

# Endometriosis-induced alterations in mouse metaphase II oocyte microtubules and chromosomal alignment: a possible cause of infertility

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**Objective:** To examine the effect of peritoneal fluid (PF) of patients with endometriosis on the cytoskeleton of metaphase II oocytes and correlate the results with the stage of endometriosis and the duration of infertility.

**Design:** Prospective-controlled study.

**Setting:** Center for reproductive medicine at a tertiary-care hospital.

**Patient(s):** Women with endometriosis (n=23) and tubal ligation/reversal (n=15).

**Intervention(s):** Peritoneal fluid obtained from 38 women (23 with endometriosis and 15 tubal ligation/reversal) after laparoscopy. Four hundred metaphase II oocytes were used: 165 frozen metaphase II oocytes were incubated in the PF of patients with endometriosis, 135 oocytes incubated in the PF of nonendometriosis patients (control subjects) and 100 oocytes incubated in human tubal fluid (HTF) media.

**Main Outcome Measure(s):** Spindle abnormalities (microtubule and chromosomal) were evaluated by confocal imaging.

**Result(s):** In the endometriosis group, the cytoskeleton had a higher frequency of abnormal meiotic spindle and chromosomal misalignment (score  $\geq 3$ ), indicating severe damage compared with the control groups. The proportions of abnormalities in microtubule and chromosome alterations in endometriosis (67.9% and 63.6%, respectively) were significantly higher than for oocytes incubated with PF of the nonendometriosis group (24.4% and 14.8%) as well as the HTF group (13% and 13%). Oocyte cytoskeleton damage positively correlated with the duration of infertility and the stage of endometriosis.

**Conclusion(s):** Alteration of oocyte cytoskeleton might be one of the causes of poor oocyte quality in patients with endometriosis. (Fertil Steril® 2009; ■: ■–■. ©2009 by American Society for Reproductive Medicine.)

**Key Words:** Endometriosis, infertility, peritoneal fluid, oocyte quality, microtubules, chromosomes

Endometriosis affects up to 10% of women of reproductive age and 35%–50% in women with pelvic pain, infertility, or both (1–5). It is associated with decreased pregnancy rate after IVF treatment (5, 6) and increased recurrent pregnancy loss (7). Oocyte quality may be one of the factors causing infertility in endometriosis patients (8–10). Endometriosis is a local pelvic inflammatory process (10–15) as well as a disease of oxidative stress (16, 17).

Exposure of the oocytes to the peritoneal fluid (PF) from women with endometriosis may affect oocyte quality. The meiotic spindle plays a critical role in maintaining chromosomal organization and formation of the second polar body (18–20). Disorganization of the meiotic spindle can result in chromosomal dispersion, failure of normal fertilization, and abnormal development (21). We recently investigated the effect of exogenous exposure to hydrogen peroxide ( $H_2O_2$ ) and tumor necrosis factor (TNF)  $\alpha$  on mouse metaphase II (MII) oocyte spindle structure and demonstrated that oxidative stress

results in concentration and time-dependent alterations in the spindle structure and that these effects are augmented by TNF- $\alpha$  (20).

The specific aims of the present study were: 1) to evaluate the effect of the peritoneal fluid (PF) of patients with endometriosis and tubal ligation patients (control) on the microtubules and chromosomal alterations in MII mouse oocytes; and 2) to correlate the damage of the oocyte spindle to the stage of the disease and the duration of infertility.

## MATERIALS AND METHODS

After approval of the study by the Institutional Review Board of the Cleveland Clinic, we conducted our study.

### Preparation of mouse oocytes

Cryopreserved mouse oocytes (Embryotech Laboratories, Wilmington, MA) were released into a Petri dish containing 500  $\mu$ L Dulbecco phosphate-buffered saline (PBS) (Irvine Scientific, Santa Ana, CA) to equilibrate for 10 minutes at room temperature. Metaphase II oocytes were carefully identified and randomized into control and experimental groups. They were all subjected to similar thawing conditions so that the effects of repolymerization would be identical in the control and treated groups. To ensure that polymerization effects were similar before addition of PF and that the subsequent effects seen were exclusively due to the factors in PF (20), the spindles were allowed to be completely repolymerized by incubating in 500  $\mu$ L human tubal fluid (HTF; Irvine Scientific) for 1 hour in 5%  $CO_2$  at 37°C and subsequently incubated in PF or HTF (control). Oocytes were randomly divided into two groups and evaluated for the presence or absence of the polar body.

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## Subjects and chart reviews

Forty-four female patients underwent laparoscopy, and indications for laparoscopy included chronic pelvic pain, infertility, tubal ligation, or tubal reversal. Of the 44 PF samples collected from the posterior cul-de-sac, only 38 (endometriosis:  $n = 23$ ; control:  $n = 15$  [tubal ligation:  $n = 12$ ; tubal reversal:  $n = 3$ ]) were included in the study (six were blood contaminated and excluded). Charts were reviewed from 23 patients with endometriosis to determine the stage of endometriosis and duration of infertility (22). Cell-free PF was stored at  $-70^{\circ}\text{C}$  until analysis.

## Incubation of oocytes with the peritoneal fluid

Different concentrations of PF (0%, 25%, 50%, and 100%) were examined to study the deleterious effect on the oocyte. Incubation with undiluted PF (100% PF) resulted in complete loss of the cytoskeleton structure. We therefore selected PF at 50% concentration (PF:HTF vol./vol. 1:1) to evaluate spindle damage. Four hundred mature MII oocytes were separated into three groups: 165 oocytes with PF from women with endometriosis; 135 oocytes with PF from nonendometriosis patients (control); and 100 with HTF (control). Oocytes were incubated with PF for 1 hour.

## Microtubules and chromosomes staining

Microtubules were detected by modified indirect immunocytochemical techniques (19, 20, 23). For microtubule staining, oocytes were fixed in fixation solution (2% formaldehyde and 0.2% Triton X-100 in 500 mL PBS) for 30 minutes and incubated in anti- $\alpha$ -tubulin monoclonal antibody (1:300; Sigma-Aldrich, St. Louis, MO) for 60 minutes, followed by incubation in fluorescein isothiocyanate (FITC)-labeled antimouse antibody (1:50; Sigma-Aldrich) for 30 minutes. For chromosome staining, oocytes were incubated in propidium iodide (PI, 10  $\mu\text{g}/\text{mL}$ ; Sigma-Aldrich) for 15 minutes.

Five oocytes were loaded on a slide with a microdrop (2  $\mu\text{L}$ ) of Antifade (Slow Fade Light Antifade; Molecular Probes, Eugene, OR) and covered with a cover slip. Alterations in the microtubule structure were observed both by epifluorescence microscopy and by confocal microscopy.

## Confocal microscopic analysis and scoring








Microtubule distribution and chromosome alignment for each oocyte were examined using a Leica TCSSP2 laser-scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with an argon ion laser for the excitation of FITC for microtubules and PI for chromosomes (excitation 488 nm and barrier 500–555 nm for FITC, excitation 568 nm and barrier 575–675 nm for PI). Scoring of microtubules and chromosomes was done according to the morphologic evaluation described by us previously (20). In brief, spindle morphology was classified as normal (scores 1 and 2) when a barrel-shaped structure with slightly pointed poles formed by organized microtubules traversing from one pole to another was observed and when chromosomes were arranged in a compact metaphase plate at the equator of the spindle (Figs. 1 and 2A–2G). Spindle structure was abnormal (scores 3 and 4) when there was a reduction in the longitudinal dimension of the spindle or when there was partial or total disorganization and complete absence or remnant of dispersing spindle and when chromosomes were displaced from the plane of the metaphase plate or were dispersed or with condensed appearance (Figs. 2H–2O).

## Statistical analysis

Differences between experimental and control samples were assessed using the Wilcoxon matched-pairs test and Kruskal-Wallis test. Correlation between variables was assessed using nonparametric Spearman  $r$ . Sample size was sufficient to detect significant difference between groups. Summary statistics are presented as median and interquartiles (25th and 75th percentiles). All hypothesis testing was two tailed, with a significance level of .05 and highly significant at  $P < .01$ . Microtubule and chromosome changes were scored as 1, 2, 3, or 4. Disease stage was also evaluated on a scale of 1 to 4. Duration of infertility was recorded in years and categorized as 0–2, 3–4, or 5+ years. A sample size of 15 patients in each group was determined to have 90% power to detect a probability of 15% that an observation

## FIGURE 1

Score sheet for microtubule and chromosome according to morphologic evaluation under the confocal microscope.

Score	Microtubule	Chromosome
1		
2		
3		
4	Missing	

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in one group is less than an observation in another group using Wilcoxon rank sum test with a .05 two-sided significance level. Statistical analyses were performed using R version 2.3.1 (<http://www.r-project.org>).

## RESULTS

No significant differences were seen in age, parity, and body mass index among the study groups.

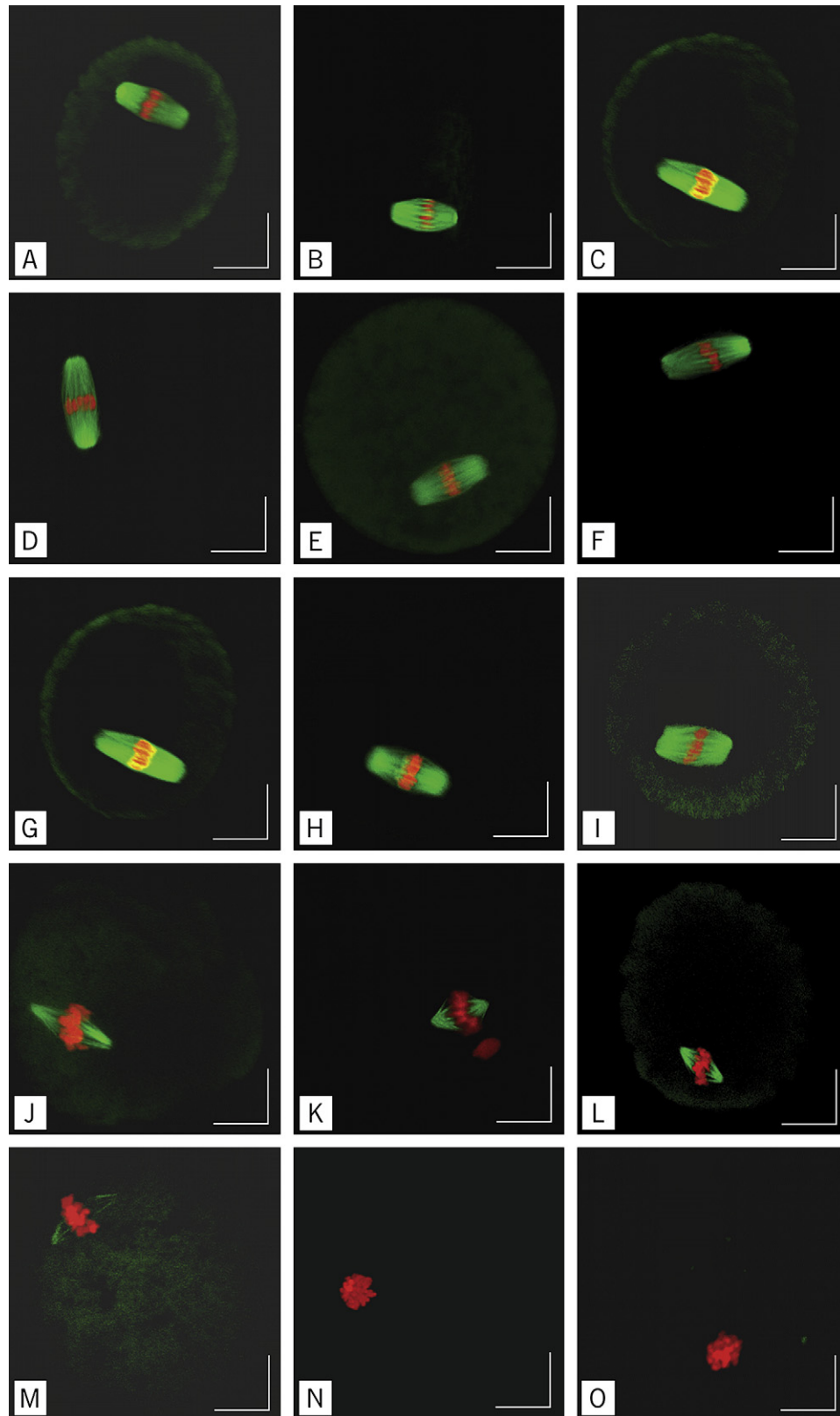
### Effect of endometriosis on the microtubules of the oocytes

Microtubules of the oocytes incubated in the PF of endometriosis patients showed significantly higher scores ( $\geq 3$ ;  $P < .001$  compared with those incubated in the PF of the tubal ligation (control) group or in HTF. There was no statistically significant difference in microtubule scores between the nonendometriosis and HTF groups ( $P = 0.37$ ; Table 1). Microtubules of the oocytes exposed to PF of endometriosis were damaged to varying degrees compared with the control groups. Normal spindle morphology (scores 1 and 2) was most seen in the control groups (75.6% of nonendometriosis group and 87% of HTF group) but in 32.1% of the endometriosis group (Table 1). Different configurations of the microtubules seen in the endometriosis group were compared with other groups (Fig. 2). Normal spindle had a barrel-shaped structure with slightly pointed poles formed by organized microtubules that traverse from one pole to another (Figs. 2A–2F). Abnormal spindle (scores 3 and 4) was most seen in the endometriosis group (67.9%) compared with 24.4% in the nonendometriosis group and 13% in the HTF group (Table 1). Abnormal spindle had a reduction in the longitudinal dimension of the spindle, or there was partial or total disorganization and complete absence or remnant of dispersing spindle (Figs. 2G–2O).

### Effect of endometriosis on the chromosomes of the oocytes

The chromosomes of the oocytes incubated in the PF of endometriosis patients showed significantly higher scores ( $\geq 3$ ;  $P < .001$ )

FIGURE 2



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## FIGURE 2 Continued

Confocal microscopy images showing structures of microtubule (green) and chromosome (red) in MII mouse oocytes stage. (A–C) Normal oocytes incubated in human tubal fluid (HTF) media (score 1 and 2). (D–F) Oocytes incubated in peritoneal fluid (PF) of tubal ligation (control). Normal-looking oocytes in either HTF media or normal PF showing normal spindle configuration: barrel-shaped structure with slightly pointed poles formed by organized microtubules that traverse from one pole to another with chromosomes arranged on a compact plate at the equator of the structure. (G–O) Oocytes incubated in PF of endometriosis patients. (G–J) Mild endometriosis, showing a reduction in the longitudinal dimension of the spindle and partial or total disorganization with more chromosomes displaced from the plane of the metaphase plate. (K–O) Severe endometriosis: complete absence or remnant of dispersing spindle as well as abnormal chromosomal organizations associated with condensed appearance and chromosome scattering. Scale bar = 18  $\mu$ m.

compared with the oocytes incubated in the PF of the tubal ligation (control) group in HTF. There was no statistically significant difference in chromosomal scores between the nonendometriosis and HTF groups ( $P=0.76$ ; Table 1). Chromosomes of the oocytes exposed to endometriosis PF showed higher damage (higher scores) compared with the control groups. Only 36.3% of the endometriosis group had a normal chromosomal configuration (scores 1 and 2), versus 80.7% in the nonendometriosis group and 87% in the HTF group (Table 1). Normal chromosomes (scores 1 and 2) were arranged in a compact metaphase plate at the equator of the spindle (Figs. 2A–2G). Chromosomal organization was considered to be abnormal (scores 3 and 4; Figs. 2H–2O) when chromosomes were displaced from the plane of the metaphase plate (Figs. 2H–2M) or when the chromosomes were dispersed or with condensed appearance (Figs. 2N–2O). Abnormal chromosomes (score 3 and 4) were most seen in endometriosis group (63.6%) compared with 19.2% in the nonendometriosis group and 13% in the HTF group.

### Stage of endometriosis and the extent of cytoskeleton damage

Endometriosis patients were categorized into different stages as follows: four in stage I; seven in stage II; five in stage III; and seven in stage IV. To simplify statistical analysis and results, stages I and II

were combined as mild endometriosis (47.8%) and stages III and IV as severe endometriosis (52.1%). Higher microtubule scores ( $\geq 3$ ) were associated with advanced stages (stage II and IV) of the disease ( $P<.001$ ; Table 2). The mean damage of the microtubules of the oocytes incubated in PF of stage I endometriosis was only  $1.88 \pm 0.82$  compared with  $3.18 \pm 0.72$  for stage IV ( $P<.001$ ; Table 2).

High chromosome scores were associated with higher stage of endometriosis ( $P=0.029$ ; Table 2). The mean damage of the chromosomes incubated in PF of stage I endometriosis was  $2.03 \pm 0.78$  compared with  $2.90 \pm 0.61$  for those incubated with PF of severe endometriosis (Table 2). Most of the microtubules and chromosomes had spindle scores of 1 and 2, whereas in stages III and IV most of the microtubules and chromosomes had scores of 3 and 4, indicating severe damage with advanced stages of the disease.

### Duration of infertility and the extent of microtubules and chromosomal damage

Of the 23 patients with endometriosis, duration of infertility was as follows: 0–2 years:  $n = 6$ ; 3–4 years:  $n = 8$ ; >5 years:  $n = 9$ . The mean damage of the microtubules and chromosomes was statistically higher in oocytes incubated in PF of women with endometriosis who were infertile for >5 years ( $3.02 \pm 0.69$ ) compared with

TABLE 1

Microtubule and chromosome scoring of the oocytes incubated in endometriosis peritoneal fluid (PF), oocytes incubated in tubal ligation (normal) PF, and oocytes incubated in plain human tubal fluid (HTF) media (control group).

Spindle score	Overall oocytes (n = 400)	Endometriosis oocytes (n = 165)	Control PF oocytes (n = 135)		Overall P value
			Tubal ligation/reversal	HTF	
Microtubule score					
1	70 (17.5%)	13 (7.9%)	34 (25.2%)	23 (23%)	< .001
2	172 (43%)	40 (24.2%)	68 (50.4%)	64 (64%)	
3	109 (27.2%)	77 (46.7%)	23 (17.0%)	9 (9%)	
4	49 (12.2%)	35 (21.2%)	10 (7.4%)	4 (4%)	
Mean $\pm$ SD	2.32 $\pm$ 0.90	2.81 $\pm$ 0.86	2.07 $\pm$ 0.85	1.95 $\pm$ 0.72	
Chromosome score					
1	89 (22.2%)	6 (3.6%)	47 (34.8%)	36 (36%)	< .001
2	167 (41.7%)	54 (32.7%)	62 (45.9%)	51 (51%)	
3	116 (29%)	85 (51.5%)	20 (14.8%)	11 (11%)	
4	28 (7%)	20 (12.1%)	6 (4.4%)	2 (2%)	
Mean $\pm$ SD	2.19 $\pm$ 0.86	2.72 $\pm$ 0.72	1.89 $\pm$ 0.82	1.84 $\pm$ 0.75	

Note: A P value of < .001 was considered to be significant in endometriosis vs. control and endometriosis vs. HTF. There was no significant difference between HTF and control groups (microtubules:  $P = .37$ ; chromosomes:  $P = .76$ ).

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TABLE 2

Correlation between the microtubule and chromosome scores and stage of endometriosis and duration of infertility (DOI) in oocytes incubated in the peritoneal fluid (PF) of women with endometriosis.

Spindle score	Stage of endometriosis					DOI (yrs)			
	I (n = 4)	II (n = 7)	III (n = 5)	IV (n = 7)	P value	0–2 (n = 6)	3–4 (n = 8)	> 5 (n = 9)	P value
Microtubule score	1.88 ± 0.82	2.64 ± 0.90	3.02 ± 0.57	3.18 ± 0.72	<.001	2.00 ± 0.86	2.94 ± 0.75	3.02 ± 0.69	<.001
Chromosome score	2.73 ± 0.78	2.44 ± 0.91	2.70 ± 0.59	2.91 ± 0.61	.029	2.56 ± 0.81	2.65 ± 0.74	2.82 ± 0.61	<.001

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oocytes that were incubated in PF of endometriosis patients who were infertile for 0–2 years ( $2.00 \pm 0.86$ ;  $P < .001$ ; Table 2). There was also an increase in the mean damage of microtubules and chromosomes with the increase in duration of infertility.

Higher microtubule and chromosome scores tended to be associated with longer duration of infertility ( $P < 0.001$ ). Microtubules and chromosomes with scores of 1 and 2 were higher in oocytes incubated in PF of patients who had infertility of 0–2 years; whereas microtubules and chromosome score of 3 and 4 were seen largely in patients who were infertile for  $\geq 3$  years and progressively increased in patients who were infertile for  $> 5$  years.

## DISCUSSION

The present results indicate that quality of oocytes from women with endometriosis is poor compared with nonendometriosis patients. The PF environment is host to the process of ovulation, oocyte transportation, fertilization, and early embryonic development. Peritoneal fluid in endometriosis is an inflammatory media, and there is increased inflammation both in the pelvic cavity and in the peripheral blood, because it is rich in cytokines, cells, and many other constituents that have a negative effect on embryo quality and infertility (11, 14, 24–29).

There is also marked evidence in the literature that endometriosis is a disease of oxidative stress (16, 26, 30). In addition, some studies have reported the presence of reactive oxygen species (ROS) in the PF of patients with endometriosis (26, 30). Reactive oxygen species have detrimental effects on oocytes (31, 32). Oocytes in vivo are severely affected by the surrounding peritoneal environment. The meiotic spindle is fundamental to ensuring correct chromosome segregation at MI and MII. For the past decade, the meiotic spindle has been extensively investigated as a possible predictive feature for oocyte quality (23). Our group recently demonstrated that oxidative stress results in concentration- and time-dependent alterations in the spindle structure (20).

Although earlier studies showed the effect of the PF of endometriosis on the reproductive outcome in an in vivo model (33), there are no reports evaluating the effect of endometriosis on the microtubules and chromosomal alignment in MII oocytes. Therefore, we evaluated the effects of the PF obtained from women with endometriosis with all its constituents on the oocyte spindle and chromosomal alignment using mature MII mouse oocytes as our model.

In this study, we used cryopreserved oocytes as in our earlier study (20). No significant differences in the cytoskeleton or the chromosomal alignment of the oocytes have been reported as a result of cryopreservation (34, 35). When using thawed oocytes, recovery of spindles was shown to depend on the repolymerization of the microtubules and incubation time ranging from 1–3 hours at 37°C (20, 35–37). Complete repolymerization of spindles is seen in mouse oocytes

that underwent slow freezing and were incubated for 1 hour after thaw (20–21).

We found that oocytes incubated in PF from women with endometriosis underwent significantly more severe damage to the microtubules and chromosomes compared with oocytes incubated in normal PF (from tubal ligation/tubal reversal patients) and HTF ( $P=0.37$ ). There was increased concentration of PF cytokines TNF- $\alpha$  and interleukin (IL) 8 in patients with endometriosis compared with patients without the disease (27, 38, 39). Similarly, increased concentration of oxidative stress markers and antibodies have been reported in PF from women with endometriosis compared with those without the disease (17, 40, 41).

One of the study limitations was that we did not measure the actual levels of ROS in the peritoneal fluid. We therefore do not know which of the constituents of the PF in particular had an effect on the oocyte cytoskeleton. Earlier studies show that PF of endometriosis is rich in a variety of cytokines and oxidative stress markers. In addition, reports show alterations in cytoskeleton after exposure of the oocytes to H<sub>2</sub>O<sub>2</sub> as well as TNF- $\alpha$ , glutathione oxidized with diamide, and tertiary butyl hydroperoxide (20, 32, 42, 43). However, the present results showed that chromosomes in MII oocytes were more resistant to damage than the microtubules. In each stage of endometriosis, we observed that the percentage of microtubular damage was higher compared with chromosomal damage, although this was not statistically significant. Alterations to the microtubules and chromosomes induced by endometriosis showed statistically significant positive correlation with the duration of infertility and a weak correlation with the stage of the disease. These findings are in agreement with some earlier studies that have shown that IL-8, TNF- $\alpha$ , and IL-17 have been related to the stage, severity, and activity of the disease (39, 44–47). Weak correlation seen between the degree of cytoskeleton damage and the stage of endometriosis warrant further studies with a large sample size to verify the effect of the stage of endometriosis on the microtubules and the chromosomes.

We used confocal imaging to evaluate the meiotic spindle and the chromosomes, unfortunately this technique requires a fixation step that causes loss of oocyte viability. Alternately, spindle can also be visualized with Polscope which offers the unique advantage of being totally noninvasive, preserving oocyte viability, and allowing repeated observations (48); however it is not commonly available nor cost-effective. Despite these limitations, to our knowledge this is the first study to address the effect of the PF of endometriosis on the oocyte spindle and chromosomes.

In conclusion, we analyzed the effect of endometriosis on the quality of the oocytes in terms of the microtubules and the chromosomes. Alterations of the spindle may be one of the many causes related to infertility and/or recurrent pregnancy loss in patients with endometriosis. There is a need for further studies to examine the effects of each constituent in the PF, to measure it and investigate its

effect on oocyte quality, to obtain new insight into this disease and help develop novel diagnostic and therapeutic remedies. The inflammatory factors as well as the presence of ROS may offer new therapeutic options, because selective inhibition of TNF- $\alpha$ , anticytokines, or antioxidants may be beneficial in preventing the

development of endometriosis and/or helping to preserve oocyte quality.

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