Am J Clin Exp Urol 2021;9(6):469-478
www.ajceu.us /ISSN:2330-1910/AJCEU0135588

Original Article

Effect of testicular morphology on embryo development to the blastocyst stage after round spermatid injection

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Received May 23, 2021; Accepted August 25, 2021; Epub December 15, 2021; Published December 30, 2021

Abstract: If spermatozoa cannot be found after testiculer sperm extraction (TESE) in patients followed up due to nonobstructive azospermia (NOA) and the patients do not want donor spermatozoa, performance of round spermatid injection (ROSI) with the current technology seems to be the last resort. This retrospective study was conducted to evaluate the effect of testicular morphology on the development of embryos to the blastocyst stage obtained from ROSI. Between September 2019 and March 2020, after TESE and biopsy 29 patients who had only spermatid were taken to study. Tubular appearance, basal membrane appearance, Johnson score, peritubular fibrosis, interstitial fibrosis, and Leydig cell proliferation were pathologically examined. Following egg collection, ROSI was applied to the oocytes using the piezoelectric method. The embryos were monitored until the blastocyst stage. The mean age of the 29 patients was 36.3±5.01 years. Also, 7 patients had not previously undergone TESE, 20 had previously undergone once, and 2 had previously undergone twice. It was observed that having a history of TESE and a high Johnson score increased the likelihood of the embryo remaining in the blastocyst stage (P=0.021 and 0.014, respectively). However, other parameters do not affect the likelihood of blastocyst formation (P>0.05). Low TESE history and high Johnson score were associated with embryo development to the blastocyst stage. If spermatozoa are not found in patients with nonobstructive azoospermia, ROSI performed during initial TESE increases the likelihood of blastocyst formation.

Keywords: Spermatid, leydig cell, in vitro fertilization, testicular interstitial cells, blastocyst

Introduction

Azoospermia, defined as the failure to detect spermatozoa during semen analysis, occurs in about 1% of the male population and is observed in about 15% of infertile men [1]. Although patients with azoospermia were previously considered as sterile, it was found that they were able to have children with intracytoplasmic sperm injection (ICSI) after obtaining sperm from the testicles [2]. The ICSI method requires the presence of sperm for oocyte fertilization. Vloeberghs et al. [3] found the initial rate of spermatozoa detection in patients with nonobstructive azoospermia (NOA) to be 40.5%. Schlegel et al. [4] found this rate to be 62% after performing testicular sperm extraction (TESE), and Ashraf et al. reported it to be 50% after micro-TESE [5]. If men who do not have spermatozoa in the micro-TESE procedure and do not accept donor sperm, the embryo will not be obtained because the ICSI procedure cannot be performed. In these patients, spermatid injection may be an alternative option.

There are several etiologies for hypogonadism leading to NOA, including genetic disorders (e.g. chromosome abnormalities, translocations, or Y chromosome microdeletions), cryptorchidism, radiation, and/or gonadal toxins. Despite this, the goal is to obtain sperms by finding the focus containing focal spermatogenesis [6]. Despite the successful percentages of obtaining sperms during TESE, a much smaller minority of patients with spermatogen-

ic insufficiency eventually become a biological father.

Tanaka et al. included 76 out of 730 (10.3%) male patients in whom spermatids were found following TESE; they reported that 734 oocytes were fertilized with piezoelectric stimulation, and about 367 embryos were ready in the blastocyst stage [7]. Despite these encouraging results, round spermatid injection (ROSI) is still controversial, with a previous study [8] reporting no embryos. Developments in human embryo culture [7, 9, 10] having achieved successful ROSI results encouraged us to perform ROSI using the piezoelectric method.

In this study, we present the preliminary results of embryo development to the blastocyst stage after ROSI using the piezoelectric method.

Material and methods

This retrospective study was performed after patients gave written informed consent for research purposes and ROSI procedure, and in compliance with the Helsinki Declaration [11] with the approval of the ethics committee of ethics committee of Ataturk University (B.30.2.ATA.0.01.00/4). The data of 57 azoospermic patients who were underwent micro-TESE were retrospectively analyzed between September 2019 and March 2020 in our clinic. Spermatozoa were not found in these partipiciants and round spermatids in 29 of the 57 men (50.87%) were found. A total of 244 transfer cycles were created with round spermatids obtained from the 29 male patients.

Collection and cryopreservation of spermatogenic cells from azoospermic men

We performed a semen analysis at least twice for each male participant before the procedure. They were included in the study after confirming the absence of spermatozoa. Following an incision on a single testicle under general anesthesia, MESA (microsurgical epididymal sperm aspiration) was applied to the caput epididymis. Micro-TESE was performed under a microscope after making a 1-cm incision in the upper part of the tunica albuginea. Testicular tubular specimens (20-50 mg each) were obtained from the surface and from the depths of the testicle until spermatozoa or spermatids were found. Subsequently, the collected seminiferous tubules were placed in 1 ml mHTF (Human tubal fluid medium) and cut into small pieces with a pair of fine needles. Part of the suspension that contained tubule parts, the part containing 10% polyvinylpyrrolidone (PVP-360; Sigma, St Louis, MO, USA), was thoroughly mixed with 0.9% sodium chloride solution. Spermatogenic cells were released from the tubular parts and distributed in the medium by repeated pipetting. The final suspension contained various spermatogenic cells in different developmental stages; all tissues were cryopreserved. During the procedure, tubule biopsy was performed in all participants who underwent micro-TESE, and the samples obtained were placed in formaldehyde and sent to the pathology laboratory.

Histopathology

The tissue sections were fixed in Bouin solution and stained with hematoxylin-eosine. They were examined by the same specialist pathologist under a microscope. Testicular histology involved investigation of tubular diameter, number of tubules, basal membrane, peritubular fibrosis, interstitial fibrosis, and Leydig cell hyperplasia. Scores (points) were given according to the level of severity: 0 (normal), 1 (mild), 2 (moderate), and 3 (severe) [12]. Spermatogenesis was assessed according to the Johnson score. Histology was scored on a scale of 1-10 according to the Johnson method [13]. Histological appearance of the patient's seminiferous tubules showed in **Figures 1** and **2**.

Ovulation induction, collection, and cryopreservation of oocytes

Induction of ovulation was performed using a gonadotropin-releasing hormone antagonist and a recombinant follicle-stimulating hormone. Human chorionic gonadotropin was administered 24-36 hours before oocyte collection. The procedure for oocyte collection was performed transvaginally with ultrasound. After aspiration of the follicular fluid, the cumulus/oocyte complex was transferred into the medium, which was frozen after oocyte collection.

Thawing frozen testicular cell samples and ıdentification of round spermatids ımmediately before ROSI

Thawing was performed at 37°C. Morphological and physical characteristics of individual cells were examined using a differential inter-

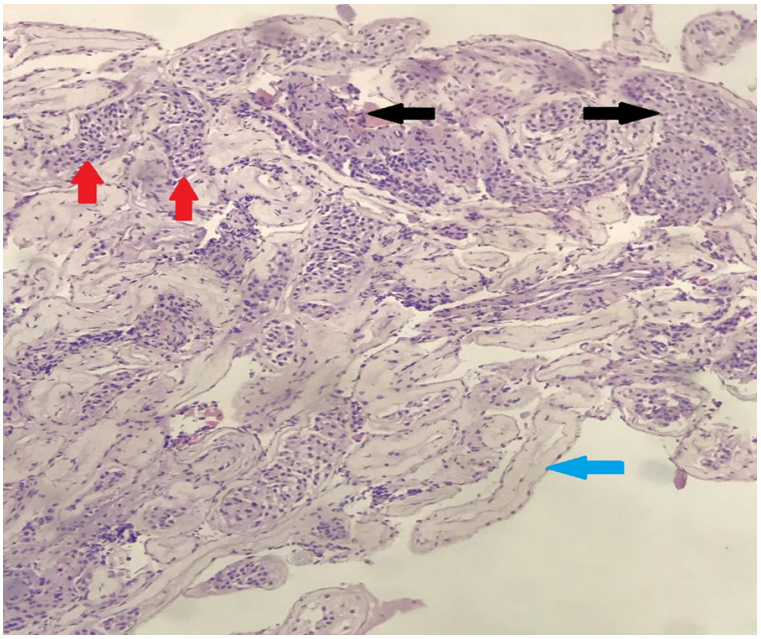

Figure 1. Pathological appereance of patient who have TESE history before surgery. Black arrow shows severe leydig cell hyperplasia. Red arrow shows severe basal membrane thickness and fibrosis. Blue arrow shows empty tubules (Johnson score: 1).

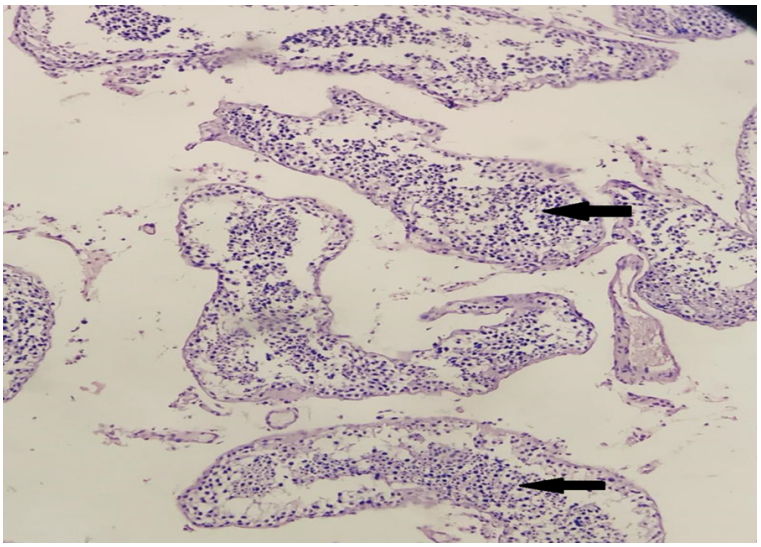

Figure 2. Pathological appereance of patient who have no TESE history before surgery. Black arrow shows spermatocytes (Johnson score: 7). There is no fibrosis around the tubules.

ference microscope. The key features of round spermatids included their small size (6-8 μm diameter), the thin edge of the cytoplasm around the nucleus, and the easy separation of the cytoplasm from the nucleus (showed in **Figure 3**). The accuracy of visual identification was checked by FISH and karyotyping, which was proved to be nearly 100%.

ROSI using piezo micromanipulator

ROSI was performed under an inverted microscope (Zeiss, Oberkochen, Germany). The angle of deviation of the injection pipettes was 30° and the outer diameter was 6 μm. The grip pipettes, with fire-polished tips, had an internal diameter of 10-15 μm and an outer diameter of 100 μm. Oocytes with a polar body visible under a stereo microscope (NIKON SMZ745T, Tokyo, Japan) were considered to be in metaphase II (MII) and were used for microinjection. Two drops of frozen spermatid 12% PVP (polyvinylprrolidone, molecular weight: 36,000, Vitrolife, Gothenburg, Sweden) in MOPS (3-N-morpholino propane sulfonic acid, Vitrolife, Gothenburg, Sweden) suspension and six 5 μL MOPS drops containing oocytes were placed in the reservoir under liquid paraffin (Origio, Malov, Denmark). The reservoir consisted of 40 mm ×50 mm ICSI dishes (Vitrolife, Gothenburg, Sweden).

MII oocytes were electrically activated using Zeus Pulse Stimulator ZPS-19 (Zeus Bioscience, Ankara, Turkey), and incubated for 20 minutes with Sage 1-Step (Origio, Malov, Denmark) under liquid paraffin in a plastic petri dish (4-well, Thermo Nunc, USA) at 37°C with 5.8% CO_2 and 5% O_2. To avoid damage to the oocytes, the polar body was held in the holding pipette so that the body of the oocyte was at a 12 or 6 o'clock position and the injection pipette was at 3 o'clock position. A round spermatid was absorbed into the microinjection pipette (inner diameter of 5-6 μm at the tip). The zona pellucida of each oocyte was punctured with the spermatid injection pipette (showed in **Figure 4**). After the spermatid was pushed forward, the pipette tip was moved for-

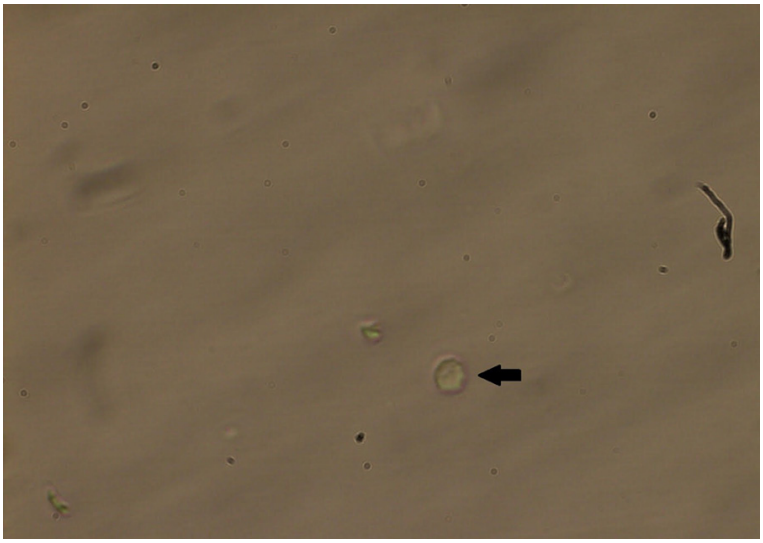

Figure 3. Round spermatid, showed with black arrow.

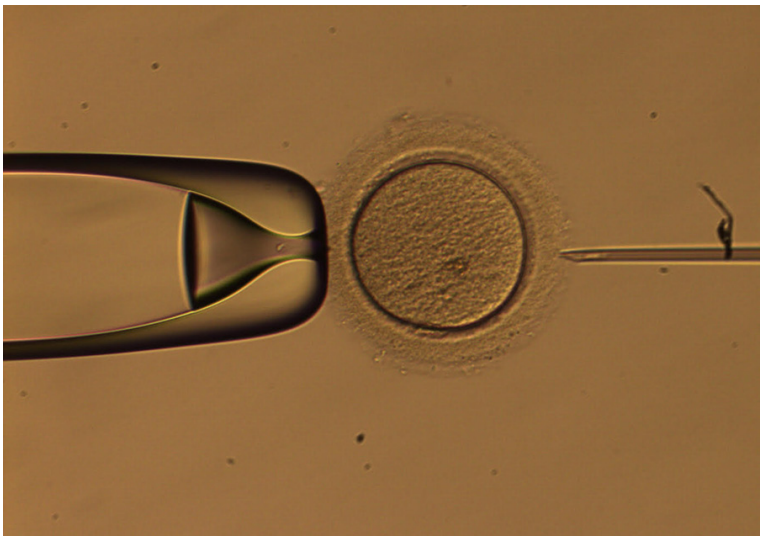

Figure 4. The zona pellucida of oocyte was punctured with the spermatid injection pipette.

ward almost until it reached the opposite side of the oocyte cortex. Oolemma was broken with a single suction, and the entire spermatid was expelled into the ooplasm before the pipette was slowly pulled out. All procedures were performed at room temperature. The injected oocytes were kept at room temperature for about 10 minutes.

Examination of oocytes after ROSI and monitoring embryonic development

After the injection, oocytes were incubated for 18 hours, and a pronucleus check was performed in the oocytes. Spermatid-injected oocytes were continuously cultured up to 120 hours in Sage 1-Step for examining embryonic development up to the morula and blastocyst stages. The loaded and injected oocytes were placed in a drop (~50 µl) of Sage 1-Step covered with liquid paraffin in a 4-chamber petri dish.

Embryo culture

After microinjection and activation, oocytes were cultured in Sage 1-Step under mineral oil in a plastic petri dish at 37°C with 5.8% CO_2 and 5% O_2. The percentages of divided embryos, morulas, and blastocysts (depending on the number of oocytes injected) were evaluated on days 3, 5 (showed in **Figure 5**), and 6 after microinjection. After the procedure, the embryos that reached the blastocyst stage were frozen.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM, New York, USA) software. Continuous variables were presented as mean ± standard deviation, and categorical variables were presented as percentage values. Variables with normal distribution were analyzed using the independent t-test and non-normally distributed variables were analyzed using the chi-square test. $P<0.05$ was considered statistically significant.

Results

As shown in **Table 1**, the mean age of the 29 patients was 36.3±5.01 (29-48) years. While 7 patients had not previously undergone TESE, 20 had previously undergone TESE once and 2 had previously undergone TESE twice. In testicular histopathology of the patients, mean severity appearance of tubular diameter and

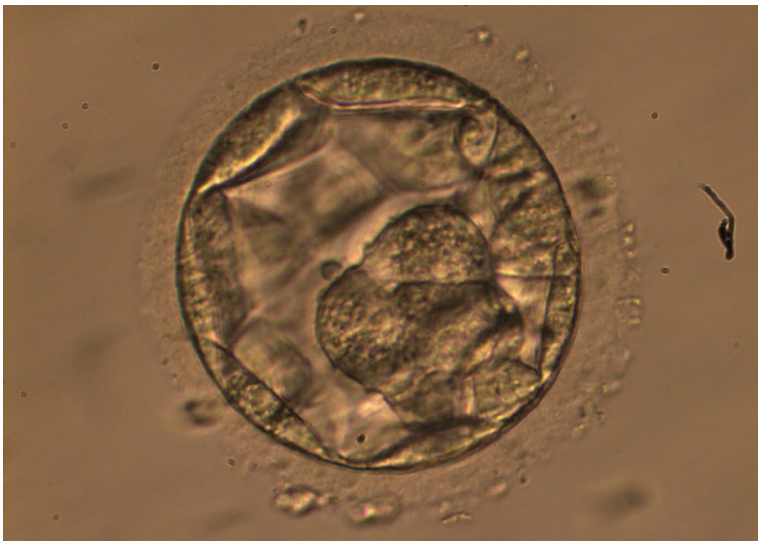

Figure 5. Embryo in fifth day.

number of tubules was 1.79±1.2 (0-3), mean basal membrane severity was 1.89±0.77 (0-3), mean peritubular fibrosis was 2.03±0.86 (0-3), mean interstitial fibrosis was 1.9±0.94 (0-3), and mean Leydig cell proliferation was 1.93±1.03 (0-3). The mean Johnson score was 2.51±1.52 (1-10).

It was observed that the increase in the number of TESEs significantly affected the success of embryo development (P=0.021). Changes in tubular diameter and number of tubules as well as changes in the basal membrane did not affect the success of embryo development (P<0.05). Additionally, it was found that an increase in the Johnson score significantly affected the success of embryo development (P=0.014). Increase in peritubular fibrosis, increase in interstitial fibrosis, and Leydig cell hyperplasia did not play a role in the success of embryo development (P<0.05) (showed in **Table 2**).

Total number of transferred embryos was nineteen. Pregnancy rate was 10.5% (2/19) and no live of birth was observed in our study.

Discussion

After obtaining encouraging results with injection of spermatids in animals [14] injection of spermatids into human oocytes resulting in fertilization and embryo formation was performed for the first time by Edwards et al. [15] and Wanderzwalmen et al. [16]. In these studies, ROSI was reported to result in very low rates of fertilization and embryonic development in patients with completely unsuccessful spermatogenesis. Although the quality of the embryo was poor, Fishel et al. [17] reported that there was no difference in the quality of embryos resulting from ROSI compared to traditional ICSI. However, it is difficult to distinguish and recognize round spermatids among other cell types in addition to ensuring their viability and genetic normality. Therefore, ROSI is typically associated with weak rates of fertilization and embryonic development, and the clinical outcomes have been reported to be significantly lower [18].

Ghazzawi et al. [19] performed ROSI in 87 patients in whom spermatogenesis was completely unsuccessful and compared the results with the results from 93 patients with mature spermatozoa. Fertilization rates were 22% in the ROSI group and 72% in ICSI group, and the number of embryos developed was 62% in ROSI group and 95.8% in ICSI group. Although the results for ROSI were not as successful as in traditional ICSI, it has been noted that it may be recommended in patients with a maturation arrest.

In a study by Tanaka et al., 14 healthy newborns were obtained using ROSI, and the mean age of the fathers of these newborns was 35.08 (26-43) years. The mean Johnson score was 4.95 in the biopsy results obtained from these patients, and it was observed that higher scores were not significant in the success of the embryo [7]. In contrast, based on the results of the present study, we believe that high Johnson scores affect the success of the embryo.

In a study by Vicdan et al. [8], no embryos developed in six patients after ROSI. It was noted that waiting for a period as short as 3 days for the blastocyst stage could have been the reason behind this finding. They also tried the same method in round spermatozoa injection

Table 1. Patients clinical results

Age, mean ± SD (range; min-max)	36.3±5.01 (29-48)
Tese number	No (%)
0	7 (24.1%)
1	20 (69.0%)
2	2 (6.9%)
Tubules diameter, mean ± SD (range; min-max)	1.79±1.2 (0-3)
No, (%)	
0 (Normal apparence)	6 (20.7%)
1 (Mild deformation)	6 (20.7%)
2 (Moderate deformation)	5 (17.2%)
3 (Severe deformation)	12 (41.4%)
Bazal membrane, mean ± SD (range; min-max)	1.89±0.77 (0-3)
No, (%)	
0 (Normal apparence)	0
1 (Mild thickness)	10 (34.5%)
2 (Moderate thickness)	12 (41.4%)
3 (Severe thickness)	7 (24.1%)
Johnson score, mean ± SD (range; min-max)	2.48±1.40 (1-10)
No, (%)	
1 No cell in tubule	4 (13.79%)
2 No germ cell, sertoli cells are existed	18 (62.06%)
3 Spermatogonium is existed	2 (6.89%)
4 A few spermatocyte is existed	1 (3.44%)
5 A lot of spermatocyte is existed	3 (10.34%)
6 A few spermatid is existed	0
7 A lot of spermatid is existed	1 (3.44%)
8 A few spermatozoa is existed	0
9 Almostly complete spermatogenesis, but irregularity in tubule	0
10 Complete spermatogenesis	0
Peritubular fibrosis, mean ± SD (range; min-max)	2.03±0.86 (0-3)
No, (%)	
0 (None)	2 (6.9%)
1 (Mild)	4 (13.8%)
2 (Moderate)	14 (48.3%)
3 (Severe)	9 (31.0%)
İnterstitial fibrosis, mean ± SD (range; min-max)	1.9±0.94 (0-3)
No, (%)	
0 (None)	2 (6.9%)
1 (Mild)	8 (27.6%)
2 (Moderate)	10 (34.5%)
3 (Severe)	9 (31.0%)
İncrease of Leydig cell, mean ± SD (range; min-max)	1.93±1.03 (0-3)
No, (%)	
0 (None)	3 (10.3%)
1 (Mild)	7 (24.1%)
2 (Moderate)	8 (27.6%)
3 (Severe)	11 (37.9%)

Table 2. Success of embryo development

	Success (%)	P
Tese number		0.021*
0	62.7	
1	31.2	
2	53.4	
Tubules diameter		0.344
0 (Normal apparence)	49.8	
1 (Mild deformation)	23.9	
2 (Moderate deformation)	54.2	
3 (Severe deformation)	38.3	
Bazal membrane		0.462
0 (Normal apparence)	-	
1 (Mild thickness)	38.9	
2 (Moderate thickness)	39.7	
3 (Severe thickness)	44.0	
Johnson score		0.014*
1 No cell in tubule	53.5	
2 No germ cell, sertoli cells are existed	35.3	
3 Spermatogonium is existed	31.3	
4 A few spermatocyte is existed	20	
5 A lot of spermatocyte is existed	47.1	
6 A few spermatid is existed	-	
7 A lot of spermatid is existed	100	
8 A few spermatozoa is existed	-	
9 Almostly complete spermatogenesis, but irregularity in tubule	-	
10 Complete spermatogenesis	-	
Peritubular fibrosis		0.375
0 (None)	70	
1 (Mild)	44.3	
2 (Moderate)	34.9	
3 (Severe)	40.8	
İnterstitial fibrosis		0.166
0 (None)	70	
1 (Mild)	30.6	
2 (Moderate)	46.0	
3 (Severe)	36.5	
İncrease of Leydig cell		0.191
0 (None)	60	
1 (Mild)	34.99	
2 (Moderate)	39.5	
3 (Severe)	39.3	

(*) $p<0.05$ statistical significant.

in their study because they believed that the culture medium used in spermatozoa increased the probability of success. However, embryos could not be obtained successfully. We believe that the success of the embryo in the present study is associated with the use of piezo method. Tsujimoto et al. [20], compared sperm injection into cat oocyte by piezo-ICSI method and sperm injection by the conventional ICSI method. It was found that the rate of embryos reaching the blastocyst stage increased by 5.4% with the piezo method.

Despite the fact that the interstitial tissues of the testicles of normal males consist mainly of Leydig cells and vessels, the testicular histology of males with spermatogenic insufficiency is usually characterized by proliferation of Leydig cells and an increase in fibrous connective tissue. Although the effect of testicular interstitial fibrosis on spermatogenesis is not clearly known, it was observed that spermatogenesis was adversely affected after fibrotic reactions like orchitis [21]. In a study by Oka et al. [22], the number of interstitial lesions other than Leydig cells increased by approximately 10 times in NOA compared with obstructive azoospermia (OA). Additionally, in patients with NOA, the testicular interstitium was primarily filled with collagen fibers and seminiferous tubules were wrapped with thick collagen fibers. Furthermore, they also reported that interstitial fibrosis had a negative correlation with Johnson scores.

Leydig cell proliferation was associated with spermatogenic failure in a previous study [23], but another study reported no significant difference in Leydig cell count and size between OA and NOA [22].

About 22 studies on ROSI have been found in the literature review. Most of the studies were conducted between 1996 and 2003, which were low-volume and had a mean egg fertilization rate of 39.6 percent. However, after 2015, high-volume studies with a mean fertilization rate of 57.6% were conducted by Tanaka et al. [10]. The authors noted that the results showed improvements in comparison with the initial studies and were on a larger scale. It was indicated that in order to improve the process, a wider adoption of technology and improvements in the identification of round spermatids were needed [24].

Since most males with NOA have only a few foci of spermatogenesis in their testicles, multiple TESEs may have to be performed for finding these foci and obtaining a sufficient amount of spermatozoa. However, spermatozoa cannot be found in more than 50% of the cases that underwent multiple TESEs [25]. Additionally, procedures, such as multiple blind random biopsies and TESE, may result in various complications, such as persistent pain, swelling, infection, hydrocele, hematoma, transient or even stable decrease in serum testosterone, and rarely, testicular atrophy due to incorrect performance and invasive nature of the procedure [26]. We also believe that cases of multiple TESEs reduce the applicability of ROSI or any other method that may be performed in the future.

There were several potential limitations in our study. The main limitation of our study is that it coincided with the time when the corona virus epidemic started, so the number of participants was watched as low. Another limitation was that testicular morphology of the patients in the group without spermatids and spermatozoa were not included in the study. Samples were taken from dilated tubules to find spermatozoa or spermatids. We think that the samples taken from the point of view of pathology are also preferred from these tubules, so it is sufficient to evaluate the effectiveness of interstitial tissue. Only patients without spermatozoa with micro-TESE were included in our study. Considering the literature, spermatozoa cannot be found in approximately 50% of NOA patients, so we think that ROSI can be presented as an alternative option in couples who do not accept donor spermatozoa.

Conclusion

If spermatozoa cannot be found after micro-TESE in patients followed up due to NOA, performance of ROSI with the current technology seems to be the last resort. It is known that the oocyte is prepared before the ROSI procedure by electrical stimulation with piezo. However, there is no study investigating the effect of testicular morphology on oocyte fertilization and blastocyst formation after ROSI in the existing literature. As a result of this study, it was observed that multiple TESEs adversely affect the development of embryo to the blastocyst stage in patients scheduled to undergo ROSI. Additionally, changes in tissues other than tubules do not affect the development of the embryo to the blastocyst stage. The results would be more successful with ROSI by the piezo method if spermatozoa cannot be found in the first micro-TESE. Prospective studies with a larger number of patients could give a definite judgement on these issues.

Disclosure of conflict of interest

None.

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