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From Cell Biology to Tissue Engineering

Blind aspiration biopsy versus a guided hysteroscopic technique for investigation of the endometrium in infertile women

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Summary. Embryo implantation failure and recurrent abortion are common indications for endometrial evaluation to determine the implantation window and diagnose endometrial anomalies. There are few research studies comparing the efficacy of different techniques used for endometrial sampling in infertile females during the luteal phase. Likewise, morphometric studies of the endometrium through aspiration biopsy are scant. A cross-sectional study of 30 infertile and 10 fertile females was carried out. The study participants underwent hysteroscopic and aspiration biopsies (pipelle) at the midluteal phase. Computer-assisted morphometric and pathological anatomy analyses were conducted independently by two pathologists blinded to the study. The two endometrial sampling biopsy techniques were compared through morphometric and pathological anatomy analyses using three parameters: a) the amount of material collected for the endometrial studies; b) the scope and origin of sampled materials; and c) the quality of the sample.

Both biopsy techniques produced sufficient material for analysis. The directed biopsies yielded higher quality samples from targeted segments of the uterine cavity because samples were homogeneous and had no architectural distortion ($p<0.05$). Blood was present only in the samples obtained through a Pipelle. Endometritis was detected in 10% of the infertile women. Our findings suggest that hysteroscopic biopsies are superior to blinded aspiration biopsies.

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Introduction

Endometrial analysis, especially during the implantation window, provides important data for estimating the most suitable phase for embryo transfer and possibly for the detection of endometritis (Chan et al., 2013; Garrido-Gómez et al., 2014). Such information is essential to understand and thus be able to improve assisted reproductive treatments (Revel, 2012; Garrido-Gómez et al., 2014). Endometrial biopsies are still performed through aspiration without optical visualization of the uterine cavity (Liu et al., 2014; Kitiyodom, 2015). This raises concerns about the biopsy site, which is vulnerable to infiltration of extraneous matter such as blood. Literature addressing endometrial morphometry in infertile females during the luteal phase is scant (Revel, 2012). These limitations are particularly problematic because extraction of inadequate endometrial samples is likely. The attendant faulty evaluations negatively impact the efficacy of infertility treatments (Farrell et al., 1999; Edi-Osagie et al., 2004).

No reports were found (web of science/pubmed/Cochrane) on hysteroscopic biopsy for endometrial evaluation in infertile women, nor any contrasting the procedure with aspiration biopsy. Therefore, the main goal of this study was to compare the two techniques (hysteroscopically directed biopsy and aspiration biopsy without direct vision) to determine which was the more adequate for obtaining endometrial samples. The secondary purpose was to make a histomorphometric

assessment of the endometrium in infertile women during the midluteal phase.

Materials and methods

Sample

Thirty infertile females from the Center for Human Reproduction, Gynecology Discipline, Department of Obstetrics and Gynecology, Clinics Hospital, School of Medicine, University of São Paulo, who met the study criteria and were eligible to participate in the infertility group gave their written informed consent. The infertile women, aged 21 to 42 years, were diagnosed with primary infertility, had no other medical conditions, were not taking medication, had a normal ovarian reserve (FSH <12, estradiol <80 pg/mL and at least eight antral follicles throughout the three-day cycle of pelvic ultrasound exposure), and regular menses of up to 30-day intervals. Patients were excluded if they had had previous endometrial surgery, had used hormone contraceptives in the previous three months, and were not found to have endometrial polyps or submucosal myomas or synechiae at hysteroscopy.

For the sake of a secondary analysis, the group of infertile women was subdivided according to the cause of infertility as follows: endometriosis, tubal abnormality (from pelvic inflammatory disease [PID]), and male infertility.

The control group comprised 10 fertile females enrolled in the Family Planning Section, Gynecology Discipline, Department of Obstetrics and Gynecology, Clinics Hospital, School of Medicine, University of São Paulo. The inclusion criteria for the control group were the following: age 21 to 42 years; a history of successful pregnancies; regular menses at intervals of up to 30 days; a healthy state and a normal ovarian reserve (FSH <12, estradiol <80 pg/mL and at least eight antral follicles throughout the three-day cycle of pelvic ultrasound exposure). The exclusion criteria for the control group were as follows: previous endometrial surgery; use of any hormonal contraceptive methods or intrauterine devices in the previous three months; and presence of endometrial polyps or submucosal myomas or synechiae diagnosed by hysteroscopy. This research study was approved by the Institutional Review Board (protocol number 144/09).

Procedures

Each woman underwent sonographic monitoring starting on the 6th day of menses to monitor folliculogenesis and identify the ovulation phase (Shoupe et al., 1989; Mcgovern et al., 2004). Sonography was performed at most every three days. Sonographic scanning and hysteroscopy were always conducted by the same author (Dani Ejzenberg) in the same cycle.

First, a diagnostic hysteroscopy was carried out during the embryo implantation window (between the 20th and 24th day of the patient's menstrual cycle). Prior to the performance of any endometrial biopsies and sampling, the patient's uterine cavity was inspected with a Bettocchi hysteroscope with serum used as the distension medium. Bettocchi grasping forceps were used to perform the directed biopsy of the upper third of the posterior uterine wall (near the fundus) and then of the medium third of the anterior wall (Fig. 1a) (Bettocchi et al., 2002). Endometrial tissue processing was immediately started for histological evaluation.

After collection by directed biopsies, blinded aspiration biopsies of the endometrium were performed with a Pipelle de Cornier (CCD Laboratories, Paris, France) placed with its opening facing the posterior wall (single track of endometrial tissue) in the same patient and on the same day.

The samples were fixed with 4% formaldehyde in tri-buffered saline for 24 hours and then dehydrated with graded ethyl alcohol (EtOH) in serially increasing concentrations (30%, 50%, 70%, 80%, 90%). Next, they were diluted in TBS and finally in 100% EtOH. The EtOH was replaced with isopropyl alcohol before the samples were embedded in paraffin wax and mounted. The paraffin block was cut into thin 5-μm-thick sections using a sledge microtome (Leica Microsystems, Wetzlar, Germany). This procedure was carried out by an author (Manuel Jesus Simões) who did not participate in the histological analyses. The independent pathologist received only the histological sections of the endometrium and was blinded to the groups and techniques.

Hysteroscopic biopsy preceded aspiration because a preliminary study showed endometrial bleeding when procedures were reversed. The same fragments were used for the histological, histopathologic, and histomorphometric analyses.

The histomorphometric analysis was conducted with a direct digital imaging system (Axion Vision Rel-4 software; Carl Zeiss, Jena, Germany) allowing optical microscope images to be simultaneously captured by a video camera and scanned. The histomorphometric analysis evaluated the density of the surface epithelium and the height of the gland cell composite. The number of glands in targeted areas and the circular area of the gland (740,000 μm^2) were recorded.

Slide analysis determined the day of the menstrual cycle and detected histopathologic abnormalities. The procedure was carried out by one histologist and two independent pathologists, who were completely blinded to the method of endometrial tissue collection, the sample source (anterior or posterior uterine wall), and the subject's group. Also, the pathologist received histological sections with special identification to avoid breaking the blind method of our study. These investigators did not have any information on patient data.

The histopathologic analysis identified the day and phase of the menstrual cycle according to Noyes' classification and determined the number of endometrial fragments, the homogeneity of the sample, the amount of

sampled material (an 0.5 mm^2 minimum was considered sufficient), and the presence of plasmacytes and stromal bleeding (Rock and Bartlett, 1937; Noyes et al., 1975).

Statistical methodology

The fertile group (control) was contrasted with the infertile subgroups. The Shapiro Wilk tests were performed to assess variable distribution with a view to parametric (the Student *t* test) and non-parametric (the Mann-Whitney and Kruskall-Wallis tests) testing. Quantitative analyses involved the calculation of the mean value, standard deviation, median, and value range.

To evaluate qualitative variables, the absolute and variable scales were compared. The statistical analysis of variance (ANOVA) was applied to compare the different endometrial areas. The level of significance was set at 5% ($p \leq 0.05$). The power calculation of 80% was calculated based on the endometrial thickness of a previous study (Sabry et al., 2014) with a minimum total of 22 patients.

Results

Causes of infertility included the tubal peritoneal factor (n=10), endometriosis (n=10), and male infertility (n=10). The volunteers' mean age was 32.2±7.1 in the control group and 35.3±4.8 in the infertile group, a difference of no statistical significance (p=0.27). Of the 90 biopsies conducted in the 30 subjects, 59 were directed by optical imaging and 30 were blind

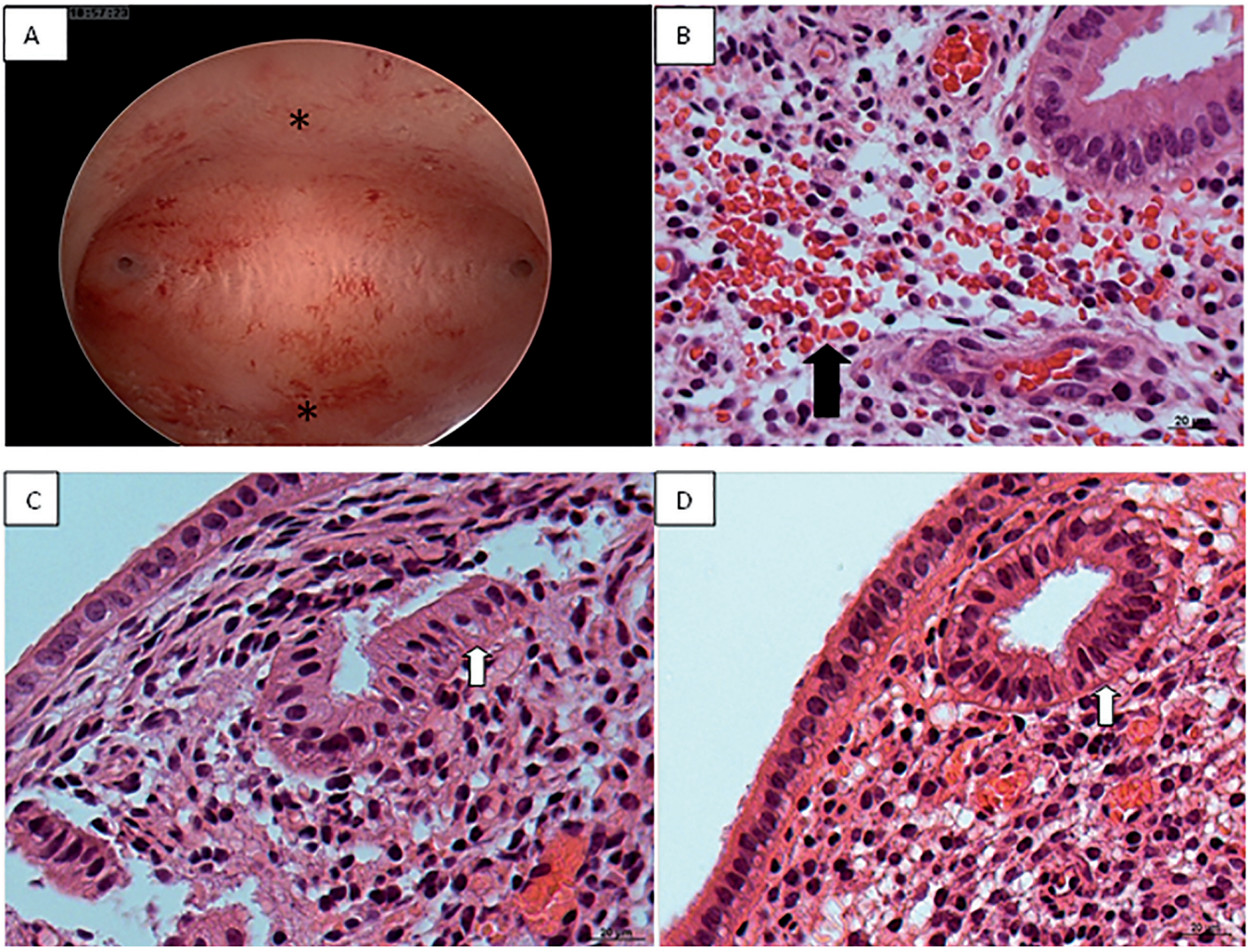

Fig. 1. A. Sites of directed hysteroscopic biopsy (asterisks). **B.** Microscopic hemorrhage in the endometrial stroma in a sample obtained through aspiration. Red blood cells can be seen among stromal cells (black arrow). **C, D.** Photomicrographs of endometrial samples from the anterior and posterior wall, respectively, showing simple ciliated columnar epithelium on a loose stroma. Minor secretory glandular changes can be seen (white arrow) (hematoxylin and eosin).

aspirations; one directed sample from the anterior wall (infertile group) could not be utilized for technical reasons.

Endometrial dating

Two independent pathologists performed a blinded analysis of endometrial biopsies and a histological evaluation. There were no significant differences in the menstrual phase results examined by the two pathologists, except in five cases of one-day range infertile subjects, a number which did not change the classification of the menstrual cycle phase. Furthermore, endometrial dating matched the menstrual phase dating based on clinical features and ultrasound follow-up.

Histological analysis

In each case, endometrial homogeneity was assessed according to Noyes' histological criteria. Biopsy samples containing at least one endometrial fragment with characteristics similar to those from the proliferative phase were considered heterogeneous. Histological analysis revealed that all fertile patients (control group, n=10) and 65% of the infertile women (n=13) presented endometrial homogeneity. The fact that heterogeneity was found solely among infertile patients (n=7) did not translate into a statistically significant difference between the groups (p=0.11).

Endometrial heterogeneity was found in 46% (n=14 samples) of the aspiration samples but not in the directed biopsy samples of the posterior wall with more than one fragment. Only one case of a heterogeneous sample with more than one fragment occurred among the 13 patients (65% of infertile group) who underwent directed biopsies of the anterior wall (p<0.01).

Endometritis was found in 10% of the patients in the infertile group (n=2). Further examination revealed that those patients had adhesions caused by PID (20% of the patients with tubal peritoneal factor). Moreover, these findings did not result from the same biopsy technique: one sample was obtained through aspiration biopsy, and the other through a biopsy directed at the anterior wall.

Microscopic blood suffusion was observed in 70% of the aspiration samples (n=21) from the endometrial stroma. This result was similar in both groups (control and infertile, Fig 1b). However, the alteration was not detected in the directed-biopsy samples from either group (Fig. 1c,d). Multiple directed biopsies of the same wall were not carried out. Also, no atypical cells or neoplasias were found in our samples.

Histomorphometry

Table 1 shows the results of the histomorphometric analysis. The values of the structures of interest (endometrial epithelium thickness, endometrial gland cell height, and gland surface area) in the case of aspiration biopsy were significantly higher than those obtained from the directed biopsy. The number of glands per field was an exception. Finally, no significant difference was found between the infertile and fertile (control) groups.

Discussion

Despite the obstacles preventing the extraction of better biopsy samples for a more accurate evaluation of the endometrium, the findings of the present study show that directed biopsies are superior to aspiration biopsies. The former provided data about the exact site from which the endometrial sample was obtained, produced homogeneous samples, and preserved tissue integrity.

Aspiration has been the method most used to study the endometrium, including the genes and proteins related to embryo implantation (Garrido-Gómez et al., 2014). Aspiration biopsy using a Pipelle for endometrial sampling was the method adopted in our study. The Pipelle is considered superior to all other blind biopsy instruments, regardless of its previous failures to identify focal endometrial lesions in cases of suspected neoplasia (Ceci et al., 2002; Polena et al., 2007; Williams et al., 2008). Another study used ultrasound to improve aspiration biopsy sensitivity. However, the authors found that the ultrasound-directed technique was inferior to hysteroscopy (Metzger et al., 2004). Therefore, a hysteroscopy-assisted biopsy seems to be the better technique.

The hysteroscopy-assisted endometrial biopsy is the recommended technique to evaluate focal endometrial

Table 1. Histomorphometric analysis of the infertile and fertile (control) groups.

	Infertile Group			Fertile Group (controls)		
	PA	PP	ASP	PA	PP	ASP
EE thickness (μm)	20.6±4.9	22.2±5.4	24.3±4.0*	20.9±2.4	18.5±4.4	22.6±3.5*
Gland area(μm^2)**	7.3±2.7	8.6±2.9	11.1±2.9*	9.0±3.1	8.0±3.9	12.5±1.9*
Cell Height (μm)	20.8±3.2	21.3±4.1	24.2±2.7*	21.3±5.5	20.3±3.9	25.2±3.0*
Glands/field (n)	14.7±5.8	15.8±5.1	17.0±4.1	17.2±6.0	18.0±6.7	17.1±2.4

EE thickness, epithelial endometrium thickness; PA, directed biopsy of the anterior wall; PP, directed biopsy of the posterior wall; ASP, aspiration biopsy of the posterior wall; *p<0.05 ASP X PA and X PP; **Gland area (*10^3)

changes as part of diagnosing neoplasias (Ceci et al., 2002; Polena et al., 2007; Williams et al., 2008). Moreover, the diagnostic potential of directed biopsies has not yet been fully investigated in infertile women (Ceci et al., 2002; Goncharenko et al., 2013; Panzan et al., 2013; Lopes et al., 2014).

The directed biopsies produced adequate samples of homogeneous tissue with no artifacts. The lower histomorphometric measures of the directed biopsies as opposed to the Pipelle samplings suggest less cellular distortion of the original gland architecture. This finding strengthens our position that directed biopsies are superior to aspiration biopsies for endometrial evaluation studies in infertile women. An additional benefit of hysteroscopy-assisted biopsy is the possibility of viewing lesions or intracavitary alterations, such as polyps, synechiae, and leiomyomas, capable of compromising endometrial quality and negatively influencing the embryo implantation rate. Imaging methods do not always reveal such changes. Another disorder which may also be diagnosed is endometrial hyperplasia (Vilos et al., 2015). However, no hyperplastic alterations were found in this study.

The exact influence of endometritis on fertility has not yet been fully determined. Many authors have reported that endometritis may negatively impact implantation rates due to the inflammatory process and activation of T-Helper 1 cells (Kasius et al., 2011). In this study, both the directed and the aspiration biopsies detected asymptomatic endometritis in two patients in the infertility subgroup. These results are similar to those of other papers in the literature. Polisseni et al. found chronic endometritis in 12% of the infertile patients given the lower accuracy of visual examination and diagnosis (Polisseni et al., 2003).

In addition, a slightly higher percentage of endometritis (30%) was reported in a survey involving the immunohistochemical analysis of patients with repeated and failed implantation attempts. Furthermore, this study found that implantation success rates improved after treatment, but outcomes were persistently lower than those of patients without endometritis (Johnston-MacAnanny et al., 2010; Galgani et al., 2015). The rate of endometritis detection might have been higher had immunohistochemical and molecular biology techniques also been used. Another limitation in this study was a failure to consider the economic aspects of the two techniques (aspiration and directed).

In short, this study suggests that infertile women who are prescribed an endometrial evaluation could benefit from hysteroscopic biopsy for the following reasons: 1) it supplies homogeneous tissue samples in satisfactory amounts for analysis, 2) it prevents artifacts such as blood from infiltrating tissue samples, and 3) it preserves the integrity of tissue architecture. Further studies, particularly those using molecular biology, are necessary to confirm our data. If our results are corroborated by other studies, directed biopsy could become an alternative for evaluation of the endometrial cavity in infertile women.

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