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Comparison of immunohistochemical characteristics of endometriomas with non-endometriotic benign ovarian cysts

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Abstract. – OBJECTIVE: The aim of the study was to investigate the existence of neuroendocrine cells and to compare the density of those in normal ovarian tissue, endometriotic and non-endometriotic benign ovarian cysts.

PATIENTS AND METHODS: Twenty patients with the diagnosis of endometrioma and 30 control subjects consisting of ovarian serous cystadenoma (n=10), ovarian mucinous cystadenoma (n=10) and normal ovarian tissue (n=10) were included. The tissues were prepared and assessed according to staining density by using the H-score method.

RESULTS: Tissues with mucinous cystadenoma were significantly more stained with PAS and VanGieson, when compared to women with endometrioma. Macrophage deposition was higher in cyst samples with endometrioma and in normal ovarian tissue when compared to serous cystadenoma and mucinous cystadenoma. Normal ovarian tissue was significantly more stained with PGP9.5, NSE and SYN when compared to endometrioma and non-endometriotic benign ovarian cyst. PGP9.5 staining was higher in normal ovarian tissue when compared with endometriotic lesions ($p<.001$). Endometrioma samples were significantly more stained with p53 when compared to non-endometriotic cysts and normal ovarian tissue. c-Kit staining was mild and not statistically significant among all groups.

CONCLUSIONS: During endometrioma transformation, expression intensity of neuroendocrine markers decreases compared to normal ovarian tissue and other benign ovarian cysts.

Key Words:
Endometriosis, Immunohistochemistry, Mucinous-cystadenoma, Neuroendocrine cells, Serous cystadenoma.

Introduction

Endometriosis is a chronic, estrogen-dependent and multi-factorial disease with a small malignancy potential[1]. Pain is the most common and specific symptom for women across a wide age range. Although there is substantial evidence concerning the role of immunologic factors in the pathogenesis of endometriosis[2], the pathophysiology of the association between endometriosis and related pain is poorly understood[1].

The relationship between the nerve content and endometriosis is an important area of investigation[2-4]. The endometrium is innervated by small nerve fibres in patients with endometriosis, but not in other ovarian pathologies and, therefore, this unique innervation was suggested as a possible diagnostic marker for endometriosis[5-8]. It was previously shown that deep infiltrative endometriotic lesions expressed higher levels of nerve growth factor compared to superficial peritoneal lesions. This finding suggests that infiltration or inflammation in the nerve tissue adjacent to the affected regions can play a critical role in the occurrence of pain related with endometriosis[9]. Immunohistochemical studies showed that the rate and the density of immune-reactive nerve fibres in endometriotic lesions of the ovary were associated with the severity of the pain symptoms but not with disease severity[1].

The accumulated evidence in the literature suggests that the innervation of ovarian endometriotic lesions may have an important role to play in the mechanism of pain generation in women with ovarian endometriosis[10,11]. This study aimed at investigating the existence of the neuroendo-

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crine cells in ovarian endometriomas and to compare the density of those in normal ovarian tissue and non-endometriotic benign ovarian cysts including serous and mucinous cystadenoma.

Patients and Methods

This prospective study was undertaken at a tertiary hospital of a Medical School between 2009 and 2011 following approval from the Local Institutional Ethical Board of Inonu University School of Medicine (Approval No: 2011/166). Twenty patients with endometrioma and 30 controls consisting of ovarian serous cystadenoma (n=10), ovarian mucinous cystadenoma (n=10) and normal ovarian tissue (n=10) were included. Patients, who were scheduled for endometrioma surgery irrespective of the surgical route and whose final pathology results were confirmed as endometrioma were included in the study arm. Patients, who were scheduled for ovarian cyst surgery or oophorectomy were included in the study as the control group. Control groups were designed as three arms: (1) ovarian serous cystadenoma, (2) ovarian mucinous cystadenoma and (3) normal ovarian tissue. Postmenopausal patients and patients with a suspicion of gynaecological malignancy were excluded. Patients who were operated on *via* laparoscopy or laparotomy were noted, as well as demographic features, sonographic data and obstetrical history.

Endometriotic tissues collected during the endometrioma cystectomy were embedded in paraffin blocks, then cut into 6 μM sections. The staining methods used in this study included Hematoxylin and Eosin (HE), picric acid-acid fuchsin mixture in Van Gieson's stain and Periodic Acid Schiff (PAS). The tissues were deparaffinized in 56°C for one hour and in xylol twice (5 minutes duration each). Then, it was consecutively soaked in ethanol absolute (twice for 3 minutes), ethanol 95% (twice for 3 minutes each), and ethanol 70% (for 3 minutes). The tissue was washed with aqua bides (H_2O_2), and then, sprayed with proteinase K solution for 5 minutes. Afterward, it was double washed with PBS, sprayed with hydrogen peroxidase 3% (H_2O_2) for 5 minutes, and then, double washed using PBS 2 times.

Two observers, specialized in histopathology, classified the sections with light microscopy. Tissues were assessed according to their density as 0 (none), +1 (trace), +2 (moderate) and +3 (dense). The observers were blinded to the endometriotic status of the patients. Pathology slides were evaluated at 40x zoom in the eye piece graticule. H-scores that were based on the staining density were calculated using the Pi index and I values [H-Score= $\sum pi(1+i)]^{12}$. The I value was defined with respect to staining density; weak staining (i=1), moderate staining (i=2) and strong staining (i=3). The Pi index was defined as the rate of stained cells in all density categories. Further sub-group analysis was performed to compare the tissues with endometrioma to other lesions, including serous cystadenoma, mucinous cystadenoma and normal ovarian tissue.

Informed consent was obtained from all participants.

Statistical Analysis

The collected data were analyzed with IBM SPSS Statistics version 22.0 (IBM Corporation, Armonk, NY, USA). The conformity of the data to the normal distribution was evaluated with the Shapiro-Wilk Francia test. Data were summarized as the median and interquartile range for non-normally distributed data. The Kruskal-Wallis H Test, one of the nonparametric tests, was used for the comparison of the groups according to the histochemical and immunohistochemical staining results, and the Dunn's Test was used for post-hoc analyses. A p-value <0.05 was considered to indicate a significant difference.

Results

A total of 50 patients were included to the final analysis. The mean age was 28.5 years (±3.2) in the study group. The mean age of the control group including those with serous cystadenoma, mucinous cystadenoma and normal ovarian tissue was34.7 (±3.6), 33.7 (±4.1) and 44.5 (±3.8) years, respectively. The median gravida of the patients was 0 and 3 in the study and control groups, respectively. The mean body-mass index was 22.8±2.6, 26±2.1, 27.1±2.8 and 26.7±2.7 kg/m^2 in endometrioma, serous cystadenoma, mucinous cystadenoma and normal ovarian tissue groups, respectively. Laparoscopy was used in 80% of the patients (n=16) in the study group, and in40% of all the patients in both the serous cystadenoma(n=4) and mucinous cystadenoma (n=4) control groups. Normal ovarian tissue samples were collected *via* laparotomy (n=10).

The comparison of the study group (endometrioma) and control groups (serous cystadeno-

ma, mucinous cystadenoma and normal ovarian tissue) according to their staining outcomes are given in Table I. Tissues with mucinous cystadenoma were significantly more stained with PAS (Table I) and Van Gieson, when compared to those with endometrioma. Macrophage deposition was higher in tissues with endometrioma and normal ovarian tissue when compared to serous cystadenoma and mucinous cystadenoma (Figure 1A, Table I).

Normal ovarian tissue was significantly more stained with PGP9.5, NSE (Figure 1B) and SYN (Figure C) when compared to endometriotic and non-endometriotic benign ovarian lesions (Figure 1B and C, Table I). PGP9.5 staining was found to be lesser in serous cystadenoma when compared to mucinous cystadenoma and endometrioma. In addition, PGP9.5 staining was higher in normal ovarian tissue when compared with those with endometriotic lesions ($p<.001$, Table I).

Endometriotic lesions were significantly more stained with p53 when compared to non-endometriotic lesions and normal ovarian tissue (Figure D, Table I). c-Kit staining was mild and not statistically significant among all groups (Table I). Further sub-group analysis that compared the tissues with endometrioma with other lesions, including serous cystadenoma, mucinous cystadenoma and normal ovarian tissue is set out in Table I.

Discussion

This prospective immunohistochemical study comparing endometrioma with normal ovarian tissue and non-endometriotic benign cysts, including serous cystadenoma and mucinous cystadenoma showed that endometrioma samples collected during cystectomy in patients with endometrioma without severe painful symptoms was found to lack neuroendocrine cells when compared to normal ovarian tissue.

PGP9.5-immunoactive nerve fibers were seen in ovarian endometrioma samples particularly in fibrotic interstitium[1]. It was suggested that the density of PGP9.5-immunoactive nerve fibers in women with ovarian endometrioma may be involved in the pathophysiology of pain generation and pelvic adhesions, but not with disease severity[1]. Another study showed that the density of PGP9.5-immunoreactive nerve fibers in painful endometriosis patients with peritoneal lesions was associated with the severity of pain but not with active lesions, site and staging[13].

In our study, normal ovarian tissue was stained with PGP9.5 more than other endometriotic and non-endometriotic benign ovarian cysts. This unexpected and interesting finding maybe explained by the fact that the PGP9.5 staining is more likely associated with the pain mechanism itself rather than the existence of endometrioma. Zhang et al[14] showed the presence of PGP9.5-immunoactive nerve fibers in the functional layer of the endometrium in women with pain symptoms. PGP9.5 staining was not seen in women without pain symptoms irrespective of the underlying utero-ovarian pathology, including endometrioma, adenomyosis, uterine fibroids, or endometriosis with adenomyosis[14]. Therefore, PGP9.5-immunoactive nerve fibers may play an important role in pain generation in the functional layer of the endometrium, regardless of underlying pathology.

Apart from neuroendocrine tumors, a recent animal study[15] showed that PGP9.5-immunoreactive nerve fibers existed throughout the ovary at all stages of the cycle. They showed that the PGP9.5 stained immunoreactive nerve fibers were mainly distributed around the follicles and, therefore, the authors proposed that the interventions should have focused more on the maturation of oocyte and subsequent ovulation[15].

Synaptophysin (SYN) is a neuroendocrine marker that presents as a membrane component of synaptic vesicles[16]. A recent systematic analysis[17] of neuroendocrine tumors of the ovary showed that ovarian neuroendocrine tumors expressed at least one neuroendocrine marker, such as Cg-A, CD56 or SYN. SYN seemed to be more sensitive and specific than others. In our study, SYN was found to be significantly more positively stained in normal ovarian tissue when compared to other groups. This finding suggests that the normal ovarian tissue has its own neuroendocrine cells. We believe that the transformation from normal ovarian tissue to the endometriotic and non-endometriotic cysts may be associated with regression in neuroendocrine cells in the ovary when injury occurs.

Neuron specific enolase (NSE) is a general neuroendocrine marker and the role played by it in the reproductive tract is not well attested in the scientific literature[18]. Higher staining with NSE when compared to endometriotic and non-endometriotic cysts may be due to the presence of possible neuroendocrine pathways in the ovary, and possible ovarian injury during the cyst formation.

Table I. Evaluation of histochemical and immunohistochemical staining results of the groups.

	Group I (n = 20) Endometrioma median (q1-q3)	Group II (n = 10) Serous cystadenoma median (q1-q3)	Group III (n = 10) Mucinous cystadenoma median (q1-q3)	Group IV (n = 10) Normal ovary median (q1-q3)	*p*-values		
					I *vs.* II	I *vs.* III	I *vs.* IV
PAS	1 (0-3)	1 (1-1)	3 (1-3)	1 (1-2)	0.114	0.001	0.481
Macrophages	1 (0-3)	0 (0-0)	0 (0-0)	1 (0-2)	< 0.001	< 0.001	0.311
Van Gieson	1 (0-2)	1 (1-2)	2 (1-2)	1 (1-2)	0.954	0.058	0.954
Vimentin	3 (3-3)	3 (3-3)	3 (3-3)	3 (3-3)	0.999	0.999	0.999
NSE	0 (0-0)	0 (0-0)	0 (0-1)	1 (0-2)	0.999	0.157	< 0.001
SYN	0 (0-1)	0 (0-2)	0 (0-2)	2 (1-3)	0.412	0.933	< 0.001
PGP9.5	1.5 (0-3)	1 (0-2)	2 (0-3)	3 (1-3)	0.308	0.122	< 0.001
p53	1.5 (0-3)	0 (0-3)	1 (0-3)	0 (0-2)	0.082	0.853	0.025
c-Kit	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-0)	0.999	0.933	0.309

Kruskal-Wallis H-Test; Post-Hoc Test: Dun's, q1: 1st quartile, q3: 3rd quartile.

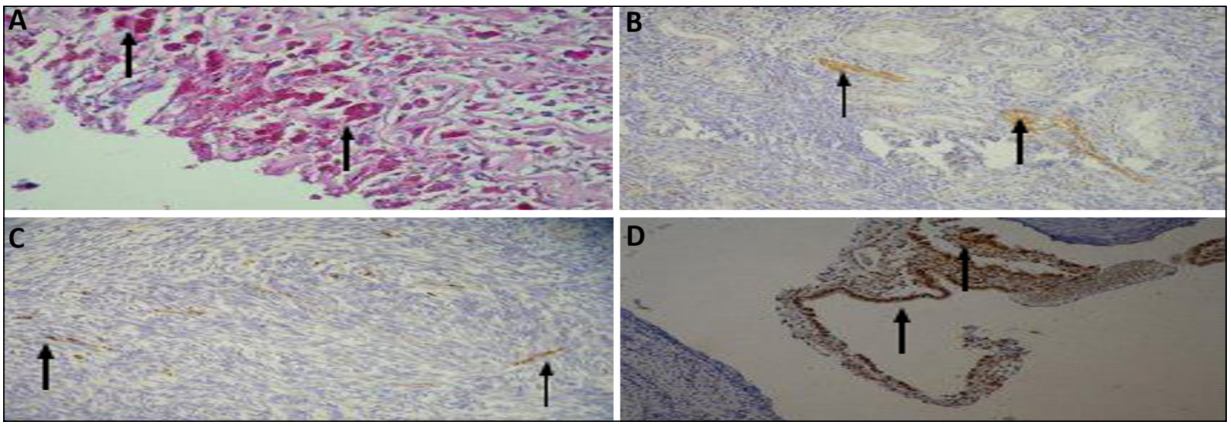

Figure 1. A, Shedding endometrial tissue and prominent PAS-positive macrophages (*arrows*) in the wall at high magnification (PAS × 400). **B,** Peripheral nerves (*arrows*) reacted with NSE antibody in the ovarian stroma (NSE × 200). **C,** Synaptophysin positive neurons (arrows) (SYN × 200). **D,** Diffuse severe nuclear staining (arrows) on the endometrioma surface (p53 × 250).

Some studies[19,20] investigating NSE levels in small cell neuroendocrine carcinoma of the cervix found a very high NSE staining in those patients.

Future research should focus on the role of neuroendocrine markers, including NSE and SYN for early diagnosis of endometrioma transformation. Moreover, patient should be counselled for a fertility-sparing treatment to preserve their fertility in case of early diagnosis of malignancies or endometriosis. Later diagnosis can be resulted with morbidity, and therefore, a multidisciplinary approach may be needed. They usually need an *in vitro* fertilization treatment by using antagonist protocol to freeze their gametes through a vitrification system for future pregnancy[21-25].

In the present study, macrophage deposition was found to be higher in tissues with endometrioma and normal ovarian tissue when compared to that in serous cystadenoma and mucinous cystadenoma. It is known that macrophages are involved in the pathogenesis of endometriosis[26]. Additionally, peritoneal macrophages play an important role in increasing cytokine production, growth and angiogenic factors during endometriosis development[26]. In this study, the lower density of PAS staining in endometrioma when compared to mucinous cystadenoma may point to presence of mild or moderate endometriosis with respect to the cohort of this study.

The main limitations of this study include the lack of standardized classification of disease severity and pain scores of patients, and the relatively small sample size. The main strength of our study was that we used a standardized method (H-index) to compare the staining density between the endometrioma and other benign ovarian samples. A large case-control study is warranted between patients with different pain scores and disease severity.

Conclusions

Current study provides evidence of lower levels of neuroendocrine markers in the endometrioma cyst samples compared to normal ovarian tissue suggestive of a possible injury mechanism during the endometrioma transformation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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