In search of an efficient injection technique for future clinical application of spermatogonial stem cell transplantation: infusion of contrast dyes in isolated cadaveric human testes

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Objective: To develop an efficient infusion technique for human spermatogonial stem cell transplantation.

Design: A mixture with ultrasonic contrast, computerized tomography (CT) contrast, and Chinese ink was injected into isolated human testes through different sites (the rete testis, the head of the epididymis, the deferent duct, and blind testicular infusion). Ultrasound transducer was used to visualize the injection site and to observe the flow of the mixture injected in the testes. Then, micro-CT scan was used to construct three-dimensional images, allowing the calculation of the testicular volume filled by the mixture. Finally the efficiency of the infusion was evaluated on histologic sections.

Setting: Research laboratory.

Patient(s): Cadaver testes obtained from autopsied bodies at the department of pathology.

Intervention(s): Ultrasound-guided infusion of contrast liquid.

Main Outcome Measure(s): Contrast liquid-filled testis volume and presence of ink in seminiferous tubules.

Result(s): Ultrasonography clearly visualized the flow when seminiferous tubules were injected from the rete testis. No flow was observed when infusions were made either blindly, into the deferent duct, or into the head of the epididymis. On micro-CT no significant differences were observed between the different volumes. After rete testis infusion, ink particles were found in the lumen of the rete testis and in tubules, close and distant from the rete testis.

Conclusion(s): A single ultrasound-guided injection of 800 μL in the rete testis may provide a promising method to transplant human spermatogonial stem cells in a clinical setting.

Key Words: Infertility, spermatogonial stem cell, radiology, rete testis, transplantation

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Spermatogonial stem cell (SSC) loss is an important cause of male factor infertility (1). Stem cell loss can occur after chemotherapy and total body radiotherapy or can be associated to specific genetic disorders (2–4). Because children do not have the possibility to bank spermatozoa, preservation and eventually transplantation of SSCs may become an important strategy to prevent
reproductive stem cell loss disorders [5]. In the past few years, research has been done on SSC preservation and transplantation. Our research group has shown in a mouse model that reproduction after SSC transplantation is efficient and safe in case of genetically related donors and recipients (6–8). As a consequence, the banking of testicular tissue from prepubertal patients has been introduced in a clinical setting. In the next few years we may expect some of these patients to return for transplantation. Therefore, it is very important to develop techniques that allow successful transplantation of SSC suspensions.

In mouse and rat, the efferent duct has been shown to be an efficient site for infusion (9). However, in animals with larger testes (e.g., goat and monkey), this technique is not feasible. Ultrasound-guided infusion into the intratesticular rete testis may be more appropriate (10–13).

Schlatt et al. (14) showed that ultrasound-guided intratesticular rete testis infusion was the best and least invasive infusion technique with maximal infusion efficiency for larger testes (monkey, bovine, and human). Labeled cells could be observed in tubules close to the rete testis but not in tubules far from the rete testes. Brook et al. (15) injected isolated human testes through the rete testis with single and multiple infusions using trypan blue dye and erythrocytes. The efficiency of the infusion was examined macroscopically and microscopically, showing 55% of the testis volume being filled. However, different puncture sites were not examined and there was no proof whether the dye was injected either into the seminiferous tubules or in the interstitial space. At present, only two studies reported on injecting human testis and it is still far from clear which technique (i.e., single or multiple infusions) and which volume are the most efficient for infusing cell suspensions into the testis. Therefore, in the present study, we used an infusion solution containing ultrasonic contrast, computerized tomography (CT) contrast, and Chinese ink (a dye that cannot pass into the basal membrane), enabling us to monitor and to evaluate the volume injected by a catheter. Transfusion was performed using the technique described for pigs by Honaramooz et al. (11) (Supplemental Fig. 1, available online). Ultrasonography was performed using a 15–7-MHz linear transducer, attached to an iE33 scanner (Philips Medical System). After exposure of the testis by careful surgical dissection, ultrasound scanning was used to guide the insertion of the injection needle into the rete testis (mediastinum: 0.15–0.20 mm in diameter). We used 23-gauge needles (0.64 mm, outer diameter), as smaller needles could not penetrate the tunica. The needle was inserted close to the caput epididymis into the rete testis. The gradual movement of the needle was continuously monitored ultrasonographically to guide and ensure the position of the needle in the center of the rete testis. Once the needle was inserted, an infusion set containing the contrast liquid was connected to the catheter. Transfusion was performed under gravity by positioning the syringe at 75 cm above the testis. A total dose of 800 μL mixed contrast was transfused during 2.5–4.5 minutes. For multiposition infusions, 15 minutes elapsed between two infusions. Ultrasonography data were acquired for later analysis.

CT Scan and Three-Dimensional Reconstruction Scan

The CT images were acquired using a micro-CT system (SkyScan-1178; SkyScan) using digital X-ray cameras that scanned more than 180 degrees at a resolution of 83 μm, a rotation step of 1.08°, at 50 kV, and 615 μA. The system comprises a metalloceramic tube and two 1,280 × 1,024 pixels digital X-ray cameras. The scanner contains a fixed 0.5-mm Al filter. Images were reconstructed using SkyScan’s volumetric reconstruction software (Nrecon, SkyScan) and processed using Amide (amide.exe.0.9.1). The three-dimensional movies were processed by Image J version 1.33 u (National Institutes of Health) and OsiriX Medical Imaging Software version 2.3.1 (David Geffen School of Medicine, University of California Los Angeles). The volume of contrast was evaluated by livvol software (Department of radiology, Vrije Universiteit Brussel). To compare the efficiency of the different infusion sites and dosages, statistical analysis was carried out using Mann-Whitney analysis (SPSS Statistics 17.0). A P value <.05 was considered significant.

Histology

After micro-CT scan, the testes were fixed in 4% formaldehyde, and sectioned in total at 4–5 mm. All sections were embedded in paraffin and from each slice 5-μm sections were cut and stained with hematoxylin, eosin, and safranin. Slides

**MATERIALS AND METHODS**

**Tissue Source**

Cadaver testes (n = 20) were obtained from autopsied bodies at the department of pathology of the Universitair Ziekenhuis Brussel. All testes were isolated within 3 hours after death and injected and fixed within 2 hours after isolation. All experimental procedures were approved by the Institutional Review Board of the Universitair Ziekenhuis Brussel.

**Experimental Design**

In the first experimental series, a single dose (800 μL) of combined contrast liquid was injected into the testis through a 23-gauge needle at different sites: the rete testis (n = 3), the head of the epididymis (n = 3), the deferent duct (n = 3), and blind infusion in the testis (n = 3). In a second experiment, different volumes of contrast liquid (1 × 800 μL: n = 3; 2 × 400 μL: n = 2; 4 × 200 μL: n = 3) were injected through the rete testis.

**Ultrasound-guided Infusion of Contrast Liquid**

Optison (Mallinckrodt Medical GmbH), Visipaque (GE Healthcare Inc.), and 10% Chinese ink (Pelikan) were combined at a volume ratio of 1:3:1 and suspended in an equal volume of phosphate-buffered saline (PBS) before transfusion. The mixture solutions were prepared by shaking the syringe for 20 seconds. Transfusion was performed using the technique described for pigs by Honaramooz et al. (11) (Supplemental Fig. 1, available online). Ultrasonography was performed using a 15-7-MHz linear transducer, attached to an iE33 scanner (Philips Medical System). After exposure of the testis by careful surgical dissection, ultrasound scanning was used to guide the insertion of the injection needle into the rete testis (mediastinum: 0.15–0.20 mm in diameter). We used 23-gauge needles (0.64 mm, outer diameter), as smaller needles could not penetrate the tunica. The needle was inserted close to the caput epididymis into the rete testis. The gradual movement of the needle was continuously monitored ultrasonographically to guide and ensure the position of the needle in the center of the rete testis. Once the needle was inserted, an infusion set containing the contrast liquid was connected to the catheter. Transfusion was performed under gravity by positioning the syringe at 75 cm above the testis. A total dose of 800 μL mixed contrast was transfused during 2.5–4.5 minutes. For multiposition infusions, 15 minutes elapsed between two infusions. Ultrasonography data were acquired for later analysis.
were examined by a Zeiss Axioscope 40 microscope. The localization of the Chinese ink in the deferent duct, rete testis, epididymis, seminiferous tubules, blood vessels, and interstitial was recorded.

**RESULTS**

**Experimental Series 1: Selection of the Most Efficient Infusion Site**

The microbubble could be detected in the testis by the ultrasonic detector during the infusions through the rete testis. Sonographies showed clear fluid streams from the rete testis into the parenchyma testis in three of three testes (Fig. 1A; Supplemental Video 1, available online). Ultrasonography showed no infusion of the parenchyma testis after injections through the deferent duct (n = 3) or through the head of the epididymis (n = 3). Few infusions of the parenchyma testis were observed after blind infusions (n = 3). These results were confirmed by CT scan 30 minutes after infusion (Fig. 1B; Supplemental Video 2, available online).

Histology shows ink particles located in the lumen of the rete testis and seminiferous tubules. No ink particles were
FIGURE 2

Testis of patient 3 injected with Chinese ink through the rete testis. (A) Overview of a testis section. Ink particles were observed in the lumen of the rete testis (B) and the seminiferous tubules, close to the rete testis (C). No ink particles were observed in the lumen of the epididymis (D). In the testes of patient 2, ink particles could be observed in the lumen of the epididymis (E) and the interstitium close (F) and distant (G) from the rete testis, indicating liquid reflux and leakage during infusions.

observed in the lumen of the epididymis and the interstitial tissue after a successful infusion in the rete testis [Fig. 2A–2D]. In other testes, ink particles were also observed in the lumens of the epididymis and in the interstitial tissue close to or distant from the rete testis, showing liquid reflux and leakage during infusions (Fig. 2E–2G). Few ink particles could be found in the testicular interstitium but not in the lumen of seminiferous tubules after blind infusions into testes. There were no ink particles in the seminiferous tubules that were injected into the epididymis and deferent duct.

**Experimental Series 2: Selection of the Most Efficient Injection Volume through the Rete Testis**

Micro-CT visualized the contrast dye present in the testes after infusions through the rete testis with different volume. Three-dimensional reconstruction and statistical analysis did not show significant differences between average testis volumes filled by contrast liquid (2,541.7 ± 98.8 mm³ vs. 2,884.7 ± 106.8 mm³ vs. 2,055.1 ± 282.9 mm³ for 1 × 800 μL, 2 × 400 μL, and 4 × 200 μL, respectively; Table 1). There was no advantage of using a multiple site infusion technique compared to a single site infusion technique.

Ink particles could be found in the lumen of the rete testis in seven of eight testes, proving the efficacy of ultrasonic-guided injection (87.5%). Ink particles could also be found in the seminiferous tubules close to the rete testis in two of three with one infusion of 800 μL. (Table 1).

**DISCUSSION**

Spermatogonial stem cell transplantation is a promising technique for fertility preservation in prepubertal boys (5). In rodents, the microinjection of SSCs into the seminiferous tubules through the efferent duct was shown to be feasible. However, infusion by this route is not applicable in human testes due to anatomical differences. Human testes are bigger, the seminiferous tubules are more fibrous, and the lamina propria is more resistant (14). In the past, injection methods for transferring (cell) suspensions into the seminiferous tubules of human testes have been described. Hirsh (16) reported on historic specimens of testes in the Glasgow Hunterian collection where fixed human testes were infused with mercury through the deferent duct by William and John Hunter 200 years ago. Schlatt et al. (14) tried three different sites of infusion using human orchidectomized testes and showed that ultrasound-guided intratesticular rete infusion was the most effective approach to allow the infusion of a large injection volume into the testis. However, in their experiments, very few seminiferous tubules near the rete testis area contained labeled cells and these cells were also observed in the interstitial tissue. Brook et al. (15) confirmed that the rete testis may be an efficient site for germ cell reintroduction. Although erythrocytes were found in the lumen of the seminiferous tubules, it was not clear how much of the dye was introduced in the seminiferous tubules.

In the present study, contrast dyes were introduced into human orchidectomized testes under ultrasonic visualization. Different injection sites were evaluated for a single injection of 800 μL (i.e., the rete testis, the deferent duct, the head of the epididymis, and blind infusion). Ultrasound-guided rete testis infusion proved to be the most effective. Ink particles were observed in the lumen of the rete testis and in the seminiferous tubules. Compared with the study by Brook et al. (15), ours clearly shows the infusion of seminiferous tubules. In our set-up, the infusions were driven by the gravity of 75 cm water and continued for 2.5–4.5 minutes (30 minutes in the study by Schlatt et al. [14]). On the one hand, the high speed of infusion gave a clear vision in ultrasonography; on the other hand, this high pressure might be the reason of liquid leakage from the injection site to the interstitium. Ultrasound-guided injection improves the efficiency of puncturing the rete testis. It may allow the location of the rete testis in future clinical applications so that a cell suspension can be injected directly into the rete testis through the scrotal skin, which makes this strategy minimally invasive and potentially efficient to reintroduce SSCs into the testes of a cured cancer patient. Alternatively, this approach can be performed through a very small scrotal incision exposing the zone between the head of the epididymis and the upper pole of the testis as has been described for collecting sperm by microsurgical epididymal sperm aspiration (17). The injury caused by the injection is expected to be similar to that caused by

**Table 1**

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<th>Chemo</th>
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<th>Ink in seminiferous tubules</th>
<th>Ink in interstitial tissue</th>
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*Note: Infusion of contrast dyes in isolated cadaveric human testes. Fertil Steril 2012.*
testicular fine needle aspiration (also using 23-gauge needles), which is reported to be minor (18). Testes of various species have been used to explore the efficiency of SSC transplantation, but there were no reports about the iatrogenic injury caused by rete testis puncture in these studies. It would be interesting to further investigate this in animal models.

Because of the results of the study by Brook et al. (15), we assumed that multiple infusions into the rete testis could be more efficient than a single infusion. However, according to our CT results, there were no significant differences between single and multiple infusions. In addition, pathology results showed that multiple infusions filled the interstitial compartment but failed to fill the tubular compartment. Based on these preliminary results, and considering the preference for a simple and minimally invasive technique, a single injection of a volume of 800 μL through the rete testis may be preferred versus multiple infusions.

However, this injection method has its limitations. First, the accuracy of the first puncture is very important. Injecting too deep in the rete testis or injecting not deep enough may induce leakage of the liquid toward the interstitium. Thus, this technique has its learning curve. Second, the consistency of the testes seems crucial. We experienced that testes from aging donors (aged 40–65 years) were more difficult to inject than the ones from aged donors (aged 65–83 years). The older testes have less tension and are prone to be filled by liquid. Testes of boys who had radiotherapy or chemotherapy do not contain differentiated germ cells (19, 20). Their testes have less tension and are prone to be filled by liquid.

In conclusion, the present study, together with the work of other investigators, shows that ultrasound-guided single infusion with a volume of 800 μL through the rete testis is an efficacious method to infuse an acellular suspension into human seminiferous tubules. Further research should focus on the injecting of cell suspensions.

REFERENCES

Set-up for infusion. Transfusion was performed under gravity by positioning a syringe at 75 cm above the testis. A 23-gauge needle was inserted close to the caput epididymis into the rete testis under continuous monitoring by ultrasonography. A total dose of 800 μL mixed contrast was transfused in 2.5–4.5 minutes.