RBM ONLINE

ELSEVIER

Article

PTGS2 down-regulation in cumulus cells of infertile women with endometriosis

Caroline M da Luz [a,*], ***Michele G da Broi*** [a], ***Flávia Cappello Donabela*** [a], ***Cláudia Cristina Paro de Paz*** [b], ***Juliana Meola*** [a], ***Paula A Navarro*** [a]

[a] *Human Reproduction Division, Department of Obstetrics and Gynecology, Ribeirão Preto School of Medicine, University of São Paulo, 3900 Bandeirantes Avenue, Ribeirão Preto, SP 14049-900, Brazil*

[b] *Department of Genetics, Ribeirão Preto School of Medicine, University of São Paulo, 3900 Bandeirantes Avenue, Ribeirão Preto, SP 14049-900, Brazil*

Caroline Mantovani da Luz has a master's degree in Medical Sciences, Gynaecology and Obstetrics, obtained from the University of São Paulo, Ribeirão Preto Medicine School, Human Reproduction Division, in 2016. Her project focused on endometriosis and oocyte quality. She has experience in experimental research and molecular biology, and an interest in reproductive medicine, endometriosis and infertility.

KEY MESSAGE

This study revealed decreased expression of *PTGS2* in cumulus cells of infertile women with endometriosis compared with controls. It is postulated that, based on *PTGS2* down-regulation, lower levels of cyclooxygenase 2 in cumulus cells might be involved in the impairment of oocyte development.

A B S T R A C T

A deleterious effect of endometriosis on oocyte quality has been proposed. Evidence suggests that cumulus cells could be used as indirect biomarkers of oocyte quality. The *PTGS2* gene, which encodes cyclooxygenase 2 (COX-2), is deregulated in endometriotic lesions and plays a crucial role in the acquisition of oocyte competence. To date, research evaluating *PTGS2* expression in cumulus cells of infertile patients with endometriosis has not been conducted. The aim this study was to compare the expression levels of *PTGS2* in cumulus cells of infertile women, with and without endometriosis, undergoing ovarian stimulation for intracytoplasmic sperm injection (ICSI). Therefore, a case–control study compared *PTGS2* gene expression in the cumulus cells of 38 infertile patients with endometriosis and 40 without, using real-time polymerase chain reaction. For the first time, decreased expression of *PTGS2* was found in cumulus cells of infertile women with endometriosis compared with controls (7.2 ± 10.5 versus 12.4 ± 15.7), which might be related to reduced levels of COX-2 in the cumulus cells of women with the disease. Consequently, we hypothesize that lower transcript levels of *PTGS2* in cumulus cells may be involved in the impairment of oocyte quality, suggesting a possible mechanism involved in disease-related infertility.

* Corresponding author.
E-mail address: carollinemantovani@gmail.com (CM da Luz).

http://dx.doi.org/10.1016/j.rbmo.2017.06.021

Introduction

Endometriosis is a gynaecologic disease, characterized by the presence of functional endometrial-like tissue outside of the uterine cavity (Ozkan et al., 2008). About 10% of women of reproductive age are diagnosed with this oestrogen-dependent disorder (Ahn et al., 2015). They may display diverse clinical conditions, ranging from asymptomatic to symptoms such as dysmenorrhoea, chronic pelvic pain and subfertility, which significantly degrades the patient's quality of life (Bulun, 2009; Giudice and Kao, 2004). The incidence of the disease is increased in 40–60% of women with subfertility, and around 30–50% of the affected women are estimated to be infertile (Holoch and Lessey, 2010).

The mechanisms underlying endometriosis-related infertility remain to be elucidated, especially in the initial stages, when patients do not exhibit distortions and adhesions in the reproductive tract (Da Broi and Navarro, 2016). Several hypotheses have been proposed to justify the fertility impairment in these women, and some studies suggest a significant role of oocyte quality in this condition (Andrade et al., 2010; Barcelos et al., 2009, 2015; Da Broi and Navarro, 2016; Da Broi et al., 2014; Donabela et al., 2015; Garcia-Velasco and Arici, 1999; Simon et al., 1994; Sung et al., 1997). Oocyte analysis in these patients, however, is not routinely feasible, as human oocytes are rarely donated to research centres, and their application in invasive techniques precludes subsequent use in assisted reproduction technique procedures. Therefore, the indirect evaluation of oocyte quality (Assou et al., 2010; Ouandaogo et al., 2011, 2012), for follicle assessment, may contribute to the understanding of endometriosis-related infertility (McKenzie et al., 2004). In this context, evidence suggests that cumulus cells are intimately associated with oocyte development (McKenzie et al., 2004), contributing to cytoplasmic oocyte maturation (Furger et al., 1996; Tanghe et al., 2002). Moreover, it is believed that impaired cumulus cell functions may culminate in impaired fertility (Hizaki et al., 1999).

Endometriosis is known to be associated with several deregulated molecules related to the pathogenesis of the disease, such as cyclooxygenase-2 (COX-2) and aromatase (Banu et al., 2008; Gupta et al., 2008). The COX-2 enzyme, encoded by the *PTGS2* gene (prostaglandin–endoperoxide synthase 2), is naturally induced by aromatase and is involved in the conversion of arachidonic acid into prostaglandins (Sugimoto et al., 2007), which, in turn, regulate aromatase levels in endometriotic tissue (Bulun et al., 2001). In the endometrial tissue of patients with endometriosis, aberrant aromatase is induced via cyclooxygenase-2 prostaglandine-2 (COX-2–PGE_2) pathway deregulation, with a positive feedback cycle (Bukulmez et al., 2008; Bulun et al., 2002). It is also related to proliferative and inflammatory properties of ectopic implants (Bulun et al., 2001). Supporting these data, several studies have demonstrated COX-2 upregulation in eutopic (Chen et al., 2012; Cho et al., 2010; Ota et al., 2001; Wang et al., 2012) and ectopic (Ota et al., 2001; Rakhila et al., 2013) endometrial tissues from women afflicted by the disease.

In contrast to what occurs in the endometrial tissue, aromatase activity is decreased in granulosa cells (Barcelos et al., 2015), and lower expression of the aromatase gene (*CYP19A1*), not only in luteinized mural granulosa cells (Lu et al., 2012) but also in cumulus cells of infertile women with endometriosis (Barcelos et al., 2015; Hosseini et al., 2016) was demonstrated. On this basis, decreased aromatase activity may reflect other disorders in the cumulus cells of these patients, such as reduced PGE_2 in response to low levels of COX-2; however, further investigation is required. Interestingly, it was verified that COX-2 levels in cumulus cells are associated with cumulus expansion and matured oocytes in cattle (Nuttinck et al., 2002), equine (Dell'Aquila et al., 2004) and mice (Calder et al., 2001) models. Moreover, the higher expression of *PTGS2* in cumulus cells was previously correlated to human oocyte competence acquisition and higher-grade embryos (McKenzie et al., 2004). Therefore, we wonder if this gene may be deregulated in the cumulus cells of infertile women with the disease, which might be related to the impairment of oocyte competence.

In cumulus cells, COX-2 plays a significant role during oocyte competence acquirement. Moreover, aromatase, and consequently COX-2, seem to be altered in the endometrial tissue of women with endometriosis; aromatase has also been reported to be decreased in granulosa and cumulus cells of endometriosis patients. On the basis of this, we hypothesize that the *PTGS2* gene may be deregulated in cumulus cells of women with endometriosis, consequently compromising oocyte quality. To date, however, published research evaluating *PTGS2* gene expression in cumulus cells of infertile patients with the disease is lacking. Therefore, the objective of the present study was to compare the transcript levels of the *PTGS2* gene in cumulus cells of infertile women, with and without endometriosis, undergoing ovarian stimulation for intracytoplasmic sperm injection (ICSI).

Materials and methods

Ethics

A prospective case-control study was conducted in the Human Reproduction Sector of the Department of Obstetrics and Gynecology, at the University Hospital of the Ribeirão Preto School of Medicine, at the University of São Paulo (FMRP-USP). The study was approved by the Research Ethics Committee of the University Hospital (HCRP 10187/2007) on 17 January 2008. All patients who met the eligibility criteria, and who agreed to participate in the study, provided written informed consent.

Settings and duration

Between February 2009 and October 2010, patients who participated in the Assisted Reproduction Program of the University Hospital, FMRP-USP, and underwent ovarian stimulation for ICSI, were evaluated according to eligibility criteria, and those who were considered eligible were interviewed. Cumulus cells from the human cumulus-oocyte complex (COC) were collected on the day of oocyte retrieval. The patients were then followed up until oocyte analysis to obtain the number of retrieved mature oocytes, included in the clinical data assessment. The samples were analysed at the Human Reproduction Core Laboratory of FMRP-USP. The cDNA processing and quantitative polymerase chain reaction (PCR) analysis were carried out at the beginning of 2011.

Participants and eligibility criteria

The inclusion criteria for the endometriosis group was the presence of infertility, exclusively associated with endometriosis, diagnosed by video laparoscopy according to ASRM criteria (Revised American Society for Reproductive Medicine classification of endometriosis: 1996,

1997). The diagnostic laparoscopy was carried out as part of our routine service to investigate the cause of infertility before any assisted reproduction treatment, even in asymptomatic women. Surgical treatment was carried out in all patients during the laparoscopy, at least 9 months before the patients were recruited for the present study. The control group consisted of patients displaying male, tubal factor infertility, or both, which underwent diagnostic video laparoscopy, as part of our routine service to investigate the cause of the condition, ruling out the presence of endometriosis.

The eligibility criteria for both groups were as follows: age 38 years or younger; body mass index <30 kg/m^2; serum level of basal FSH <10 mIU/ml; non-smoker; lack of alcohol consumption; absence of polycystic ovary syndrome and other causes of chronic anovulation; lack of diseases such as diabetes mellitus or any other endocrinopathy, cardiovascular disease, dyslipidaemia, systemic lupus erythematosus and other rheumatologic diseases; any active infection; hydrosalpinx; absence of invasive methods to obtain spermatozoa, and use of medication, such as non-steroidal anti-inflammatory drugs and corticosteroids, which could interfere in ovarian folliculogenesis, 3 months before ovarian stimulation. These criteria aimed to avoid situations related to the worsening of oocyte quality, which could be considered as confounding factors (Di Emidio et al., 2014; Hou et al., 2016; Ma et al., 2013; Rambags et al., 2014; Simsek-Duran et al., 2013).

Ovarian stimulation

Menstruation programming was applied to determine the beginning of the ovarian stimulation cycle. This procedure consists of daily administration of combined oral contraceptives, starting during the menstrual period of the preceding cycle until about 5 days before the scheduled day for the beginning of ovarian stimulation, after undergoing basal transvaginal ultrasound.

Ovarian stimulation was carried out according to the protocol adopted in the Human Reproduction Sector (FMRP-USP). The procedure consists of pituitary desensitization using the gonadotrophin-releasing hormone (GnRH, Lupron®, Abbott, Brazil) after the extended protocol, ovarian stimulation using recombinant follicle-stimulating hormone (FSH, Gonal F®, Serono, Geneva, Switzerland; Puregon®, Organon, The Netherlands), and the administration of recombinant HCG (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®, Abbott, Brazil), starting 10 days before the basal ultrasound, and maintained throughout the ovarian stimulation period until the day of HCG (Ovidrel®, Serono, Brazil) administration.

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 7th day of stimulation and was carried out daily or every 2 days, and the gonadotrophin dose was adjusted according to observed follicular growth. 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 HCG was subsequently administered (Ovidrel®, Serono, Brazil). Oocytes were retrieved 34–36 h after the administration of recombinant HCG.

Oocyte retrieval and ICSI

Oocytes were retrieved from patients under general intravenous anaesthesia with propofol (Diprivan®, Astra-Zeneca, Brasil) and fentanyl (Fentanil®, Janssen-Cilag, Brazil). Follicles were aspirated via endovaginal route guided by a transvaginal ultrasound transducer, using a standard single-lumen needle (CDD Laboratory, France) with constant artificial aspiration pressure of 100 mmHg, provided by an electronically controlled suction pump (Craft® Suction Pump, Rocket Medical, England).

The cumulus cells for gene expression analyses were obtained individually, solely from the first aspirated follicle of the first punctured ovary, as described below. The remaining follicles were aspirated and pooled according to the adopted protocol.

After careful washing, all identified COC, including those whose cumulus cells, were obtained for gene expression analysis, were plated together on cell culture plates (Multidish 4-well Nunclon®, Delta SI, Origem) filled with human tubal fluid-HEPES culture medium (HTF, Irvine Scientific, USA), supplemented with 10% Synthetic Serum Substitute (SSS, Irvine Scientific, USA). The plates were covered with mineral oil (Sigma-Aldrich, USA) and incubated at 37°C, 95% humidity, in a 5% CO_2 environment for 3–4 h. The COC were denuded (separated from the cumulus oophorus cells) in 25 µl hyaluronidase micro drops (H4272 type IV-S, Sigma, USA) at a concentration of 80 IU/ml in HTF-HEPES (Irvine Scientific, USA) for a maximum of 30 s, and washed two or three times with modified HTF medium (HTF/HEPES, Irvine Scientific, USA), supplemented with 10% SSS.

Mature oocytes, characterized by the extrusion of the first polar body, were submitted to ICSI 2–3 h after oocyte retrieval. Immature oocytes (germinal vesicle and metaphase I) were discarded.

Sample collection and processing

Cumulus cell samples

Immediately after the identification of the COC of the first aspirated follicle, cumulus cells were separated from the oocyte by microdissection, using two insulin needles placed within a cryotube and immediately frozen in liquid nitrogen until RNA extraction. All procedures were conducted under RNase-free conditions.

Total RNA extraction

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

Real-time polymerase chain reaction

One microgram of total RNA was reverse transcribed according to the protocol provided by the High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Each sample was submitted to 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 TaqMan® assay FAM-MGB probes and primers used for the genes were as follows: *GAPDH* Hs 99999905-m1, *ACTB* Hs 99999903-m1 (reference genes) and *PTGS2* Hs 01573474-g1 (target gene) (ThermoFisher Scientific, Warrington, UK).

The expression level (quantitative PCR) of the analysed gene was calculated for each sample using the 2-ΔΔCt (or 2-Ct) method (Livak et al., 2000; Livak and Schmittgen, 2001). An aliquot of each control sample was pooled and used as a sample calibrator for the calculation of normalization.

Study size

Because of the lack of data in the literature of expression of the *PTGS2* gene in cumulus cells from COC for estimating the sample size, a pilot study was designed. All eligible patients who consented to participate were analysed during the 21-month recruitment period (from February 2009 to October 2010), in which all infertile patients were submitted to diagnostic laparoscopy as part of the routine service infertility investigation. A sample size of 24–50 patients per group was previously considered suitable for pilot studies (Julious, 2005; Lancaster et al., 2004; Sim and Lewis, 2012) being enough to detect or discard at least a large (24 patients per group) or moderate effect size (50 patients per group) between these two groups with a power of 80%.

Statistical methods

Quantitative continuous variables (number of 14–17 mm follicles, ≥18 mm follicles, retrieved oocytes and mature oocytes) were characterized as counting data, adjusted by Poisson distribution using the GENMOD procedure (Generalized Linear Models), and statistically analysed using the T test.

The relative quantification values (quantitative PCR) of *PTGS2* were $\log_{10}$ transformed. Logarithmic transformation was necessary as one of the assumptions (linearity) in the linear model analysis was not satisfied. The T-test was applied to compare mean *PTGS2* gene expression among the endometriosis and control groups.

The data in graphic are represented in logarithmic transformation (mean and standard deviation). In the Results section, relative quantification values are shown without log transformation. All analyses were conducted using the 9.3 SAS® software (SAS Institute Inc., USA). Significance was defined as $P < 0.05$ in all analyses.

Results

Flowchart

Betweek February 2009 and October 2010, 451 patients participated in the Assisted Reproduction Program at the University Hospital, in the School of Medicine of Ribeirao Preto, and underwent ovarian stimulation for ICSI. Of these, 203 were deemed ineligible. The 248 eligible patients (126 with endometriosis and 122 controls) were interviewed, and 32 of them did not agree to participate in the study. Therefore, 216 patients provided written informed consent (112 with endometriosis and 104 controls) and began the ovarian stimulation cycle for ICSI. Fifty-eight patients did not undergo oocyte retrieval, whereas 158 did. In 19 of those who did, oocyte retrieval did not occur (nine with endometriosis and 10 controls), and six patients exhibited few cumulus cells, impeding donation for the study (three with endometriosis and three controls). Therefore, the obtained cumulus cells were donated by 133 patients (68 with endometriosis and 65 controls). Total RNA was extracted from cumulus cells, and RNA integrity was assessed, although the latter was inappropriate in 55 samples. In the end, 78 samples were analysed (40 controls and 38 from the endometriosis group), of which 25 displayed minimal to mild and 13 exhibited moderate to severe endometriosis (**Figure 1**).

Clinical variables for infertile patients with endometriosis and controls

No significant differences were observed between groups in age, basal FSH, number of follicles, number of retrieved oocytes and number of mature oocytes (**Table 1**).

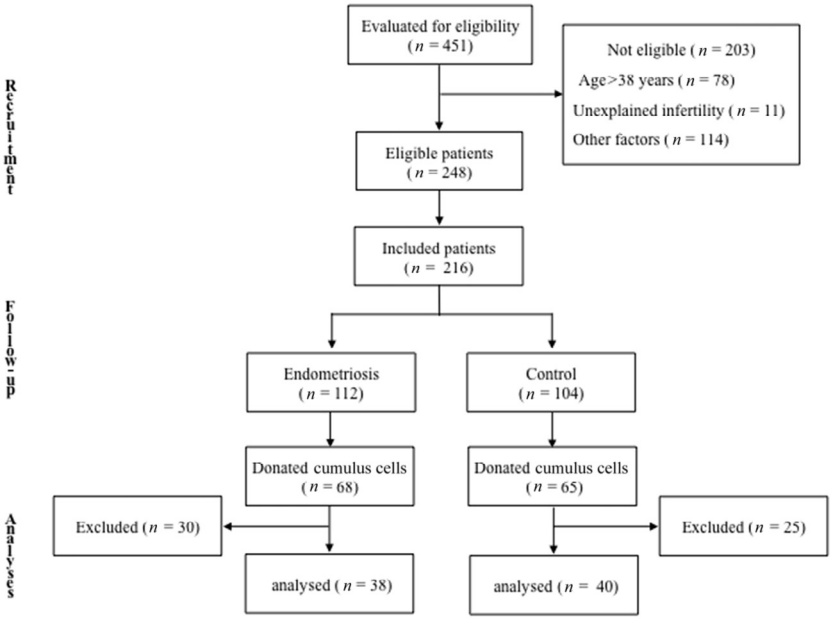

Figure 1 – Patient inclusion.

Table 1 – Clinical variables for infertile patients with endometriosis and controls undergoing intracytoplasmic sperm injection.[a,b]

Parameter	Control mean (SD)	Endometriosis 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)	2.7 (0.3)	2.7 (0.2)
Number of oocytes retrieved	7.1 (0.6)	6.7 (0.8)
Number of mature oocytes	5.7 (0.5)	4.9 (0.6)

[a] Data presented as mean and standard deviation (SD).
[b] No statistically significant differences were observed between the two groups.

Expression of the PTGS2 *gene in cumulus cells of infertile patients with endometriosis and controls*

The expression of *PTGS2* was significantly lower ($P = 0.046$) in cumulus cells from infertile women with endometriosis compared with control patients (7.2 ± 10.5 versus 12.4 ± 15.7) (**Figure 2**).

Discussion

To the best of our knowledge, this is the first study to show lower transcript levels of *PTGS2* in cumulus cells from infertile patients with endometriosis compared with infertile controls submitted to ovarian stimulation for ICSI. The results suggest possibly reduced levels of COX-2 in cumulus cells of patients with the disease. COX-2, a key enzyme in the biosynthesis of prostaglandins (Lim et al., 1997), has a well-described role in the pathogenesis of endometriosis (Chen et al., 2012; Wang et al., 2012). With pelvic endometriosis, higher activity of aromatase was shown in ectopic lesions, leading to increased oestradiol production in the pelvic environment, and consequent COX-2 induction (Bulun et al., 2001, 2002). Interestingly, our results were contrary to what is regularly verified for COX-2 in eutopic and ectopic endometrial tissues of endometriosis patients (Cho et al., 2010; Ota et al., 2001; Rakhila et al., 2013). In cumulus cells, *PTGS2* seems to follow *CYP19A1* regulation, which has previously been shown by our group as decreased (Barcelos et al., 2015), using the same samples evaluated at the present study. This may be explained by the close relationship between aromatase (*CYP1A1* gene) and COX-2 (Bulun et al., 2001, 2002; Zeitoun and Bulun, 1999). Aromatase converts androstenedione into oestrone, which is then converted into oestradiol by the type 1 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme in the granulosa cell. Oestradiol stimulates COX-2 to convert arachidonic acid into PGE_2 (Bulun et al., 2001), the most potent inducer of aromatase activity (Zeitoun and Bulun, 1999). Considering the reduced follicular aromatase activity in patients with endometriosis, the consequent lower levels of oestradiol (Lu et al., 2012; Wunder et al., 2005) might explain lower *PTGS2* expression in these women, which should be evaluated in future research using appropriate methodologies.

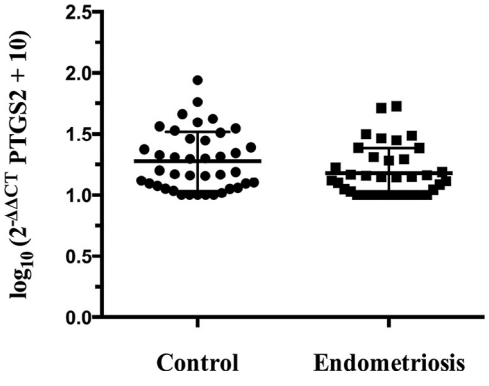

Figure 2 – Expression of *PTGS2* in cumulus cells from the first aspirated follicle obtained from an oocyte retrieval in endometriosis patients ($n = 38$) and controls ($n = 40$). Each sample was tested in triplicate. *PTGS2* expression was normalized to *GAPDH* and *ACTB*. Logarithmic transformed mean ± SD values are indicated by the horizontal lines. For endometriosis patients versus controls $P = 0.046$.

Several studies developed in animals described the importance of COX-2 in various aspects of final differentiation of ovarian follicles (Calder et al., 2001; Dell'Aquila et al., 2004; Nuttinck et al., 2002). In studies using knockout mouse models, the ablation of *PTGS2* culminated in multiple reproductive failures, including ovulation, fertilization, implantation, and decidualization damage, confirming that prostaglandins produced by COX-2 play a crucial role in reproductive processes (Langenbach et al., 1999; Lim et al., 1997). Also, supporting this hypothesis, higher levels of in-vitro COX-2 were associated with greater rates of cumulus expansion and mature oocytes in cattle (Nuttinck et al., 2002). Lack of cumulus expansion and oocyte maturation were additionally observed in ewe after the administration of prostanoid synthesis inhibitors (Murdoch, 1988, 1996). It is well-established that gap junctions mediate the association between cumulus cells and oocyte, contributing to gamete development and maturation (Furger et al., 1996; Tanghe et al., 2002). Nuttinck et al. (2002) suggested that COX-2-derived-prostanoids could modulate the expression level of connexins between cumulus-oocyte, directly influencing oocyte quality in cattle (Nuttinck et al., 2002). Furthermore, McKenzie et al. (2004) demonstrated that cumulus cells *PTGS2* expression was greater in oocytes that developed into higher quality embryos, compared with lower quality embryos, in women who underwent IVF cycles. Therefore, *PTGS2* gene expression might be correlated with morphological and physiological embryo characteristics (McKenzie et al., 2004). Therefore, we hypothesize that reduced *PTGS2* expression in cumulus cells of infertile women with endometriosis may lead to reduced COX-2 levels, and impaired oocyte competence in these patients, being considered a possible mechanism involved in endometriosis-related infertility. Nevertheless, further appropriate studies should be conducted to better understand the influence of lower *PTGS2* expression in cumulus cells of infertile women with endometriosis on oocyte quality obtainment.

To date, studies evaluating *PTGS2* expression in cumulus cells of infertile women, with and without endometriosis, have not yet been conducted. One single study evaluated *PTGS2* expression in granulosa cells obtained from the follicular fluid of women with and without endometriosis, revealing no significant differences between the groups. The cells were aspirated during oocyte retrieval, thus being represented mainly by mural granulosa cells (Du et al., 2013). Nonetheless, it is important to state that granulosa cells and cumulus cells have

functionally distinct roles. Cumulus cells surround the oocyte in the ovarian antral follicle (Russell et al., 2016), contributing directly to the process of oocyte cytoplasmic maturation through a network of gap junctions between them and the oocyte (Tanghe et al., 2002; Thomas and Vanderhyden, 2006). In turn, granulosa cells carry out endocrine functions as well as cellular architectural diversity (Park et al., 2004; Shimada et al., 2006; Zhang et al., 2010). It is also relevant to point out that the used eligibility criteria and gene expression analysis methodology displayed deficiencies, once they included women of advanced ages (<42 years), which could interfere with oocyte quality. Also, they did not describe whether control patients underwent diagnostic laparoscopy to exclude the presence of endometriosis. In the quantitative PCR analysis of *PTGS2*, Du et al. (2013) used a SYBR™ Green dye, without providing the dissociation curve. This methodology is less accurate than the TaqMan™ probes used in the present study. Therefore, the differences between their results and ours may be due to the distinct types of cells evaluated and also the differences in the adopted methodologies.

Five primary limitations of the present study should be pointed out. First, owing to the restrictive eligibility criteria used, the study was carried out on a small sample size, compromising the generalizability of the study and affecting the comparison between different stages of endometriosis. Notwithstanding, this principle was required to elevate internal validity, eliminating other factors that are potentially related to compromised oocyte competence. Therefore, further investigations using a larger cohort of patients are needed to increase the external validity of our findings. Second, data obtained from studies using samples collected after controlled ovarian stimulation may not necessarily be extrapolated to natural cycles. Nevertheless, ICSI procedures in natural cycles are not performed in our Assisted Reproduction Program and are possibly related to decreased cumulative live birth rates compared with procedures performed after ovarian stimulation, which also requires further investigation (Allersma et al., 2013). Third, it was not possible to clarify whether the analysis of cumulus cells from a single follicle was representative of the set of follicles that responded to ovarian stimulation. As it is not certain whether longer periods of anaesthesia, repeated ovarian punctures, or both, might interfere with cumulus cells gene expression, in order to increase the internal validity of the present study we chose to only use cumulus cells of the first follicle from the first ovary punctured. Future studies evaluating all cumulus cells of each patient will be necessary to confirm our findings. Fourth, because of the low concentration of RNA and proteins present in the cumulus cells, it was not possible to analyse both the expression of the *PTGS2* gene and enzymatic levels of COX-2, in the same sample. Moreover, new approaches that investigate the levels and mechanisms of COX-2 are needed. And fifth, our study did not investigate the molecular process resulting from the decrease of *PTGS2* regarding oocyte quality. Nevertheless, our findings may guide future studies on the mechanisms involved in endometriosis-related infertility.

In summary, to the best of our knowledge, the present data demonstrate for the first time a down-regulation of *PTGS2* in cumulus cells of infertile patients with endometriosis, compared with infertile controls. On the basis of these results, as consequence of the *PTGS2* lower expression, we hypothesize that lower levels of COX-2 in cumulus cells might be involved in the impairment of oocyte development, suggesting a possible mechanism involved in disease-related infertility, which needs to be evaluated in future studies with larger numbers of enrolled participants, and with suitable methodologies.

Acknowledgements

The authors would like to thank the staff of the Human Reproduction Division of the Department of Obstetrics and Gynecology at the Ribeirão Preto School of Medicine, at the University of São Paulo, particularly Maria Cristina Picinato MSc and Roberta Cristina Giorgenon for cumulus cells collection, Cristiana Padovan for technical assistance, and Ionara Diniz Evangelista Santos Barcelos MD MSc for her valuable contribution in data analyses. We also thank Professor Rui Alberto Ferriani MD Ph.D., Clinical Director of the Human Reproduction Center, for granting the support and the laboratory to carry out this work.

ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form 26 June 2017
Accepted 27 June 2017
Declaration: This study received financial support from the National Council for Scientific and Technological Development (CNPq; 474858/2009-0; 309056/2009-8; 503867/2010-1) and the Research Support Foundation of the State of São Paulo (FAPESP; 2008/58197-6).

Keywords:
Cumulus cells
Endometriosis
Infertility
Oocyte quality
PTGS2

REFERENCES

1997. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil. Steril. 67, 817–821.
Ahn, S.H., Monsanto, S.P., Miller, C., Singh, S.S., Thomas, R., Tayade, C., 2015. Pathophysiology and immune dysfunction in endometriosis. Biomed Res. Int. 2015, 795976.
Allersma, T., Farquhar, C., Cantineau, A.E., 2013. Natural cycle in vitro fertilisation (IVF) for subfertile couples. Cochrane Database Syst. Rev. (8), CD010550.
Andrade, A.Z., Rodrigues, J.K., Dib, L.A., Romao, G.S., Ferriani, R.A., Jordao, A.A., Junior, Navarro, P.A., 2010. [Serum markers of oxidative stress in infertile women with endometriosis]. Rev Bras Ginecol Obstet 32, 279–285.
Assou, S., Haouzi, D., De Vos, J., Hamamah, S., 2010. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. Mol. Hum. Reprod. 16, 531–538.
Banu, S.K., Lee, J., Speights, V.O., Jr., Starzinski-Powitz, A., Arosh, J.A., 2008. Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms. Endocrinology 149, 1180–1189.
Barcelos, I.D., Vieira, R.C., Ferreira, E.M., Martins, W.P., Ferriani, R.A., Navarro, P.A., 2009. Comparative analysis of the spindle and chromosome configurations of in vitro-matured oocytes from patients with endometriosis and from control subjects: a pilot study. Fertil. Steril. 92, 1749–1752.

Barcelos, I.D., Donabella, F.C., Ribas, C.P., Meola, J., Ferriani, R.A., de Paz, C.C., Navarro, P.A., 2015. Down-regulation of the CYP19A1 gene in cumulus cells of infertile women with endometriosis. Reprod. Biomed. Online 30, 532–541.

Bukulmez, O., Hardy, D.B., Carr, B.R., Word, R.A., Mendelson, C.R., 2008. Inflammatory status influences aromatase and steroid receptor expression in endometriosis. Endocrinology 149, 1190–1204.

Bulun, S.E., 2009. Endometriosis. N. Engl. J. Med. 360, 268–279.

Bulun, S.E., Yang, S., Fang, Z., Gurates, B., Tamura, M., Zhou, J., Sebastian, S., 2001. Role of aromatase in endometrial disease. J. Steroid Biochem. Mol. Biol. 79, 19–25.

Bulun, S.E., Gurates, B., Fang, Z., Tamura, M., Sebastian, S., Zhou, J., Amin, S., Yang, S., 2002. Mechanisms of excessive estrogen formation in endometriosis. J. Reprod. Immunol. 55, 21–33.

Calder, M.D., Caveney, A.N., Westhusin, M.E., Watson, A.J., 2001. Cyclooxygenase-2 and prostaglandin E(2)(PGE(2)) receptor messenger RNAs are affected by bovine oocyte maturation time and cumulus-oocyte complex quality, and PGE(2) induces moderate expansion of the bovine cumulus in vitro. Biol. Reprod. 65, 135–140.

Chen, Q., Zhang, C., Chen, Y., Lou, J., Wang, D., 2012. Identification of endometriosis-related genes by representational difference analysis of cDNA. Aust. N. Z. J. Obstet. Gynaecol. 52, 140–145.

Cho, S., Park, S.H., Choi, Y.S., Seo, S.K., Kim, H.Y., Park, K.H., Cho, D.J., Lee, B.S., 2010. Expression of cyclooxygenase-2 in eutopic endometrium and ovarian endometriotic tissue in women with severe endometriosis. Gynecol. Obstet. Invest. 69, 93–100.

Da Broi, M.G., Navarro, P.A., 2016. Oxidative stress and oocyte quality: ethiopathogenic mechanisms of minimal/mild endometriosis-related infertility. Cell Tissue Res. 364, 1–7.

Da Broi, M.G., Malvezzi, H., Paz, C.C., Ferriani, R.A., Navarro, P.A., 2014. Follicular fluid from infertile women with mild endometriosis may compromise the meiotic spindles of bovine metaphase II oocytes. Hum. Reprod. 29, 315–323.

Dell'Aquila, M.E., Caillaud, M., Maritato, F., Martoriati, A., Gerard, N., Aiudi, G., Minoia, P., Goudet, G., 2004. Cumulus expansion, nuclear maturation and connexin 43, cyclooxygenase-2 and FSH receptor mRNA expression in equine cumulus-oocyte complexes cultured in vitro in the presence of FSH and precursors for hyaluronic acid synthesis. Reprod. Biol. Endocrinol. 2, 44.

Di Emidio, G., Falone, S., Vitti, M., D'Alessandro, A.M., Vento, M., Di Pietro, C., Amicarelli, F., Tatone, C., 2014. SIRT1 signalling protects mouse oocytes against oxidative stress and is deregulated during aging. Hum. Reprod. 29, 2006–2017.

Donabela, F.C., Meola, J., Padovan, C.C., de Paz, C.C., Navarro, P.A., 2015. Higher SOD1 gene expression in cumulus cells from infertile women with moderate and severe endometriosis. Reprod. Sci. 22, 1452–1460.

Du, Y.B., Gao, M.Z., Shi, Y., Sun, Z.G., Wang, J., 2013. Endocrine and inflammatory factors and endometriosis-associated infertility in assisted reproduction techniques. Arch. Gynecol. Obstet. 287, 123–130.

Furger, C., Cronier, L., Poirot, C., Pouchelet, M., 1996. Human granulosa cells in culture exhibit functional cyclic AMP-regulated gap junctions. Mol. Hum. Reprod. 2, 541–548.

Garcia-Velasco, J.A., Arici, A., 1999. Is the endometrium or oocyte/embryo affected in endometriosis? Hum. Reprod. 14 (Suppl. 2), 77–89.

Giudice, L.C., Kao, L.C., 2004. Endometriosis. Lancet 364, 1789–1799.

Gupta, S., Goldberg, J.M., Aziz, N., Goldberg, E., Krajcir, N., Agarwal, A., 2008. Pathogenic mechanisms in endometriosis-associated infertility. Fertil. Steril. 90, 247–257.

Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., Ichikawa, A., 1999. Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). Proc. Natl. Acad. Sci. U.S.A. 96, 10501–10506.

Holoch, K.J., Lessey, B.A., 2010. Endometriosis and infertility. Clin. Obstet. Gynecol. 53, 429–438.

Hosseini, E., Mehraein, F., Shahhoseini, M., Karimian, L., Nikmard, F., Ashrafi, M., Afsharian, P., Aflatoonian, R., 2016. Epigenetic alterations of CYP19A1 gene in Cumulus cells and its relevance to infertility in endometriosis. J. Assist. Reprod. Genet. 33, 1105–1113.

Hou, Y.J., Zhu, C.C., Duan, X., Liu, H.L., Wang, Q., Sun, S.C., 2016. Both diet and gene mutation induced obesity affect oocyte quality in mice. Sci. Rep. 6, 18858.

Julious, S.A., 2005. Sample size of 12 per group rule of thumb for a pilot study. Pharm. Stat. 4, 5.

Lancaster, G.A., Dodd, S., Williamson, P.R., 2004. Design and analysis of pilot studies: recommendations for good practice. J. Eval. Clin. Pract. 10, 307–312.

Langenbach, R., Loftin, C., Lee, C., Tiano, H., 1999. Cyclooxygenase knockout mice: models for elucidating isoform-specific functions. Biochem. Pharmacol. 58, 1237–1246.

Lim, H., Paria, B.C., Das, S.K., Dinchuk, J.E., Langenbach, R., Trzaskos, J.M., Dey, S.K., 1997. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91, 197–208.

Livak, F., Burtrum, D.B., Rowen, L., Schatz, D.G., Petrie, H.T., 2000. Genetic modulation of T cell receptor gene segment usage during somatic recombination. J. Exp. Med. 192, 1191–1196.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) Method. Methods 25, 402–408.

Lu, X., Wu, Y., Gao, X.H., Wang, Y.W., Wang, L., Sun, X.X., 2012. Effect of letrozole on estradiol production and P450 aromatase messenger RNA expression of cultured luteinized granulosa cells from women with and without endometriosis. Fertil. Steril. 98, 131–135.

Ma, J.Y., Li, M., Luo, Y.B., Song, S., Tian, D., Yang, J., Zhang, B., Hou, Y., Schatten, H., Liu, Z., Sun, Q.Y., 2013. Maternal factors required for oocyte developmental competence in mice: transcriptome analysis of non-surrounded nucleolus (NSN) and surrounded nucleolus (SN) oocytes. Cell Cycle 12, 1928–1938.

McKenzie, L.J., Pangas, S.A., Carson, S.A., Kovanci, E., Cisneros, P., Buster, J.E., Amato, P., Matzuk, M.M., 2004. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Hum. Reprod. 19, 2869–2874.

Murdoch, W.J., 1988. Disruption of cellular associations within the granulosal compartment of periovulatory ovine follicles: relationship to maturation of the oocyte and regulation by prostaglandins. Cell Tissue Res. 252, 459–462.

Murdoch, W.J., 1996. Differential effects of indomethacin on the sheep ovary: prostaglandin biosynthesis, intracellular calcium, apoptosis, and ovulation. Prostaglandins 52, 497–506.

Nuttinck, F., Reinaud, P., Tricoire, H., Vigneron, C., Peynot, N., Mialot, J.P., Mermillod, P., Charpigny, G., 2002. Cyclooxygenase-2 is expressed by cumulus cells during oocyte maturation in cattle. Mol. Reprod. Dev. 61, 93–101.

Ota, H., Igarashi, S., Sasaki, M., Tanaka, T., 2001. Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis. Hum. Reprod. 16, 561–566.

Ouandaogo, Z.G., Haouzi, D., Assou, S., Dechaud, H., Kadoch, I.J., De Vos, J., Hamamah, S., 2011. Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. PLoS ONE 6, e27179.

Ouandaogo, Z.G., Frydman, N., Hesters, L., Assou, S., Haouzi, D., Dechaud, H., Frydman, R., Hamamah, S., 2012. Differences in transcriptomic profiles of human cumulus cells isolated from oocytes at GV, MI and MII stages after in vivo and in vitro oocyte maturation. Hum. Reprod. 27, 2438–2447.

Ozkan, S., Murk, W., Arici, A., 2008. Endometriosis and infertility: epidemiology and evidence-based treatments. Ann. N. Y. Acad. Sci. 1127, 92–100.

Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L., Conti, M., 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303, 682–684.

Rakhila, H., Carli, C., Daris, M., Lemyre, M., Leboeuf, M., Akoum, A., 2013. Identification of multiple and distinct defects in prostaglandin biosynthetic pathways in eutopic and ectopic endometrium of women with endometriosis. Fertil. Steril. 100, 1650–1659, e1-2.

Rambags, B.P., van Boxtel, D.C., Tharasanit, T., Lenstra, J.A., Colenbrander, B., Stout, T.A., 2014. Advancing maternal age predisposes to mitochondrial damage and loss during maturation of equine oocytes in vitro. Theriogenology 81, 959–965.

Russell, D.L., Gilchrist, R.B., Brown, H.M., Thompson, J.G., 2016. Bidirectional communication between cumulus cells and the oocyte: old hands and new players? Theriogenology 86, 62–68.

Shimada, M., Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Richards, J.S., 2006. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. Mol. Endocrinol. 20, 1352–1365.

Sim, J., Lewis, M., 2012. The size of a pilot study for a clinical trial should be calculated in relation to considerations of precision and efficiency. J. Clin. Epidemiol. 65, 301–308.

Simon, C., Gutierrez, A., Vidal, A., de los Santos, M.J., Tarin, J.J., Remohi, J., Pellicer, A., 1994. Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum. Reprod. 9, 725–729.

Simsek-Duran, F., Li, F., Ford, W., Swanson, R.J., Jones, H.W., Jr., Castora, F.J., 2013. Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. PLoS ONE 8, e64955.

Sugimoto, T., Koizumi, T., Sudo, T., Yamaguchi, S., Kojima, A., Kumagai, S., Nishimura, R., 2007. Correlative expression of cyclooxygenase-1 (Cox-1) and human epidermal growth factor receptor type-2 (Her-2) in endometrial cancer. Kobe J. Med. Sci. 53, 177–187.

Sung, L., Mukherjee, T., Takeshige, T., Bustillo, M., Copperman, A.B., 1997. Endometriosis is not detrimental to embryo implantation in oocyte recipients. J. Assist. Reprod. Genet. 14, 152–156.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., de Kruif, A., 2002. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol. Reprod. Dev. 61, 414–424.

Thomas, F.H., Vanderhyden, B.C., 2006. Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. Reprod. Biol. Endocrinol. 4, 19.

Wang, D., Chen, Q., Zhang, C., Ren, F., Li, T., 2012. DNA hypomethylation of the COX-2 gene promoter is associated with up-regulation of its mRNA expression in eutopic endometrium of endometriosis. Eur. J. Med. Res. 17, 12.

Wunder, D.M., Mueller, M.D., Birkhauser, M.H., Bersinger, N.A., 2005. Steroids and protein markers in the follicular fluid as indicators of oocyte quality in patients with and without endometriosis. J. Assist. Reprod. Genet. 22, 257–264.

Zeitoun, K.M., Bulun, S.E., 1999. Aromatase: a key molecule in the pathophysiology of endometriosis and a therapeutic target. Fertil. Steril. 72, 961–969.

Zhang, M., Su, Y.Q., Sugiura, K., Xia, G., Eppig, J.J., 2010. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. Science 330, 366–369.