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ORIGINAL ARTICLE

Distribution and number of Cajal-like cells in testis tissue with azoospermia

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Abstract We investigated the number and distribution of Cajal-like cells in patients with azoospermia. A total of 99 patients with non-obstructive azoospermia were divided into subgroups [19 patientsin hypospermatogenesis group (S1), 40 patients in maturation arrest group (S2), 20 patients in a Sertoli cell-only syndrome (S3), and 20 patients in a testicular atrophy and fibrosis group (S4)], and 20 patients with obstructive azoospermia group (S0). Sections stained with a c-kit antibody were studied by light microscopy to determine the number and distribution of Cajal-like cells in peritubular and perivascular areas of testis. The number of Cajal-like cells were higher in all the non-obstructive groups than in the obstructive group (S0: 2.43 cells/mm^2, S1: 3.14 cells/mm^2, S2: 4.00 cells/mm^2, S3: 4.57 cells/mm^2, S4: 3.86 cells/mm^2) but statistically significantly different ($p < 0.05$) in the S2 and S3 subgroups only. Distribution of Cajal-like cells were similar in all groups. The number and distribution of Cajal-like cells in non-obstructive groups suggest that these cells may affect spermatogenesis. This cellular type can be responsible for the regulation of cellular motility or spermatogenesis. Electrophysiological and electron microscopic studies are needed to better define morphology and function of Cajal-like cells in the testis, especially totally the normal testis tissue.
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Introduction

In 1893, Cajal cells were described by Ramon Y. Cajal as primitive neurons in the gastrointestinal (GI) tract [1]. Currently, it is known that in the GI tract, these cells have important physiological functions in generating slow surge currents (pacemaker cell function), radiating electrical transmissions, and mediating neurotransmission between enteric nerves and smooth muscle cells [2]. Reduction of these cells or distribution anomalies can affect the GI motility [3].

Cajal-like cells were demonstrated in the urinary system in 1999 [4]. Sanders [2] showed that these cells are located between neurons and smooth muscle cells in the upper urinary system and are in charge of the transmission of slow surge electrical currents for peristaltic movements. Moreover, their electrical transmissions play a role in the passing of urine in the calyceal system to the ureter and bladder [5]. Cajal-like cells are present in the vas deferens and ureter of rats, prostate and bladder of guinea pigs, and urethra of rabbits. In humans, in addition to the ureteropelvic junction, they are found in the renal pelvis, ureter, vesicoureteral junction, bladder, and urethra [6—10].

c-Kit is a proto-oncogene that encodes the tyrosine kinase receptor, which is expressed by Cajal and mast cells but not by smooth muscle cells or fibroblasts [11]. The discovery that Cajal cells could be labeled with anti-c-Kit provided a reliable method for identifying Cajal cells in smooth muscle tissues with light microscopy [12].

Approximately 15% of infertile men have azoospermia, of which many have non-obstructive azoospermia (NOA). NOA derived from intrinsic testicular pathology is difficult to treat. Testicular etiologies depend on many factors, although there are cases without an identified causative agent. It was demonstrated that the membrane receptors of c-kit (+) cells in the fetal testis were expressed by somatic cells (Sertoli and Leydig cells) and germ cells (type A and B spermatogonium) [13].

Rodríguez et al [14] examined orchiectomy material of prostate cancer patients. Cajal-like cells positively stained by anti-c-kit were demonstrated in the testis interstitium. Furthermore, it was found that these cells were close neighbors to myoid cells, which are considered to be responsible for contractility in the peritubular area [14]. In another related study, it was suggested that c-kit receptor expression can regulate the proliferation of seminiferous tubule epithelium and tubular motility and can further affect factors involved in spermatogenesis [15]. To date, the pacemaker Cajal-like cells of azoospermic patients have not been investigated.

In the first phase of this study, we investigated whether c-kit positive Cajal-like cells were expressed in biopsy materials taken from patients on whom testicular sperm extraction (TESE) was performed for their azoospermia. In the second phase, we assessed whether these cells in the tissues taken from the patients differed from each other, in terms of number and distribution, in male patients with obstructive azoospermia (OA) and NOA. Due to the fact that Cajal-like cells were found to be reduced in patients in whom spermatozoa could not be obtained, we believe that these cells control induction of spermatogenesis.

Materials and methods

Between January 2004 and December 2013 in our clinic, 99 out of 644 patients on whom TESE was performed, owing to NOA, were included in the study. Patients who had a history of cryptorchidism, genetic abnormalities, post-chemotherapy azoospermia, and history of orchitis were excluded from the study. All of the cases consisted of idiopathic NOA patients. In addition, 20 out of 104 patients received TESE because of OA. Patients who had obstruction secondary to the infection were included in the OA group. In testis biopsies, we utilized the Johnsen Classification System of normal spermatogenesis, hipospermatogenesis, maturation arrest, Sertoli cell-only syndrome (SCOS), and testicular atrophy and fibrosis [16]. The distribution of the patients in the NOA group included 19 in the hipospermatogenesis group, 40 in the maturation arrest group, 20 in the SCOS group, and 20 in the testicular atrophy and fibrosis group. To determine the number of Cajal-like cells in peritubular and perivascular areas of the samples obtained from the case testis biopsies, CD117 staining was performed using an immunohistochemical system.

The same sections were stained with toluidine blue so that Cajal-like cells located in the interstitium could be distinguished from mast cells, which are known to be expressed with c-kit. Due to their morphological round oval shape and metachromatic granular cytoplasm, mast cells have a different appearance in blue-purple stains. Their granules contain proteoglycans that bind to histamine and heparine. Through this technique, we distinguished the Cajal-like cells from mast cells.

Histopathological examination

Five micron sections from paraffin wax-embedded blocks belonging to each case were prepared and placed on polysine microscope slides. The samples placed on polysine microscope slides were deparaffinized twice in xylene (Xylene, CAS No: 1330-20-7, Birpa, Turkey) and twice in 10% ethyl alcohol in the immunohistochemical study. After the sections were treated for antigen retrieval and endogenous peroxidase in a Dako PT Link, the sections were processed by peroxidase blocking, primary antibody application (CD117, C-kit, Code A4502, 1/1500 dilution, Dako, Denmark), EnVision FLEX/HRP (Code K8000, Dako, Denmark) kit application, and substrate DAB chromogen and hematoxylin staining in a Dako Autostainer Link 48.

For light microscopy analysis, an Olympus BX51 microscope (Olympus, Tokyo, Japan) was used and one cell-counter was blinded to our experimental conditions. Gastrointestinal stromal tumor cells were used as a positive control for CD117 immunohistochemical staining. For each patient, four sections were examined and the highest quality image was viewed at a magnification of 400X. One section per sample was evaluated randomly. The magnification area was calculated as 0.88 mm^2, and peritubular

and perivascular CD117 positive Cajal-like cells were counted randomly in four magnification areas (40X, total area = 3.5 mm^2). The average number of Cajal-like cells per 1 mm^2 was then calculated.

Statistical analysis

Data are presented as number of observations (n), mean ± standard deviation, median, or minimum–maximum values. The results of homogeneity (Levene's test) and normality (Shapiro–Wilk test) were used to decide the statistical methods for comparing the NOA group. Among normally distributed groups with homogeneous variances, dependent groups were compared using Student t test and independent groups (3 or more) were compared using analysis of variance. According to the test results, parametric test assumptions were not available for some variables and the independent groups were therefore compared using the Kruskal–Wallis test. For multiple comparisons, the adjusted Bonferroni test was used [17]. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago IL, USA.) A p value < 0.05 (two sided) was considered statistically significant.

Results

The average age of the patients was 34.35 (26–44) years in the OA group and 34.22 (19–45) years in the NOA group.

Cajal-like cells appeared brown in color because of the chromogen used in immunohistochemical staining. These cells stained by CD117 included extensions having cytoplasmic radial branches. Samples were analyzed using a light microscope. The cells that were stained brown due to the chromogen were identified as Cajal-like cells. The distribution and number of Cajal-like cells by groups in the microscopic analysis are shown in Table 1.

Table 1 Distribution of Cajal-like cells by groups.

	Cajal-like cell counts	Distribution (%)	
	cell/mm^2	peritubular	perivascular
Group S0 (n = 20)	2.43 (1.14–5.14)	88.2	11.8
Group S1 (n = 19)	3.14 (2–6) [a,*]	90.9	9.1
Group S2 (n = 40)	4 (1.43–7.43) [a,**]	85.7	14.3
Group S3 (n = 20)	4.57 (3.43–9.43) [a,***]	87.5	12.5
Group S4 (n = 20)	3.86 (1.14–6.29) [a,*]	85.2	14.8

Data are presented as the median (minimum–maximum), n = the number of cases, a = Compared with Group S0.
* $p > 0.05$, ** $p < 0.05$, *** $p < 0.001$.
Group S0 = obstructive azoospermia group; Group S1 = hypospermatogenesis group; Group S2 = maturation arrest group; Group S3 = sertoli cell-only syndrome group; Group S4 = fibrosis group.

Number of Cajal-like cells

The number of Cajal-like cells was significantly higher in the maturation arrest and SCOS groups (Table 1) than in the OA group. Although not statistically significant in hypospermatogenesis and testicular fibrosis groups, an increase in the number of Cajal-like cells was observed.

Distribution of Cajal-like cells

It was observed that Cajal-like cells were mainly located around the seminiferous tubule in the testis with fewer numbers localized to the perivascular area. The distribution of Cajal-like cells in the testis in the NOA and OA groups displayed similar features. Furthermore, the Cajal-like cells in all groups were not different with regard to c-kit staining density (Table 1).

Cajal-like cells in peritubular and perivascular areas in the OA, hypospermatogenesis, maturation arrest, SCOS, and testicular atrophy and fibrosis sub-groups are shown in Figures 1, 2, and 3.

Discussion

In the last 20 years, extensive findings have been made regarding the morphological and physiological functions of the interstitial cells of Cajal. This progress was facilitated by the fact that interstitial cells of Cajal express c-kit proto-oncogene, which encodes for the tyrosine kinase receptor. The activation of the Kit signal is necessary for the growth, differentiation, and function of the interstitial cells of Cajal [2,11]. Since the c-kit receptor does not exist in smooth muscle cells, nerve cells, and fibroblasts, Cajal-like cells are likely to be identified in the intestine and other organs with an immune reaction [11,18]. Interstitial cells that exist in the urinary system display morphological and electrical characteristics of interstitial cells of Cajal. Di Benedetto et al [19] showed that Cajal-like cells are pacemaker cells that produce electrical slow-wave currents and trigger peristaltic activity in the upper urinary system.

The hyperpolarization-activated cyclic nucleotide-gated channel in the lamina propria of rat and human bladder was also found in Cajal-like cells [20,21]. Furthermore, ZD7288, which is a specific inhibitor of this channel, reduces stimulation of the bladder. In addition, Johnston et al [22] suggested that the Cajal-like cells in guinea pig bladder are reduced after spinal cord damage, which supports the argument that these cells function as pacemaker cells in the bladder.

There may be a similar relationship in the testis tissue. It is known that the testis tissue consists of seminiferous tubules, interstitium, and peritubular areas. Different cellular relationships in the seminiferous tubules and different stages of the germinal cells can be observed. These complex cellular relationships can be well regulated by Cajal-like cells. If Cajal-like cells are pacemaker cells, they then may control the maturation or movement of spermatids like GI system. The Cajal cells are localized in the GI system close to the mesenteric plexus, and multiple cytoplasmic projections are scattered between the cells [23]. Additionally, Cajal-like cells were seen to be

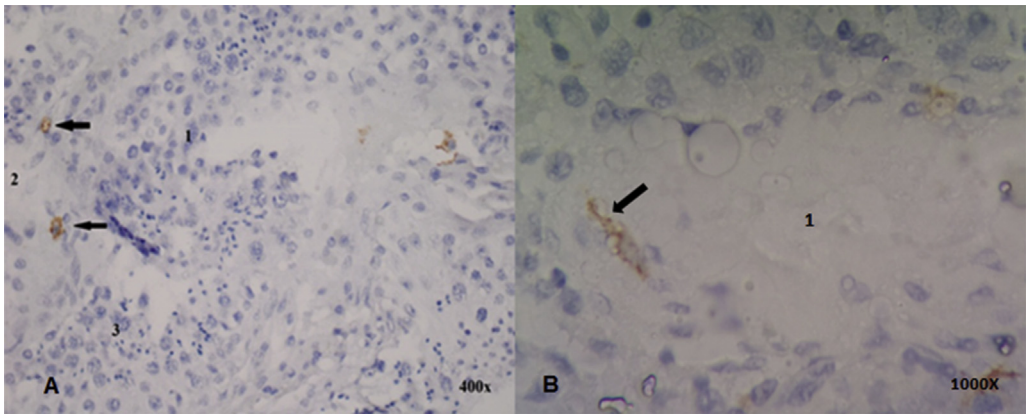

Figure 1. Distribution of Cajal-like cells in testis of the obstructive azoospermia group. The seminiferous tubules showed spermatogenetic maturation reaching to the elongated spermatids. The arrows indicate peritubular Cajal-like cells: (1) tubular compartment, (2) peritubular compartment, and (3) interstitial compartment. (hematoxylin staining, magnification, 400x and 1000x).

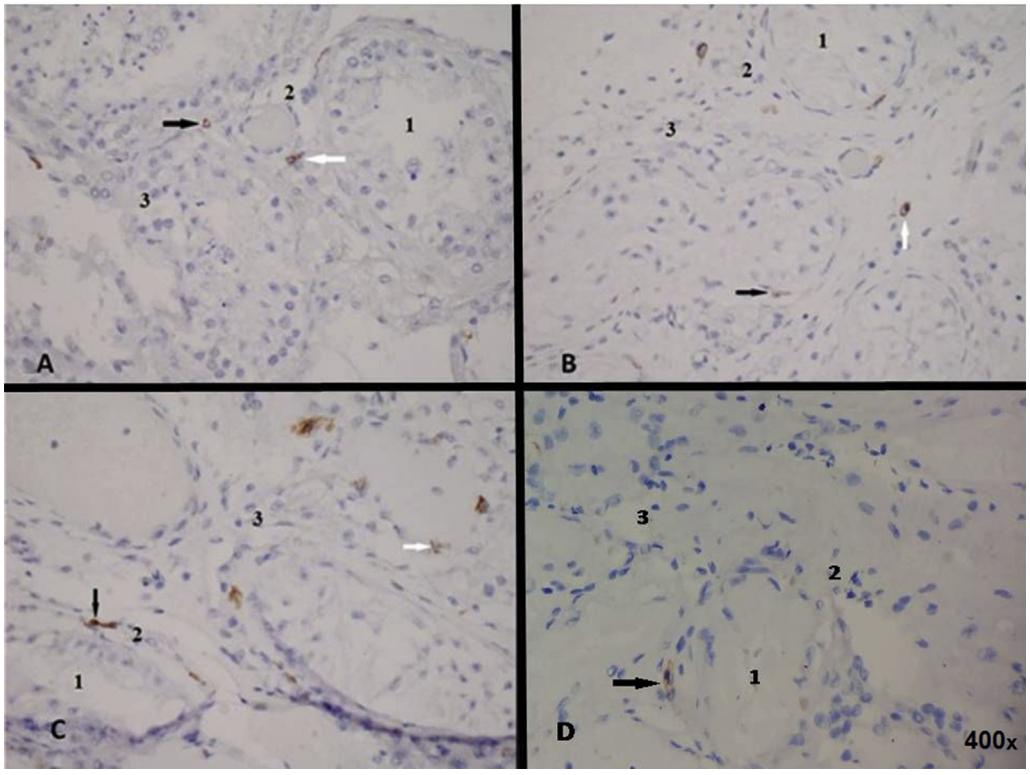

Figure 2. (A) Distribution of Cajal-like cells in the testis in the hypospermatogenesis group; the semininiferous tubules showed slight ectasis and numerical reduction in all germ cell types. (B) In the maturation arrest group, the seminiferous tubules showed reduction in the number of spermatogonia. (C) In the SCOS group, the seminiferous tubules contained only mature sertoli cells without germ cells. (D) In the and athropy and fibrosis group, athropic seminifer tubules were observed. The black arrows indicate peritubular Cajal-like cells, the white arrows indicate perivascular Cajal-like Cells, (1) tubular compartment, (2) peritubular compartment, and (3) interstitial compartment (hematoxylin staining, magnification, 400x).

scattered close to the peritubular area in the interstitial compartment of the human testis [14]. Similar results were obtained in our study (Table 1).

Rodriguez et al. showed that c-kit positive Cajal-like cells existed in testis tissue in the interstitial area [14]. Using CD117, they found that the number of Cajal-like cells in the peritubular area of testis tissue of patients with prostate cancer was 0.4 cells/0.25 mm^2. We have found similar results in the OA group without any testicular pathology. In our study, while the cell number in the OA group was 2.4 cells/mm^2 in the peritubular area, it was markedly increased up to 4.5 cells/mm^2 in the SCOS group. Rodriquez et al. found that the number of Cajal-like cells in the peritubular area was 1.6 cells in 1 mm^2 [14]. This number is

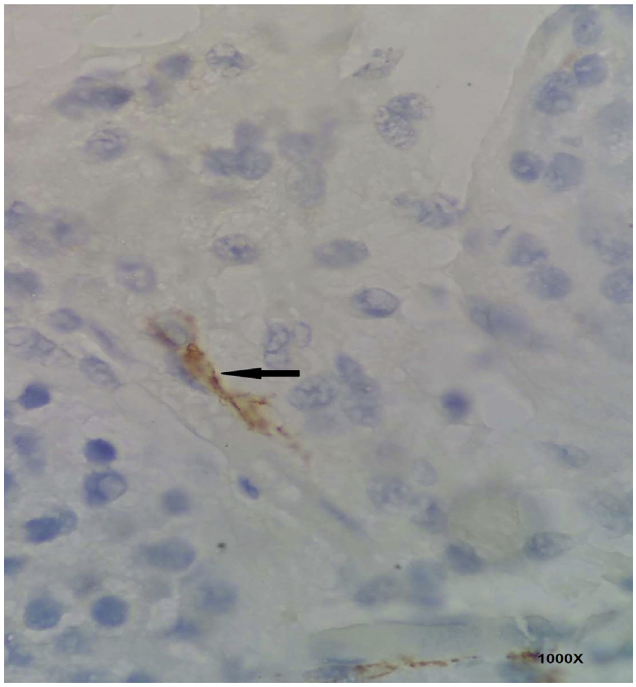

Figure 3. Peritubular Cajal-like cell. The black arrow indicates peritubular Cajal-like cell (hematoxylin staining, magnification, 1000x)

approximately similar to the average cell number (2.4 cells/mm^2) determined in the OA group of our study. The reason why the number of Cajal-like cells was lower in the study by Rodriguez et al. compared with our study might be due to model and calibration differences of microscopes and digital cameras. Regarding cellular reactions, cell motility and spermatogenesis in elderly patients with prostate tumor in the Rodriguez working group might have been compromised. Therefore, due to these factors, the number of Cajal-like cells may have been reduced compared that in our groups.

Rodríguez et al [14] suggested that these cells are in close contact with peritubular myoid cells and function in the motility and secretion of intertubular fluid and cells. This suggestion has become the starting point of our study. In fact, Cajal-like cells have close neighboring peritubular myoid cells; thus, we should not ignore the hypothesis that these cells can also function as pacemakers in the testis. It is likely that the stem cell factor, which interacts with kit protein existing on the spermatogonia, has a role in the maturation of spermatogonia in the early stages of germinal cell development by also affecting Cajal-like cells in the same way. However, it also seems possible that the stem cell factor has an effect on the motility of germ cells in the seminiferous tubule after maturation is complete. In our study, Cajal-like cells have been shown to be localized to the seminiferous tubule and perivascular area in the testis. Furthermore, these cells were higher in number in the NOA group than in the OA group. The increase in number of Cajal-like cells in hipospermatogenesis, maturation arrest, and SCOS groups compared with the OA group is remarkable. Moreover, this increase in the maturation arrest and SCOS groups is statistically significant. Conversely, it was found that the number of Cajal-like cells decreases in testicular atrophy and fibrosis groups.

In our study, we aimed to show that Cajal-like cells exist in the testis and to analyze quantitative anomalies in different stages of spermatogenesis. Although NOA subgroups have different clinical presentations, the decrease in the number of Cajal-like cells suggests that these cells might be in charge of the induction of spermatogenesis. However, we assessed more Cajal-like cells in the NOA subgroups than in the OA group. These results indicate that it is difficult to estimate the role of these cells in spermatogenesis. It is known that Cajal-like cells are pacemaker cells in the urinary and GI systems. The increase in the number of these cells in azoospermic patients may show that these cells proliferate for the induction of spermatogenesis. The reduction of the number of Cajal-like cells in testicular atrophy and fibrosis groups may depend on the extent of deformation of the testicular tissue. Electrophysiological studies in testis should be performed to confirm this hypothesis. On the other hand, these cells may negatively affect spermatogenesis given that the count of these cells in the normal tissue (in OA group) was less than that of the broken spermatogenesis groups. Nonetheless, previous studies show that these cells are pacemaker cells and possibly serve to induce spermatogenesis.

Cajal cells can be considered to be pacemaker cells in light of studies conducted on the GI and, more recently, the urinary systems. Interestingly, the number of Cajal-like cells has shown an inversely proportional relationship to the possibility of finding spermatozoa in hipospermatogenesis, maturation arrest, and SCOS groups, which displayed a lower probability of finding spermatozoa than the OA group in TESE. Thus, it may be possible that these cells might proliferate in order to overcome a stop in spermatogenesis phases. In testicular atrophy and fibrosis groups, which were different from other groups, the decreased number of cells may mean that the number of Cajal-like cells decreased due to the disappearance of seminiferous tubules in this group. Nevertheless, Cajal-like cells in the testis should be further analyzed by means of electrophysiological studies to confirm whether they have a spermatogenesis inductive role. Nonetheless, the fact that these cells show an increase in number in the NOA group, in which spermatogenesis was reduced or did not exist at all compared to the OA group that had spermatozoa, presents a different possibility. These cells might be among cell groups that negatively affect spermatogenesis or inhibit it, and are thus increased in number in the NOA group. However, when looking at recent publications on the roles of Cajal-like cells as pacemaker cells in other parts of the urinary GI systems, the potential function of these cells may be different in the testis. Nevertheless, the function of Cajal-like cells in the urinary system has not yet been definitively determined despite the numerous studies. Therefore, Cajal-like cells might not function in cell contraction, intercellular neurotransmission, cell motility regulation, or peacemaking activities in this particular area compared to other organ tissues. However, it must not be overlooked that these cells might be completely independent from spermatogenesis regulation and catalyze reactions in many different levels in the peritubular area.

This study was initially planned to have five groups, including hypospermatogenesis, incomplete maturation arrest, complete maturation arrest, germ cell aplasia (SCOS), and fibrosis. However, the incomplete and complete maturation arrest groups were later combined into a single maturation arrest group. Therefore, 40 patients were examined in this group. The results may have been affected for this reason. In addition, assessment was only made by light microscopy. The results of this study would have been strengthened by the use of fluorescent microscope evaluation and Western blot.

In addition, the patients' hormone levels (such as estrogen and testosterone) were ignored in this study because of the lack of an adequate patient sample size. Thus, the study was likely underpowered to show statistically significant differences among all NOA phenotypes.

Furthermore, the NOA group was compared with the OA. Comparison of the NOA group with normal testicular tissue samples would have strengthened this study. Normal testicular tissue could be taken from patients with testicular cancer or orchiectomy material of prostate cancer patients. However, we did not include these patients because we thought that spermatogenesis was affected in these patients. On the other hand, orchiectomy material could be taken from patients who have undergone orchiectomy due to testicular trauma. Nonetheless, we preferred comparisons with patients of the OA group because we could not achieve a sufficient number of trauma patients.

In conclusion, Cajal-like cells have been clearly identified in the testis and urinary system. Understanding whether these cells are related with spermatogenesis or not can be made possible with a study that includes a greater number of patients, which will also be done to incorporate normal testis tissue with electron microscopy.

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