Original Article:

Title: Likelihood of sperm retrieval is increased four-fold when Sertoli-cell only histology is observed in combination with another histological pattern during microdissection testicular extraction (mTESE) for non-obstructive azoospermia

Running Title: Mixed Sertoli cell only histology and NOA

Authors:

Sheba Jarvis*, Rong S Luo*, Josca M Schoonejans, Ethna Mannion, Shiama Balendra, Waljit S Dhillo, Jonathan WA Ramsay, Channa N Jayasena

Correspondence address:

Dr. Channa N Jayasena

Department of Investigative Medicine

6th Floor Commonwealth Building

Imperial College London

Hammersmith Hospital

London W12 0NN

Key words:

Microdissection TESE, testicular biopsies, Sertoli cell only, Hypospermatogenesis, Maturation arrest, sperm retrieval rates,

Abstract:

Study question:

In men with non-obstructive azoospermia (NOA), does the concurrence of Sertoli Cell Only (SCO) histology with another histological pattern change the probability of sperm retrieval following microdissection testicular extraction (mTESE) when compared with SCO histology alone?

Summary answer:

The probability of confirmed sperm retrieval following mTESE for NOA is four-fold higher when SCO is found in combination with at least one other major histological pattern when compared with SCO alone.

What is known already:

It has been reported that sperm retrieval is successful in a minority of patients with NOA with Sertoli-cell only (SCO) histology undergoing microdissection testicular extraction (mTESE); this uncertain prognosis for successful sperm retrieval may drive some couples to seek multiple sperm retrieval procedures if the first procedure has been unsuccessful, which may aggravate the psychological trauma associated with infertility. SCO can co-exist with other histological patterns such as complete spermatogenesis (CS), hypospermatogenesis (HS) and/ or maturation arrest (MA). No previous study has investigated whether, and to which extent, the concurrence of SCO with other histological patterns modify the prognosis for sperm retrieval.

Study design, size and duration

Review of 196 cases of NOA undergoing mTESE at a single centre by a single surgeon (JR) between 2002 and 2015. Ninety-six of the 196 patients with NOA had SCO histology.

Participants/materials, setting, methods:

The study was performed in a tertiary hospital with established Andrology clinic. Confirmation of sperm retrieval was defined as the visualisation of mature spermatozoa during microscopic examination of suspended, mechanically digested testicular tissue collected during mTESE.

Main results and role of chance

Following mTESE, SCO histology was observed without any other histological subtype (i.e. pure SCO) in 50 patients with NOA. SCO histology was observed with at least one other histological subtype (i.e. mixed SCO) in 46 patients (1 with CS, 9 with HS, 15 with MA, 4 with CS+MA, 16 with HS+MA, 1 with CS+HS+MA). The probability of sperm retrieval following mTESE was four-fold higher in mixed SCO when compared with pure SCO (proportion of patients with sperm retrieval: 9/50, pure SCO; 23/46, mixed SCO, OR 4.6 [1.8-11.5] *vs.* pure SCO, $P<0.01$). The probability of sperm retrieval following mTESE was not significantly different in mixed forms of CS, HS or MA when compared with pure forms of CS, HS or MA, respectively.

Limitations, reasons for caution:

Results of this small study require confirmation in a larger patient group. More than one histopathologist reported histology results, but they were blinded to sperm retrieval findings.

Wider implications of the findings:

Couples affected by NOA associated with SCO face an uncertain prognosis for sperm retrieval, which may drive them to seek multiple sperm retrieval procedures such as mTESE if the first procedure has been unsuccessful. Results of our study suggest that the prognosis for sperm retrieval associated with mixed SCO is much higher when compared with SCO alone in patients with NOA. Our data may improve the information given to couples affected by NOA and SCO to guide their management.

Study funding/competing interests:

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No competing interests**.**

Introduction

Ten to twenty percent of infertile men have azoospermia (Jarow *et al.*, 1989; Wosnitzer *et al.*, 2014), and most of these cases are due to deficient spermatogenesis which is referred as non-obstructive azoospermia (NOA). Major histological features that may be observed within the testes of patients with NOA include: complete spermatogenesis (CS); hypospermatogenesis (HS), maturation arrest (MA) and Sertoli-cell only syndrome (SCO). Whereas CS and HS confers a good prognosis for sperm retrieval, MA and SCO are classically associated with lower sperm retrieval rates (SRR) (Schulze *et al.*, 1999; Su *et al.*, 1999; Meng *et al.*, 2000; Colpi *et al.*, 2005, Aydin *et al.*, 2015; Sokmensuer *et al.*, 2015). However, it is recognised that pockets of spermatogenesis may be found within the testis in NOA (Turek et al., 1999) and thus multiple histological patterns may be observed in some patients with NOA undergoing surgical sperm retrieval (Schlegel *et al.*, 1997; Silber *et al.*, 1997; Meng *et al.*, 2000; McLachlan *et al.*, 2007; Berookhim and Schlegel, 2014). This raises the possibility that patients with mixed histological features may have a different prognosis in terms of sperm retrieval when compared to a pure histological class.

There is a paucity of data investigating the prevalence of mixed histological features in patients with NOA. Furthermore, no previous study has compared sperm retrieval rates in patients with NOA associated with mixed *versus* patients with NOA associated with pure histological patterns. If mixed pattern histology was shown to modify the prognosis for sperm retrieval during NOA, such information could enhance the counselling given to couples with NOA commencing fertility treatment.

This is particularly important for histology such as SCO which is typically associated with the poorest of prognosis in terms of sperm retrieval and there is no study to date that carefully reviews the impact of mixed SCO on SRR.

We performed a single centre study investigating the prevalence of co-existing histological features in patients undergoing microdissection testicular sperm extraction (mTESE) for NOA. We also compared sperm retrieval rates associated with mixed versus pure histological patterns in patients following mTESE for NOA.

Materials and methods

Subjects and exclusions:

Following departmental approval, the records of 213 men with men with NOA undergoing mTESE between 2002 and 2015 at the Hammersmith Hospital Andrology unit, UK were retrospectively audited. Men with serious concurrent pathology such as testicular cancer, renal pathology or cardiac disease were excluded (n=14). Records without complete histological data (n=36) were also excluded. Therefore, 163 men with NOA were included in the study; SCO was observed on testicular histology in 97 of these patients. Two men with a diagnosis of testicular atrophy in all biopsies were excluded.

Protocol:

A microsurgical sperm aspiration (MESA) was first performed by the same surgeon (JWAR) to exclude patients with obstructive azoospermia from this study. Upon a negative MESA result, multiple mTESE biopsies were undertaken. The use of multiple biopsies allows investigation of heterogeneity in histological features within the testes. Testicular biopsies were sent to Andrology laboratory for detailed assessment and cryopreservation for future use in ICSI cycles. Tissue was also taken and fixed in Bouins solution overnight and processed using standard methods for histological assessment. Findings from both departments were independently registered onto the hospital database.

Sperm retrieval microTESE technique: The microdissection procedure has previously been described (Ramasamy and Schlegel, 2007). In brief, a midline incision was made

in the scrotum and the testis. The tunica vaginalis was opened and the tunica albuginea visualised. After careful incision of the tunica albuginea, the testicular parenchyma was methodically assessed with at least 5-10-fold magnification. Larger more opaque and favourable seminiferous tubules were selected at various sites within the testis (Ramasamy *et al.*, 2005). Seminiferous tubules were then assessed under the light microscope (Nikon) intra-operatively to look for the presence of sperm. If no sperm are identified, the surgeon continues testicular dissection process. On average around 4-10 biopsies are taken per testis (ranging between 1-15mg).

Histological analysis of testicular tissue: Experienced histologists microscopically examined each histological specimen under high-power objectives (20x and 40x) and reported their histological characteristics. The histopathologists used the Johnsen scoring system in order to grade the testicular biopsies (Johnsen, 1970). The maximum Johnsen score observed in either testis was also recorded for each patient.

Confirmation of sperm retrieval in testicular tissue prior to cryopreservation: Sperm retrieval was defined as the identification of sperm during dissection of seminiferous tubules harvested during mTESE, by an embryologist under light microscopy.

Data analysis:

Data all presented as mean ± SD. Fisher's exact test was used to compare proportions. Histological analysis and confirmation of sperm retrieval were performed independently of each. Odds ratio was calculated for successful sperm retrieval in mixed histology compared with pure histology, $p<0.05$ was considered statistically significant.

Results

Patient characteristics:

A total of 163 patients undergoing mTESE for NOA were included in this study. The mean age of the study population was 36 ± 7 years. Each patient received a combined mean of 4.9 ± 2.7 biopsies across both testes and the mean Johnsen score was 4.8 ± 2.8.

Prevalence of mixed histological patterns in patients with NOA:

We initially reviewed the prevalence of mixed histological patterns in patients with NOA following mTESE. Overall, mixed pattern histology was observed commonly. CS, HS, MA and SCO histology was observed with at least one other histological subtype (i.e. mixed CS, HS, MA or SCO) in 11/41 (27%), 44/53 (83%), 56/63 (89%) and 46/96 (48%) patients with NOA, respectively (Table 1).

Histological combinations associated with mixed SCO: Of the 96 patients with SCO histology, no other histological subtype was observed (i.e. pure SCO) in 50 patients. SCO histology was observed with at least one other histological subtype (i.e. mixed SCO) in the remaining 46 patients (1 with CS, 9 with HS, 15 with MA, 4 with CS+MA, 16 with HS+MA, 1 with CS+HS+MA) (Figure 1).

Probability of sperm retrieval with pure and mixed histological patterns:

We next investigated the probability of confirming successful sperm retrieval in patients with pure and mixed histological features following mTESE for NOA (Figure 2). The probability of sperm retrieval following mTESE was four-fold higher in mixed SCO when compared with pure SCO (proportion of patients with sperm retrieval: 9/50, pure SCO; 23/46, mixed SCO, OR 4.6 [1.8-11.5] *vs.* pure SCO, P<0.01). The probability of sperm retrieval following mTESE was not significantly

different in mixed forms of CS, HS or MA when compared with pure forms of CS, HS or MA, respectively.

Histological combinations associated with mixed SCO: Having observed that mixed SCO was associated with improved prognosis for sperm retrieval when compared with pure SCO, we investigated whether individual histological patterns improved the probability of sperm retrieval in SCO (Table 2). When compared with pure SCO, the probability of sperm retrieval following mTESE was significantly higher when SCO occurred in combination with CS (4/6, OR 9.1 [1.4-57.6] *vs.* pure SCO, $P<0.05$) or HS (15/26, OR 6.2 [2.1-17.9] *vs.* pure SCO, $P<0.001$) and MA (16/36, OR 3.6 [1.4-9.8] *vs.* pure SCO, $P<0.01$) (Figure 3).

Discussion

NOA with SCO histology confers an uncertain prognosis for sperm retrieval. Affected couples may be driven to seek multiple sperm retrieval procedures if the first procedure has been unsuccessful. This may, in turn, aggravate the devastating psychological consequences experienced by affected couples. Sperm retrieval using mTESE allows histological examination of testicular tissue and direct visualisation of sperm under light microscopy. Since SCO histology implies the absence of germ cells in the testicular microarchitecture, it has been classically assigned a poor prognosis for sperm retrieval with rates around 14-16% (Seo and Ko, 2001; Weedin *et al.*, 2011). However, in one meta-analysis, sperm retrieval rates of 32.8% were found from SCO biopsies (Yang *et al.*, 2008). More recent study showed sperm retrieval rates of 40% following mTESE from SCO biopsies (Aydin *et al*., 2015). By inference, zones of residual spermatogenesis are likely to exist in many patients with SCO histology and thus this may explain such variability in the literature. In accordance with this model, we report that the histological patterns CS, HS, MA and SCO are observed in approximately half cases of NOA with SCO histology. Importantly, by carefully reviewing the mixed histological patterns coexisting with SCO histology, we report for the first time that successful sperm retrieval is four-fold more likely in patients with mixed SCO when compared with pure SCO.

Observing mixed histological patterns following single unilateral or bilateral testicular biopsies is a well-known phenomenon (Johnsen *et al*., 1970; Meng *et al.*, 2000). However, the clinical impact of mixed histological patterns on sperm retrieval rates during NOA and frequency of mixed histological patterns have not been reported previously. In the current study, mixed histological patterns (a biopsy during which

different histological features coexist in adjacent regions) were observed in 40% of patients undergoing mTESE. We may speculate whether mixed histological patterns actually exist in all patients with NOA; however, we failed to observe that the probability of observing mixed histological patterns was associated significantly with the number of histological samples collected during mTESE (data not shown). Further studies are required to further define the optimum number of histological samples required to determine the presence of mixed histological patterns in patients with NOA.

Johnsen scoring is an established method for analysing testicular histology, which we observed to be associated with sperm retrieval outcomes in agreement with other studies. Johnsen scoring is labour intensive, and provides a mean score which when calculated may mask variations in histological features. By observing a four-fold increased probability of sperm retrieval in mixed SCO when compared with pure SCO in patients with NOA, our data augments information within the Johnsen score, by informing how combinations of histological features affect sperm retrieval.

A range of sperm retrieval rates associated with SCO following mTESE have been reported in the literature. Clearly, local protocols, technical expertise and population characteristics might also affect these results. Our data also suggest that the variability in published sperm retrieval outcomes following mTESE might be partially explained by the presence or absence of mixed histology; sperm retrieval was confirmed in 47% of patients with NOA and mixed SCO histology but only 16% of patients with NOA and pure SCO histology. It would be interesting to confirm whether mixed SCO

histology associated with improved sperm retrieval outcomes at other centres performing mTESE.

It is important to consider the strengths and weaknesses of the study. We report a 13-year experience of a large tertiary centre where mTESE procedures are well established and undertaken by a single surgical operator. Although variable numbers of biopsies were taken per case, our data suggest this did not significantly affect the results. Multiple experienced histopathologists reported the findings of the testicular biopsies, and we recognise this may be subject to inter-observer variation.

In summary, our data suggest that approximately 40% of patients with NOA undergoing mTESE with multiple biopsies have mixed histology pattern. Mixed histological patterns did not significantly modify the chance of sperm retrieval following mTESE in patients with NOA associated with CS, HS or MA. However, we suggest that sperm retrieval was four-times more likely in patients with NOA with mixed SCO when compared with pure SCO. These data have important potential implications for the management of couples affected by NOA, by implicating mixed SCO histology as a favourable prognostic group for sperm retrieval when compared with pure SCO histology.

Author contributions:

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Conflict of Interest:

There are no conflicts of interest

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Figure Legends

Figure 1: Venn diagram showing histological combinations observed in patients with non-obstructive azoospermia using overlapping circles. The number in each area shows the number of patient(s) with that histological combination.

Figure 2: Comparison of overall sperm retrieval rates (SRR) in relation to (A) the maximum Johnsen score and according to (B) histological patterns (pure and mixed).

Figure 3: Odd ratios (OR) of sperm retrieval associated with Sertoli cell only (SCO) + complete spermatogenesis (CS, n=6), SCO + hypospermatogenesis (HS, n=26), and SCO +maturation arrest (MA, n=36), when compared with pure SCO (n=50). Pure SCO was assigned an OR=1. * denotes $p<0.05$; ** denotes $p<0.01$, *** denotes $p<0.001$.

Table 1. Prevalence of pure and mixed histological patterns with observed mean Johnsen scores in men undergoing mTESE

Histological classes	Mean Johnsen score	Number of patients
Pure CS	8.7	30
Mixed CS	7.5	11
Pure HS	5.1	9
Mixed HS	5.1	44
Pure MA	4.9	7
Mixed MA	5.2	56
Pure SCO	1.9	50
Mixed SCO	4.4	46

Table 2. Comparison of sperm retrieval rates according to histological combinations. Each cell denotes the number (and %) of patients with successful sperm retrieval. Shaded cells represent pure histological patterns.

	CS	**HS**	**MA**	**SCO**
CS	25/30 (83.3)	2/3 (66.7)	7/9 (77.8)	4/6 (66.7)
HS		5/9 (55.6)	20/34 (58.8)	15/26 (57.7)
MA			3/7 (42.9)	16/36 (44.4)
SCO				9/50 (18.0)

Supplemental table. Total and motile sperm retrieval rates (SRR) in distinct histological combinations found in patient cohort (C=complete spermatogenesis, H=hypospermatogenesis, M=maturation arrest, S=Sertoli cell only).

Histological combination	n total	n sperm	n motile sperm	% sperm retrieved	% motile sperm retrieved	% of population (n=163)
C H M S	1	0	0	0.0	0.0	0.6
H M S	16	9	6	56.3	37.5	9.8
C M S	4	3	1	75.0	25.0	2.5
C H M	1	1	1	100.0	100.0	0.6
M S	15	4	0	26.7	0.0	9.2
H S	9	6	2	66.7	22.2	5.5
H M	16	10	3	62.5	18.8	9.8
C S	1	1	0	100.0	0.0	0.6
C M	3	3	1	100.0	33.3	1.8
C H	1	1	0	100.0	0.0	0.6
S	50	9	1	18.0	2.0	30.7
M	7	3	0	42.9	0	4.3
H	9	5	2	55.6	22.2	5.5
C	30	25	16	83.3	53.3	18.4