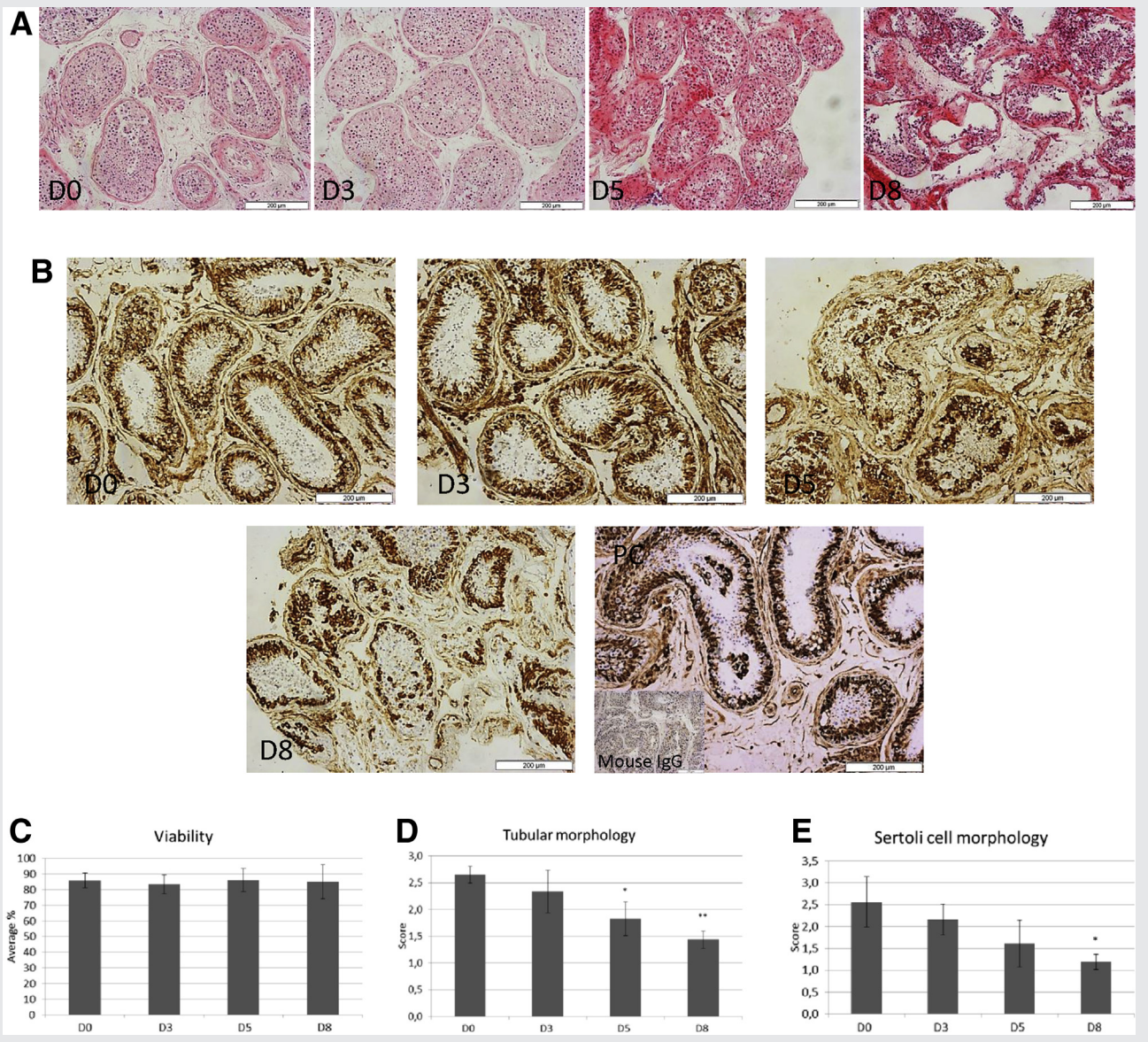
DISCUSSION

Our data reveal that human testicular tissue can be maintained for 3 days in DMEM/F12 without compromising the viability of the testicular cells, the morphology of the tubules and the Sertoli cells, and the number of spermatogonia. Preservation solutions can be classified in two categories: intra- and extracellular-type solutions. Intracellular-type solutions are typically hypertonic (e.g., HTS-FRS), and extracellular-type solutions are usually isotonic (13). For hypothermic storage extracellular-type solutions are mostly used, like PBS, DMEM or L15 (7–9). From a clinical viewpoint, we opted to only include DMEM with varying concentrations of has, to establish a simple storage medium. Additionally, DMEM and HSA are widely available components and normally already present in fertility laboratories, which should facilitate clinical implementation.

The normal HSA concentration in human plasma varies between 35 and 50 g/L (11). However, none of the tested concentrations (20 mg/mL, 50 mg/mL, and 100 mg/mL) established a beneficial effect on the tissue. It could be that the stabilizers, added to protect HSA against oxidative stress (17), have a toxic effect on the tissue. Their negative effect on the blastocyst development in mouse has been shown very recently (18). Therefore, the beneficial effect of HSA could have been counteracted so that only the diminishing buffering capacity of DMEM/F12 supplemented with HSA

FIGURE 3



(**A**) Comparison of tubular morphology between the different storage periods. Significant changes start to occur as of day 5 of preservation, with detachment of spermatogenic cells from the basement membrane, ruptures of the membrane, and loss of germinal epithelium. (**B**) Comparison of Sertoli cell morphology between D0 and the different storage periods. The longer the tissue was stored, the more the Sertoli cells detached from the basement membrane and obtained a round appearance. Mouse IgG isotype control as negative control (human adult testicular tissue); PC = positive control (human adult testicular tissue). (**C**) Viability percentage (n = 4) for the different storage periods. Error bars show the standard deviation. (**D**) Scoring for the tissue morphology based on hematoxylin-eosin data. Data shown as mean ± SD. Statistical analysis was performed according to one-way ANOVA followed by post hoc Bonferroni correction and compared with D0: **P*=.007, ***P*<.001. (**E**) Scoring for Sertoli cell morphology by vimentin staining. Data shown as mean ± SD. Statistical analysis was performed according to one-way ANOVA followed by post hoc Bonferroni correction and compared with D0: **P*=.005.

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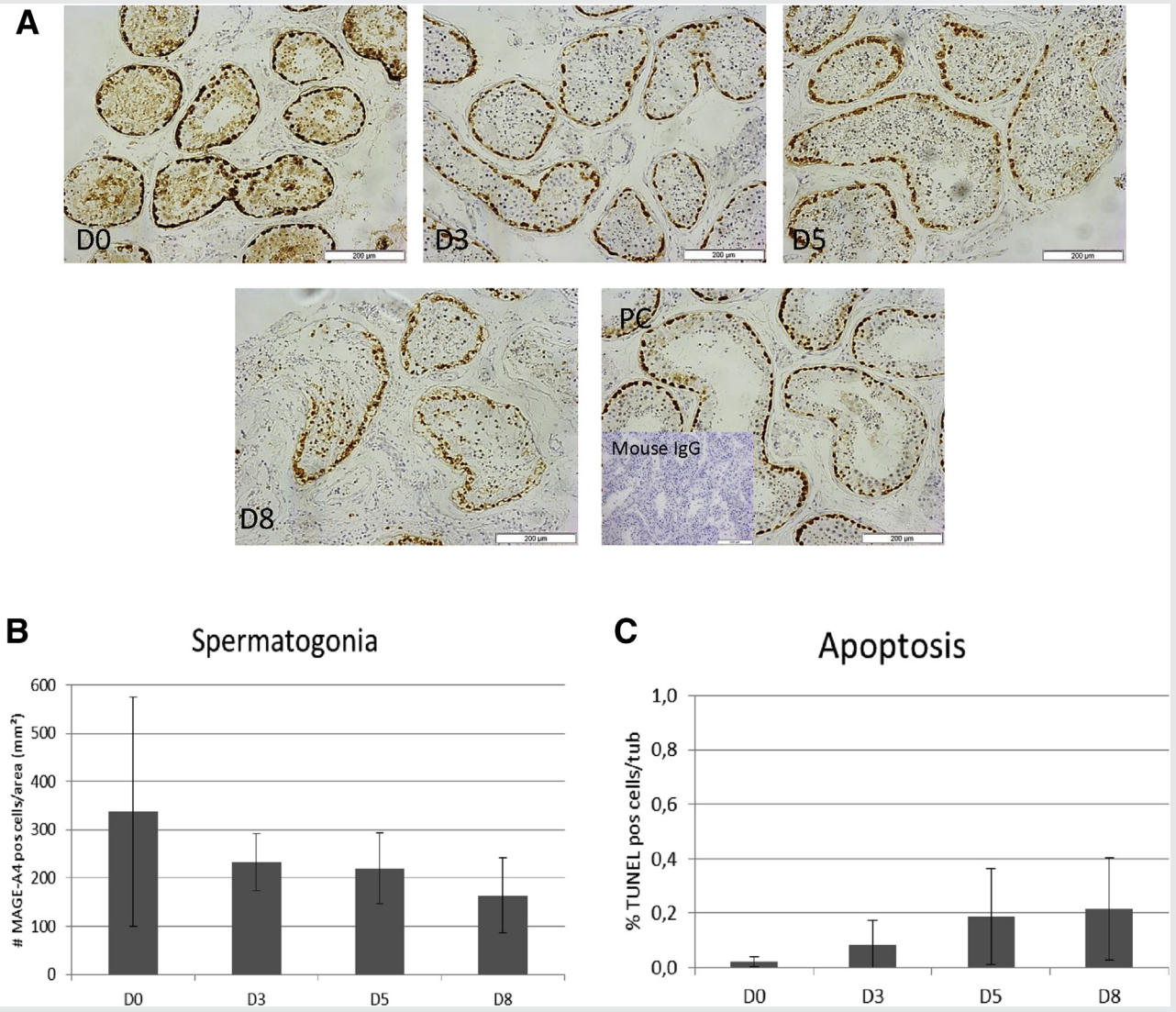
was observed. Possibly, this buffering capacity would not have been lost if the manufacturer had reconstituted the HSA in PBS instead of water. Therefore, all solutions containing HSA, were hypotonic. Because this causes cell swelling, it could also explain the significant deterioration of the tubular morphology with increasing amount of HSA in the medium.

Apoptosis is referred to as the programmed cell death and is associated with cell shrinkage, membrane blebbing, protease (caspase) activation, nonrandom DNA degradation, and ultimately phagocytosis by neighboring cells in vivo (19). Apoptosis is a homeostatic mechanism to maintain cell populations in tissues during normal development and aging (20),

FIGURE 4



(**A**) Comparison of MAGE-A4 positivity (*brown* cells) between D0 and the different storage periods. Mouse IgG isotype control as negative control (human adult testicular tissue); PC = positive control (human adult testicular tissue). (**B**) Number of spermatogonia per mm^2 according to MAGE-A4 immunostaining. Data shown as mean ± SD. (**C**) Overview of the TUNEL evaluations of D0 and the different storage periods. Error bars show the standard deviation.

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but it can also be triggered by stressful conditions like hypothermia (21, 22).

As revealed by the TUNEL staining, apoptosis was not the key determining factor for spermatogonial loss. However, apoptotic spermatogenic cells could have been eliminated already (23) even within 2 to 3 hours after onset of apoptosis (20). On the other hand, necrosis ("accidental" cell death), which is associated with cell swelling, loss of plasma membrane integrity, and eventually cell rupture (cell lysis), could also have occurred. Both apoptotic and necrotic cells undergo DNA fragmentation, thus it is not ruled out that the obtained results are related to necrosis (20) because the transition between both stages depends on the storage time (21). In general, the longer a sample is preserved hypothermically, the more necrotic cells will be present (21). Additionally, the early stages of apoptotic cells are missing because the TUNEL assay overlooks cells in which DNA fragmentation has not started yet (24).

To evaluate the storage period for short-term preservation, DMEM/F12 was selected as the storage medium. Only the tubular morphology already decreased significantly after 5 days, and the Sertoli cell morphology was affected after 8 days. Possibly, the tissue morphology restores after grafting. Unfortunately, functional assays are still lacking for human testicular tissue, whereas in animals, tissue grafting (7, 9, 25, 26) or in vitro maturation into sperm (27) are available.

Our results are slightly different from those obtained in pigs. However, Zeng et al. (7) observed good overall viability and germ cell viability up to 48 hours of short-term preservation in Dulbecco's phosphate-buffered saline. The study from Yang et al. (8) showed that porcine tissue retained a high cell survival rate for 3 days and that tissue morphology was preserved for 6 days in HTS-FRS medium.

In the latter study, DMEM was not selected as the best storage medium (8). However, the DMEM used was a basic medium, whereas our DMEM/F12 medium is a 1:1 mixture of DMEM and Ham's F12 medium, supplemented with 15 mM HEPES. During hypothermic storage, many putative stress factors could be involved, like transmembrane ionic imbalances and intracellular acidosis. The synthetic zwitterionic buffer HEPES, which is an effective impermeable anion, has been shown to possess superior buffering capacities at low temperatures and also contributes to osmotic support in the extracellular compartment because of its molecular size (238 Da) (5). Its presence in our medium might be the reason for the high viability and conservation of tissue morphology, as well as Sertoli cell morphology and the number of spermatogonia.

Future parameters that should be addressed for the short-term storage of human tissue are the tissue size and ideal storage temperature. Notwithstanding the scarcity of prepubertal material, this study should also be validated with immature testicular tissue fragments, as this is our target group.

These findings can and should readily be implemented in the clinic, because transportation or preservation under suboptimal conditions will already influence the tissue quality before cryopreservation and reduce the chances for successful fertility restoration.

In conclusion, we have proven that adult human testicular tissue can be preserved in hypothermic conditions for 3 days in DMEM/F12 without affecting the viability, tissue morphology, Sertoli cell morphology, or number of spermatogonia.

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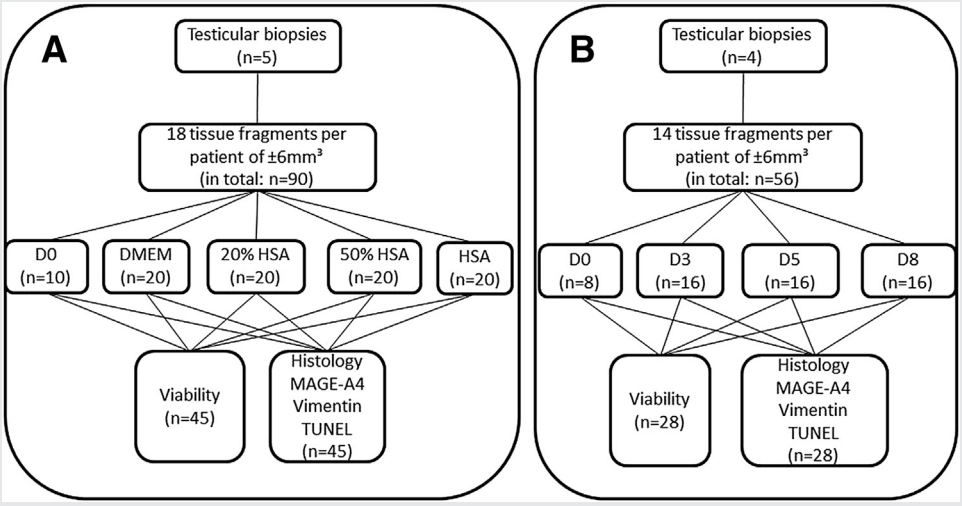
SUPPLEMENTAL FIGURE 1



(**A**) Experimental design for the different media studied. Testicular tissue was obtained from five patients were evaluated. From each biopsy, 18 fragments were obtained and divided over all conditions: 2 fragments for day 0 and 4 fragments for the experimental conditions. For evaluation, per experimental group, half of the fragments were analyzed for viability, and the other half was evaluated for histology/immunohistochemical stainings. (**B**) Experimental design for the different storage periods studied. Testicular tissue was obtained from four patients. From each biopsy, 14 fragments were obtained and divided over all conditions: 2 fragments for day 0 and 4 fragments for the experimental conditions. For evaluation, per experimental group, half of the fragments were analyzed for viability, and the other half were evaluated for histology/immunohistochemical stainings.

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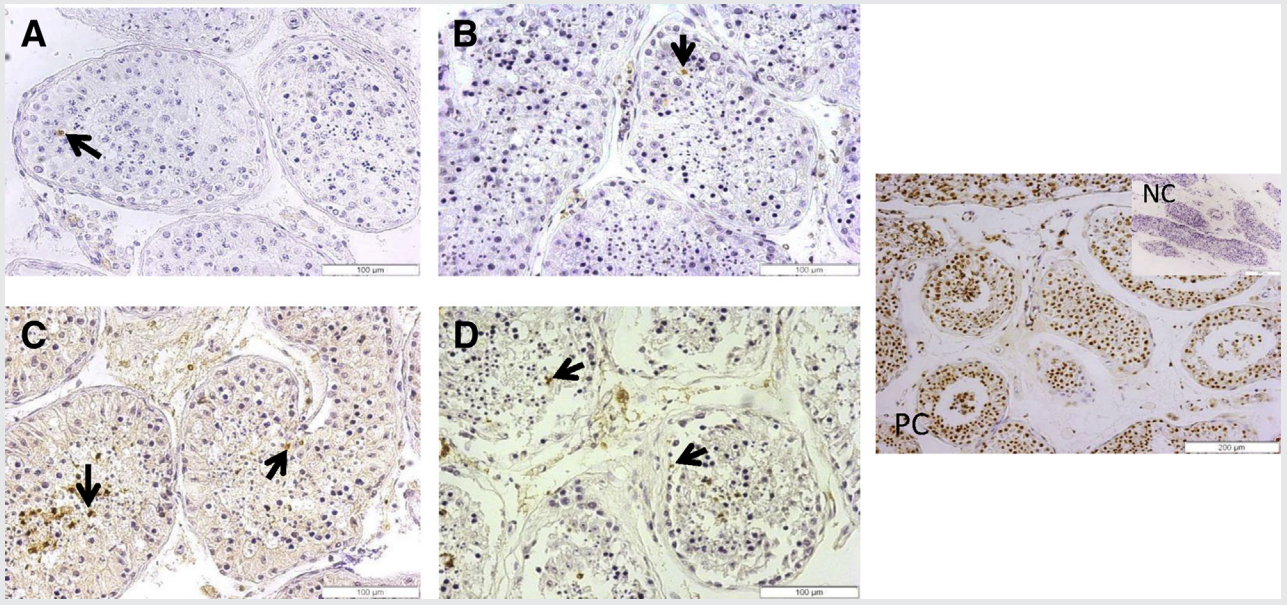
SUPPLEMENTAL FIGURE 2



Apoptotic cells (*brown*; indicated by *arrow*) shown by TUNEL staining in fresh control (**A**) and after 3 days of storage in DMEM/F12 (**B**). Only few apoptotic cells could be found when storing the samples in DMEM/F12. Apoptosis was also determined after several storage periods. Apoptotic cells (*brown*) after 5 days (**C**) and after 8 days of hypothermic storage (**D**). After 8 days of storage, only few apoptotic cells could be detected. NC = negative control (no rTdT enzyme added); PC = positive control (DNase I treated).

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SUPPLEMENTAL TABLE 1

Osmolarity of the different storage media.

	Osmolarity (mOsmol/kg)	
Medium	**Average[a]**	**SD**
DMEM	315	10
DMEM + 20% HSA	260	12
DMEM + 50% HSA	211	17
HSA	106	17

[a] Average of three measurements.

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SUPPLEMENTAL TABLE 2

Scoring for histologic evaluation.

Structure	Score	Rupture from basal membrane	Score	Swelling	Score	Tubular cell loss	Score
Structure intact	3	No rupture	3	No swelling	3	No cell loss	3
All cell types present although slightly disordered structure	2	Partly ruptured	2	Most cells ok	2	Some cell types lost	2
Random distribution of remaining cells	1	Mostly ruptured	1	Some cells ok	1	Most cell types lost	1
No cells present	0	Fully ruptured	0	No cells ok	0	All cell types lost	0

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SUPPLEMENTAL TABLE 3

Scoring for vimentin staining.

Description	Score
Sertoli cells all normal	4
Some Sertoli cells are round or detached from basal membrane	3
Most Sertoli cells are round or detached from basal membrane	2
All Sertoli cells are round or detached from basal membrane	1
No Sertoli cells left	0

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