(MP44-15) Efficient selection method for human round spermatid in TESE negative men

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INTRODUCTION

Around 1% of general population of men suffer from azoospermia who divided into two categories: Obstructive (OA) and non-obstructive azoospermia (NOA). The most precise surgical biopsy methods (Micro-TESE) have been succeeded up to 50% to find sperm in testis tissue of NOA patients. The rest of NOA patients without any spermatozoa in their testis biopsies (TESE negative) are mostly faced with two main options: Adoption or donation (either Sperm or Embryo). In past few years, a well-known Japanese group (Tanaka et al 2015 and 2018) introduced a novel method of round spermatid injection (ROSI) and consequently over 90 healthy babies were born. This ROSI method is expected to be used in the United Stated soon. The most challenging issue utilizing ROSI is separating of spermatids (haploid cells) from other testicular cells (diploid cells). Therefore, a practical method to enrich and select human round spermatid is required.

MATERIALS & METHODS

Frozen testis tissue samples (from brain-dead patients via NDRI) were thawed and subjected for two-step enzymatic digestion with collagenase NB4, Trypsin and DNAse I. Cells were filtered with 40 micron cell strainer and washed with Quinn's advantage medium supplemented with 10% SPS (M10 Medium). Then cells were separated at a discontinuous density gradient column, prepared from a Gradient medium (100%) that is routinely used at IVF clinics. Different concentrations of gradient medium were prepared (70%, 35% and 15%) with dilution in M10 medium. Cell suspension was layered over the top of the column and centrifugation was done to force the cells to put at the different layers. After centrifugation, cells were collected from each layer separately and washed to remove gradient medium. Cytospine slides were prepared fro microscopic evaluation and around 100k cells of each layer was fixed with 66% Ethanol for Cell cycle Flow-Cytometry analysis (using Propidium Iodide staining). Slides were subjected to X and Y chromosome Fluorescence in Situ Hybridization (FISH). For FISH analysis, cells with expected morphology and just one sex chromosome (either X or Y) were considered as spermatid.

RESULTS

FISH slides were assessed with fluorescence microscope (DAPI, FITC and TX red) and five pictures were taken from different field of each slides. At least 200 cells were counted totally. Counting data indicated up to 65% spermatid enrichment in 15% gradient layer which was double in compared with initial cell suspension (32%). Cell cycle flow cytometry showed four different peaks for 4N (dividing cells), 2N (diploid cells), N (spermatid) and also sub-N (sperms) cells. Percentage of cells in N peak were also double in 15% layer in compared with initial cells suspension, this confirmed FISH counting data.

CONCLUSION & FUTURE PLANS

we were able to establish an efficient method of human round spermatid separation using clinically available gradient reagents. This can be used for ROSI in Micro-TESE patients or in vitro produced spermatid (using our recently established human 3D testis organoid system).

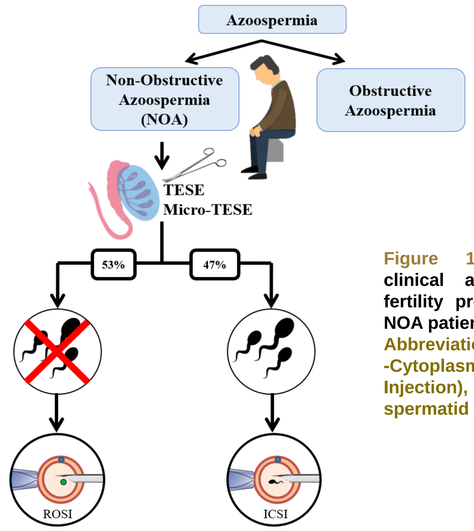

Figure 1. Proposed clinical algorithm for fertility preservation in NOA patients. Abbreviations:ICSI(Intra-Cytoplasmic Sperm Injection), ROSI(round spermatid injection)

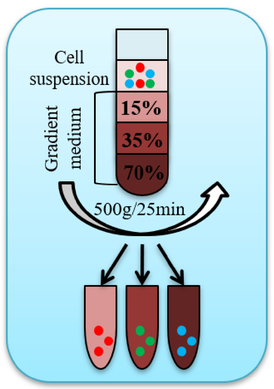

Figure 2. Schematic picture of spermatid isolation via a density gradient method. Cells were digested by a two-step enzymatic method. Single cell suspension was prepared and washed. Different concentrations of the gradient medium 100 were prepared, then cell suspension was layered over the columns and centrifuged at 500g/25min. Layers were gathered in the separated tubes and analyzed.

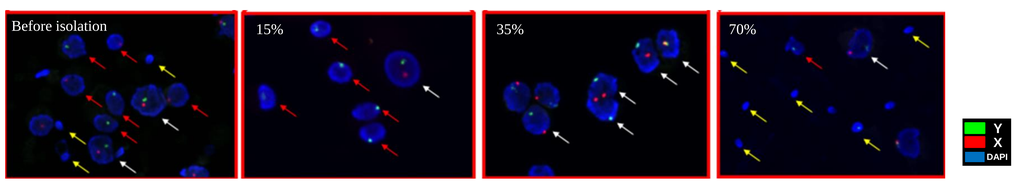

Figure 3. X/Y Fluorescence in-situ hybridization (FISH) in testis cell suspension. **A)** Cells suspension after digestion (no isolation). **B)** cells were extracted from 15% layer **C)** cells were extracted from 35% layer. **D)** Cells were extracted from 70% layer. Arrows are showing spermatids (red), diploid cells (white) and late elongating spermatids and sperm (yellow).

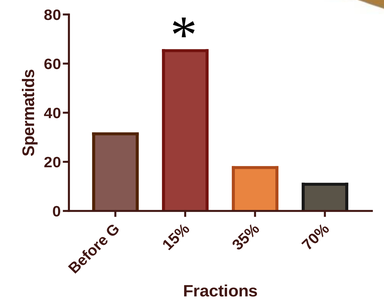

Figure 4. Spermatids were counted based on FISH staining at the different gradient fractions (15%, 35% and 70%) and also cell suspension before gradient.

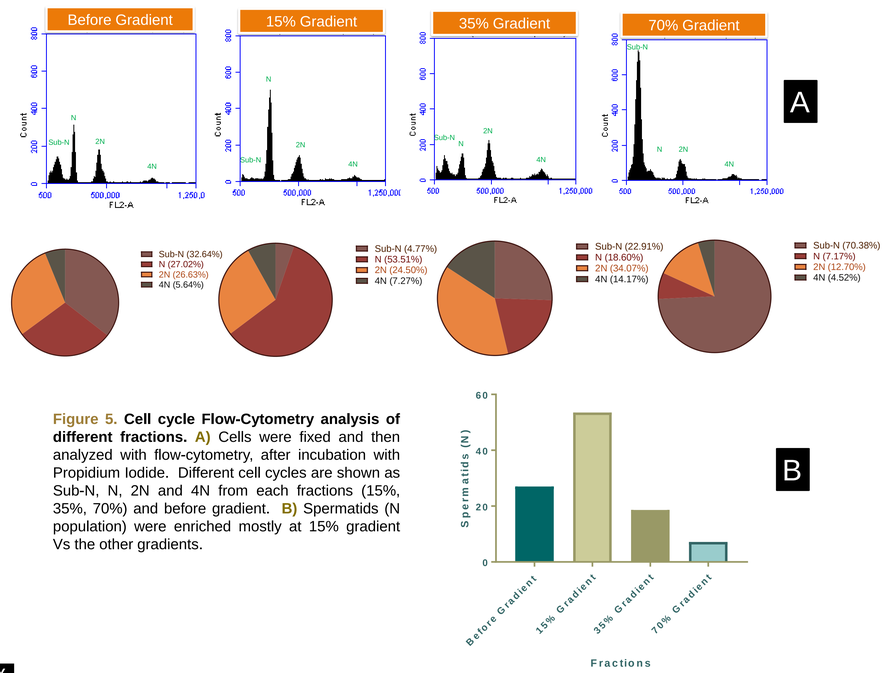

Figure 5. Cell cycle Flow-Cytometry analysis of different fractions. A) Cells were fixed and then analyzed with flow-cytometry, after incubation with Propidium Iodide. Different cell cycles are shown as Sub-N, N, 2N and 4N from each fractions (15%, 35%, 70%) and before gradient. **B)** Spermatids (N population) were enriched mostly at 15% gradient Vs the other gradients.

Acknowledgment

Special thanks to:

Bethy Jackle (FISH staining) at Department of Pathology, Wake Forest School of Medicine