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Comparison of enzymatic digestion and mechanical dissociation of human testicular tissues

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Objective: To compare mechanical dissociation, employing the Medimachine system, and enzymatic digestion of human testicular tissues with respect to the proportion of spermatogonia and somatic cells, with the long-term objective of establishing human spermatogonial cultures.
Design: Experimental basic science study.
Setting: Reproductive biology laboratory.
Patient(s): Testicular tissues were obtained from patients with gender dysphoria on the day of sex reassignment surgery. On the basis of the histological evaluation, tissue samples with complete spermatogenesis (fresh, n = 6; cryopreserved, n = 7) and with meiotic arrest (cryopreserved, n = 4) were selected.
Intervention(s): None.
Main Outcome Measure(s): The composition of testicular cell suspensions was assessed performing quantitative real-time polymerase chain reaction (qPCR) analyses for germ cell–specific (*FGFR3*, *SALL4*, *UTF1*, *MAGE-A4*) and somatic marker genes (*ACTA2* and *VIM*). Additionally, flow-cytometric analyses were used to evaluate the percentage of SALL4-and vimentin-positive cells.
Result(s): While Medimachine dissociation yielded higher cell numbers in all patient groups, viability of cells was highly variable and correlated with the histological status of the tissue. Interestingly, qPCR analysis revealed a significantly decreased expression of the somatic marker genes *ACTA2* and *VIM* and an increased expression of the spermatogonial marker genes *FGFR3* and *SALL4* after Medimachine dissociation. These findings were corroborated by flow-cytometric analyses that demonstrated that the proportion of SALL4-positive cells was up to 4 times higher after mechanical dissociation.
Conclusion(s): Medimachine dissociation of human testicular tissues is comparably fast and leads to an enrichment of SALL4-positive spermatogonia. The use of this method may therefore constitute an advantage for the establishment of human spermatogonial cell cultures. (Fertil Steril® 2015;104:302–11. ©2015 by American Society for Reproductive Medicine.)
Key Words: Human testis, Medimachine dissociation, SALL4, spermatogonia

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Spermatogonial stem cells (SSCs) form the basis of spermatogenesis as they can self-renew and give rise to differentiating daughter cells. They are located at the basal membrane of seminiferous tubules, which also contain the somatic Sertoli cells, and are enclosed by a tubular wall containing myoid cells and fibroblasts. The second compartment of the testicular tissue, the interstitium, is composed of a variety of different cell types, including macrophages, fibroblasts, and Leydig cells (1).

As testicular cell suspensions always consist of different cell types, the use of cell type–specific markers is essential for the analysis of these suspensions. Recent studies have identified a variety of germ cell, as well as somatic cell–specific markers. The FGFR3 is a marker for undifferentiated spermatogonia, and colocalization studies have revealed that it is only expressed by a subpopulation of UTF1-positive spermatogonia. Furthermore, SALL4 has been shown to be expressed by a population of undifferentiated spermatogonia (2, 3), whereas

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MAGE A4 is expressed in spermato-gonia and spermatocytes (4, 5). In contrast, vimentin (VIM) and smooth muscle actin (SMA) facilitate detection of somatic Sertoli, peritubular, and mesenchymal cells; and peritubular and endothelial cells, respectively (6, 7).

In particular, the population of human SSCs is of high interest for basic research focusing on growth factors regulating SSC proliferation and mechanisms of self-renewal and SSC differentiation. Moreover, the ability to isolate and propagate SSCs in vitro would provide options for up to now experimental approaches of fertility preservation, including autologous germ cell transplantation. Prepubertal cancer patients who are at risk of becoming infertile after a gonadotoxic treatment and who do not have the option to cryopreserve semen would benefit from this treatment option as would Klinefelter patients, who often lose the entire germ line peripubertally (8, 9). To provide these patients with an opportunity for fertility preservation, testicular biopsies containing the SSCs need to be obtained before gonadotoxic cancer treatment. The underlying idea is that the SSCs could be enriched from these tissues, propagated in vitro, and then be retransplanted autologously after successful cancer therapy (10).

As only small testicular biopsies containing a limited number of SSCs can be obtained from prepubertal patients, the following processes need to be optimized to obtain the highest possible number of viable SSCs from each biopsy: [1] Cryopreservation protocols, [2] the dissociation procedure of testicular tissues, [3] the enrichment of spermatogonia, and [4] the propagation of SSCs. With regard to process 1, comparative results were obtained comparing cell viability after enzymatic digestion of testicular tissues cryopreserved in four commonly used cryoprotective agents (11). With regard to process 2, for the preparation of single-cell suspensions from testicular tissues, typically a combination of mechanical dissociation and enzymatic dissociation involving the use of collagenase, hyaluronidase, and DNAase or trypsin is used (5,12–16). While this method has been shown to yield high cell numbers and to result in representative cell type proportions, the use of enzymes bears the risk of impairing cell integrity, the protocols are costly and time consuming, and the results are highly variable. Alternatively, single-cell suspensions can be obtained using solely mechanical dissociation. Recently, Rodriguez-Casuriaga et al. (17) demonstrated that the mechanical dissociation of testicular tissues from rodents using the Medimachine system is very fast, provides reproducible results, and does not selectively damage certain cell types. With regard to process 3, the most commonly applied approaches for the enrichment of human spermatogonia include differential plating (5, 15), magnetic-activated cell sorting (MACS) (12, 16, 18), and fluorescence-activated cell sorting (FACS) (19). It is important to note, however, that no human SSC-specific antibody has been identified so far, so that depending on the selected marker, only a subpopulation of spermatogonia or a combination of spermatogonia and somatic cells can be obtained. Differential plating has the advantage that it can be applied even if the amount of testicular tissue is very limited. However, as this approach does not allow for a pure isolation of a specific cell population, resulting cultures need to be monitored for the presence of testicular somatic cell types (5, 20). Finally, with regard to process 4, the successful culture of human spermatogonia and their propagation in vitro represent a further milestone in the transfer of human spermatogonial cultures into a clinical application (5, 15, 21).

Human testicular biopsies present the starting material for basic research and future clinical applications focusing on human spermatogonia. Duration of the dissociation procedure, costs, and the yield of spermatogonia present crucial factors in testicular tissue processing. As recent rodent studies have suggested that the mechanical dissociation of testicular tissues is advantageous compared with enzymatic digestion, we aimed to compare these methods using human testicular tissues. As the access to normal human testicular tissues is limited and high cell numbers were required to perform downstream analyses, testicular tissues from patients undergoing sex reassignment surgery were used in this study. To address the aim, the total cell numbers and the proportion of germ cells in particular spermatogonia and somatic cells were determined in resulting cell suspensions, with the long-term objective to establish human spermatogonial cell cultures.

MATERIALS AND METHODS

Selection of Testicular Tissues on the Basis of Preliminary Results

Preliminary experiments have shown that the Medimachine does not yield cell suspensions that are representative of the initial tissue, in contrast with the enzymatic technique. It was rather observed that a higher amount of spermatogonia was obtained. To perform a systematic comparison of both methods, tissues from patients with gender dysphoria undergoing sex reassignment surgery were used in this study as this material is available in amounts that allow developing techniques and conditions for later use in target tissues.

Ethical Approval

Testicular tissues were obtained from patients with gender dysphoria undergoing sex reassignment surgery from three urological departments in Germany (Kinikum Osnabrück, Asklepios Westklinikum Hamburg, and the University Clinic of Essen). Ethical approval was received from the ethics committee of the Ärztekammer Westfalen-Lippe (no. 2012-555-f-S) and the Ärztekammer Hamburg (no. MC-131/13) before study initiation. Written informed consent was obtained from each patient before study participation. As positive controls for immunohistochemical analyses, testicular tissues with normal spermatogenesis were selected from archived testicular tissues stored at CeRA. These samples were obtained from patients who underwent orchiectomy as part of prostate cancer treatment. For preliminary experiments, testicular biopsies were obtained from infertile male patients presenting with obstructive or nonobstructive hypergonadotropic azoospermia at the Department of Clinical Andrology, University Hospital Münster, Germany. After ethical approval (Ethics Committee of the Medical Faculty of Münster and the State Medical Board no. 2008-090-f-S) and written informed consent, research biopsies were obtained at the same time as

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