The gametotoxic effects of the endometrioma content: insights from a parthenogenetic human model

Alessio PAFFONI [a,c], Valentina BOLIS [a] , Stefania FERRARI [a], Laura BENAGLIA [a], Paolo VERCELLINI [a,b], Edgardo SOMIGLIANA [a,b]

[a] Fondazione Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy

[b] Dept of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy

[c] To whom correspondence should be addressed

Alessio PAFFONI

Infertility Unit – Fondazione Ca' Granda, Ospedale Maggiore Policlinico

Via M. Fanti, 6 - 20122 - Milan - Italy

Phone: +39-02-55034304

Email: alessio.paffoni@alice.it

Funding: This work was partially financed by Italian fiscal contribution "5x1000" 2012 devolved to Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico

Abstract

The present randomized controlled *in vitro* study was designed to evaluate the effects of the exposure of human cryopreserved oocytes to endometriotic fluid. Twenty-three women aged 36 ± 4 years donated a total of 147 vitrified supernumerary metaphase-II oocytes. Warmed oocytes were randomly assigned to exposure to endometriotic fluid or unexposed control. Thereafter oocytes were parthenogenetically activated and cultured up to five days. The rate of activation on day 1 and the developmental rates on day 3 and day 5 did not significantly differ between the two groups. The rate of day 3 good quality parthenotes per oocyte was lower in exposed compared to unexposed oocytes, being 22% (13/60) and 41% (25/61), respectively. Moreover, in the exposed parthenotes, a significantly higher proportion of parthenotes failing to develop to the blastocyst stage showed cellular fragmentation (RR=0.64, 95%CI: 1.04-2.57). Exposure of human oocytes to endometriotic fluid has a negative effect on the morphology of deriving embryos/parthenotes mainly due to an excess of cellular fragmentation.

Keywords

Endometrioma, follicular fluid, parthenogenesis, IVF, oocyte

Introduction

The American Society of Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE) have recently stressed the risk of reduced ovarian function after surgery for ovarian endometriomas in their guidelines for the management of endometriosis[1-2]. These guidelines are based on several evidences highlighting that the excision of the endometrioma can severely impair ovarian reserve and affect the subsequent fertility[3-7]. Accordingly, an increasing proportion of infertile women with the disease currently undergoes in vitro fertilization (IVF) in the presence of one or more endometriomas.

However, from an IVF-centered perspective, the conservative management of ovarian endometriomas is not without drawbacks and risks[8]. The presence of these cysts can make oocyte aspiration more difficult and the endometriotic fluid may damage oocytes, especially in case of direct contact after inadvertent contamination[9]. In fact, even if it is generally claimed that these cysts should not be punctured during oocytes retrieval, unintentional aspiration of endometrioma content and follicular fluid contamination can occur. The incidence has been estimated to be 5% (95% confidence interval-CI: 3-7%)[8].

Exposure to the content of endometriotic cysts may be harmful to the oocyte and may alter its subsequent developmental potential as an embryo due to the presence of several potentially toxic substances such as growth factors and interleukins[10-12], matrix metalloproteinases[13], catalytic iron, and lipid peroxide[14]. In particular, the great amount of free iron in endometriotic cysts, up to 10000-fold higher than in serum[15], is a matter of concern because of the risk of local production of the highly toxic reactive oxygen species (ROS) via the Fenton reaction[16].

On the other hand, experimental data supporting these potential concerns are scanty. In an *in vitro* study in the mouse model, oocyte exposure to endometrioma content did not affect

fertilization, cleavage and blastocyst formation but lowered the proportion of hatching/hatched blastocysts[17]. Evidence in humans is derived from retrospective and underpowered studies comparing embryological variables and pregnancy outcome between exposed and non-exposed cases[18-20]. Data from *in vitro* studies are inconsistent, as the embryological process appears impaired in the study by Suwajanakorn *et al.*[18], unaffected in that by Khamsi *et al.*[19], and even paradoxically improved in that by Benaglia *et al.*[20]. Pregnancy rate was reduced in the two studies reporting this outcome[18,20]. These conflicting results may be explained by different kinds of exposure to endometriotic fluid in terms of duration or relative amount of contamination, together with suboptimal study designs. In particular, the retrospective collection of data, the small sample sizes (a total of 71 exposed cases after pooling the three studies) and possible biases in the selection of unexposed controls, actually undermine the validity of human studies.

Given the difficulties in obtaining clear embryological data in the clinical settings and the debatable reliability of animal models, we designed a randomized, controlled, *in vitro* study with the objective of evaluating the effect of exposure of human cryopreserved oocytes to endometriotic fluid. To this aim, we used a validated experimental model of human parthenogenesis[21-26].

Material and methods

Patients and oocytes

The present study was conducted between January 2016 and July 2016 at the Infertility Unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy). Oocytes used for the study protocol were previously cryopreserved during IVF procedures as supernumerary ones and subsequently donated by women after completion of their reproductive program. In

Italy, the obtainment of supernumerary embryos is limited by law. Therefore, when a large amount of good quality oocytes is available, part of them should be destined to cryopreservation according to clinical conditions and couples' willing. Women no longer interested in using their supernumerary vitrified oocytes were asked to donate them for research purposes through a specific informed consent. A diagnosis of endometriosis was an exclusion criterion. Only oocytes retrieved from women under 42 years age at cryoconservation were considered. The reasons for cryoconservation waiver varied, the most common being a successful pregnancy achieved in the fresh cycle. The study was approved by the local Institutional Review Board. (Comitato di Etica Milano Area B).

Oocytes were obtained following controlled ovarian hyperstimulation as described elsewhere (27). Following a 3±1 hours incubation at 37 °C in an atmosphere of 5% CO2, supernumerary oocytes were denuded from cumuli oophori by pipetting them out through a 140-170 mm (internal diameter) pipette (Flexipet; Cook, Bloomington, Ind), after a brief exposure to 40 IU/mL of hyaluronidase in HEPES buffered gamete handling medium. Metaphase II-oocytes without morphological abnormalities showing a clear cytoplasm with fine granularity were vitrified using the cryotop method according to Kuwayama *et al.*[28]. At the time of the experimental protocol, donated oocytes were thawed and cultured together for 2 hours in 1 ml of Quinns Advantage Fertilization Medium (SAGE, Trumbull, CT USA). Thereafter, the viability of oocytes was checked and non viable ones were discarded.

Exposure to endometriotic fluid

Endometriotic fluid was collected during laparoscopy for ovarian endometrioma excision from three different patients who gave an informed consent. Once terminated the initial diagnostic part of the laparoscopy, the ovary carrying the endometrioma was punctured and aspirated with a 14-16 gauge needle. The procedure was done prior to initiate the adhesyolysis because of the

risk of subsequent cyst rupture and inevitable contamination of the endometrioma content with the peritoneal fluid. The endometriotic fluid was stored untreated at – 20 °C (3-5 weeks). It appearance was “dark chocolate”.

During the incubation of warmed oocytes, endometriotic fluid was thawed at 37 °C. Thereafter, it was added to one well (20% v/v) of a four-well dish containing 500 microlitres aliquotes of pre-equilibrated fertilization medium. For every set of experiments, after 2 hours of post-thawing culture, a maximum of 12 intact oocytes belonging to a single patient were collected from the incubator and randomly separated to the fertilization medium containing endometriotic fluid (exposed oocytes) or to the fertilization medium (unexposed oocytes) in a 4-well culture dish (ratio 1:1). In order to exclude selection biases based on morphological features of the oocytes, the procedure was performed using a stereo-microscope with minimum magnification power (8x). Therefore, oocyte belonging to every women were randomly allocated half to treatment and half to control.

In case of odd number of oocytes, the balance between groups was obtained with the following set of experiments. Oocytes were then incubated for 3 minutes at 37 °C, 5% CO_2. Afterwards, exposed oocytes were carefully rinsed in the wells containing clean fertilization medium and moved to the parthenogenetic activation dish; similarly, unexposed oocytes were moved to the parthenogenetic activation dish. Contact between unexposed oocytes and endometriotic fluid was avoided using separate wells/pipettes for cases and controls.

Parthenogenetic activation

The parthenogenetic activation was conducted on exposed/unexposed oocytes using a 4-wells dish through sequential exposure to 5 mM ionomycin in IVF medium for 5 minutes at 37°C, 5% CO_2 in the dark followed by an incubation in 2 mM 6-dimethylaminopurine (DMAP; Sigma-Aldrich, Milan, Italy) in cleavage medium for three hours at 37 °C. Afterwards, oocytes

were washed three times in fresh cleavage medium, placed separately in 40 mL drops of the same medium under mineral oil and cultured in standard conditions (37°C, 5% CO_2). For every set of experiments, exposed and unexposed oocytes were cultured in the same dish using different wells or drops. After 18 to 20 hours (day 1) from the activation with ionomycin, oocytes were checked with an invertoscope for signs of activation.

The oocytes showing one enlarged pronucleus and no extrusion of the second polar body were considered correctly activated (parthenotes). Parthenotes deriving from exposed and unexposed oocytes were washed twice and kept separately in culture using 40 microliters drops of fresh cleavage medium in the same dish until day 5 from the activation, when parthenogenetic blastocysts were expected to develop. Culture media was renewed on day 3. The cleavage of parthenotes was checked daily. Using embryological criteria[29], morphological data based on equal-sized blastomeres and pattern of fragmentation were recorded after 66 to 68 hours of activation (day 3) while criteria for blastocyst scoring (based on the degree of expansion, and inner cell mass/trophectoderm development) were used on day 5. On day 3 of culture, parthenotes belonging to grade 1 or 2 (fragmentation less than 10% with equal size blastomeres) were classified as “good quality” ones. Blastulation was registered in the presence of blastocoel filling more than 50% of the volume of parthenote; in case of fully enlarged size with thin zona pellucida (or hatching) and distinguishable inner cell mass, the blastocyst was considered as “good quality-expanded” (Figure 1).

The length (days) of viability in culture was recorded for every parthenote together with the cause of developmental failure: 1) fragmentation in case of few cells and >50% of parthenote showing fragments or 2) cleavage arrest in case of unchanged number of cells for at least 24 hours with no or moderate fragmentation. All morphological evaluations were made by a single expert biologist (AP) who was blinded to the previous exposure.

Data analysis

Analysis of data was performed using the Statistical Package for Social Sciences (SPSS 18.0; Chicago, Ill). Statistical significance was set at $p < 0.05$. Fisher's exact test and the unpaired Student *t* test were used where appropriate to compare the two groups. Relative risks (RRs) and their 95% CI for activation, cleavage and blastulation rates are reported to compare parthenotes deriving from exposed and unexposed oocytes. Proportions are reported as percentages. The 95% CI of proportions were calculated using a binomial distribution model.

To obtain the sample size of the study, for power calculation purposes, a three-fold~~s~~ decrease in the rate of day 3 good quality parthenotes for exposed oocytes compared to unexposed ones was considered embryologically important. Based on our previous data[21], the sample size was then calculated considering a good quality parthenotes rate on day 3 equal to 37% per oocyte in the group of unexposed oocytes, and setting type I and II errors at 0.05 and 0.20, respectively. Based on these assumptions, the number of required intact oocytes were at least 55 per group. Considering a survival rate of 80% after warming, the total number of oocytes to be used for the study were thus 140.

Results

Twenty-three women aged 36 ± 4 years donated a total of 147 vitrified metaphase-II oocytes (range 2-17). After warming and culture, 26 oocytes (18%) showed signs of degeneration/lysis and were therefore discarded leaving 121 oocytes for randomization. Intact oocytes were treated in 13 sets of experiments. A total of 60 oocytes were randomized to the exposure group while 61 oocytes were randomized to the non exposure group. Endometriotic fluid from three patients were used for 4, 4 and 5 sets of experiments, respectively. Results are summarized in

Table 1. The day after treatment and activation, 42 parthenotes (70%) among exposed oocytes and 46 parthenotes (75%) among unexposed oocytes were observed (p=0.51). The developmental rates on day 3 and day 5 did not significantly differ between the two groups. The RRs (95%CI) of exposed oocytes for activation, cleavage on day 3 and blastulation on day 5 were 0.93 (0.75-1.16), 0.88 (0.65-1.19) and 0.47 (0.19-1.15), respectively.

Conversely, data on morphology tend to support a detrimental effect of endometrioma fluid exposure (Table 1). The rate of day 3 good quality parthenotes was 22% (13/60) and 41% (25/61) in exposed and unexposed oocytes, respectively (RR=0.53; 95%CI: 0.30-0.93, p=0.031). A trend was observed also in the development of good quality-expanded blastocysts on day 5, 5% (3/60) and 13% (8/61) of oocytes, respectively; RR=0.39, 95%CI: 0.11-1.37). Moreover, a significantly higher proportion of parthenotes failing to develop to the blastocyst stage showed cellular fragmentation in the exposed compared to unexposed parthenotes (RR=0.64, 95%CI: 1.04-2.57; p=0.024).

Discussion

In the present randomized *in vitro* study we observed, by means of the parthenogenesis experimental model, that exposure of human oocytes to the content of endometriotic cysts negatively impacts on the first phases of *in vitro* embryogenesis compared to controls, with specific regards to the percentage of oocytes developing to good morphology embryos. In particular, although activation and cleavage rates were similar between exposed and unexposed oocytes, the chance of developing into a good quality embryo was nearly halved when oocytes were exposed to 20% (v/v) of endometriotic fluid in culture medium for three minutes. Moreover, although not statistically significant, a reduction in terms of length in culture and blastulation rate was observed for exposed oocytes.

Our results partially diverge from data derived from the heterogeneous available studies[18-20]. Two of these studies actually failed to identify detrimental effects during the *in vitro* phases[19,20]. Of note, in the clinical setting all efforts are made to limit the exposure of oocytes to endometriotic fluid. In case of contamination of follicular fluid during aspiration, oocytes are promptly and accurately washed and the needle is flushed with clean culture medium[8]. Moreover, the contamination is often quantitatively reduced with endometriotic fluid highly diluted into follicular content. As a consequence, the exposure of oocytes to potentially toxic substances is kept to a minimum both in time and quantity. In our experimental model, on the contrary, time and endometriotic fluid concentration were settled to fixed values in order to resemble a substantial exposure of oocytes. It is therefore plausible that the embryological parameters are affected in a dose-dependent manner with earlier effects on cleaving embryos discernible at higher doses only.

On the other hand, our results tend to be in line with those reported in the experimental study in mouse[17], overall showing that the detrimental effects of endometrioma fluid exposure cannot be observed immediately but tend to become evident when extending the embryo culture. In our study, an excess of fragmentation was the main visible effect of endometriotic fluid on parthenotes, as seen both in the lower percentage of grade 1 and 2 parthenotes deriving from exposed oocytes, and in the higher proportion of parthenotes failing to develop to blastocyst for massive fragmentation.

The fragmentation at the cleavage stage as a non-immediate effect of oocyte exposure to a toxic substance deserves particular consideration. Blastomere cytofragmentation is very common in humans with more than 80% of embryos produced by *in vitro* fertilization exhibiting some degree of cellular fragmentation[30]. Although fragmented embryos can produce full term pregnancies and healthy babies, cytofragmentation is associated with lower embryo

viability and developmental potential[31]. Therefore, embryo fragmentation in *in vitro* produced embryos is a fundamental aspect of embryo grading systems[29,32]. Cellular fragments could impair the developmental potential of embryos by inducing cell death of surrounding blastomeres[30] or altering the blastomeres' division planes[33]. Morphological features of cytofragmentation appear to be a robust indicator of apoptosis, supporting the possible involvement of programmed cell death in early human embryo arrest and demise[30,34].

Aside from apoptosis, the disruption of the correct control of the contractile apparatus/actin metabolism can represent an alternative mechanism of fragmentation directly exerted on the cytoskeleton[35]. Beyond the ooplasmic or genetic origin of embrionic fragmentation, it has been showed that fragmentation may reflect cellular responses to suboptimal culture conditions[36-39] and, of note, this propensity is programmed very early, at the one-cell stage[40,41]. Our experimental model, although not designed to explore the mechanisms of cell death or fragmentation, supports the hypothesis that environmental factors in the very early stages of development can modulate the predisposition of an oocyte to arrest by cytofragmentation after fertilization with an exclusive effect on the maternal cellular armamentarium.

The study design is the main strength of the present study. In fact, oocytes were randomly allocated to the treatment groups with a donor based stratification. This design is particularly favorable in order to exclude possible and important confounding factors linked to clinical parameters of the donors. From every single women a comparable number of oocytes was allocated to case and control groups thus excluding the need for statistical adjustment to rule out possible biases related to oocyte quality.

However, some limitations of our study have to be acknowledged. Firstly, our study

actually evaluates a fixed experimental model that cannot mimic all the variability of every possible clinical conditions; therefore caution is needed when extrapolating our results to the IVF routine. Secondly, our control group is represented by non exposed oocytes; the exposure to a control medium would have been methodologically more rigorous. However, we preferred the use of unexposed oocytes in order to create a condition more similar to the real one. Thirdly, endometriotic fluids from three different patients were used; even though we did not observed differences in the main outcome based on the donor of the endometriotic fluid (data not shown), we cannot exclude that its composition may vary in a patient-specific manner implying possible different biological effects. Lastly, as an experimental approach, the use of vitrified oocytes for parthenogenetic activation may seem far from the typical IVF setting, where fresh oocytes are inseminated with spermatozoa. However, it should be mentioned that, with the present vitrification protocol, warmed oocytes have been demonstrated to perform similarly to corresponding fresh ones[42] and that parthenotes, whose chance to develop *in vitro* is mainly dependent on oocyte quality, show no significant differences in embryonic development compared to fertilized oocytes[21]. It also has to be recognized that the exposure of denuded oocytes could overestimate the toxic effect of endometriotic fluid since the interaction between the oocyte and surrounding cumulus cells complex can be protective from external (environmental) factors[43].

Our results have some potential clinical implications. Even if robust inferences for clinical practice cannot be drawn, because our findings were obtained in an experimental and artificial context, we believe that our study should be interpreted as a proof of concept. Up to now, the common dogma that ovarian endometriomas should not be punctured or aspirated during oocytes retrieval lacked scientific support[8]. Detrimental effects were presumed but not demonstrated. In this context, the present study should be viewed as the first contribution that

justifies the common cautious attitude to do all the efforts to avoid puncturing and aspirating endometriomas during oocytes retrieval. Moreover, our results highlight the importance of limiting as much as possible the exposure of aspirated oocytes to endometriotic fluid when an accidental contamination occurs. This goal can be achieved through immediate control of follicular fluids during oocyte aspiration in women affected by ovarian endometrioma and with prompt interruption of the aspiration, rapid flushing of needle and washing of oocytes with fresh culture medium in case of contamination[20]. In order to reduce the risk of cross-contamination one may also consider to change test tube for each follicle during oocyte retrieval. Moreover, in case of contamination, we suggest to culture exposed and unexposed oocytes separately and to favor the transfer of embryos derived from uncontaminated follicular fluids.

In conclusion, our experimental model shows that the exposure of human oocytes to endometriotic fluid can have a negative effect on the morphology of deriving embryos/parthenotes mainly due to an excess of cellular fragmentation. A definitive demonstration that this effect can lead to reduced chances of pregnancy in IVF clinical practice is lacking, but can be presumed. On these bases, we support the common clinical attitude aimed at avoiding endometrioma puncture during oocytes retrieval and at reducing as much as possible the exposure of the oocytes when accidental contamination occurs.

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Table 1. Embryological development in exposed and unexposed oocytes

Variables	Exposed	Unexposed	p
Metaphase-II oocytes	60	61	
Day 1			
Parthenotes	42 (70%)	46 (75%)	0.51
Day 3			
Parthenotes (cleavage rate)	33 (55%)	38 (62%)	0.42
Good quality parthenotes	13 (22%)	25 (41%)	0.031
Number of blastomeres	6.8 ± 2.9	6.6 ± 3.0	0.81
Day 5			
Parthenogenetic blastocysts (Blastulation rate)	6 (10%)	13 (21%)	0.13
Good quality-expanded blastocyst	3 (5%)	8 (13%)	0.21
Total lenght in culture (days)	2.6 ± 1.2	3.0 ± 1.3	0.09

Data is reported as number (percentage) or mean ± SD. P values were calculated using the Fisher's exact test or the Student's *t*-test

Table 2. Baseline characteristics of oocyte donors (n=23)

Characteristics	Women
Age (years)	35.7 ± 3.7
BMI (Kg/m^2)	22.4 ± 1.8
Previous pregnancies	1 (4%)
Previous IVF cycles	7 (30%)
Duration of infertility (years)	3.3 ± 2.0
Cause of infertility	
Male factor	7 (30%)
Tubal factor	3 (13%)
Unexplained	6 (26%)
Ovulatory	2 (9%)
Mixed	5 (22%)
Current smoking	4 (17%)
Abnormal karyotype	0 (0%)
Embryological parameters in the IVF cycle	
Fertilization rate	68%
Cleavage rate/zygote	91%

Data are reported as percentage, mean ± SD or number (%).

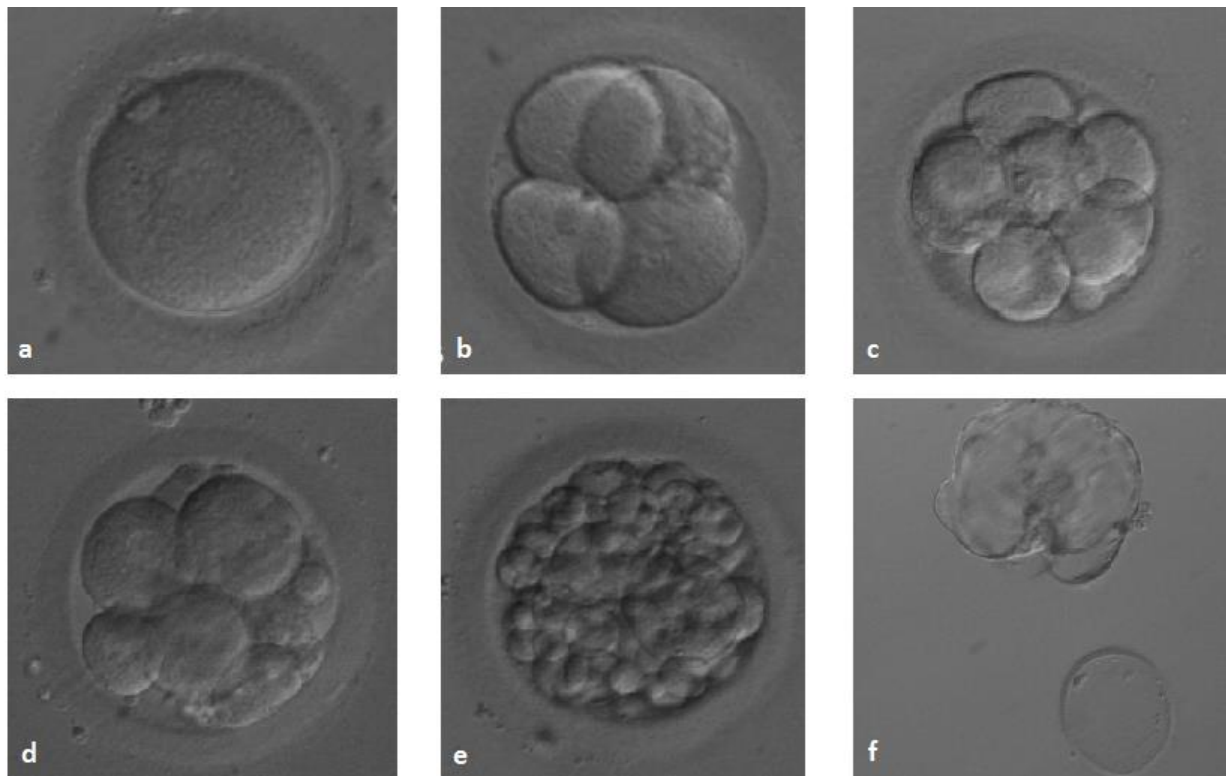

Figure 1: Parthenotes at various stages of development. a) a parthenote on day 1, showing one pronucleus and one polar body; b-c) “good quality” parthenotes on day 2 and day 3; d) poor quality parthenote on day 3 showing uneven sized blastomeres; e) a parthenote on day 3 failing to develop to the blastocyst stage for massive fragmentation. f) good quality hatched parthenogenetic blastocysts on day 5