Effect of induced peritoneal endometriosis on oocyte and embryo quality in a mouse model

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Abstract

Purpose To assess the impact of peritoneal endometriosis on oocyte and embryo quality in a mouse model.

Methods Peritoneal endometriosis was surgically induced in 33 B6CBA/F1 female mice (endometriosis group, $N=17$) and sham-operated were used as control (sham group, $N=16$). Mice were superovulated 4 weeks after surgery and mated or not, to collect E0.5-embryos or MII-oocytes. Evaluation of oocyte and zygote quality was done by immunofluorescence under spinning disk confocal microscopy.

Results Endometriosis-like lesions were observed in all mice of endometriosis group. In both groups, a similar mean number of MII oocytes per mouse was observed in non-mated mice (30.2 vs 32.6), with a lower proportion of normal oocytes in the endometriosis group (61 vs 83 %, $p<0.0001$). Abnormalities were incomplete extrusion or division of the first polar body and spindle abnormalities. The mean number of zygotes per mouse was lower in the endometriosis group (21 vs 35.5, $p=0.02$) without difference in embryo quality.

Conclusions Our results support that induced peritoneal endometriosis in a mouse model is associated with a decrease in

oocyte quality and embryo number. This experimental model allows further studies to understand mechanisms of endometriosis-associated infertility.

Keywords Endometriosis · Female infertility · Oocyte development · Embryo quality · Meiosis

Introduction

Endometriosis is a well-known gynecological disorder affecting young women characterized by chronic pelvic pain and infertility [1]. It is thought to affect 5 to 15 % of women of reproductive age [2] and up to 40 % of infertile women [3].

A potential cause of infertility is the peritoneal inflammatory response causing adhesions affecting tubo-ovarian function as well as alterations in gamete transport. Moreover, previous randomized studies and a meta-analysis have demonstrated that removal of minimal and mild endometriosis enhanced spontaneous fertility [4–6]. However, in the absence of adhesions, the pathophysiology of endometriosis-associated infertility remains poorly understood. The peritoneal fluid of endometriotic women reveals various aberrations in the inflammatory response such as increased macrophage activation [7], pro-inflammatory conditions, secretion of growth and angiogenic factors [8], presence of reactive oxygen species [9] or dysfunction in natural killer lymphocyte activity [10]. As fertilization occurs in the fallopian ampulla, which is in contact with the peritoneal fluid, several hypotheses link infertility with the inflammatory features of the peritoneal fluid. These include sperm alterations such as reduced motility [11], DNA damage [12] and apoptosis [13]. Oocyte quality, fertilization and early embryo development could also be impaired in endometriosis.

Mouse oocytes incubated in peritoneal fluid from women with endometriosis [14] or in an inflammatory protein

Capsule In a mouse model, peritoneal endometriosis was responsible for decrease in oocyte quality and embryo quantity. Number of ovulated oocytes was not impaired.

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enriched medium BanerjeeSharmaAgarwalMaitraDiamond and Abu-Soud [15] displayed meiotic spindle abnormalities, chromosomal misalignment and alterations in blastocyst development [16]. Moreover, in a rat model of endometriosis, alterations in folliculogenesis with an increase in luteinized unruptured follicles [17, 18], reduced ovulation [19] and spindle abnormalities in metaphase II (MII) oocytes [18] have been described. While Vernon et al. found a reduced number of day-14 embryos and litter size [20] and Barragan et al. a decrease in pregnancy rate, no difference was found in duration of gestation or number of pups [21]. More recently, Stilley et al. have reported in vivo abnormalities in oocyte and pre-implantation embryo development in endometriotic rats [18]. In addition, Furukubo et al. found a decrease in the fertilization rate of rats with artificial endometriosis after IVF [22]. Moreover, a mouse model of surgically induced endometriosis has been used to assess the pathogenesis of endometriosis [23, 24], angiogenesis [25, 26] and response to medical treatments [27, 28]. However, this specific model has not yet been used to assess fertility parameters such as oocyte or embryo quality or embryo implantation.

The aim of our study was, therefore, to assess fertility in a mouse model of surgically induced peritoneal endometriosis and to compare the number and quality of MII oocytes and embryos in endometriotic mice versus sham-operated mice after ovarian stimulation.

Materials and methods

Ethical approval

All experiments were conducted according to the European Communities Council Directive (2010/63/UE) for the care and use of animals for experimental procedures, complied with the regulations of the French Ethics Committee in Animal Experimentation “Charles Darwin”, and were registered at the “*Comité National de Réflexion Ethique sur l'Experimentation Animale Charles Darwin n°5*” (Ile-de-France, Paris, no5). All efforts were made to minimize suffering.

Animals

Five-week-old (recipients) and 9-week-old (donors) female B6CBA/F1 mice (Janvier, France) were used for the experiments. The study population consisted of 33 female B6CBA/F1 mice: 17 with surgically induced peritoneal endometriosis and 16 sham-operated. They were housed five per cage in a temperature-controlled environment under a 12.5 h light–dark cycle and received standard pellet food and water ad libitum.

Induction of intraperitoneal endometriosis

Endometriosis-like lesions were induced using a previously described surgical technique [29] and widely used by Lashke et al. [25] and Becker et al. [26]. For syngenic transplantation, 9-week-old B6CBA/F1 donor mice were anesthetized by isofluorane inhalation (Aerane, Baxter, USA). Both uterine horns were removed and transferred in a Petri dish containing 37 °C warm Dulbecco's modified Eagle's medium (10 % fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin; (Gibco, Life Technologies, USA). Subsequently, the uterine horns were opened longitudinally with micro scissors under a stereomicroscope, and 2 mm tissue samples were removed using a dermal biopsy punch (Kai Medical, USA). Then two tissue samples were attached with a 7–0 polypropylene suture (Prolene; Ethicon Products, USA) to the right and left abdominal wall of 5-week-old recipient female B6CBA/F1 mice through a 10 to 15 mm midline incision initiated 1 cm above the urethral opening. Finally, the peritoneum was closed with a 7–0 polypropylene suture and skin with a 4–0 polypropylene suture. Sham-operated mice were used as control, following exactly the same procedures as in recipient mice, except that two simple stitches were sutured to the abdominal wall without any tissue transplantation.

Oocyte and embryo recovery

Four weeks after surgery, the mice were superovulated by an intraperitoneal (IP) injection of 5 IU of pregnant mare serum gonadotrophin (PMSG, Intervet, France), followed 48 h later by an IP injection of 5 IU of human chorionic gonadotrophin (hCG, Intervet).

On the day of the hCG injection, 22 mice were mated (11 in each group), one female for one male in order to obtain embryos and 11 females were not placed with males (6 in the endometriosis group and 5 in the sham-operated group) in order to obtain MII oocytes. After hCG injection, mating was confirmed by the presence of a vaginal plug for the 22-mated mice. At day 31 post surgery, all the mice were sacrificed to collect either MII oocytes (not mated mice) or embryos at E0.5 stage (mated mice). Sham and endometriotic mice were sacrificed at the same time and oviducts were quickly removed and placed in warm M2 medium (Sigma, USA).

MI oocytes and embryos were collected into a drop of M2 medium by tearing the oviductal ampulla with a 30-gauge needle. To remove cumulus cells, the oocytes and embryos were repeatedly pipetted for 30 s in M2 medium containing 0.01 % hyaluronidase (Sigma) and were then rinsed in several drops of M2 medium under stereomicroscope.

Microtubule and chromosome staining

For microtubule immunostaining, the oocytes were fixed in fixation solution (1 % BSA, 2 % paraformaldehyde and 0.5 % Saponine in PBS) for 45 min and incubated in anti- β -tubulin monoclonal antibody (1:50; Merck Millipore, Germany) for 45 min, followed by incubation in Alexa fluor 488-labelled anti-mouse antibody (1:50; Invitrogen, USA) for 30 min. For chromosome staining, the oocytes were incubated in 4',6'-diamidino-2-phenylindole (DAPI; Sigma, USA) for 15 min. All the oocytes and embryos were then observed by spinning disk confocal microscopy (Leica DMI4000, Germany) with a CSU22 spinning head (Yokogawa, Japan) and pictures were taken. Then, according to previously determined criteria [14, 18, 30, 31], two observers classified separately oocytes and embryos. The observers were blinded to the endometriotic status of the mouse.

Histology

At day of sacrifice, 31 days after endometriosis induction, endometriotic implants were harvested concomitantly to oviducts removal and immediately frozen. Six-micron-thick sections were cut and stained with hematoxylin and eosine according to standard procedures and examined by light microscopy (Nikon eclipse 90i, Japan).

Statistical analysis

The data were normally distributed and are reported as the mean $\pm$ standard error of the mean (SEM) or as percentages. Differences between the number of ovulated oocytes and the number of zygotes per mouse in endometriotic versus sham-operated mice were assessed using the unpaired *t*-test. Correlation between qualitative variables (oocytes and embryos quality parameters) was assessed using nonparametric Chi-square or Fisher's exact test, as appropriate. All hypothesis testing was two tailed, with a significance level fixed at less than 0.05. An anonymous statistician, blinded to the status of the mice, verified all data.

Results

Endometriotic graft development

In the endometriosis group ($n=17$), all mice displayed two lesions; one on the left and one on the right abdominal wall while no endometriotic implant were noted in the sham-operated group ($n=16$). Macroscopically, the endometriotic implants were cystic in all cases and adhesions were observed between the graft and intra-peritoneal pelvic fats (Fig. 1). No

endometriotic implants were detected on the uterine horns or other pelvic organs. Histology of the lesions showed a cyst surrounded by endometrial tissue (stroma and glandular epithelium) and no myometrial cells (Fig. 1). No adhesions or endometriotic implants at the stitch level were observed in the sham group (Fig. 1).

Effects of endometriotic implants on oocyte quality

The endometriotic ($n=6$) and sham mice ($n=5$) had similar mean numbers of ovulated oocytes (30.2 ± 2.5 vs. 32.6 ± 2.2 , respectively) (Table 1). The endometriotic mice had fewer normal MII oocytes than the sham-operated mice (110/181 (60.8 %) vs. 136/163 (83.4 %); $p<0.0001$) (Table 1, Fig. 2). The following abnormalities were over-represented in the endometriotic mice: the first polar body (PB-1) was either not completely extruded (13/181 (7 %) vs. 4/163 (2.5 %), $p<0.05$; Table 1, Fig. 2b) or divided (16/181 (8.8 %) vs. 5/163 (3 %), $p<0.05$; Table 1, Fig. 2c), some oocytes displayed spindle abnormalities (16/181 (8.8 %) vs. 5/163 (3 %), $p<0.05$) such as scattered chromosomes (Fig. 2e) or arciform spindles (Fig. 2f) and other oocytes appeared as activated with a second meiotic division (26/181 (14.4 %) vs. 13/163 (8 %), not significant, Table 1, Fig. 2d).

Effects of endometriotic implants on E 0.5 embryo development

The endometriotic mice ($n=11$) had significantly fewer zygotes per mouse than the sham-operated mice ($n=11$) (21.0 ± 3.8 vs. 35.5 ± 4.6 ; $p<0.05$) (Table 1). Among the recovered zygotes we found typical E 0.5 zygotes with 2 pronuclei (2-PN) (Fig. 3a) as well as unfertilized oocytes (Fig. 3b), fertilized oocytes with a decondensed sperm head and second polar body (PB-2) (Fig. 3c), 2-PN embryos with a delayed PB-2 extrusion (Fig. 3d), and fragmented or dead embryos (Fig. 3e–f). The same proportions of each of these different configurations were seen in both the endometriotic and sham-operated mice embryos as described in Table 1.

Discussion

Our data demonstrate that peritoneal endometriosis decreases oocyte quality but not the number of ovulated oocytes in a mouse model. Moreover, the number of embryos is decreased in mice with endometriosis.

We found a significant increase in spindle abnormalities in mice with induced endometriosis. Oocyte quality is mainly assessed by analyzing spindle morphology, which is altered in experimental pathological conditions such as oocyte cryoconservation [30, 32] or oocyte exposure to oxidative

Fig. 1 Macroscopic and microscopic aspect of lesions at Day 31 after endometriosis induction. **a:** Typical macroscopic aspect of the sham-operated group, with no endometriotic tissue around the stitch (*white arrow*) and no adhesion. **b:** Typical appearance of the lesions developed in the endometriotic group, after induction of endometriosis by fixation of uterine tissue samples to the peritoneal wall: a cystic lesion (*white arrowhead*) and adhesion between the lesion and the peritoneal pelvic fats (*white asterisk*). **c:** Hematoxylin-eosin stained cross section of endometriotic lesion: a cyst (©) surrounded by endometrium-like tissue (glandular epithelium (*e*) and stroma (*s*)) without myometrial tissue

Table 1 Metaphase II and E 0.5 embryo in endometriotic mice versus sham-operated mice

		Sham-operated mice	Endometriotic mice	<i>p</i>
Metaphase II oocytes	Number of mice	5	6	
	Total number of oocytes	163	181	
	Mean number $\pm$ SEM of oocytes per mouse	32.6 $\pm$ 2.2	30.2 $\pm$ 2.5	NS ^(a)
	Delayed PB-1 extrusion; <i>n</i> (%)	4 (2.5)	13 (7)	0.04 ^(b)
	Divided PB-1; <i>n</i> (%)	5 (3)	16 (8.8)	0.025 ^(c)
	Activated oocyte; <i>n</i> (%)	13 (8)	26 (14.4)	0.06 ^(b)
	Spindle abnormalities; <i>n</i> (%)	5 (3)	16 (8.8)	0.025 ^(c)
	Normal metaphase II oocyte; <i>n</i> (%)	136 (83.4)	110 (60.8)	<0.0001 ^(b)
E 0.5 embryos	Number of mice	11	11	
	Total number of embryos	389	231	
	Mean number $\pm$ SEM of embryos per mouse	35.5 $\pm$ 4.6	21.0 $\pm$ 3.8	0.02 ^(a)
	2-PN embryos; <i>n</i> (%)	171 (44)	105 (45.3)	0.74 ^(b)
	Fertilized oocytes with PB-2; <i>n</i> (%)	62 (16)	49 (21.4)	0.1 ^(b)
	Unfertilized embryos; <i>n</i> (%)	117 (30)	55 (23.9)	0.096 ^(b)
	Fragmented or dead embryos; <i>n</i> (%)	39 (10)	22 (9.4)	0.89 ^(b)

NS not significant

^(a) Unpaired *t*-test

^(b) Chi-2 test

^(c) Fisher exact test

Fig. 2 Metaphase II oocytes: chromosomes and spindle staining. Spinning disk confocal microscopy images showing structures of microtubule (*green*) and chromosome (*blue*) in MII mouse oocytes stage. Normal oocyte with typical spindle morphology: barrel-shaped structure with slightly pointed poles formed by organized microtubules that traverse from one pole to the other with chromosomes arranged on a compact plate at the equator of the structure (**a**). Oocytes displaying abnormal configurations: first polar body (*white asterisk*) not totally extruded (**b**), or divided (*white asterisk*) (**c**); activated oocytes with a second meiotic division (*white arrow*) (**d**). Spindle abnormalities: scattered chromosomes (*white arrowheads*) (**e**) or arciform spindle (**f**). The oocyte represented in **a** originates from a sham-operated mouse and those represented in **b**, **c**, **d**, **e** and **f** originate from endometriotic mice. Scale bar: 10 μ m

stress [33, 34]. Meiotic spindles ensure chromosomal organization and second polar body formation [31]. Meiotic spindle impairment results in chromosomal dispersion, reduced fertilization, and arrest of embryo development [30]. Our findings are partially in agreement with those of Banerjee et al. showing deterioration in microtubule and chromosomal alignment in MII mouse oocytes after exposure to recombinant mouse IL-6 [15], a cytokine expressed in peritoneal fluid of women with inflammatory disease and endometriosis [35, 36]. Mansour et al. found that the cytoskeleton had a higher frequency of abnormal meiotic spindles and chromosomal misalignment after exposing MII mouse oocytes to women endometriotic peritoneal

fluid [14]. However, in contrast to our *in vivo* study, these studies were performed *in vitro*. Another interesting finding of our study concerned the increase in first polar body abnormalities. We found an increased number of delayed polar body extrusion and first polar body division in the endometriotic group. First polar body abnormalities are associated with decreased potential of oocyte fertilizability [37–39] and may provide conditions for parthogenesis [40]. Other oocyte abnormalities have been observed in endometriotic rats; spontaneous oocyte activation and formation of pseudopronuclei or karyomeres surrounded by a *de novo* formed nuclear envelope with nuclear pore complexes [18].

Fig. 3 Two-PN Embryos: chromosomes and spindle staining. Spinning disk confocal microscopy images showing structures of microtubule (*green*) and chromosome (*blue*) in 2-PN embryos. **a**: typical E 0.5 zygotes with 2 PN (*white arrowheads*), **b**: unfertilized oocytes, **c**: fertilized oocytes with a decondensed sperm head (*white arrow*) and second polar body (*white asterisk*), **d**: E 0.5 zygotes with 2PN (*white arrowheads*) and a delayed PB-2 extrusion. **e–f**: fragmented or dead embryos. Scale bar: 10 μ m. Embryos represented in **a**, **b** and **d** originate from sham-operated mice and those represented in **c**, **e** and **f** originate from endometriotic mice. Scale bar: 10 μ m

We also found that the number of 2-PN embryos was reduced in the endometriosis group without difference in the ratio of good and bad quality embryos. The specific 2-PN stage was chosen for two reasons. First, it is the most relevant stage to assess the quality of PB-2 extrusion and pronuclei localization. Second, at this step of the fertilization process, embryos are still in the distal part of the fallopian tube, the ovarian bursa, which is in contact with the peritoneal fluid at this stage. In mouse, pre-ovulation hormonal stimulation (PMSG-primed hCG injection) induces a rapid fluid accumulation and re-absorption within the ovarian bursa, [41] and recent findings suggest that the ovarian bursa could be regulated or affected by outside tissues as well as by the peritoneal

fluid [42]. These assertions support our hypothesis that the peritoneal fluid might negatively affect oocyte and embryo quality in mouse. Our results suggest that endometriosis affects oocyte development but not embryo quality until E0.5 stage.

In a mixed model of mouse oocytes exposed to human endometriotic fluids, the oocytes had lower rates of hatching/hatched blastocysts than control oocytes [16]. Finally, in an in vivo rat model, Stilley et al. report a reduction in the number of zygotes as well as a decrease in their quality [18]. Unfortunately, the authors only gave embryo quality parameters (pronuclear size, chromosomes alignment, nuclear fragmentation, cytoplasmic fragmentation delayed or arrested cleavage)

without quantitative data. Shahine et al. have shown that surgical removal of endometriosis did not increase embryo quality in women (57 % with stage I-II ASRM and 43 % with endometriomas) [43]. However, they assessed embryo quality at day 3 and day 5 and provide no data on oocyte and 2-PN embryo quality. In contrast, Brizek et al. found that some abnormalities including the presence of cytoplasmic depletions, condensations or voids and aberrant cleavage of enucleated cytoplasm, were over-represented in 2-PN embryos from women with endometriosis [44]. Furthermore, Pellicer et al. have reported an increased number of arrested embryos in vitro [45].

To our knowledge, this is the first study to focus on the in vivo impact of endometriosis on oocyte and embryo quality in a mouse model of surgically induced endometriosis. However, some limits of the present study deserve to be mentioned. First, caution should be exercised when transposing the implications of our results to the human [46, 47]. Endometriosis has to be induced artificially in the mouse, as they do not menstruate. We thus have a model of endometriosis in animals with no genetic, immune or environmental susceptibility to endometriosis. Second, superovulation in mouse has been shown to impair oocyte/embryo quality [48], however in our study, since both groups received the same stimulation regimen, its impact was equivalent in endometriotic and sham-operated mice. Third, as we sacrificed mice early in pregnancy, at day E0.5, no information on implantation alteration, foetal losses or on litter size and pregnancy outcomes was available. However, numerous studies already showed impairment in natural fertility in rats with surgically induced endometriosis [20–22, 49] without evaluating oocyte quality that is a crucial step in fertility process. Fourth, in the other studies using a murine model of surgically induced endometriosis, some authors chose to implant more than two uterine implants on the peritoneal wall or on the intestinal mesentery. In the studies of Lashke et al. [25] and Rutzitis-Auth et al. [28], four implants were engrafted in 10 to 16 weeks-old mice. Our mice were younger, 5 weeks old, and this is the reason why we engrafted only two implants. We chose to engraft tissues only in the peritoneal cavity to be as close as possible to the human peritoneal endometriosis.

In conclusion, our data indicate that the quality of the oocyte is a crucial factor affecting fertility in a mouse with induced endometriosis. Further studies will clarify the molecular mechanisms responsible for this impaired quality. Our model could be used to assess the impact of endometriosis on pre-implanted embryo development as well as on the implantation process.

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