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Evaluation of FAS and caspase-3 in the endometrial tissue of patients with idiopathic infertility and recurrent pregnancy loss

Michele Q. Panzan [a], Rosiane Mattar [b], Carla C. Maganhin [a], Ricardo dos Santos Simões [c], Alexandre Guilherme Zabeu Rossi [a], Eduardo Leme Alves da Motta [a], Edmund C. Baracat [c], José Maria Soares-Jr [a,*]

[a] Department of Gynaecology, Federal University of São Paulo, São Paulo, Brazil
[b] Department of Obstetrics, Federal University of São Paulo, São Paulo, Brazil
[c] Gynaecology Division, Department of Obstetrics and Gynaecology, University of São Paulo, São Paulo, Brazil

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ABSTRACT

Objective: To investigate expression of BcL-2, FAS, FAS ligand (FASL) and cleaved caspase-3 in the endometrial tissue of women with idiopathic infertility (with two consecutive failed cycles of in vitro fertilization) and women with idiopathic recurrent pregnancy loss. The control group consisted of fertile women.

Study design: Endometrial tissue samples from fertile women ($n = 25$), women with idiopathic infertility ($n = 25$) and women with idiopathic recurrent pregnancy loss ($n = 25$) were collected on the seventh or eighth postovulatory day of their menstrual cycles for evaluation. Expression of BcL-2, FAS, FASL and cleaved caspase-3 was assessed using immunohistochemical methods.

Results: Expression of BcL-2 and FAS was significantly higher and lower, respectively, in the women with idiopathic infertility than in the other groups ($p < 0.01$). Expression of cleaved caspase-3 was significantly lower in the women with idiopathic infertility than in the other groups ($p < 0.01$). Expression of FASL was similar in all three groups.

Conclusion: Disturbances in endometrial apoptosis may be a contributing factor in patients with idiopathic infertility and recurrent pregnancy loss.

1. Introduction

Infertility is defined as one year of frequent unprotected intercourse that does not result in pregnancy. According to data from the National Survey of Family Growth, an estimated 10–15% of couples in the USA are infertile [1]. Idiopathic infertility is diagnosed when a couple does not conceive and no definitive causes of infertility can be diagnosed following a complete evaluation. Despite improved diagnostic techniques, the average incidence of idiopathic infertility has been reported to be approximately 15% in infertile couples [2], with a range of 0.1–37% [3–9]. Conventional treatments have not been effective in some cases of idiopathic infertility [9,10], and these patients were not able to conceive following these procedures [9,10]. It is important to understand the potential changes in endometrial physiology, particularly following the failure of consecutive cycles of in vitro fertilization (IVF), in cases of idiopathic infertility.

Idiopathic recurrent pregnancy loss is a serious reproductive problem. Most of these losses occur at the time of implantation, and 15–20% of cases end in spontaneous abortion. Recurrent pregnancy loss is defined as at least three consecutive miscarriages, and represents a frustrating clinical challenge for both clinicians and patients. Recurrent pregnancy loss affects 0.5–3% of women of reproductive age. Approximately 50% of recurrent pregnancy losses are idiopathic [11,12].

The human endometrium has the unique ability to undergo extensive and rapid remodelling [13]. It is fascinating to study, and has been used to understand normal and pathological uterine functions and as a model for rapid tissue remodelling events, such as embryonic development, tumorigenesis and wound healing.

As a mucosal tissue, the endometrium provides defence against infection [13]. It has a unique immunological capability that allows implantation of a semi-allogeneic embryo [14,15]. During the process of implantation, cellular differentiation and changes in the extracellular matrix and endometrial apoptosis may be important [16,17]. Apoptosis is regulated by various gene products, including

* Corresponding author at: Rua Sena Madureira, 1245 Ap 11, São Paulo, SP 04021-051, Brazil. Tel.: +55 11 5081 3681; fax: +55 11 5081 3681.
E-mail address: jsoares415@hotmail.com (J.M. Soares-Jr).

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cytokines, interleukins and steroid hormones. Many promoters and inhibitors of apoptosis have been described. Tumour necrosis factor-α and the FAS/FAS ligand (FASL) system have been shown to promote apoptosis [18], while BcL-2 appears to inhibit the process [19–27]. Several studies have suggested that apoptosis of human endometrial glandular cells is blocked by BcL-2 expression during the proliferative phase, and is induced by the FAS/FASL system in an autocrine or paracrine manner during the secretory phase. In general, downstream signalling of the FAS/FASL system in human endometrial glandular cells is influenced by cleaved caspase-3, which represents an irreversible step during apoptosis [24]. Information regarding this process during the embryo implantation window is scarce, particularly in cases of idiopathic infertility and recurrent pregnancy loss.

2. Materials and methods

Between October 2004 and February 2007, 160 women were recruited as volunteers to participate in this study at the Gynaecology Endocrinology Outpatient Clinic at the Federal University of São Paulo, São Paulo, Brazil. Written informed consent was obtained from the participants, and the local ethics committee approved the study.

Case histories, complete physical and gynaecological examinations, transvaginal pelvic and complete abdominal ultrasonographies, and laboratory and hormone tests were performed. The women with idiopathic infertility had previously undergone a laparoscopy that did not reveal any signs of endometriosis or other anatomical disorders that could explain their infertility. All potential participants underwent a standard diagnostic work-up to rule out verifiable causes of recurrent pregnancy loss or idiopathic infertility prior to inclusion in the study. Diagnostic procedures included hysterosalpingography, hysteroscopy, paternal and maternal karyotypes, cervical cultures for chlamydia, ureaplasma and mycoplasma, a comprehensive hormonal status [oestradiol, progesterone, testosterone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstendione, follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), free T4 and prolactin], evaluation of antiphospholipid syndrome using IgM and IgG anticardiolipin antibodies, and lupus anticoagulant testing. Blood was collected to test for toxoplasmosis, listeriosis, brucellosis, rubeola, cytomegalovirus, hepatitis (B and C), human immunodeficiency virus and VDRL (for syphilis). The exclusion criteria included: diabetes or high blood levels of fasting glucose (>110 mg/dl); anaemia (low levels of haematoglobin); leukocytes; liver and kidney disturbances; systemic arterial hypertension; a history of abnormal menses, anovulation or endometriosis (or high levels of CA-125); luteal body insufficiency; or other endocrinological disturbances. Women with endometrial tissues that were histologically incompatible with the secretory phase were also excluded from this study.

To avoid confounding factors due to ethnicity, only caucasian women were included in this study. Women with parents of the same ethnicity were included to avoid confounding factors due to genetic admixture.

In total, 75 volunteers were selected and divided into three groups of 25 women: (a) fertile women with at least two normal pregnancies and no history of infertility (control group); (b) women with idiopathic recurrent pregnancy loss; and (c) women with idiopathic infertility and two consecutive failed IVF cycles.

The specific inclusion criteria for each group were as follows: control group, at least two consecutive pregnancies and labours without any history of infertility or pregnancy loss; women with idiopathic recurrent pregnancy loss, a documented history (based on ultrasound examination) of at least three spontaneous consecutive miscarriages prior to 20 weeks of gestation with the same partner; and women with idiopathic infertility, a documented history of infertility with at least two consecutive failed IVF cycles using good-quality embryos (e.g. at least two embryos with eight cells (Grade 4) on the third day of embryonic life). The partners of the women with idiopathic infertility and recurrent pregnancy loss were normospermic and presented with negative semen and urethral cultures. The exclusion criteria in these two groups included the presence of thrombophilic factors (e.g. PT mutation G20210A, AT-III, R506Q mutation of Factor V Leiden, proteins C and S, lupus anticoagulant and anticardiolipin antibodies).

On the second clinic visit, patients underwent endometrial biopsies using a Pipelle catheter (Unimar, Bridgeport, CT, USA) under sterile conditions to remove tissue from the uterine fundus during the seventh or eighth postovulatory day of their menstrual cycles. Extreme care was taken to ensure that a sufficient tissue sample was obtained from each biopsy. The sample was fixed in 10% formalin and processed for histological evaluation using haematoxylin–eosin (H–E), and immunohistochemical analysis of BcL-2, FAS, FASL and cleaved caspase-3.

For endometrial dating, 4 μm sections stained with H–E were evaluated. All of the endometrial biopsies were coded, and evaluated blindly by an experienced pathologist according to the histopathological criteria of Noyes et al. [28]. If the samples were not compatible with the secretory phase, the tissues were not included for immunocytochemical analysis.

All endometrial samples investigated in the study were tested using: mouse monoclonal antibody FAS (B-10, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:50), rabbit polyclonal FASL (N-20, Santa Cruz Biotechnology; dilution 1:50), a BcL-2 oncoprotein kit [Dako, 124, MxH (M0887-1)], and cleaved caspase-3 (Asp175) (Antibody #9661, Cell Signaling Technology, Beverly, MA, USA; dilution 1:200).

The biopsy samples were embedded in paraffin and cut into 4 μm sections. The sections were dewaxed in Bioclear (Bio-Optica, Milan, Italy) and rehydrated in decreasing concentrations of ethanol followed by distilled water. Sections were pretreated in a 0.01 mol/l citrate buffer (Merck, USA) in a microwave oven (700 W) for two incubations of 12 min each and cooled at room temperature for 20 min. The slides were rinsed with Tris-buffered saline.

Endogenous peroxidases were blocked using 3% hydrogen peroxide in methanol for 10 min. The sections were washed in phosphate buffered saline (PBS), covered in 100 μl of blocking serum, and incubated for 30 min. The sections were incubated overnight with the primary antibody at 4 °C in PBS buffer with 1% bovine serum albumin (Sigma A 9647, USA). After washing with PBS buffer, localization of the antibodies was visualized by incubating the sections with secondary antibodies using a Dako kit (Dako LSAB + Sys, Peroxidase Universal K0690-1). After 30 min of incubation followed by washing, the sections were incubated with a freshly prepared solution of streptavidin-biotin immunoperoxidase (Dako LSAB kit) according to the protocol provided by the manufacturer. After washing, the bound enzyme was visualized following incubation with the enzyme in the presence of 3,3-diaminobenzidine tetrahydrochloride (Dako K3468-1), 1% DMSO (Synth, Brazil) and 0.06% hydroxide peroxide in 100 ml of PBS buffer for 5 min at 37 °C in a dark room. The slides were washed in PBS with 0.1% Triton X-100. The sections were counterstained with haematoxylin and dehydrated prior to mounting with Entellan (Merck 107961, Germany). Palatine tonsil tissue and BcL-2 were positive controls for FAS/FASL/cleaved caspases-3 and BcL-2, respectively. Bovine serum albumin was substituted for the primary antibody and used as a negative control. Other negative controls were applied using non-specific goat antibodies with the same concentration of primary antibody for each immunohistochemical reaction (BcL-2, FAS, FASL and cleaved caspase-3).