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human reproduction **ORIGINAL ARTICLE** ***Infertility***

Spermatogenesis in tumor-bearing testes in germ cell testicular cancer patients

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STUDY QUESTION: What are the factors that might indicate a greater likelihood of success in oncologic testicular sperm extraction (onco-TESE)?

SUMMARY ANSWER: Smaller tumor diameter and greater noncancerous testicular tissue width (NCTW) are positive predictors of spermatogenesis in patients with testicular germ cell tumors (TGCTs).

WHAT IS KNOWN ALREADY: Onco-TESE is a key modality for fertility preservation in cases of inadequate pretreatment sperm collection and azoospermic men with testicular cancer. TGCTs are known to reduce sperm quality such that ~10% of these patients are azoospermic, making surgical TESE at the same time as orchiectomy their only means of fertility preservation.

STUDY DESIGN, SIZE, DURATION: This study is a retrospective analysis performed in a single university hospital from 2002 to 2014.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were 102 male patients (104 testes) who underwent inguinal orchiectomy and were diagnosed with a germinoma. In each specimen, the Johnsen Score Count (JSC) in seminiferous tubules at each established distance from the tumor margin (1, 2.5, 5, 7.5, 10 and 12.5 mm) was determined. We analyzed the relations between age, tumor histopathologic type, tumor size (maximum diameter), distance from the tumor, non-tumor tissue width and JSC.

MAIN RESULTS AND THE ROLE OF CHANCE: The 104 specimens consisted of 78 seminomas and 26 non-seminomatous TGCTs. The mean ± SD JSC was 4.7 ± 2.4 in seminomas and 3.9 ± 2.5 in non-seminomatous germ cell tumors, with no significant difference between the two subtypes. Single regression analysis showed that tumor diameter was significantly negatively correlated with spermatogenesis ($RC = -0.422$, $P < 0.001$). Multiple linear regression analysis also showed that tumor diameter had a negative influence on spermatogenesis ($RC = -0.437$, $P < 0.001$). The greater the distance the seminiferous tubules from the tumor, the better the preservation of spermatogenesis. Mature spermatozoa were identified in 93.0% of patients with a NCTW ≥7.5 mm and in 41.3% of those with NCTW <7.5 mm ($P < 0.001$).

LIMITATIONS, REASONS FOR CAUTION: Study data were obtained retrospectively, which might have affected the quality of data. We were unable to compare spermatogenesis determined using preoperative seminograms with that determined histopathologically. It was not possible to evaluate spermatogenesis in the total volume of noncancerous testicular tissue.

WIDER IMPLICATIONS OF THE FINDINGS: When Onco-TESE is conducted at sites distant from tumors, the rate of sperm extraction is high and contamination by tumor cells can be prevented. By measuring non-testicular cancerous margin before the operation, the possibility of sperm extraction can be predicted and biopsy of the contralateral testis can be considered based on the results.

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TRIAL REGISTRATION NUMBER: none.

Key words: seminiferous tubules / spermatogenesis / testicular germ cell tumor / testicular sperm extraction / infertility

Introduction

Generally, testicular germ cell tumors (TGCTs) occur more frequently in patients of reproductive age. Therefore, their influence on spermatogenesis is an important consideration. Patients with TGCTs have a comparatively high survival rate and radical cure is possible, but post-operative chemotherapy and radiotherapy significantly reduce fertility, sometimes permanently (Meseguer *et al.*, 2003; Dohle, 2010). Previous reports

indicate that it is difficult to predict whether spermatogenesis will recover to the preoperative level after chemotherapy and radiotherapy (Gandini *et al.*, 2006). Additionally, in a study involving 1158 patients with TGCTs, Rives *et al.* (2012) reported that the ideal time to perform sperm retrieval in patients with TGCTs was preoperatively, because the sperm concentration was the most favorable before inguinal orchiectomy. Based on this, preoperative examination of the semen is crucial and preoperative semen cryopreservation is highly recommended. However, previous studies report that azoospermia has been observed in 10–15% of patients with TGCTs during preoperative semen analysis and 50% or more patients have oligozoospermia (Williams *et al.*, 2009; Fraietta *et al.*, 2010). It is possible to cryopreserve sperm from patients with oligozoospermia but not azoospermia, which is instead an indication for onco-testicular sperm extraction (onco-TESE). This technique involves collection of semen from excised testes following orchiectomy. There have been cases of pregnancy reported following ICSI with sperm collected using onco-TESE (Descombe *et al.*, 2008). Against this background, semen tests should always be conducted preoperatively in patients with TGCTs, and if azoospermia is noted, onco-TESE should be proactively recommended.

However, it will not necessarily be possible to collect semen from all patients using onco-TESE (Schrader *et al.*, 2003). There have been few studies investigating the factors that influence spermatogenesis in patients with TGCTs. If we can elucidate the factors that influence the difficulty of sperm collection during onco-TESE, the findings will be useful for establishing a strategy to allow patients with TGCTs to maintain fertility. Reports that have studied the relation between spermatogenesis in the affected side in patients with TGCTs and three factors—tumor size, tumor markers and tumor tissue type—have shown that the only relevant factor was tumor size (Choy *et al.*, 2013).

In addition, reports have stated that spermatogenesis worsens with increasing proximity to the tumor in the residual normal testicular tissue (Ho *et al.*, 1992), but no reports have investigated the relation between the actual distance from the tumor and spermatogenesis.

In this study, we investigated spermatogenesis and distance from the tumor margin to study whether it is possible to predict which sites offer the highest chances of sperm retrieval on the affected side when performing onco-TESE.

Materials and Methods

Participants

Participants were 102 male patients (104 testes) diagnosed with a TGCT, who underwent inguinal orchiectomy and were diagnosed with a germinoma at Dokkyo Medical University Koshigaya Hospital between April 2002 and April 2014. The study protocol was approved by the institutional review board and complied with all institutional guidelines of Dokkyo Medical University Koshigaya Hospital. The written informed consent was obtained from all patients.

Creation of histopathology specimens and evaluation of spermatogenesis

After fixing the excised testes in 10% buffered formalin, slices were cut so as to include the longest cut surface of the tumor and paraffin-embedded specimens were created. These were used to create 5-μm sections from which histopathology specimens were created after hematoxylin and eosin staining.

TGCT tissue was classified according to the Union for International Cancer Control guidelines. Tumor size was measured using a pair of vernier calipers along the major axis of the tumor. In the case of multiple tumors, the major axis of the largest tumor was used as the representative value.

The Johnsen Score Count (JSC) (Johnsen, 1970) was used to evaluate spermatogenesis. Two individuals (K.S., T.S.) were responsible for the evaluation of spermatogenesis and both were blinded to patient backgrounds. Spermatogenesis was evaluated in 20 testes during the preliminary experiment and the concordance rate was 90% (data not shown). If evaluations were not in concordance, a third individual (H.O.) was included, discussions were conducted and a decision was made in the final evaluation.

The distance from the tumor margin to the seminiferous tubules was measured using a micrometer. The mean JSC value was calculated using 25 or more seminiferous tubules observed in the cross section at each established distance from the tumor margin (1, 2.5, 5, 7.5, 10 and 12.5 mm) to evaluate spermatogenesis at each location. The JSC score was considered 0 in the event the testis was completely replaced by tumor tissue with no residual normal testicular tissue.

We used the median JSC value at each distance from the tumor margin, as described above, as the representative value for spermatogenesis in each patient. The noncancerous testicular tissue width (NCTW) was defined as the distance between the tumor margin and the tunica albuginea, measured by a micrometer.

Statistics

The relation between spermatogenesis and patient age, maximum tumor diameter, and distance from the tumor margin was investigated using multiple regression analysis. Spermatogenesis on the affected side was compared using the Mann–Whitney test between patients in the following groups: age <35 years versus ≥ 35 years, seminoma versus non-seminomatous tumors, and NCTW <7.5 mm versus NCTW ≥ 7.5 mm.

The presence of spermatogenesis on the affected side in patients aged <35 years was compared with that in patients aged ≥ 35 years using the chi-square test. The relationship between spermatogenesis and patient age, maximum tumor diameter, and tissue type was investigated using multiple regression analysis. The Statistical Package for the Social Sciences version 18.0 (IBM, Armonk, NY, USA) was used for statistical analysis. A P-value of <0.05 was considered statistically significant.

Results

Patient backgrounds of the 102 individuals (104 testes) in the study are shown in Table I. Table II shows whether the patients desired a baby at the time of diagnosis and whether they had already fathered a child. We obtained the preoperative semen values in seven patients only (Table III). Of these seven patients, spermatozoa were cryopreserved in five for fertility preservation. Tissue type breakdown revealed 78 cases of seminoma and 26 cases of non-seminomatous tumors (19 mixed germ cell tumors, 4 embryonal carcinoma, 1 choriocarcinoma, 1 yolk sac tumor, and 1 teratocarcinoma). In 21 cases, no seminiferous tubules and only tumor was found in the specimen. The mean $\pm$ SD age and the average maximum tumor diameters for these 21 patients were 43.0 $\pm$ 9.7 years and 7.88 $\pm$ 2.79 cm, while those of the other 83 patients were 37.8 $\pm$ 8.8 years and 4.23 $\pm$ 1.64 cm, respectively. The average age was significantly higher ($P = 0.032$) and the maximum tumor diameter significantly larger ($P < 0.0001$) in the 21 patients without seminiferous tubules versus those with seminiferous tubules. In this study we excluded these 21 cases and we used the remaining 83

cases for regression analysis. Single regression analysis showed that maximum tumor diameter but not age was significantly negatively correlated with spermatogenesis (age: regression coefficient [RC] = −0.017, $P = 0.37$, Fig. 1; maximum diameter: RC = −0.422, $P < 0.001$, Fig. 2).

Spermatogenesis in the testes on the affected side in patients aged <35 years was evaluated as a JSC score of 4.9 ± 2.0 (mean ± SD), which was significantly higher than the JSC score of 3.5 ± 2.6 ($P = 0.031$) for those aged ≥35 years. Spermatozoa were more frequently observed in patients aged <35 years (74%) than in those aged ≥35 years (44%; $P = 0.024$). Table IV shows the data for the two groups separated by age. We found no significant difference in spermatogenesis (JSC) between seminoma (4.7 ± 2.4) and non-seminomatous tumors (3.9 ± 2.5). Multiple regression analysis showed that tumor diameter had a negative influence on spermatogenesis (RC = −0.437, $P < 0.001$), but age and tissue type had no significant influence.

In terms of the distance from the tumor margin and spermatogenesis, spermatogenesis was maintained to a greater degree in the seminiferous tubules that were more distant from the tumor (Fig. 3). Spermatozoa were observed in 93% ($n = 30$) of patients when NCTW was 7.5 mm or greater, but was seen in only 41% ($n = 74$) of patients when NCTW was less than 7.5 mm ($P < 0.001$).

Table I Characteristics of patients with germ cell testicular cancers.

	Seminoma ($n = 26$)	Non-seminoma ($n = 78$)
Age (years)		
Mean ± SD	40.1 ± 9.2	34.2 ± 7.3
Range	20–62	22–48
Maximum diameter (cm)		
Mean ± SD	7.14 ± 1.91	6.63 ± 3.02
Range	4.0–2.0	3.3–14.0
Location		
Left	11	37
Right	12	40
Bilateral	3	1

Table II Desire for a baby at time of diagnosis and whether the patients had already fathered a child.

		Desire for a baby	
		Yes	No
Already fathered a child	Yes	12	49
	No	30	11

Discussion

Due to advances in multidisciplinary healthcare, TGCTs have become curable, and maintaining fertility presents a challenge for cancer survivors (Ginsberg, 2011). If spermatozoa are observed in the semen preoperatively, it should ideally be cryopreserved for preservation of fertility (Loren *et al.*, 2013). However, if preoperative azoospermia precludes sperm retrieval, then onco-TESE (Schrader *et al.*, 2003; Descombe *et al.*, 2008), which is the collection of sperm from the excised testes at the time of surgery, should be performed. At this time, knowing which sites in the testes to search preferentially is important for ensuring a maximum sperm retrieval rate.

For this reason, we investigated spermatogenesis-influencing factors on the affected side in 102 TGCT patients. First, we examined spermatogenesis using three parameters; namely, patient age, tumor tissue type, and maximum tumor diameter, and then investigated the relation between spermatogenesis and distance from the tumor margin, NCTW, and the presence of spermatogenesis.

The results of this study show no correlation between tumor tissue type (seminoma/ non-seminomatous tumors) and spermatogenesis. The results of a study by Rives *et al.* involving 1158 TGCT patients indicated that sperm concentration was lower in patients with seminomas than in patients with non-seminomatous tumors (Rives *et al.*, 2012), but no statistically significant difference due to tissue type was observed in our study.

The negative effect of female age on fertility is well known. Meanwhile, the effect of age on male fertility has been controversial. Okada and his

Table III Preoperative semen values obtained from seven patients in the study.

Case	Age (years)	Pathology	Maximum diameter (cm)	JSC (average)	Vol (ml)	Cons (×10^6/ml)	Mo (%)	Malformation rate (%)
1	40	Seminoma	10	2	5.3	8	50	<15
2	30	Seminoma	4.2	6.9	6.5	52	54	<15
3	34	Seminoma	7.5	5.5	1.2	11	80	19
4	29	Seminoma	5	2	4.6	27	70	17
5	57	Seminoma	2.1	5.8	3.3	1	10	15
6	33	Seminoma	3.4	7.8	4.6	7	50	<15
7	31	Seminoma	5.2	3.6	2.9	1	15	<15

JSC, Johnsen's score count; Vol, volume; Cons, concentration; Mo, motility.

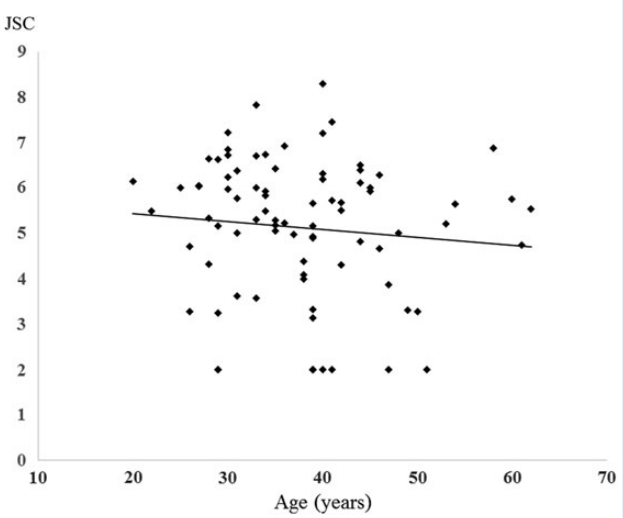

Figure 1 Influence of age on spermatogenesis, assessed by Johnsen score count (JSC). Single linear regression was performed. Age was not associated with spermatogenesis in tumor-bearing testes ($P = 0.37$, regression coefficient RC $= -0.017$) ($n = 83$).

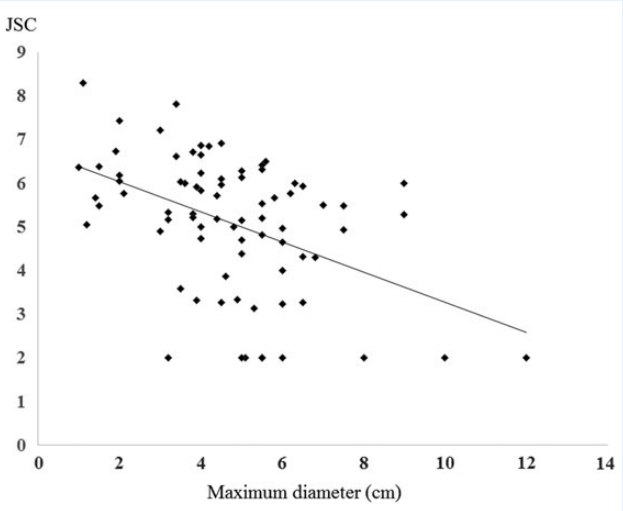

Figure 2 Influence of tumor size on spermatogenesis assessed by JSC. Single linear regression was performed. Tumor size was negatively associated with spermatogenesis in the tumor-bearing testes ($P < 0.001$, RC $= -0.422$) ($n = 83$).

colleagues previously reported that in azoospermic patients with non-mosaic Klinefelter's syndrome, the percentage of successful recovery of spermatozoa decreased after the age of 35 years (Okada *et al.*, 2005). This fact suggested a negative effect of male age on fertility. In this study, we separated the patients based on the cut off age of 35 years, the age when a woman's fertility starts to decrease (Maheshwari *et al.*, 2008). Spermatogenesis was significantly higher and spermatozoa were observed significantly more frequently in patients aged <35 years, compared with those ≥35 years.

Table IV Characteristics of the two patient groups according to age.

	<35 years ($n = 41$)	≥35 years ($n = 61$)
Mean ± SD (years)	30.3 ± 3.7	43.7 ± 8.9
Maximum diameter (cm)	6.3 ± 1.9	7.5 ± 2.9
Location		
Left	17	31
Right	22	28
Bilateral	2	2
Married		
Yes	21	51
No	20	10
Already fathered a child		
Yes	16	44
No	25	17
Desire for a baby	32/41 (78%)	13/61 (21%)

Choy *et al.* reported that maximum tumor diameter had a significant negative influence on spermatogenesis in their study of 77 patients (Choy *et al.*, 2013) and Delouya *et al.* reported a moderately negative correlation between tumor volume and spermatogenesis in their study of 77 patients (Delouya *et al.*, 2010). Similar to these past studies, multiple linear regression analysis in our study indicated that maximum tumor diameter had a significant negative influence on spermatogenesis.

When we investigated the distance from the tumor margins and spermatogenesis, we found that spermatogenesis increased with distance from the tumor margins. In addition, spermatozoa were observed in 93% of patients when NCTW was ≥7.5 mm, but sperm was observed significantly less often (41%) when patients' NCTW was <7.5 mm. Based on these findings, spermatogenesis on the affected side in patients with TGCTs is more likely to be maintained in the seminiferous tubules that are farther from the tumor and spermatozoa are more likely to be present when the NCTW is higher. Ho *et al.* also reported that spermatogenesis worsened in the seminiferous tubules surrounding the tumor in an investigation of 28 TGCT patients (Ho *et al.*, 1992). NCTW is highly dependent on size, location and shape of the tumor. Unfortunately, we could not analyze the location and shape of the tumor in this retrospective study. Table V shows the information about tumor diameter and proportion relative to the whole testis in patients with NCTW <7.5 mm and in those with NCTW ≥7.5 mm. Imaging evaluation of the residual testicular tissue using ultrasound of the testes or magnetic resonance imaging may become an effective preoperative step for predicting the results of sperm retrieval during onco-TESE.

To date, Hadded *et al.* have mentioned tumor toxicity, high temperature, mass effect, and paracrine effects (interleukin-1, interferon-γ, leukemia-inhibiting factor) as factors affecting testicular tissue with residual tumor (Haddad *et al.*, 2014). In our study, there was no additional investigation of these factors, but we have begun experimentation in animal models to elucidate the mechanism of this spermatogenic dysfunction.

Surgically recovering spermatozoa from the contralateral testis is an alternative method for fertility preservation in unilateral testicular

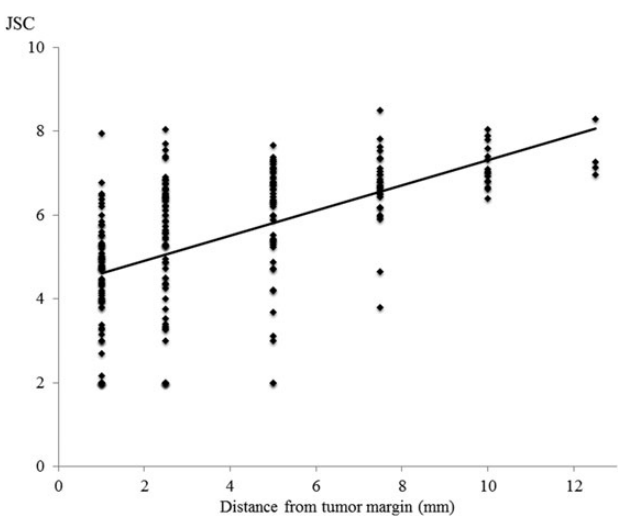

Figure 3 The average JSC in terms of the distance from the tumor margin. The average JSC in the seminiferous tubules at 1, 2.5, 5, 7.5, 10, and 12.5 mm distance from tumor margin. The farther from the tumor seminiferous tubules were located, the better the preservation of spermatogenesis ($n = 83$).

Table V Tumor diameter and proportion relative to the whole testis in patients according to noncancerous testicular tissue width (NCTW).

	NCTW (mm)	
	<7.5 (*N* = 74)	≥7.5 (*N* = 30)
Maximum diameter (cm)	3.28 ± 2.45	6.05 ± 1.02
Tumor proportion (%)	72.8 ± 17.1	58.4 ± 15.6

cancer patients. Some of the recovered spermatozoa from the ipsilateral testis may have been affected by the biochemical malignant environment, or they may come from seminiferous tubules with *in situ* carcinoma. Biopsy of the contralateral testis and recovering spermatozoa could overcome these problems, and could exclude the presence of a simultaneous carcinoma *in situ*. Sofikitis and his colleagues indicated that autotransplantation of frozen/thawed germ cells isolated from the contralateral healthy testis as a promising method of fertility preservation (Sofikitis *et al.*, 2003). In this procedure, frozen–thawed germ cells from the neoplastic testis can be transplanted back to the rete testis of the contralateral testis after chemotherapy. Semen samples should be evaluated for the presence of spermatozoa several months after transplantation. They suggested that autotransplantation of testicular frozen–thawed germ cells after chemotherapy may represent a means of colonizing the human testis with its own cells, with the overall target of appearance of spermatozoa in the ejaculate. However, we should bear in mind that biopsy of the contralateral testis has the potential risk of post-operative hypogonadism, and it cannot be performed in patients with a single testis.

Limitations of our study include unknown preoperative semen findings in several cases. We were unable to compare spermatogenesis determined using preoperative semen findings with that determined histopathologically. Furthermore, the evaluation of spermatogenesis involved the use of histopathology specimens that included the largest cut surface of the tumor, so it was not possible to evaluate spermatogenesis in the total volume of noncancerous testicular tissue. When onco-TESE is performed, all of the excised noncancerous testicular tissue is examined in detail using a surgical microscope and sperm collection is attempted, so it is possible that the presence of spermatogenesis detected in the present study was less than it would have been during onco-TESE in practice. Another limitation of this study is that patients with probably normal spermatogenesis were included in this study. When patients have sperm in their ejaculate, sperm cryopreservation would be performed for fertility preservation. Onco-TESE would not be offered for those patients. Only azoospermic or severely oligoasthenoteratozoospermic patients are candidates for onco-TESE. Therefore, a study that limits subjects to those whose seminograms are azoospermic or severely oligoasthenoteratozoospermic would be more preferable for the analysis. Going forward, we believe a prospective study is required to compare the results of sperm retrieval with the results of spermatogenesis determined using histopathology specimens.

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Authors' roles

K.S. contributed to study concept and design, acquisition of data and drafting of the manuscript, statistical analysis. T.S. contributed to critical revision of the manuscript for important intellectual content. Y.S. and T.I. conducted analysis and interpretation of data. H.O. conducted supervision.

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Conflict of interest

None declared.

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