Follicular fluid from infertile women with mild endometriosis may compromise the meiotic spindles of bovine metaphase II oocytes

M.G. Da Broi1,*, H. Malvezzi1, C.C.P. Paz2,3, R.A. Ferriani1,4, and P.A.A.S. Navarro1,4

1Human Reproduction Division, Department of Gynecology and Obstetrics, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, SP, Brazil 2SAA/APTA, Ribeirão Preto, SP, Brazil 3Department of Genetics, Ribeirão Preto School of Medicine, University of São Paulo, Ribeirão Preto, SP, Brazil 4National Institute of Hormones and Woman’s Health, CNPq, Porto Alegre, RS, Brazil

*Correspondence address. Avenida Bandeirantes, 3900 – Ribeirão Preto, SP, CEP 14049-900, Brazil. Tel: +55-16-3602-28-21; Fax: +55-16-3633-1028; E-mail: michi.dabroi@gmail.com

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STUDY QUESTION: What is the potential impact of follicular fluid (FF) from infertile women with mild endometriosis (ME) on oocyte quality, especially on nuclear maturation and the meiotic spindle?

SUMMARY ANSWER: FF from infertile women with ME may compromise nuclear maturation and the meiotic spindles of in vitro matured bovine oocytes.

WHAT IS KNOWN ALREADY: Controversial studies have suggested that impaired oocyte quality may be involved in the pathogenesis of endometriosis-related infertility. Moreover, some studies have demonstrated alterations in the composition of FF from infertile women with endometriosis. However, to date no study has evaluated the effect of FF from infertile women with ME on the genesis of meiotic oocyte anomalies.

STUDY DESIGN, SIZE, DURATION: We performed an experimental study. Samples of FF were obtained from February 2009 to February 2011 from 22 infertile women, 11 with ME and 11 with tubal or male factors of infertility (control group), who underwent ovarian stimulation for ICSI at our university IVF Unit. From March 2011 to February 2012 we performed in vitro maturation (IVM) experiments using immature bovine oocytes as described below.

PARTICIPANTS/MATERIALS, SETTING, METHODS: FF free of blood and containing a mature oocyte was obtained from 22 infertile women during oocyte retrieval for ICSI. Immature bovine oocytes underwent IVM in the absence of FF (No-FF) and in the presence of four concentrations (1, 5, 10 and 15%) of FF from infertile women without endometriosis (C-FF) and with ME (ME-FF). Eleven replicates were performed, each one using FF from a control patient and a patient with ME. Each FF sample was used in only one experiment. After 22–24 h of IVM, oocytes were denuded, fixed and immunostained for morphological visualization of microtubules and chromatin by confocal microscopy.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 1324 cumulus—oocyte complexes were matured in vitro. Of these, 1128 were fixed and 1048 were analyzed by confocal microscopy. The percentage of meiotically normal oocytes was significantly higher for oocytes that underwent IVM in the absence of FF (No-FF; 76.5%) and in the presence of 1% (80.9%), 5% (76.6%), 10% (75%) and 15% (76.2%) C-FF than in oocytes that underwent IVM in the presence of 1% (44.4%), 5% (36.7%), 10% (45.5%) and 15% (51.2%) ME-FF (P < 0.01). No differences were observed among FF concentrations within each group. When the four concentrations from each group were pooled, the number of oocytes in metaphase I stage was significantly higher in the ME-FF (50 oocytes) than in the C-FF (29 oocytes) group and the percentage of meiotic abnormalities was significantly higher when oocytes were matured with ME-FF (55.8%) than with C-FF (23.1%), P < 0.01.

LIMITATIONS, REASONS FOR CAUTION: Owing to the strict selection criteria for FF donors, this study had a small sample size (11 cases and 11 controls), and thus further investigations using a large cohort of patients are needed to confirm these results. In addition, data obtained from studies using animal models may not necessarily be extrapolated to humans and studies evaluating in vivo matured oocytes from infertile women with ME are important to confirm our results.
Endometriosis is a benign, estrogen-dependent gynecological disease (Minici et al., 2008), characterized by the implantation and growth of endometrial tissue (glands and/or stroma) outside the uterine cavity (Gupta et al., 2006). The condition is highly prevalent, affecting up to 10–15% of the female population of reproductive age (Augoulea et al., 2012). Endometriosis is also frequently associated with infertility, being present in more than 30% of infertile women (Augoulea et al., 2012), with 30–50% of affected women experiencing difficulties in having children (Bulletti et al., 2010). However, the mechanisms involved in the pathogenesis of endometriosis-associated infertility, especially in women with early stage pelvic disease (i.e. minimal and mild endometriosis, defined as Stages I and II, respectively), who show no anatomical changes in the reproductive tract, have not been fully elucidated (Holoch and Lessey, 2010). Understanding of these etiopathogenic mechanisms may be valuable in designing effective therapeutic approaches to improve the natural fecundity of these patients.

New approaches to the treatment of endometriosis-related infertility include the use of assisted reproduction techniques. Contradictory results of IVF in patients with endometriosis have been reported (Garcia-Velasco and Arici, 1999; Garrido et al., 2000; Kumbak et al., 2008). Several studies have found that the rates of fertilization, implantation and pregnancy are lower in women with than without endometriosis that undergo IVF (Barnhart et al., 2002; Al-Fadhli et al., 2006). These reduced rates may be due to impaired oocyte quality and, consequently, to impaired embryo quality, to endometrial defects and/or to impaired interactions between the endometrium and the embryo (Brizek et al., 1995; Pellicer et al., 1995; Kumbak et al., 2008). However as that the presence of endometriosis in oocyte recipients does not decrease implantation or pregnancy rates it is possible that impaired implantation in women with endometriosis was mainly related to worsening of oocyte quality (Garrido et al., 2000; Pellicer et al., 2001; Katsoff et al., 2006), which needs to be confirmed in future studies.

Oocyte quality depends on the appropriate acquisition of cytoplasmic and nuclear maturation, where the latter depends on the presence of a normal cell spindle (Mattson and Albertini, 1990; Albertini, 1992). The meiotic spindle of human oocytes in metaphase II (MII) is a temporary dynamic structure consisting of microtubules associated with the oocyte cortex and their network of subcortical microfilaments (Liu et al., 2000; Wang and Keefe, 2002; Navarro et al., 2005). This microtubular structure functions primarily in assisting chromatid segregation, concomitant with the extrusion of the second polar body, ensuring the end of the meiotic process (Coticchio et al., 2010). The meiotic spindle of the oocyte is extremely sensitive to factors such as oxidative stress (Hu et al., 2001; Eichenlaub-Ritter et al., 2002; Mullen et al., 2004), which, in turn, promotes meiotic anomalies and chromosome instability, and is associated with increased apoptosis and impairments of preimplantation embryo development (Liu et al., 2003; Navarro et al., 2004, 2006).

Endometriosis may be associated with oxidative stress (Agarwal et al., 2003; Szczepanska et al., 2003; Gupta et al., 2008). Mansour et al. (2009) demonstrated that the peritoneal fluid (PF) of women with endometriosis promotes meiotic oocyte anomalies and embryo apoptosis in an experimental murine model, with oxidative stress being the potential mediator. However, due to the scarcity of human oocytes that can be donated for research using invasive methodologies that prevent the subsequent utilization of these cells in assisted reproduction techniques, there are no conclusive data to date about the association between endometriosis and meiotic oocyte anomalies.

Since the follicular environment is extremely important for the process of oocyte maturation, changes in the composition of the follicular fluid (FF) may influence the maturation and quality of oocytes, affecting fertilization, early embryo development and subsequent pregnancy (Ma et al., 2010). Differences in the constituents of FF have been reported between women with and without endometriosis (Garrido et al., 2000; Jackson et al., 2005; Campos Petean et al., 2008; Fuji et al., 2008; Gupta et al., 2008), suggesting that the FF may influence the acquisition of oocyte competence in women with this condition.

To our knowledge, however, no study to date has evaluated the role of FF from infertile women with mild endometriosis in the genesis of meiotic oocyte anomalies. Studies on FF may provide evidence about the mechanisms involved in the etiopathogenesis of endometriosis-related infertility and may help in the development of new therapeutic approaches in the treatment of infertility. Thus, the objective of the present study was to evaluate the potential impact of different concentrations of FF from infertile women with and without mild endometriosis on the in vitro maturation (IVM) of bovine oocytes, especially on nuclear maturation and the genesis of meiotic anomalies.

Materials and Methods

Samples of FF were obtained between February 2009 and February 2011 from 22 infertile women who underwent ovarian stimulation for ICSI at the Sector of Human Reproduction, Department of Gynecology and Obstetrics, Ribeirão Preto Medical School, University of São Paulo (FM-RP-USP). The study was approved by the Research Ethics Committee and the Ethics Committee for Animal Experimentation of the University Hospital, FM-RP-USP, and all participating patients provided written informed consent.

The endometriosis group consisted of patients with infertility associated only with mild endometriosis; these patients had been diagnosed and classified by videolaparoscopy, performed by an experienced surgeon, according to the 1997 criteria of the American Society for Reproductive Medicine (1997). The control group consisted of women with infertility due to male and/or tubal factor. All control patients underwent routine diagnostic
vidiopelaroscopy to determine the cause of marital infertility, with absence of endometriosis. Exclusion criteria included age ≥38 years; body mass index (BMI) ≥30 kg/m²; serum follicle stimulating hormone (FSH) concentration ≥10 mIU/ml on the third day of the menstrual cycle; chronic anovulation; presence of hydrosalphinx or of chronic diseases such as diabetes mellitus or another endocrinopathy, cardiovascular disease, dyslipidemia, systemic lupus erythematosus or another rheumatologic disease, HIV infection or any active infection; smoking habit; and use of vitamins, hormonal medications or hormonal or non-hormonal anti-inflammatory agents during the 6 months prior to inclusion in this study.

There was no significant difference between endometriosis and control groups regarding mean age (32.72 ± 0.52 and 30.63 ± 1.36 years, respectively), body mass index (23.10 ± 1.09 and 23.16 ± 0.69, respectively), FSH concentration on the third day of the menstrual cycle (5.02 ± 0.90 and 5.79 ± 0.62, respectively), number of follicles between 14–17 mm (10.09 ± 1.43 and 6.11 ± 1.52, respectively) and number of follicles ≥18 mm (4.89 ± 0.72 and 3.11 ± 0.76, respectively) after controlled ovarian stimulation (data presented as ± SEM).

Protocol for controlled ovarian stimulation
Controlled ovarian stimulation (COH) followed the sector protocol, which consists of pituitary desensitization with gonadotrophin-releasing hormone (GnRH, Lupron®, Abbott, Sao Paulo, Brazil) using the long protocol, COH with recombinant FSH (Gonal F, Serono, Geneva, Switzerland; Puregon, Organon, Oss, The Netherlands) and administration of recombinant human choric gonadotrophin (hCG; Ovidrel®, EMD Serono, Rockland, MA, USA) to induce ovulation, followed by oocyte retrieval 34–36 h later.

Each patient received a daily subcutaneous injection of 0.5 mg leuprolide acetate (Lupron®; Abbott), starting 10 days prior to the initial ultrasound examination before ovarian stimulation. Each patient was administered 200–225 units/day of recombinant FSH (Gonal-F®; Puregon®) during ovarian stimulation, according to the observed follicular growth. Ovulation was triggered with Ovidrel® and oocyte retrieval was performed 34–36 h later.

Collection and processing of FF samples
FF was collected in individual sterile tubes pre-heated to 37°C that contained no culture medium. The entire content of the first follicle with a mean diameter ≥15 mm from the first punctured ovary was aspirated. Only FF free of blood contamination upon visual inspection and having a mature oocyte was used. The fluid samples were centrifuged at 300 g for 7 min to separate cell remnants and the supernatant was stored at −80°C in two aliquots for later use.

FF was collected from 22 infertile women, 11 with mild endometriosis and 11 with male and/or tubal factor of infertility.

Oocyte collection
Cattle ovaries were collected soon after slaughter and transported in saline at 35–38.5°C. In the laboratory, 2–8 mm follicles were aspirated and only cumulus–oocyte complexes (COCs) with a homogeneous cytoplasm and at least three layers of cumulus oophorus cells were selected (Adona and Lima Verde Leal, 2004; Ferreira et al., 2009).

In vitro maturation
COCs were cultured in 200 ml droplets of IVM medium (± 20 oocytes per droplet), at 38.5°C, 95% humidity. 5% CO2 in air (Hashimoto et al., 2002; Adona and Lima Verde Leal, 2004; Ferreira et al., 2009), in a culture system without mineral oil. IVM medium consisted of M199-containing Earle salts and bicarbonate (Invitrogen, Gibco Laboratories Life Technologies, Inc., Grand Island, NY, USA) supplemented with 0.2 mM pyruvate, 10.0 mg/ml gentamicin, 0.5 μg/ml FSH, 5.0 μg/ml LH, 1.0 μg/ml estradiol and 10% fetal calf serum (FCS; Gibco). All culture media and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), except where otherwise indicated.

Fixation and immunofluorescence staining to visualize microtubules and chromosomes
After IVM, oocytes were denuded, fixed and extracted for 30 min at 38.5°C in a microtubule-stabilizing buffer, as described (Liu et al., 1998; Ferreira et al., 2009). The oocytes were washed extensively, blocked overnight at 4°C in wash medium (PBS supplemented with 0.02% NaN3, 0.01% Triton X-100, 0.2% non-fat dry milk, 2% goat serum, 2% BSA and 0.1 M glycine) and incubated overnight at 4°C with mouse monoclonal anti-β-tubulin antibody (1:1000). The oocytes were washed, incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:500; Zymed Laboratories, Invitrogen Corporation, Carlsbad, CA, USA) at 38.5°C for 2 h, washed again and then incubated with rhodamine-phalloidin (1:1000; Molecular Probes, Invitrogen Corporation, Eugene, OR, USA). After further washing, the samples were stained for DNA with Hoechst 33342 (10 μg/ml) in Vectashield mounting medium (H-1000, Vector, Burlingame, CA, USA) on a glass slide and sealed. The samples were viewed under a high-performance confocal microscope (Confocal Leica TCS SP5, Leica Microsystems, Mannheim, Germany).

Oocyte classification based on meiotic spindle morphology and chromosome configuration
The oocytes were classified as being in metaphase I (MI), anaphase I (AI), telophase I (TI) or MIi, or as activated (PA; telophase II or with two polar bodies). For better accuracy, oocytes in MIi were subdivided as analyzable and non-analyzable. Oocytes were termed analyzable when their spindles were present in a lateral or sagittal view and non-analyzable when their spindles were present in a polar view (Ju et al., 2005), preventing an overall evaluation of the spindle and only permitting a view of chromosome arrangement. MIi oocytes were classified as normal if they presented with a single barrel-shaped meiotic spindle formed by organized microtubules crossing from one pole to another with chromosomes aligned in a compact metaphase plate at the equator of the spindle and one polar body (Fig. 1A) or abnormal if they presented with an altered spindle (reduced longitudinal dimension, dispersed or disorganized microtubules, absent or remaining spindle) and/or altered chromosome configuration (dispersed or displaced from the plane of the metaphase plate) (Fig. 1B–D). Parthenogenetic activation was characterized by the extrusion of a second polar body without any fertilization.

Experimental design
Immediately after selection, the COCs were cultured in 24-well plates for 22–24 h in standard medium, in the absence of FF (No-FF); in the presence of 1, 5, 10 or 15% FF from a control patient (C-FF); or in the presence of 1, 5, 10 or 15% FF from a patient with mild endometriosis (ME-FF). After maturation, the oocytes were denuded by repeated pipetting in Tyrode’s albumin lactate pyruvate buffered with HEPES (Talp-Hepes) medium. Eleven replicates were performed, each one with the nine groups (No-FF, 1% C-FF, 5% C-FF, 10% C-FF, 15% C-FF, 1% ME-FF, 5% ME-FF, 10% ME-FF, 15% ME-FF) using the four concentrations of FF from a control patient and from a patient with endometriosis. Each FF sample was used in only one replicate.

Statistical analysis
Data were analyzed statistically using generalized linear models (PROC GENMOD) and SAS 2003 software (2002–2003, SAS Institute, Inc., Cary, NC, USA). The Poisson distribution was utilized for the numerical variables.
(total number of fixed oocytes, and numbers of oocytes in MI, TI, AI, MII and PA) and gamma distribution for the percentages (frequency of abnormal spindle and/or chromosome alignment). Groups were compared using χ² tests, with the level of significance set at P < 0.05.

**Results**

A total of 1324 COCs were matured in vitro. Of these, 1128 were fixed and 1048 were visualized by confocal microscopy (88 oocytes were in MI, 29 in TI, 921 in MII and 10 underwent PA). Of the 921 MII oocytes, 466 were considered analyzable (i.e. fixed in a sagittal or lateral view) and 455 were considered non-analyzable (Tables I and II).

A total of 462 oocytes were evaluated in the endometriosis group, with 181 in MII being analyzable; of the latter, 80 MII were normal and 101 abnormal (Tables I and II). Among the abnormal oocytes, 49 (48.51%) presented only spindle anomalies, 2 (1.99%) presented only chromosome anomalies and 50 (49.5%) presented both spindle and chromosome anomalies. A total of 116 oocytes were evaluated in the absence of FF (No-FF group), with 51 in MII being analyzable; of the latter, 39 were normal and 12 abnormal (Table I). Among the abnormal oocytes, 3 (25%) presented only spindle anomalies, 3 (25%) presented only chromosome anomalies and 6 (50%) presented both spindle and chromosome anomalies.

The percentage of meiotically normal oocytes was significantly higher for oocytes that underwent IVM in the absence of FF (No-FF; 76.5%) and in the presence of 1% (80.9%), 5% (76.6%), 10% (75%) and 15% (76.2%) FF from infertile women without endometriosis (C-FF) than in oocytes

**Figure 1** Confocal microscopy images of bovine oocytes matured in vitro in the presence of FF from infertile women without endometriosis (C-FF) or with mild endometriosis (ME-FF). (A) Normal metaphase II oocyte from the C-FF group showing chromosomes properly aligned in the median portion of a normal barrel-shaped spindle. (B) Abnormal MII oocyte from the ME-FF group with misaligned chromosomes over a normal barrel-shaped spindle. (C and D) Abnormal MII oocyte from the ME-FF group with misaligned chromosomes over an abnormal spindle. Green, spindles stained with anti-β-tubulin and fluorescein isothiocyanate-conjugated secondary antibody; blue, chromosomes stained with Hoechst 33342; red, actin filaments stained with rhodamine-phalloidin. Scale bar = 10 μm.
Table I  Stages of nuclear maturation and percentages of normal and abnormal MII oocytes matured in medium without FF (No-FF), with different concentrations of FF from infertile women without endometriosis (C-FF) or with mild endometriosis (ME-FF).

<table>
<thead>
<tr>
<th>Group</th>
<th>FF concentration (%)</th>
<th>Total number of fixed oocytes (n)</th>
<th>Total number of viewed oocytes (n)</th>
<th>MI, n (%)</th>
<th>TI, n (%)</th>
<th>PA*, n (%)</th>
<th>MI</th>
<th>Total no. of MII, n (%)</th>
<th>Analyzable, n (%)</th>
<th>Normal, n (%)</th>
<th>Abnormal, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-FF</td>
<td>0</td>
<td>119</td>
<td>116</td>
<td>9 (7.66)</td>
<td>6 (5.17)</td>
<td>1 (0.86)</td>
<td>100 (86.21)</td>
<td>51 (51)</td>
<td>39 (76.47)</td>
<td>12 (23.53)</td>
<td></td>
</tr>
<tr>
<td>C-FF</td>
<td>1</td>
<td>129</td>
<td>119</td>
<td>6 (5.04)</td>
<td>4 (3.36)</td>
<td>2 (1.66)</td>
<td>107 (89.92)</td>
<td>47 (43.93)</td>
<td>38 (80.85)</td>
<td>9 (19.15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>133</td>
<td>120</td>
<td>4 (3.34)</td>
<td>1 (0.84)</td>
<td>2 (1.66)</td>
<td>113 (94.16)</td>
<td>64 (56.64)</td>
<td>49 (76.56)</td>
<td>15 (23.44)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>126</td>
<td>119</td>
<td>13 (10.92)</td>
<td>5 (4.20)</td>
<td>0</td>
<td>101 (84.88)</td>
<td>60 (59.40)</td>
<td>45 (75)</td>
<td>15 (25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>124</td>
<td>112</td>
<td>6 (5.36)</td>
<td>4 (3.57)</td>
<td>1 (0.89)</td>
<td>101 (90.18)</td>
<td>63 (62.38)</td>
<td>48 (76.19)</td>
<td>15 (23.81)</td>
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</tr>
<tr>
<td>ME-FF</td>
<td>1</td>
<td>125</td>
<td>116</td>
<td>12 (10.35)</td>
<td>2 (1.72)</td>
<td>1 (0.86)</td>
<td>101 (87.07)</td>
<td>45 (44.55)</td>
<td>20 (44.44)</td>
<td>25 (55.56)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>124</td>
<td>115</td>
<td>16 (13.91)</td>
<td>1 (0.87)</td>
<td>2 (1.74)</td>
<td>96 (83.48)</td>
<td>49 (51.04)</td>
<td>18 (36.74)</td>
<td>31 (63.26)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>125</td>
<td>118</td>
<td>13 (11.01)</td>
<td>2 (1.70)</td>
<td>0</td>
<td>103 (87.29)</td>
<td>44 (42.72)</td>
<td>20 (45.46)</td>
<td>24 (54.54)</td>
<td></td>
</tr>
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<td></td>
<td>15</td>
<td>123</td>
<td>113</td>
<td>9 (7.97)</td>
<td>4 (3.54)</td>
<td>1 (0.88)</td>
<td>99 (87.61)</td>
<td>43 (43.43)</td>
<td>22 (51.16)</td>
<td>21 (48.84)</td>
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</tbody>
</table>

No-FF, oocytes that underwent IVM in medium containing no added follicular fluid; C-FF, oocytes that underwent IVM in medium supplemented with follicular fluid from infertile women with tubal and/or male factor; ME-FF, oocytes that underwent IVM in medium supplemented with follicular fluid from infertile women with mild endometriosis; Analyzable, oocytes with the spindle fixed in a sagittal or lateral view; MI, metaphase I; TI, telophase I; MII, metaphase II; PA, spontaneous parthenogenetic activation (extrusion of the second polar body in the absence of fertilization). Data are the results of 11 replicates. Different letters in the same column indicate a significant difference, P < 0.01, using Poisson and gamma distribution and the chi-square test.*The analysis did not converge.

Table II  Stages of nuclear maturation and percentages of normal and abnormal MII oocytes matured in medium with follicular fluid from infertile women without endometriosis (C-FF) or with mild endometriosis (ME-FF).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total number of fixed oocytes (n)</th>
<th>Total number of viewed oocytes (n)</th>
<th>MI, n (%)</th>
<th>TI, n (%)</th>
<th>PA, n (%)</th>
<th>MI</th>
<th>Total no. of MII, n (%)</th>
<th>Analyzable, n (%)</th>
<th>Normal, n (%)</th>
<th>Abnormal, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-FF</td>
<td>512</td>
<td>470</td>
<td>29 (6.2)</td>
<td>14 (3.0)</td>
<td>5 (1.1)</td>
<td>422 (89.8)</td>
<td>234 (55.5)</td>
<td>180 (76.9)</td>
<td>54 (23.1)</td>
<td></td>
</tr>
<tr>
<td>ME-FF</td>
<td>497</td>
<td>462</td>
<td>50 (10.8)</td>
<td>9 (2.0)</td>
<td>4 (0.9)</td>
<td>399 (86.4)</td>
<td>181 (45.4)</td>
<td>80 (44.2)</td>
<td>101 (55.8)</td>
<td></td>
</tr>
</tbody>
</table>

C-FF, oocytes that underwent IVM in medium supplemented with follicular fluid from infertile women with tubal and/or male factor; ME-FF, oocytes that underwent IVM in medium supplemented with follicular fluid from infertile women with mild endometriosis; Analyzable, oocytes with the spindle fixed in a sagittal or lateral view; MI, metaphase I; TI, telophase I; MII, metaphase II; PA, spontaneous parthenogenetic activation. Data are the results of 11 IVMs. Different letters in the same column indicate a significant difference (P < 0.01) using gamma distribution and the chi-square test.
Discussion

Due to inconsistent results on the association between minimal and mild endometriosis and infertility (Holoch and Lessey, 2010; Matorras et al., 2010), and to evaluate the mechanisms of infertility related to mild endometriosis, we evaluated the effect of FF from infertile women with mild endometriosis on nuclear maturation and the genesis of meiotic oocyte anomalies during IVM of bovine oocytes. We chose to use bovine oocytes because of the similar size and morphology of human and bovine oocytes, because both species are mono-ovulatory and poly-cyclic and because bovine oocytes are abundant, of low-cost and easily manipulated (Malhi et al., 2005).

Surprisingly, within the C-FF and ME-FF groups there were no significant differences among the effects of the four FF concentrations (1, 5, 10 and 15%) analyzed on the number of oocytes that reached MII or on the percentages of abnormal analyzable MII oocytes. Although the effect of FF was not dose dependent with the tested concentrations in the two groups evaluated, it is not possible to rule out the existence of a dose-dependent effect of FF on the occurrence of meiotic oocyte damage since lower concentrations (less than 1%) were not tested due to the lack of sufficient extra FF for new experiments. Thus, we cannot state that 1% was the lowest concentration of ME-FF capable to affect nuclear maturation and promote meiotic anomalies in MII oocytes matured in vitro or that there was a non-specific effect of FF, which needs to be further evaluated. Nonetheless, it is important to point out that the real concentration of FF constituents coming into contact with the oocyte in vivo during oocyte maturation is not known and we hypothesized that it is possibly higher than 1%. However, we wonder if the follicular dynamic is similar in vivo and in vitro, considering that the amount of cells surrounding the oocyte is increased in the ovary, forming a three-dimensional structure, with tight junctions which could have a role in limiting the access of FF to the oocyte, whereas some of these cells are lost by the aspiration procedure and a two-dimensional system is established in the in vitro culture. Further studies are necessary to elucidate what factor is related to the anomalies found in this study and whether oxidative stress is involved in this mechanism, since the oocyte spindle is extremely sensitive to oxidative stress (Hu et al., 2001; Eichenlaub-Ritter et al., 2002; Mullen et al., 2004). In any case, since there were no differences associated with FF concentrations, we pooled the data for each of these groups (i.e. the C-FF and ME-FF groups) and compared them with each other as following discussed.

First, we found that the number of oocytes in MII was similar when they underwent IVM in the absence of FF (No-FF) and in the presence of control FF (C-FF), indicating that the FF of infertile women without endometriosis does not impair the nuclear maturation of these bovine oocytes. In contrast, we found that the number of oocytes in MII was significantly greater when they underwent IVM in the presence of ME-FF than in the presence of C-FF, suggesting that the FF of infertile women with mild endometriosis may contain constituents that can delay or impair the conclusion of oocyte meiosis I. This finding corroborates our previous results indicating the proportion of apparently mature oocytes (with extrusion of the first polar body) in telephase I in vitro matured oocytes obtained from cycles stimulated with gonadotrophins of patients with endometriosis tended to be greater than the observed in the control group constituted by infertile patients without endometriosis (Barcelos et al., 2009). These findings, taken together with the results presented here, led us to hypothesize that the follicular microenvironment of infertile women with mild endometriosis may contain constituents that can compromise nuclear oocyte maturation, and that this may be one of the mechanisms related to infertility in this group of women.

Among the oocytes that completed nuclear maturation and reached MII stage with an analyzable spindle, the percentage with meiotic abnormalities was higher when oocytes underwent IVM in the presence of FF from patients with mild endometriosis than without this disease or in the absence of FF, suggesting that the FF of infertile women with mild endometriosis may promote meiotic oocyte anomalies, thus impairing oocyte quality. In the present study we have the results of the assisted reproduction techniques to which the patients who donated the FF were submitted. According to this analysis, no significant differences were detected between the group with stage II endometriosis and the control group regarding the number of good-quality embryos on the second and third day of development or the rates of fertilization, embryo cleavage, implantation, pregnancy and live births. However, it is important to emphasize that we do not know whether or not the embryos transferred originated from follicles whose FFs were used in our experiments.

Markers of oxidative stress have been observed in the FF of infertile women with endometriosis who underwent ovarian stimulation for assisted reproduction procedures (Prieto et al., 2012). As previously mentioned oxidative stress may promote oocyte meiotic anomalies and chromosome instability, and is associated with increased apoptosis and impairments of preimplantation embryo development (Liu et al., 2003; Navarro et al., 2004, 2006). These findings, taken together with the results presented here, led us to hypothesize that oxidative stress in the follicular microenvironment may impair nuclear maturation and may promote the genesis of meiotic oocyte anomalies in infertile women with endometriosis, a hypothesis that should be evaluated in future studies.

Although oocyte quality may be compromised in women with endometriosis, few studies have evaluated the mechanisms underlying the worsening of oocyte quality in women with this disease. Type of infertility was found to have a significant effect on meiotic oocyte errors, which are more frequent in the presence of an ovulatory factor and of endometriosis, in agreement with the findings presented here (Gianaroli et al., 2010). The PF of women with endometriosis has
been shown to promote anomalies of the microtubules and chromosomes in mature mouse oocytes (Mansour et al., 2009, 2010). These anomalies were prevented by the antioxidant l-carnitine, suggesting that oxidative stress may mediate FF-induced meiotic oocyte damage. Since oocytes remain in the follicular microenvironment during the process of maturation, in intimate contact with FF, and maintain contact with the PF after ovulation and during the initial passage through the uterine tube (Ma et al., 2010), we hypothesized that the effects of these two microenvironments may be additive, promoting a deleterious synergistic effect on oocyte maturation and on the genesis of meiotic anomalies. Thus, our findings complement earlier results (Mansour et al., 2009, 2010), providing additional evidence for the deleterious effects on oocyte quality of this reproductive microenvironment of infertile women with endometriosis.

The cell spindle is essential to guarantee faithful chromosome segregation during meiosis (De Santis et al., 2005). Moreover, meiotic anomalies may contribute to the inability of oocytes to complete maturation, causing them to become unable to be fertilized. In view of the occurrence of errors that do not prevent fertilization but impair embryo development pre- or post-implantation or the future viability of the conceptus, the meiotic oocyte abnormalities promoted by the FF of infertile women with endometriosis may explain, at least in part, both the reduced fertility and the poorer results of assisted reproduction in patients with the disease (Barnhart et al., 2002; Al-Fadhlí et al., 2006).

In this respect, it is important to emphasize that the eligibility criteria used for the present subjects intended to eliminate biases related to other potential effects associated with worsening of oocyte quality and/or results of ICSI. Thus, we reiterate that we used quite strict inclusion criteria, so that the patients with mild endometriosis included in the study, in addition to being young, non-obese, with no markers of altered ovarian reserve and with a satisfactory response to controlled ovarian stimulation, only had the presence of mild endometriosis as the single factor identified related to infertility. On this basis, we hypothesized that worsening of oocyte quality may be one of the mechanisms related to infertility, a possibility that will require further investigation.

The association between the initial stages of endometriosis and infertility has not been clearly defined. To our knowledge, only two randomized, controlled clinical studies have evaluated the relationship between surgical removal of foci of minimal and mild endometriosis and later improvement in the natural fecundity of these patients (Marcoux et al., 1997; Parazzini, 1999). A meta-analysis evaluating the data of these two studies concluded that laparoscopic surgery can improve the fertility of patients with minimal and mild endometriosis (Jacobson et al., 2010). Even after endometriotic foci are surgically removed and cauterized, however, the fecundity of women with early stage endometriosis continued to be lower than that of women without the disease during the months following surgery. It remains unclear, therefore, whether the maintenance of unfavorable conditions in the FF of infertile women with mild endometriosis might compromise the quality of oocytes, resulting in a negative impact on the natural fecundity of these women even after the surgical exeresis and cauterization of laparoscopically detected lesions, a possibility that should be evaluated in future studies.

In summary, we have shown here that the FF of infertile women with mild endometriosis can impair nuclear maturation and promote meiotic anomalies in bovine oocytes matured in vitro. These findings provide evidence about the mechanisms associated with infertility in women with early stage endometriosis. Future studies comparing the constituents of FF in these women and in infertile women without endometriosis, and evaluating the impact of these components on nuclear maturation and on the genesis of meiotic oocyte anomalies, may provide further evidence about the mechanisms underlying oocyte damage in women with mild endometriosis. Moreover, these studies may help in the design of new therapeutic approaches to improve the natural fertility of these infertile women.

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Authors’ roles

M.G.D.B. was responsible for the study design, selection of the patients, acquisition of data, methodology, interpretation of data and manuscript writing. H.M. gave substantial contributions to acquisition of data. C.C.P.P. contributed to the statistical analysis and interpretation of data. R.A.F. contributed to revising critically the manuscript for important intellectual content. P.A.N. contributed to conception and design, interpretation of data, revising critically the manuscript, final approval of the version to be published and was the coordinator of the project. All authors have approved the final version and the submission of the manuscript.

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Conflict of interest

The authors have no conflicts of interest to declare.

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