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ARTICLE

Down-regulation of the *CYP19A1* gene in cumulus cells of infertile women with endometriosis

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Abstract Aromatase plays a fundamental role in the establishment of oocyte quality, which might be compromised in infertile women with endometriosis. The expression of the *CYP19A1* gene (that encodes aromatase) was compared in cumulus cells and oestradiol concentrations in the follicular fluid of infertile women with and without endometriosis submitted to ovarian stimulation for intracytoplasmic sperm injection. Cumulus cells were isolated and the expression of the *CYP19A1* was quantitated through real-time polymerase chain reaction. Oestradiol concentrations in follicular fluid were measured by chemiluminescence immunoassay. A lower expression of the *CYP19A1* in the cumulus cells of infertile women with endometriosis was observed compared with controls (0.17 ± 0.13 and 0.56 ± 0.12, respectively), and no significant difference in the follicular fluid oestradiol concentrations was observed between groups. Our results show reduced expression of the *CYP19A1* in cumulus cells of infertile women with endometriosis, which may play a role in the pathogenesis of endometriosis-related infertility.

KEYWORDS: cumulus cells, *CYP19A1* gene, endometriosis, follicular fluid, infertility

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Introduction

Endometriosis, one of the most common gynaecologic diseases, affects about 10–15% of reproductive-age women and is associated with subfertility (Donnez et al., 2002; Holoch and Lessey, 2010). The prevalence of this condition increases to 40% in women with subfertility, and about 30–50% of affected women are estimated to be infertile (Holoch and Lessey, 2010). The mechanisms involved in the aetiopathogenesis of infertility in patients with endometriosis are likely to be multifactorial, although this complexity has not yet been fully elucidated (De Ziegler et al., 2010).

New approaches to the treatment of endometriosis-related infertility have become available, with an emphasis on assisted reproduction techniques, which are becoming increasingly common. The effect of endometriosis on assisted reproductive outcomes has been questioned, and it is unclear whether a deleterious effect of the disease occurs in assisted reproduction technique results (Aboulghar et al., 2003; Al-Fadhli et al., 2006; Barbosa et al., 2014; Barnhart et al., 2002; Harb et al., 2013; Lin et al., 2012). Some studies have shown low fertilization, pregnancy, implantation rates, or both, in women with endometriosis submitted to ovarian stimulation followed by IVF (Aboulghar et al., 2003; Al-Fadhli et al., 2006; Barbosa et al., 2014; Barnhart et al., 2002; Harb et al., 2013; Lin et al., 2012), which may be due, at least in part, to lower oocyte quality (Díaz et al., 2000; Simón et al., 1994). A previous study by our group has suggested an impairment or delay of the completion of meiosis I in in-vitro matured oocytes obtained from stimulated cycles of patients with endometriosis, although the underlying mechanisms were not elucidated (Barcelos et al., 2009). Because human oocytes are extremely rare, however, and their use in invasive studies is usually not feasible because it prevents their use in assisted reproduction techniques, few studies have evaluated the quality of oocytes from endometriosis-infertile women submitted to ovarian stimulation for assisted reproduction techniques.

Oocyte competence depends on the quality of the follicular microenvironment, and the presence of adequate bi-directional cumulus cell-to-oocyte signalling is a prerequisite for the acquisition of both oocytes and cumulus cell competence (Assou et al., 2006, 2008, 2010; Gasca et al., 2007; Ouandaogo et al., 2011, 2012). Follicular cells obtained during oocyte recovery in regular IVF cycles can be divided into two subpopulations: cumulus cells and mural granulosa cells. The cumulus cells form a group of closely associated cells that surround the oocyte in the antral follicle, and the mural granulosa cells line the follicular wall. Granulosa cells play an essential role in follicular differentiation, leading to optimal conditions for oocyte development, ovulation, fertilization and subsequent implantation (Adashi, 1994). Moreover, the bi-directional communication between the oocyte and these cells occurs throughout follicular development (Buccione et al., 1990; Eppig et al., 2002; Gilchrist et al., 2004; Makabe et al., 2006; Senbon et al., 2003; Sirard et al., 2006), and is essential for the acquisition of developmental competence in mammalian oocytes (de Loos et al., 1991; Fair, 2003; Webb et al., 2002). Therefore, the analysis of the expression of genes involved in the acquisition of oocyte competence in the cumulus cells of mature human oocytes can be used as a form of non-invasive assessment of oocyte quality and for the prediction of assisted reproduction technique results (Assou et al., 2006, 2008; Cetica et al., 2001; Hamamah et al., 2006; Hamel et al., 2008; Haouzi and Hamamah, 2009; Tesfaye et al., 2009).

A rate-limiting enzyme for the synthesis of oestrogen from androgens, P450 aromatase, is involved in the conversion of androstenedione and testosterone into oestrone and oestradiol, respectively. Aromatase is present in granulosa cells and plays a fundamental role in follicle maturation and the establishment of oocyte quality (Erickson et al., 1989; Foldesi et al., 1998; Hamel et al., 2008).

An increased expression of aromatase has been reported in endometriotic tissue (Noble et al., 1996) and endometriomas (Smuc et al., 2009). Although not detectable, aromatase expression has been observed in the eutopic endometrium from healthy women (Bulun et al., 2004; Hatok et al., 2011; Maia et al., 2012). In contrast to the increased aromatase expression in endometriotic tissue, Harlow et al. (1996) and de Abreu et al. (2006), through in-vitro studies using luteinized granulosa cell culture from women with and without endometriosis submitted to ovarian stimulation for assisted reproduction techniques, reported decreased aromatase activity in the granulosa cells of women with endometriosis, which might lead to defects in granulosa cell steroidogenesis and abnormal oocyte functioning (Harlow et al., 1996). Data on the expression of the aromatase gene (gene *CYP19A1*) in luteinized mural granulosa cells are controversial (Abreu et al., 2011; Lu et al., 2012), and no studies have evaluated the expression of the *CYP19A1* gene in cumulus cells from infertile women with endometriosis.

The main objective of the present study was, therefore, to compare the expression of the *CYP19A1* gene in cumulus cells of infertile women with and without endometriosis submitted to ovarian stimulation for intracytoplasmic sperm injection (ICSI). As secondary objective, oestradiol concentrations in follicular fluid were compared between infertile women with and without endometriosis.

Materials and methods

This prospective case-control study was approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP), Brazil on 17 January 2008 (approval reference number HCRP 10187/2007).

Setting, patients and study size

No data have been published on the expression of the *CYP19A1* gene in cumulus cells from human cumulus-oocyte complex (COC). Therefore, a pilot study was conducted in which all the women submitted to ovarian stimulation for ICSI in the Sector of Human Reproduction, Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP, SP, Brazil), were analysed consecutively from February 2009 to October 2010, a period in which all infertile patients were submitted to diagnostic laparoscopy as part of the infertility investigation at our service. Of these, all the patients who met the eligibility criteria listed

below, and who gave their written informed consent to participate in the study, were selected. In the IVF Unit, all patients undergo consultation 30–40 days before the beginning of the cycle, to clarify questions concerning the procedure. The inclusion of patients occurred during this consultation.

The endometriosis group consisted of 40 infertile patients who received the indication of assisted reproduction techniques solely because of the presence of endometriosis diagnosed through video laparoscopy and further confirmed through lesion histology according to the criteria of the American Society for Reproductive Medicine (No Authors, 1997). The control group consisted of 41 patients whose procedure was indicated exclusively owing to the presence of male-factor infertility, tubal-factor infertility, or both.

The exclusion criteria included age over 38 years; body mass index 30 kg/m^2 or over; basal FSH greater than 10 mIU/mL; smoking; alcohol consumption; the presence of diseases such as diabetes mellitus or any other endocrinopathy, cardiovascular disease, dyslipidaemia, systemic lupus erythematosus and other rheumatologic diseases; any active infection; the presence of hydrosalpinx; the use of invasive methods to obtain spermatozoa; and the use of medications such as non-steroidal anti-inflammatory drugs and corticosteroids, which might interfere with ovarian folliculogenesis, for 3 months before starting ovarian stimulation.

Ovarian stimulation and luteal phase support

Ovarian stimulation was carried out according to the protocol adopted in the Sector of Human Reproduction (FMRP-USP), which consists of pituitary desensitization with gonadotrophin-releasing hormone (GnRH, Lupron, Abott, Brazil) using the so-called long protocol, ovarian stimulation with recombinant follicle-stimulating hormone (FSH, Gonal F, Serono, Geneva, Switzerland; Puregon, Organon, The Netherlands) and the administration of human recombinant chorionic gonadotropin (Ovidrel, Serono, Brazil) to promote ovulation followed by oocyte retrieval 34–36 h later.

Each patient received daily subcutaneous injections of 0.5 mg leuprolide acetate (Lupron, Abott, Brazil) starting 10 days before the basal ultrasound examination when ultrasonographic evaluation was carried out before the beginning of ovarian stimulation. Recombinant FSH (150–225 units) was used daily (Gonal-F, Serono, Brazil; Puregon, Organon, Brazil) during the first 6 days of ovarian stimulation. Ultrasonographic monitoring of the cycle started on the seventh day of stimulation and was carreid out daily or every 2 days, and the gonadotrophin dose was adjusted according to the follicular growth observed. Gonadotrophins and the gonadotrophin-releasing hormone agonist were discontinued when at least two follicles reached a mean diameter of 18 mm, and 250 μg of recombinant human chorionic gonadotrophin (HCG) was subsequently administered (Ovidrel, Serono, Brazil). At 34–36 h after HCG administration, each patient underwent oocyte retrieval under intravenous sedation with propofol (Diprivan, Astra-Zeneca, Brazil) and fentanyl citrate (Fentanyl, Janssen-Cilag, Brazil).

Luteal phase supplementation was carried out with natural micronized progesterone (Utrogestan, Enila, Brazil) administered orally at a dose of 200 mg three times a day starting on the day of oocyte retrieval until the twelfth week of pregnancy in pregnant patients.

Oocyte retrieval

Follicle aspiration was carried out using ultrasonography with a 5 MHz transvaginal transducer coupled with a puncture guide. Cumulus cells for gene expression analyses and follicular fluid for the analysis of oestradiol concentrations were obtained individually only from the first aspirated follicle of the first punctured ovary as described below. The remaining follicles were aspirated and pooled according to the protocol adopted.

After careful washing, all COC identified, including those whose cumulus cells were obtained for gene expression analysis, were plated together on cell culture plates (Multidish 4-well Nunclon, Delta SI) filled with human tubal fluid-HEPES culture medium (HTF, Irvine Scientific) supplemented with 10% synthetic serum substitute (SSS, Irvine Scientific), covered with mineral oil (Sigma-Aldrich) and incubated at 37°C in 5% CO_2 and 95% humidity for 2–3 h. After this period, the oocytes were denuded through the exposure of COC to hyaluronidase (H4272 type IV-S, Sigma; 80 IU/mL) for 30 s, and the cumulus cells were mechanically removed in HTF-SSS with the aid of a stripper pipette (130 μm – Denuding Pipette, Cook) and discarded.

ICSI and fertilization, cleavage, implantation and pregnancy rates

Mature oocytes, characterized by the extrusion of the first polar body, were submitted to ICSI 2–3 h after oocyte retrieval. At 16–18 h after ICSI, fertilization was assessed on the basis of presence of two pronuclei and two polar bodies, and the fertilization rate was calculated for each patient as the number of fertilized oocytes divided by the number of injected oocytes. At 25–17 h after ICSI, the presence of embryo cleavage was determined, and the cleavage rate was calculated for each patient as the number of cleaved embryos divided by the number of fertilized oocytes. At 43–45 h after ICSI (day 2), the embryo quality was analysed on the basis of blastomere number, per cent fragmentation and the presence or absence of multinucleation. The embryo quality was determined again 67–69 h after ICSI (day 3).

The total numbers of retrieved mature, injected and fertilized oocytes, cleaved and produced embryos and gestational sacs were determined. The fertilization rate was calculated for each patient as the number of fertilized oocytes divided by the number of injected oocytes. The cleavage rate was calculated for each patient as the number of cleaved embryos divided by the number of fertilized oocytes. The implantation rate was also calculated for each patient as the number of gestational sacs divided by the number of transferred embryos, and the clinical pregnancy rate was determined for each patient based on the presence of an embryonic heartbeat visualized through ultrasound at 5–6 weeks after embryo transfer per transfer cycle.

Samples

Cumulus cells

Immediately after the identification of COC of the first follicle aspirated, cumulus cells were separated from the oocyte

through microdissection using two insulin needles placed within a cryotube and immediately frozen in liquid nitrogen until RNA extraction.

Follicular fluid

In the present study, the contents of the first follicle were exclusively aspirated with a mean diameter 15 mm or wider (because of the high probability that follicles will contain a mature oocyte) of the first punctured oocyte. Only follicular fluid free of blood contamination upon visual inspection (Da Broi et al., 2014; Levay et al., 1997) and presenting a mature oocyte were considered adequate for analysis. Each adequate follicular fluid sample was centrifuged at 300 × g for 7 min to remove the cell components and stored at −80°C in individual tubes for further analysis.

The stored follicular fluid samples were later submitted to the determination of oestradiol concentration as described below.

Total RNA extraction

Total RNA was extracted from the cumulus cells using TRIzol reagent (Invitrogen Life Technologies, Paisley, UK) according to manufacturer's instructions. After sample treatment with DNase I (Sigma-Aldrich, USA), the RNA integrity was determined through 1% agarose gel electrophoresis for the visualization of 28S and 18S rRNA bands. The total RNA concentration was determined using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, USA) at 260 nm optical density.

Real-time polymerase chain reaction

One microgram of total RNA was reverse transcribed according to the protocol provided for the High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK).

Each sample was submitted to quantitative polymerase chain reaction (PCR) in triplicate under the following conditions: 10 μL TaqMan® Universal PCR Master Mix (2x) (Applied Biosystems, Warrington, UK), 1 μL TaqMan® Gene Expression Assay Mix (20X) (Applied Biosystems, Warrington, UK) and 9 μL cDNA diluted 1:25 in a final reaction volume of 20 μL. The reaction conditions included an initial step at 50°C for 2 min, followed by 95°C for 10 min, 95°C for 15 s and 60°C for 1 min. The inventoried probes and primers used for the genes were as follows: *GAPDH* Hs 99999905_m1, *ACTB* Hs 99999903_m1 (reference genes) and *CYP19A1* (HS 00240671_m1) (target gene), which were obtained using Assay-on-Demand™ Gene Expression Products (Applied Biosystems, Warrington, UK).

The expression level (RQ) of the genes analysed was calculated for each sample using the $2^{-\Delta\Delta CT}$ (or 2-Ct) method (Livak and Schmittgen, 2001; Schmittgen et al., 2000). A control sample pool was used as a calibrator and normalizer for the $2^{-\Delta\Delta CT}$ calculation.

Determination of oestradiol concentrations in follicular fluid

Follicular fluid oestradiol concentrations were determined with a chemiluminescence immunoassay (IMMULITE 2000, Siemens, Los Angeles, USA). The coefficient of variability was 15 pg/mL for functional sensitivity and 9.9% for intra-assay variability. Because all determinations were performed at the same time, no inter-assay coefficient of variability was estimated.

The same technician, blinded to the clinical data of the patients or the group to which the data belonged, performed all determinations.

Statistical analysis

Data for the endometriosis and control groups were compared statistically. SAS 2003 software Cary 2003 (SAS Institute Inc., USA) was used for statistical analysis. The respiratory quotient values were $\log_{10}$ transformed. Logarithmic transformation was necessary because one of the assumptions (linearity) in the linear model analysis was not satisfied. Tukey's test in the PROC GLM software was used to compare the mean expression of the *CYP19A1* gene between the control group and the endometriosis groups. In the results section, respiratory quotient values are presented without log transformation.

Quantitative continuous variables (e.g. age, FSH levels on the third day of the cycle, the endometrial thickness and oestradiol concentration in folliculr fluid) were analysed statistically by analysis of variance followed by the Tukey test. The number of follicles measuring 14–17 mm, number of follicles measuring 18 mm or more, number of retrieved oocytes, number of mature oocytes, number of fertilized oocytes and number of formed embryos were characterized as counting data and adjusted through Poisson distribution using the GENMOD procedure (Generalized Linear Models) in SAS software for comparison of the groups studied. The variables fertilization rate, cleavage rate, and pregnancy rate were analysed by binomial regression. The level of significance was set at 5% ($P < 0.05$) in all analyses.

Results

Patient series and study flow chart

Between February 2009 and October 2010, 451 patients participated in the Assisted Reproduction Programme of the University Hospital, Faculty of Medicine of Ribeirão Preto, and underwent ovarian stimulation for ICSI. Among these individuals, 203 patients were excluded (78 patients older than 38 years, 11 with infertility of no apparent cause and 114 meeting other exclusion criteria). Of the 248 eligible patients, 122 were assigned to the control group and 126 were assigned to the pelvic endometriosis group. All eligible patients were interviewed, and 32 patients did not agree to participate in the study. Of the 216 patients who provided written informed consent, 104 were assigned to the control group and 112 were assigned to the endometriosis group. Fifty-eight of the 216 patients did not undergo oocyte retrieval, whereas 158 patients were submitted to this procedure. Among those patients who underwent oocyte retrieval, 19 had no oocyte retrieved (10 patients from the control group and nine from the endometriosis group) and six had COC with few cells, preventing cumulus cells donation for the study (three controls and three with endometriosis). Thus, cumulus cells donated from 133 patients were obtained, including 65 patients from

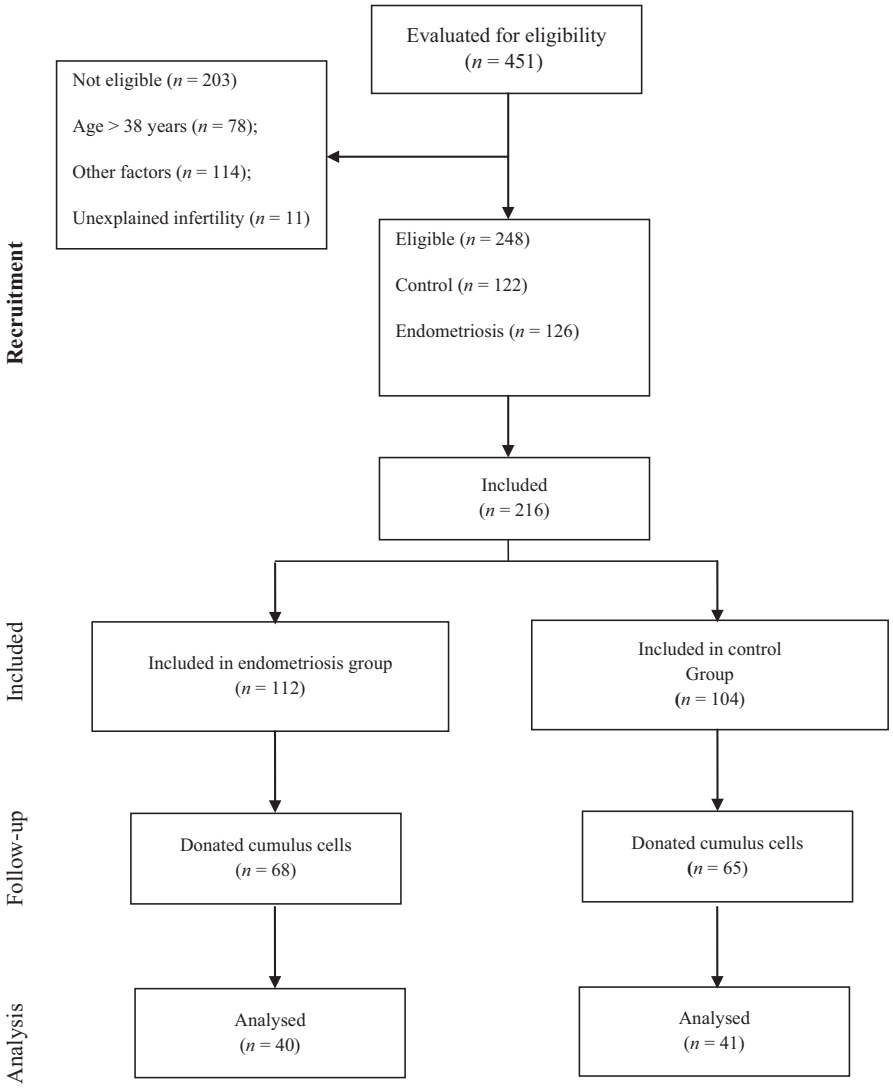

Figure 1 Study flow chart.

the control group and 68 patients from the endometriosis group. Total RNA was extracted from all 133 samples, and the RNA integrity was assessed followed by real-time PCR. In 52 samples (24 from control and 28 from endometriosis group), no RNA integrity was found, and the procedure was carried out with samples from 81 patients, including 41 samples from the control group and 40 samples from the endometriosis group (26 I/II and 14 III/IV – according to American Society for Reproductive Medicine classification), and the RNA was amplified according to the instructions provided by Applied Biosystems (Figure 1).

Clinical characterization and intracytoplasmic sperm injection results

The characteristics of the infertile patients regarding ovarian stimulation and the ICSI results are listed in Table 1. The numbers of fertilized oocytes and cleaved embryos were significantly lower in the endometriosis group (3.15 ± 0.34 and 2.40 ± 0.24, respectively) compared with the control group (4.00 ± 0.35 and 3.15 ± 0.26, respectively) (both $P = 0.04$) (Table 1). No significant differences were found between endometriosis and control groups in the other variables as presented at Table 1.

Expression of the *CYP19A1* in cumulus cells

To determine whether the presence of endometriosis is associated with the aberrant expression of the *CYP19A1* gene in cumulus cells of infertile women, *CYP19A1* gene expression in cumulus cells from infertile women with and without endometriosis who were submitted to ovarian stimulation for ICSI were compared. The expression of the *CYP19A1* was significantly lower in cumulus cells from infertile patients with endometriosis compared with control patients ($P = 0.02$) (Table 2).

Follicular fluid oestradiol concentrations

To determine whether the presence of endometriosis is associated with the compromised production of oestradiol in the

Table 1 Clinical characteristics of the patients and comparison of the ovarian stimulation and embryology parameters between infertile control patients (male factor, tubal factor infertility, or both) and patients with infertility associated with pelvic endometriosis.

Variables	*Control (n = 41) Mean (SD)*	*Endometriosis (n = 40) Mean (SD)*
Age (years)	32.4 (0.5)	33.3 (0.4)
Basal FSH (mIU/mL)	5.9 (0.3)	6.1 (0.4)
Number of follicles 14–17 mm	5.2 (0.5)	4.7 (0.4)
Number of follicles 18 mm or wider	2.7 (0.3)	2.7 (0.2)
Endometrial thickness (mm)	11.1 (0.4)	10.1 (0.3)
Number of oocytes retrieved	7.1 (0.6)	6.7 (0.8)
Number of mature oocytes	5.7 (0.5)	4.9 (0.6)
Number of injected oocytes	4.7 (0.4)	4.1 (0.4)
Number of fertilized oocytes	4.00 (0.35)	3.15 (0.34)[a]
Fertilization rate (%)	81.92 (3.92)	79.45 (3.95)
Number of cleaved embryos	3.15 (0.26)	2.40 (0.24)[a]
Cleavage rate (%)	81.3 (4.5)	80.7 (4.6)
Number of embryos produced	2.92 (0.27)	2.25 (0.21)
Implantation rate (%)	19.1 (5.31)	25.4 (6.0)
Clinical pregnancy rate (%)	26.8 (7.0)	35.0 (7.6)

The data are reported as the mean (SD).
[a]$P = 0.04$.

Table 2 Comparison of the expression of the *CYP19A1* in cumulus cells and oestradiol concentrations in the follicular fluid from infertile women with and without endometriosis submitted to ovarian stimulation for intracytoplasmic sperm injection.

Variables	*Control*	*Endometriosis*	P
CYP19A1	0.56 (0.12)	0.17 (0.13)	0.02
Follicular fluid ostradiol[a]	375,369.7 (49,026.0)	388,535.4 (45,097.4)	NS

NS = not statistically significant. Data are reported as the mean (SD).
[a]pg/ml.

follicular microenvironment of infertile women undergoing ovarian stimulation, oestradiol concentrations in follicular fluid from infertile women with and without endometriosis who were submitted to ovarian stimulation for ICSI were compared between the endometriosis and control groups. No significant difference was found in the follicular fluid oestradiol levels (**Table 2**).

We hypothesized that the analysis of the expression of the *CYP19A1* in CCs of mature human oocytes might be useful for understanding the mechanisms underlying endometriosis-related infertility. Therefore, in the present study, *CYP19A1* expression in cumulus cells from infertile patients with and without endometriosis submitted to ovarian stimulation for ICSI were compared. This study is the first assessment of the transcript levels of aromatase in cumulus cells from infertile patients with pelvic endometriosis undergoing ovarian stimulation for ICSI. We demonstrated a significant reduction in the expression of the *CYP19A1* in cumulus cells from infertile women with endometriosis compared with control infertile women. Data on the expression of the aromatase gene (*CYP19A1*) in luteinized mural granulosa cells are scarce and controversial (Abreu et al., 2011; Lu et al., 2012), and no previous studies have evaluated the expression of the *CYP19A1* gene in cumulus cells from infertile women with endometriosis.

The present study did not allow us to establish the reason of the lower expression of *CYP19A1* in cumulus cells of infertile women with endometriosis compared with controls. Antioxidants, besides exerting anti-apoptotic effect on in-vitro cultured preovulatory follicles Tsai-Turton and Luderer, 2006) are involved in the regulation of the function of steroidogenic enzymes dependent on cytochrome P450 (Verit et al., 2007). Some studies have suggested that both ascorbic acid (Murray et al., 2001), such as superoxide dismutase (LaPolt and Hong, 1995) can exert inhibitory effects on aromatase, which could favour the accumulation of androgens in follicular content, providing follicular atresia (Verit et al., 2007). Oxidative stress seems to participate in the aetiopathogenesis of endometriosis (Agarwal et al., 2012; Carvalho et al., 2012). Through mechanisms that have not been fully elucidated, oxidative stress may promote meiotic anomalies as well as impairing oocyte quality and embryo development before and after implantation (Navarro et al., 2004, 2006). The presence of oxidative stress markers in the follicular fluid of infertile women with endometriosis submitted to IVF has been recently demonstrated (Liu et al., 2013; Prieto et al., 2012; Singh et al., 2013). In a recent study by our group,

increased expression of the gene *SOD1* that encode superoxide dismutase was observed, an important antioxidant enzyme, in cumululs cells of infertile women with endometriosis in advanced stages (Navarro et al., 2013). We, therefore, hypothesized that oxidative stress in peritoneal and also follicular environment may be involved at the lower expression of *CYP19A1* in cumulus cells of infertile women with endometriosis, which needs to be further evaluated in other studies.

Owing to the strict selection criteria for cumulus cell donors, this study was carried out with a small sample size; therefore, further investigations using a large cohort of patients are needed to confirm these results. In addition, we confirmed that the amount of proteins extracted from cumulus cells of a single COC is insufficient for performing protein assay, so that it was not possible to assess whether the aromatase levels behave like their transcripts in the samples (collected over 21 months). Therefore, future studies evaluating the levels of aromatase in cumulus cells are needed. Moreover, data obtained from studies using samples collected after ovarian stimulation may not necessarily be extrapolated to natural cycles, and studies evaluating cumulus cells from natural cycles IVF are important to confirm our results. It is also important to state that the methodology used in the present study did not permit assessment of the effect of the aberrant expression of cumulus cells *CYP19A1* in women with endometriosis on the pathogenesis of infertility. We observed evidence, however, of the physiological role of aromatase in the reproductive process, suggesting a potential mechanism by which *CYP19A1* aberrant expression influences the acquisition of oocyte competence, participating in the aetiopathogeny of infertility associated with endometriosis. Oocyte quality results from a complex and synchronized process, lasting several months, from the primordial follicle phase to the preovulatory phase (Ola and Sun, 2012). Granulosa cells play an essential role in follicular differentiation, leading to optimal conditions for oocyte development, ovulation, fertilization and subsequent implantation (Adashi, 1994). Therefore, the embryo quality might depend on the final maturation of the follicle, leading to an oocyte with the ability to produce a successful pregnancy. Our understanding of the specific and temporal changes in the gene expression of follicular cells during follicular growth is far from complete in animals and humans. Evidence shows, however, that P450 aromatase is stimulated through FSH and expressed in high concentrations in dominant follicles (Sisco et al., 2003). Therefore, the increased expression of this enzyme might be associated with the induction (FSH and LH) of steroid hormone production (oestrogen and progesterone) and follicular dominance mechanisms (Tesfaye et al., 2009). In this context, Hamel et al. (2008), analysing follicular cells (both mural granulosa and cumulus cells) obtained from individual aspirated follicles from patients undergoing IVF, showed increased expression of the *CYP19A1* gene in follicular cells from follicles resulting in pregnancy. These investigators suggested that the increased expression of this gene in follicular cells could be used as a biomarker for embryonic quality and competence. Thus, we suggest that the reduced expression of the *CYP19A1* in cumulus cells of infertile patients with pelvic endometriosis might favour the impairment of oocyte quality, participating in the pathogenesis of infertility associated with this disease. The smaller number of fertilized oocytes and cleaved embryos in the endometriosis group compared with the control group might be considered as indirect evidence of lower oocyte quality associated with endometriosis, consistent with our hypothesis of lower gamete quality associated with infertility, potentially mediated through the reduced expression of the *CYP19A1* in cumulus cells. This hypothesis, however, should be confirmed in future studies using proper methodologies.

Follicular fluid is a metabolically active microenvironment intimately associated with the oocyte during the entire phase of cell growth and maturation (Attaran et al., 2000; Pasqualotto et al., 2004), and modifications of this reproductive microenvironment might result in compromised oocyte quality (Ola and Sun, 2012). A predominantly intrafollicular oestrogenic environment has been associated with good follicular growth and has anti-atresia effects. In addition, oestradiol enhances the cytoplasmic maturation of oocytes (Tesarik and Mendoza, 1997). Thus, the transformation from an androgenic to an estrogenic follicular microenvironment might be fundamental for the acquisition of oocyte competence (Revelli et al., 2009). Therefore, as a secondary objective of the present study, oestradiol concentrations in the follicular fluid were compared between infertile women with and without endometriosis. No significant difference was detected in the oestradiol concentrations in the follicular fluid between women with and without endometriosis. A possible explanation for this finding is the absence of reduced CYP19A1 expression in mural granulosa cells from infertile women with endometriosis submitted to ovarian stimulation for IVF, as Abreu et al. (2011) previously observed. As these cells are primarily responsible for the production of follicular oestradiol (Hillier et al., 1994; Whitelaw et al., 1992), the production of this hormone would be preserved even in the presence of reduced CYP19A1 expression in cumulus cells, as observed in the present study. Cumulus cells express lower levels of CYP19A1 than mural granulosa cells, and are therefore less active in steroidogenesis (Whitelaw et al., 1992). Studies comparing the expression of CYP19A1 in mural granulosa cells and the cumulus cells from infertile women with endometriosis are necessary to confirm this hypothesis. Notably, in the present study, the content of the first follicle with a mean diameter of 15 mm or wider was exclusively aspirated from the first ovary punctured and only considered follicular fluid free of blood contamination upon visual inspection and presenting a mature retrieved oocyte as adequate for analysis; therefore, the experimental sample was small, and studies with larger series will be important to confirm our findings.

In summary, we demonstrated a lower expression of the *CYP19A1* in cumulus cells of infertile patients with endometriosis undergoing ovarian stimulation for ICSI compared with infertile women without endometriosis. We also observed a lower number of fertilized oocytes in these women. On the basis of these results, we hypothesize that the reduced expression of the *CYP19A1* gene in cumulus cells might be involved in the impairment of oocyte quality associated with endometriosis, indicating a new perspective for understanding the pathogenesis of endometriosis-related infertility.

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