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Endoplasmic reticulum stress response in the spermatogenic cultures isolated from non-obstructive azoospermic patients with spermatogenic arrest

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Keywords

Spermiogenesis, spermatogenic arrest, follicle-stimulating hormone, testosterone, endoplasmic reticulum stress

ABSTRACT • Background and Aims: Cells activate a defense mechanism called endoplasmic reticulum stress response when the amount of unfolded proteins exceeds folding capacity of endoplasmic reticulum, which induces expression of heat shock proteins. As a member of heat shock protein (HSP) 70 family, HSPA5/BiP/GRP78 has been identified in mammalian spermatozoa and expressed in cytoplasm of human spermatocytes and round spermatids. However, the role of HSPA5 in spermatogenic arrest testis remains to be determined. Thus, we aimed to reveal HSPA5 immunoreactivity in the isolated mixed cell clusters from non-obstructive azospermic male by using immunocytochemical techniques and to evaluate the success of in vitro spermiogenesis. **Materials and Methods:** Spermatogenic cells were isolated from testicular biopsies of non-obstructive azospermic patients (n=18) diagnosed with maturation arrest, immunostained with HSPA5 antibody, cell suspension was cultured with G-in-vitro fertilization culture medium supplemented with 25 IU/L recombinant follicle stimulating hormone and 1 µmol/L testosterone for six days. Cultured cells were analyzed by light and electron microscopical techniques. **Results:** The rate of success by in-vitro spermiogenesis was 16.7%. Immunocytochemical analysis revealed that HSPA5 scores of all cells were significantly higher than control group ($p<0.05$) and that of spermatogenic cells was significantly higher than non-spermatogenic cells ($p<0.0001$). Some of differentiated spermatids lost their ultrastructural morphology, underwent apoptosis probably due to endoplasmic reticulum stress. **Conclusions:** Low success of in vitro spermiogenesis may be due to induced endoplasmic reticulum stress even in an ideal culture conditions acquired by follicle-stimulating hormone and testosterone. Eliminating endoplasmic reticulum stress during in vitro spermiogenesis is promising in treatment of spermatogenic arrest.

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INTRODUCTION

Male infertility has been reported to affect approximately 7% of the male population, and nearly 1% of infertile men are azoospermic (1). One of the cause of azoospermia is the spermatogenic arrest, the pause of spermatogenesis in some seminiferous tubules during the formation phase of spermatocytes or spermatids. Spermatogenic arrest has been diagnosed by testicular biopsy in men who had either severe oligospermia (partial arrest) or azoospermia (complete arrest), normal testicular volume, and depending on the etiology normal, high, or low levels of gonadotropins. The radiotherapy, heat, and chemotherapy have also been reported to cause an arrest in spermatogenesis directly or indirectly. Irreversible arrest at primary spermatocyte or spermatid level have a genetic origin due to chromosomes anomalies either in somatic cells or in germ cells (2).

In assisted reproductive techniques, microsurgical testicular sperm extraction (m-TESE) and intra-cytoplasmic sperm injection (ICSI) do not help the azoospermic patients with a complete spermatogenic arrest. However, in vitro differentiation of diploid germ cells to mature haploid germ cell has the potential to benefit these patients (1). Several experimental tests and approaches have been developed using whole testis tissue or isolated single cells from testis biopsies in two-dimensional (2D) or three-dimensional cell culture systems (3D) to investigate the reasons of spermatogenic arrest (3-5). Recent advances of in mammalian models of vitro spermatogenesis are promising in response to overcome the spermatogenic arrest in the human clinical setting.

There is a highly demand to develop an effective culture technique by which haploid productive and functional spermatozoa could be produced from diploid germ cells isolated from the azoospermic patients (6). If a successful in-vitro spermatogenesis culture method could be improved, in-vitro fertilization (IVF) treatment for patients with non-obstructive azoospermia (NOA) enable physicians to overcome the spermatogenic arrest (7). Thus, there is an urgent need for an efficient method for in-vitro spermatogenesis. One of these methods, isolated cell suspension cultures are widely used and preferred to investigate the pathological mechanisms of spermatogenic arrest (6). In order to improve the micro environment for in vitro differentiation of isolated cells, mediums supplemented with follicle stimulating hormone (FSH) and testosterone were used for the differentiation of spermatid into elongated spermatid, implicating the critical role of FSH and testosterone in spermatogenesis (6).

Molecular chaperones are able to confer cellular resistance to environmental stressors, and the majority of these chaperone families are related with the cell stress response or, more commonly known as heat shock proteins (HSPs) (8). Molecular chaperones also participate in a number of normal cellular functions, including metabolism, growth, differentiation and apoptosis (9). The regulators of HSPs, heat shock transcription factors (HSFs), are well known for their cytoprotective functions during cellular stress but less known for potential roles in gametogenesis. All HSF family members are expressed during mammalian spermatogenesis, mainly in spermatocytes and round spermatids which are characterized by extensive chromatin remodeling. Different HSFs could cooperate to maintain proper spermatogenesis (10).

In eukaryotic cells, proteins synthesized in the endoplasmic reticulum (ER) are properly folded with the assistance of HSPs. When the amount of unfolded proteins exceeds the folding capacity of the ER, cells activate a defense mechanism called the ER stress response (unfolded protein response-UPR), which induces expression of ER chaperones and transiently attenuates protein synthesis to decrease the burden on the ER (11). Along with its role in protein folding, HSPA5/BiP/GRP78 (heat shock 70kDa protein 5a- major ER chaperone) is also known to be a key component in

modulating the UPR. In certain severe conditions of ER stress, however, the protective mechanisms activated by the UPR are not sufficient to restore normal ER function and cells die by apoptosis (12, 13). HSPA5 is a member of the HSP70 family that has been identified in mammalian spermatozoa and expressed in the cytoplasm of human spermatocytes and round spermatids. Recent evidence suggests that HSPA5 may play important role(s) in the function of Sertoli cells, mature human and mouse spermatozoa (11,13-15). However, the role of HSPA5 in spermatogenic arrest testis remains to be determined. Thus, we aimed to reveal the HSPA5 immunoreactivity in the isolated mixed cell suspensions collected from TESE biopsies of NOA patients with spermatogenic arrest by using immunocytochemical techniques and to evaluate the success of in vitro spermiogenesis by light and transmission electron microscopy.

MATERIALS and METHODS

Patients

The protocol for establishing primary human cell cultures from testicular biopsies obtained during TESE was approved by the Ethics Committee of Clinical Research Center of Cerrahpasa School of Medicine, Istanbul University, Istanbul, Turkey (Date: 3rd August 2010, Approval number: 23396). This study was managed in accordance with the principles of the Declaration of Helsinki (as revised in Brazil in 2013), the International Conference on Harmonization guidelines for Good Clinical Practice and was issued legal approval by the Turkish Health Ministry. The signed written informed consent was obtained from all patients prior to the study.

Testicular biopsies of NOA patients (n=18) diagnosed with maturation arrest following TESE procedures were selected for the study. Exclusion criteria included the diagnoses of pre-testicular azoospermia, post-testicular azoospermia, obstructive azoospermia and Sertoli cell-only patterns.

All testis biopsies were dissected mechanically by hypodermal needles immediately after examination for any spermatozoa in sperm preparation medium (Medicult) conditioned by 5% CO_2 under an inverted microscope (Olympus IX71). The biopsy pieces was centrifuged and washed at 1200 rpm for 10 min inside the same medium. The supernatant containing cell suspension was divided for the four stages of experiments, namely light microscopy, immunocytochemistry, and cell culture and transmission electron microscopy.

Light Microscopy

To isolate spermatogenic cells, discontinuous density gradients of 40% and 80% were prepared by sperm separation medium (Supra Sperm, Medicult) and sperm preparation medium (Medicult). 1ml of cell suspension was placed on the gradients and centrifuged at 2000 rpm for 20 min. Separated bands of supernatant were divided into tubes and all cell suspension from 40% and 80% gradients were counted by using a Makler counting chamber (Self-Medical Instruments ltd.) under a light microscope (Nicon).

The cell suspensions were smeared on eight slides, and fixed by methanol solution of ready-to-use set (Hemadiff MGG, GBL) for 10 min. Four of slides were stained by May-Grünwald Giemsa (Hemadiff MGG, GBL) for 30 seconds and washed under running water and dried. The stained slides were examined by 100x immersion objective under a light microscope (Olympus BX 61) and photographed by a digital camera attached to microscope (Olympus DP 72). The remaining slides were used in immunocytochemical methods.

Immunocytochemical Analysis

Indirect immunoperoxidase technique was used for detection of HSPA5 protein on smears prepared from cell suspension from both gradients. The immunocytochemistry procedures were performed according to our previous study (16). For

blocking the endogenous peroxidase, 3% hydrogen peroxidase prepared by methanol and distilled water was used. 5% normal goat serum (Vector laboratories) was used to prevent non-specific binding. As a primary antibody, anti-BiP/GRP78 (Sigma Aldrich) was used as diluted for 1:1000. As a secondary antibody, biotinylated goat anti-rabbit antibody (Vector) was used. Avidin-biotin-peroxidase kit (LabVision) was used for the formation of antigen-antibody complex. 3,3-diaminobenzidine tetrahydrochloride dehydrate (DAB, LabVision) was used as a chromogen. Mayer's Hematoxylin was used for counterstaining. Negative control slides were not marked with primary antibody and positive control slide included the control cells (erythrocytes with no nuclei).

Labeled cells were assessed by two researchers and photographed by using a camera-attached light microscope (Olympus BX 61). The intensity of HSPA5 immunostaining was semi-quantitatively evaluated among spermatogenic cells (mostly spermatids) and non-spermatogenic (mostly Sertoli) cells by using H-SCORE analysis (17).

Cell Culture

0.5 ml suspension from 40% gradient including approximately 1 x 10^6 cells was washed and incubated with G-IVF culture medium (Vitrolife), and supplemented with 25 IU/L rFSH (MBL international corporation) and 1µmol/L testosterone (Nebido-BAYER) for six days in an incubator aired with 5% CO_2 at 35 °C. The mediums were changed once in two days. The doses of rFSH and testosterone were selected according to the literature (18). Pre-cultured and post-cultured cells were photographed under an inverted microscope (Olympus IX71). Matured spermatids were counted and noted.

Post-cultured cells were centrifuged at 2000 rpm for 10 min, smeared and stained with May-Grünwald Giemsa (Hemadiff MGG, GBL), and photographed.

Transmission Electron Microscopy

The cell suspension collected from 40% gradient were centrifuged at 1200 rpm for 10 min in sperm washing medium and the supernatant was expelled. The pellet was immersion fixed at 4° C for 4 h in 4% glutaraldehyde (Merck Millipore, USA) solution prepared in 0.1 M PBS (pH = 7.3). Following washing in a Milloning phosphate buffer, the pellet was postfixed in 1% Osmium tetroxide (EMS Diasum, USA) for 30 min. The pellet was washed again with the buffer and immersed in 2% Agar solution. Solidified tissues in Agar were dissected into 1 mm3 blocks, washed with the buffer and dehydrated by immersion in grading series of alcohol (10%, 30%, 50%, 70%, 80%, 96%, 100%). After application of propylene oxide, the sample was embedded in 1:1 propylene oxide + araldite mixture, 1:3 propylene oxide + araldite mixture and lastly pure araldite, respectively. Polymerization was performed at 60 °C for 48 hours. Araldite blocks were cut in 60-70 mm thickness by using an ultramicrotome (Reichert UM3) and thin-sections were placed on copper grids and stained with saturated uranyl acetate and counterstained with Reynold's lead citrate. Contrasted grids were examined by a transmission electron microscope (Jeol JEM 1001 TEM) and photographed an imaging system software (Jeol Mega View III).

Statistical Analysis

Statistical analyses of the data were performed using GraphPad InStat Software (Version 3.06). Results are presented as mean ± SEM and compared by Paired t test. $p<0.0001$ was considered significant. For post-hoc multiple comparison, Dunn Multiple Comparison test was used and $p<0.05$ was considered significant.

RESULTS

Pre-culture Findings

According to the histomorphometric counting, the percentage of round cells (potential Sa spermatids) isolated from 40% gradient was significantly higher than the percentage of round cells from 80% gradient

(p<0.05). However, the percentage of non-spermatid cells from 80% gradient was distinctly higher than that cells from 40% gradient (P<0.0001) (Table 1).

Immunocytochemical analysis revealed that H-SCORE for HSPA5 was significantly higher in all types of cells than the control group (p<0.05). H-SCORE of spermatogenic cells was significantly higher than the scores of non-spermatogenic cells in both 40% and 80% gradients (p<0.0001). However, there was no significant difference between the immunostaining of same non-cultured cell types isolated from 40% and 80% gradients (Table 2, Figure 1).

Table 1 The percentage of cell counts round cells and non-spermatid cells isolated from two different gradients

Gradient Densities	40%	80%	P value
Round cells (%)	53.16±14.96[a]	21.03±11.58	<0.05
Non-spermatid cells (%)	47.53±15.29	78.27±11.67[b,c]	<0.0001
P value	>0.05	<0.0001	

[a]P<0.05 vs 80% group; b vs 40% group. [c]P<0.001 for intragroup comparison.

Table 2 The immunoreactivity scores of spermatogenic cells and non-spermatogenic cells isolated from two different gradients for HSPA5

Groups	40 %	80 %	Control group	P value
Spermatogenic cells	263.56±27.58a	267.54±14.38[a]	56.17±14.21	<0.05
Non-spermatogenic cells	139.64±48.81[a,b]	104.83±42.77[a,b]	21.13±4.78	<0.05
P value	<0.0001	<0.0001	>0.05	

[a]P<0.05 vs control group; b P<0.001 vs spermatogenic cells.

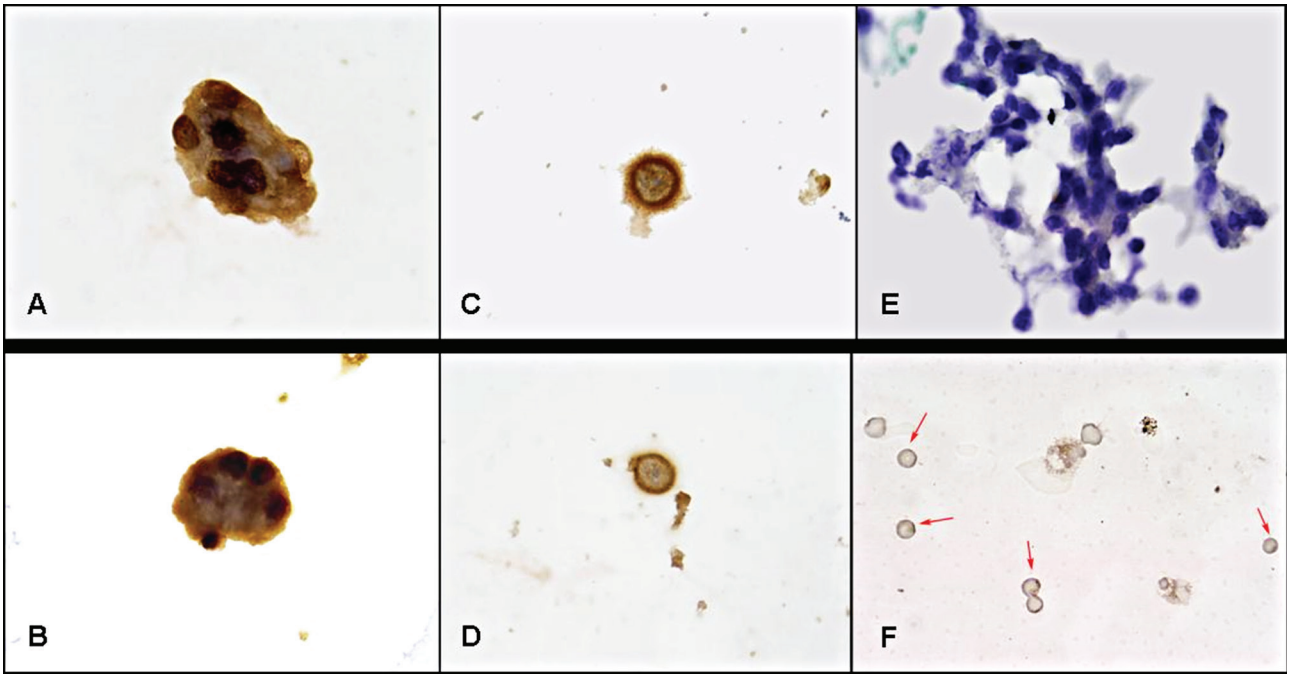

Figure 1 Immunostainings for HSPA5 in spermatogenic cells **(A&B)** and in non-spermatogenic cells **(C&D)** from 40% **(A&C)** and 80% **(B&D)** gradients. **E:** the negative (spermatids), **F:** the positive control (erythrocytes) slide, 100X

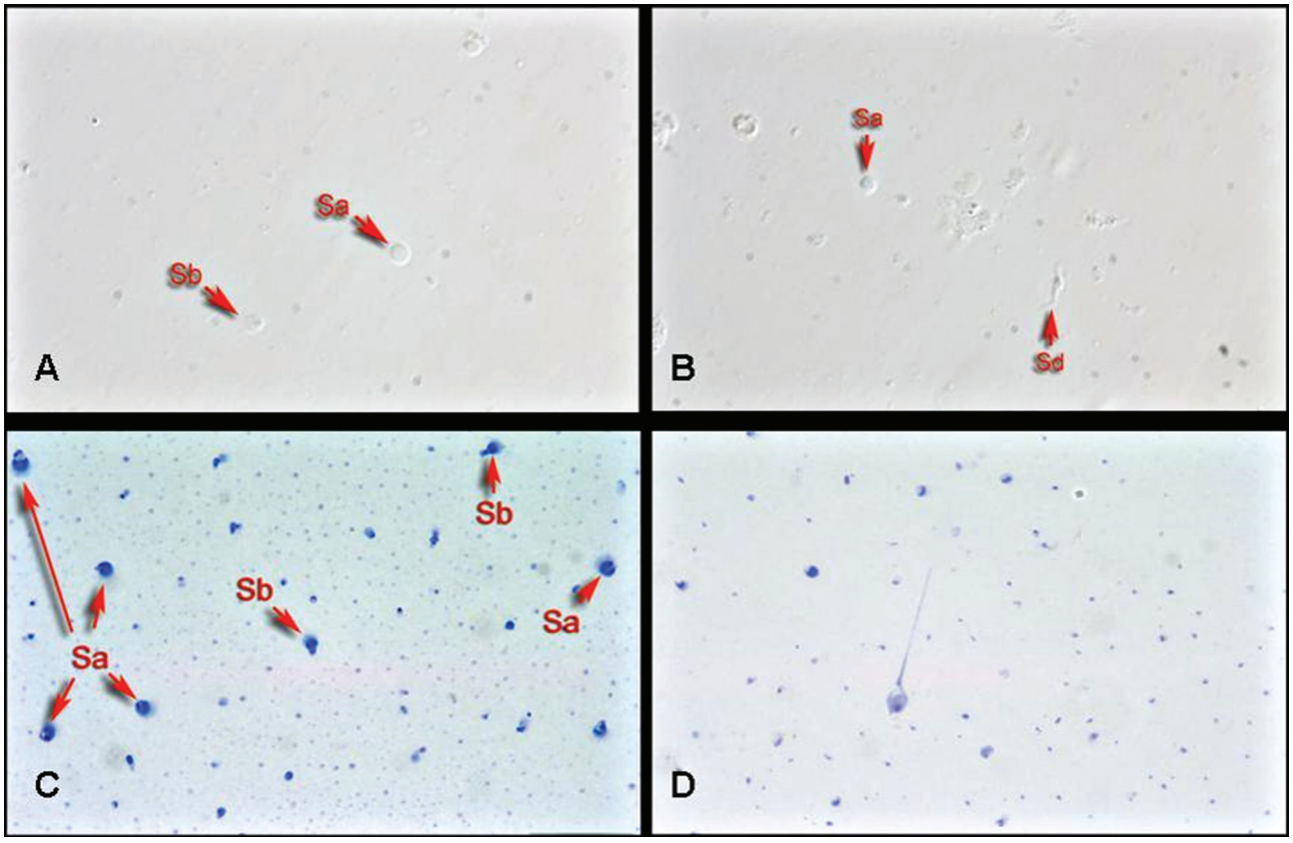

Figure 2 **A:** Round (Sa) and elongating spermatids (Sb) before culture. **B:** Round (Sa) and elongated spermatids (Sd) after culture (**A&B** were taken under inverted microscope, 60X). **C:** Round (Sa) and elongating (Sb) spermatids before culture. **D:** An elongated spermatid after culture (**C&D:** May Grünwald Giemsa, 100X)

Post-culture Findings

Round (Sa) and elongating spermatids (Sb) were observed in the cultures before the incubation. After the very first days of incubation with rFSH and testosterone containing medium, round (Sa) spermatids and elongated spermatids (Sd) were observed in the cultures. For 7 days, every examination of cultures showed elongated spermatids in three samples isolated from testicular biopsies (Figure 2). As a result, in-vitro spermiogenesis was successful in only 3 (16.7%) of 18 cultured samples up to elongated spermatid stage (Figure 3).

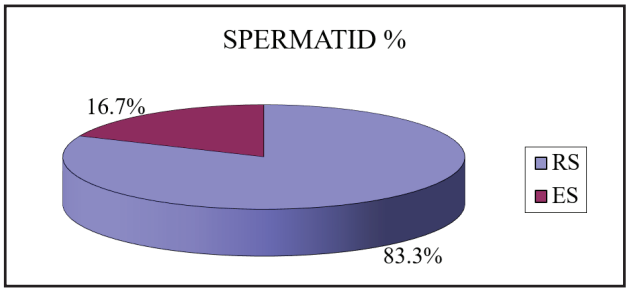

Figure 3 The ratio of the samples in which the round spermatids (RS) were matured into elongated spermatids (ES) following in vitro-spermiogenesis

Ultrastructural Findings

When isolated cells before the culture were examined under TEM, round spermatids (Sa) with condensing nuclei were observed, as parallel to light microscopic results. However, some spermatids had damaged morphology, degenerated membrane and acrosomes, and apoptotic cytoplasms. Nuclei had also apoptotic bodies (Figure 4). Connective tissue cells and collagen bundles and elastic fibers were observed in some samples.

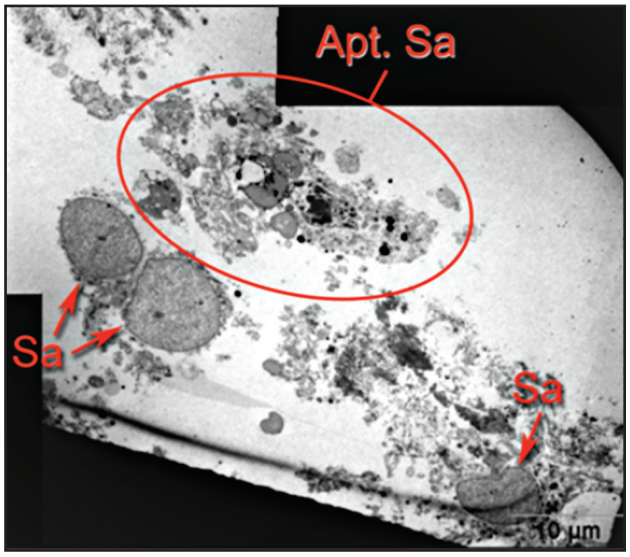

Figure 4 Ultramicrograph of pre-cultured cells. Round spermatids (Sa), apoptotic round spermatids (Apt. Sa) were observed, x2500

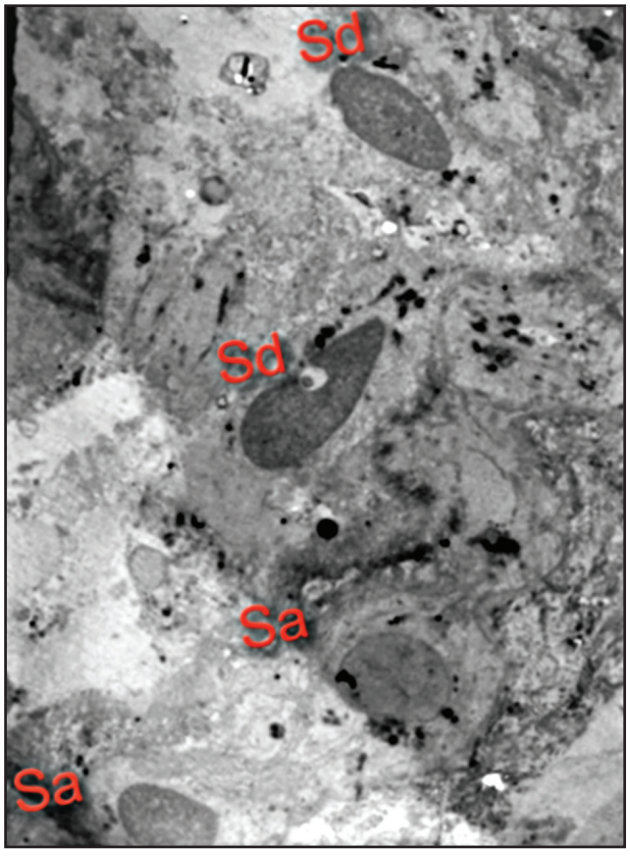

Figure 5 Ultramicrograph of post-cultured cells. Round spermatids (Sa) and elongated spermatids (Sd) were observed, x2500

Post-culture TEM micrographs occasionally showed round spermatids (Sa) and elongated spermatids (Sd) but most of spermatids were apoptotic or degenerated (Figure 5).

DISCUSSION

This work demonstrates that the protein amount of HSPA5 in spermatogenic cells (specifically spermatids) was higher than the amount in non-spermatogenic cells which were isolated from TESE biopsies of NOA patients with spermatogenic arrest, by using a density gradient method. Moreover, 40% gradient was successful to separate round spermatogenic cells from the other cells compared with 80% gradient. More importantly, our post-culture results demonstrated that in vitro maturation of isolated round spermatids, which were arrested during spermatogenic differentiation, was achieved by using rFSH and testosterone, by a success ratio of 16.7%. However, the isolation methods and culture conditions led to the ultrastructural degenerations in cells and even cellular apoptosis.

Spermatogenic arrest can occur due to a gonadotropin insufficiency or following a germ cell damage after the chemotherapy or radiotherapy. The arrest is most frequently observed at primary spermatocyte level and less frequently at spermatid level. Reversible arrest may be due to consecutive hormonal, thermic, or toxic factors and can be resolved spontaneously or by a specific treatment. Spermatogenic arrest at spermatid level is usually due to genetic factors resulting in irreversible azoospermia (2). Irriversible azoospermia can be overcome by reorganization of testicular cells isolated from TESE biopsies obtained from men enrolled in a standard clinical assisted reproduction program. The cell clusters can be cultivated with somatic cell types that are essential to support spermatogenesis for at least 3 months (2,3,6). A perfect culture model would include the combination of somatic and germ cells which mimics the seminiferous epithelium, for the maintenance of the sper-

matogonial stem cell and suitable equilibrium of self-renewal and differentiation in the pre-meiotic phase of spermatogenesis (6). However, the level of spermatogenic arrest is very crucial to understand the reason of arrest and to find a treatment. The endocrine hormones also play pivotal roles in the pathophysiology of this process. In order to improve the micro environment for in vitro differentiation of isolated cells, the effect of hormones, growth factors and feeder cells were investigated on spermatids by Movahedin et al. (19). Mouse spermatid were cultured in DMEM with FBS and supplemented with FSH, testosterone and co-culture (Feeder cell), resulted in the differentiation of spermatid into elongated spermatid at the 2nd day of culture in the hormones supplemented group. In 2010, Xie et al. cultured spermatogonia and Sertoli cells from immature buffalo testes with FBS based media supplemented with FSH, testosterone and retinoic acid (20). Spermatid-like cells with a flagellum were observed after 30 days of culture, suggesting the critical role of FSH and testosterone in spermatogenesis. Thus, we used rFSH and testosterone to improve in vitro spermiogenesis for the biopsies collected from the NOA patients with spermatogenic arrest and the spermatids of 16.7% of the samples succeeded to differentiate into elongated spermatid.

Culture conditions had promising effects on in vitro spermatogenesis by reducing the number of apoptotic germ cells (21). It was reported that the human round spermatids in co-culture with human fibroblast as feeder cells for up to 5 days experienced a spermiogenesis (22), while spermatogonia and spermatocytes co-cultured with Sertoli cells in a supplemented culture with testosterone and FSH differentiated into late spermatids (23). Iwanami et al. observed the differentiation of type-A spermatogonia of an immature (7-day-old) rat in to spermatid when these cells were co-cultured with Sertoli cells, however, the resulted spermatid was not fertile (24). Menegazzo et al. has recommended that porcine fetal Sertoli cells are proper to indorse the development of human spermatids by long-term in vitro co-culture (25). However, the production of haploid cells for fertilization in the presence of feeder cells or somatic cells also raises the problem of in vitro contamination risk and an epigenetical impact on the health of any offspring.

Recently, the use of biocompatible scaffolds is another effort to improve the efficiency of *in vitro* spermatogenesis (26). Lee et al. cultured the isolated testicular cells from immature rats on biodegradable poly scaffolds and after 18 days of culture, 65% of cells were successfully attached the scaffolds with 75% viability. The differentiation rate of germ cells was also higher compared to cells seeded on a monolayer (26). In another study, human isolated spermatogonial stem cells from NOA patients were cultured in a media supplemented with knockout serum replacement, resulted in a promoted differentiation (27). Wang et al. reported the successful generation of haploid spermatid from mouse spermatogonial stem cells when they cultured the isolated cells in 10% FBS supplemented media for 3 days, subsequently treated the cells with medium enriched with retinoic acid for differentiation (28). However, the efficiency of haploid cells production was low. Thus, we used an isolated cell suspension culture to investigate the effects of FSH and testosterone on in vitro spermiogenesis of round spermatids isolated from biopsies of patients with spermatogenic arrest but we did not check the efficiency and the fertility of haploid produced cells. Although the ultrastructure of post-cultured cells gave a valuable information about the status of spermatogenic cells, there is still need to investigate the molecular organization and functionality of these differentiated cells by further molecular analysis.

HSPA5, formerly known as GRP78/BiP is a member of the HSP70 family identified in mammalian spermatozoa. HSPA5 commonly localized in ER lumen plays a critical role in protein transport,

folding and assembly (29). As unfolded proteins accumulate in the ER lumen, HSPA5 disassociates from several stress sensors, enabling the protein folding while simultaneously promoting the activation of the released sensors and the initiation of ER-stress signaling pathways (29). It was reported that Hspa5 mRNA expression was rapidly increased in Sertoli cells following an exposure to environment contaminants linked to the disruption of testicular development and decrease male fertility (14). However, in addition to this protective role, HSPA5 may also play crucial role in the function of mature human and mouse spermatozoa since it has been identified on the surface and cytoplasm of human spermatozoa and round spermatids (11, 13, 30). However, the role of human sperm surface HSPA5 remains to be determined for NOA patients with spermatogenic arrest. In the present study, immunocytochemical analysis revealed that HSPA5 was significantly elevated especially in spermatogenic cells, suggesting induced ER stress is in pre-cultured cells. It is a controversial issue that mechanical dissection of the seminiferous tubules of azoospermic tissues and cell isolation procedures for in-vitro maturation studies can be hazardous for cell homeostasis. TEM results of this study confirmed the degenerative effects of isolation method on the spermatogenic cells. The mechanical forces raised from centrifugation cannot be rule out from the increased ER stress since spermatogenic cells are more delicate and less protected in culture conditions compared to the somatic (non-spermatogenic) cells. Low culture success may be another result of induced ER stress in spermatogenic cells. However, it is noteworthy that the application of ideal culture conditions with rFSH and testosterone conditioned medium obviously supports in vitro spermiogenesis.

The findings of this study lend support to the concept that the rough environment may modulate the formation of functional chaperone complexes during in vitro spermiogenesis and that alternations in the physiological conditions of spermatogenic cells may result in ER stress, resulting in ultrastructural changes and apoptosis. These findings may provide the cellular basis for advances in human in vitro spermatogenesis and/or the possibility for maturation of spermatids within a natural physiological environment. More intensive struggles and works are needed to develop the most optimal culture conditions for in vitro spermiogenesis, and to understand the effects of using the endocrine factors in assisted reproductive techniques.

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