

Relative Position of the Meiotic Spindle and Polar Body as a Marker of Oocyte Maturation Improves the Utilization and Pregnancy Rates

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Abstract

This research demonstrates how a mutual position of the human oocytes meiotic spindle (MS) and the first polar body (PB) correlates with the probability of obtaining high-quality embryos (utilization rates) and high pregnancy rates after intracytoplasmic sperm injection (ICSI). The quality of optically birefringent MS and the angle (α) between MS and PB (evaluated using polarizing microscopy), were used to indicate oocyte maturation and appropriate time for fertilization. In this study, 124 patients undergoing *in vitro fertilization* (IVF) whose oocytes were evaluated by MS visualization had a significantly higher clinical pregnancy rate (38% vs 26%) and utilization rate (54% vs 38%) when compared to the control group, using one standard IVF cycle without MS visualization. Significantly, in group of 79 patients > 35 years old, 34% became pregnant when α was evaluated and ICSI time adjusted to achieve the full oocyte maturation, compared to only 18% in the control group. The number of high-quality embryos in the MS visualized group was significantly higher compared to the control group, increasing the probability of pregnancy. Based on this research, we propose to incorporate monitoring the mutual position of MS and PB as a valid marker of embryo quality which can significantly improve pregnancy rate.

Introduction

In IVF process the female gamete quality is crucial parameter. Oocyte quality is not only influenced by the nuclear and mitochondrial genome, but also by the micro-environment provided by the ovary and the pre-ovulatory follicle¹. These influence transcription and translation, and consequently, cytoplasmic maturity²⁻⁹. Following the removal of the cumulus oophorus including corona radiata cells in preparation for ICSI, evaluation of the oocyte based on the nuclear maturation status, the morphology of the cytoplasm and the appearance of the extracytoplasmic structures is more accurate¹⁰⁻¹⁵.

The presence of PB is generally considered to be a marker of oocyte nuclear maturity. However, recent studies using polarized light microscopy have shown that oocytes displaying a PB may still be immature^{16,17}. Only oocytes displaying meiotic spindle (MS) in the Metaphase II (MII) stage can be considered truly mature^{16,18-20}. The presence, position and state of MS has been suggested to be related to development of oocyte competence¹⁹.

MS is specific optically birefringent structure visible in polarized light which controls oocyte maturation (proper chromosome organization). Parallel-aligned MS microtubules are birefringent and are able to shift plane polarized light inducing a retardance; birefringent MS properties enable the microscope to generate contrast and image MS structure²¹. The presence of MS gives researchers more accurate information about the nuclear stage of the oocyte. In particular, when observed with polarized light microscopy, some oocytes appear as immature (at the stage of early Telophase I)^{2,16}, despite the presence of PB in the perivitelline space. At this stage, there is continuity between the ooplasm of the oocyte and the formation of PB and MS is interposed between two separating cells^{19,22-26}. This step

physiologically has a duration of 75–90 min. MS has been found to disappear in late Telophase I, but reforms 40–60 min later²⁴ in the stage of interphase oocyte (between the first and second meiotic division; prophase II). So, when PB is present and MS of the second meiotic division is not yet visible in the cytoplasm the MII oocyte is not fully mature so should not be used for fertilization^{25,26}. Such an oocyte typically reaches full maturity (MII) after a longer time.

IVF success rate in a single cycle (up to 30% pregnancy rate) is affected by several factors^{27–31}. Apart from a women's age and the quality of oocytes and sperm, the other crucial factor is the maturation of the oocytes^{27–30}. In this paper the quality of MS is evaluated and the angle between PB and optically birefringent MS of oocytes is measured using an optical microscope with a Nikon CEE GmbH polarizing filter. During the IVF process, this research showed that the angle (α) between PB and optically birefringent MS can be used to predict the ideal time for fertilization using ICSI method. The initial angle between MS and oocytes PB was correlated with the oocytes utilization rates calculated using a number of high-quality embryos^{32,33} per oocyte with detected PB, and final clinical pregnancy rates. To prove the importance of MS visualization we compared the utilization and pregnancy rates in one IVF cycle of retrospective (R) study patients with visualized MS and a control group of patients using standard IVF treatment i.e., without MS visualization (C). Based on our results we suggested a method for determining the most suitable time for ICSI based on the relative position of PB and MS^{34,35}.

The aim of this research was to identify a novel method of assessment of oocyte maturation as an indicator of oocyte readiness for fertilization. The research was based on two major hypotheses. Firstly, that the angle between the oocyte meiotic spindle and the polar body could be used as a valuable indicator to assess the timing of fertilization using ICSI. And so, by incorporating the microscopy evaluation of oocytes to increase the probability of obtaining a high-quality embryo and significantly improve clinical pregnancy rates. Secondly, that the evaluation of MS by an optical microscope fitted with a polarizing filter does not negatively stress the evaluated oocytes, and therefore does not reduce pregnancy and utilization rates.

Results

Oocytes treated according to MS and PB positions

Based on visualisation of MS in polarized light (Figs. 1,2) all the patients in the retrospective study were treated according to meiotic spindles and PB angles α (see Supplement. Material, Table S1). The angle α between MS and PB was used as indicator of oocyte maturation and the appropriate time for fertilization. Patients from R group were treated according to the α value as follows: patients whose oocytes had angle between MS and PB more than 30° (higher probability to be fully mature) were fertilized (ICSI) typically up to 2 hours post MS evaluation (59 patients). The patients (65) who had more than 40% of oocytes with $\alpha \leq 30^\circ$ were fertilized by ICSI after 4 hours post MS evaluation.

Pregnancy and utilization rates, all patients

Generally, R group patients whose oocytes were selected for visualization of MS prior to ICSI had higher pregnancy rates compared to those without oocytes MS visualisation (C group), 48 out of 124 R group patients, which represents 38%, became clinically pregnant. Conversely, C group patients whose oocytes were not examined for α had lower pregnancy rates representing 23 out of 90, which represents 26%. These results are in correlation with data published in ²⁷⁻³¹ where pregnancy rates observed in a standard IVF cycle (without MS evaluation) was 27%. The χ square test for comparison of R group pregnancy rates and C group pregnancy rates (26%, 90 patients) showed the following results: the chi-square value was 4.0701. The p -value was 0.043 - significant at $p < .05$. (Table 1, Fig. 3)

The average oocytes utilization rate of R group patients was 54% in comparison to 38% for C group patients. The t -value (the significance of the difference between utilization rates of R and C groups) was 4.89. The p -value was < 0.001 . The oocytes utilization rate calculated for all oocytes collected from R group patients was 47% and 31% for oocytes collected from C group patients. The value of z (the significance of the difference in utilization rates between the compared oocytes groups) was 5.59. The value of p was < 0.001 .

Table 1
Average pregnancy and utilization rates calculated for R and C groups of patients.²⁷⁻³¹ For all corresponding R and C groups the significance of the difference between pregnancy and utilization rates were calculated. The p -values were due to the size of the examined sets and considered statistically significant if $p \leq 0.05$.

	Utilization rate	p value	Pregnancy rate	p value
R group	54%	< 0.001	38%	< 0.05
C group	38%	-	27%	-

Out of total number of 531 oocytes collected in R group of patients, oocytes were divided into four categories: (1) $\alpha \leq 30^\circ$ (and 250 oocytes, α corresponds to 0-5° minutes on a clock face); (2) $\alpha = 45^\circ (\pm 15^\circ)$, (220 oocytes, α corresponds to 7.5 ± 2.5 minutes on a clock face); (3) $\alpha > 60^\circ$ (31 oocytes, α corresponds to more than 10 minutes on a clock face); and (4) immature or poorly visible MS (31 oocytes). (Figs. 1, Table S1). Development of embryos (blastocysts) after oocytes ICSI is presented in Fig. 3.

The research team also found that for oocytes with MS and PB in close proximity, after two hours the α angle began to increase (Fig. 5).

There was no relevant correlation between sperm quality and utilization rates nor significant correlation between sperm quality and pregnancy rates ($p > 0.05$). The t -value representing the significance of the difference between patient's ages of the R and C groups was - 0.5, the p -value was 0.3. The t -value representing the significance of the difference between sperm quality of the R and C groups was 0.45, the p -value was 0.33.

Pregnancy and utilization rates, patients older than 35 years

For female patients > 35-year-old, there was an increased probability of significant changes in the dynamics of the oocyte maturation process. In the case of these women the accurate determination of oocyte maturity plays an important role, specifically 34% (27/79) of women older than 35 years from the study became pregnant and their utilization rate was 53%. In the control group only 18% of women > 35-year-old became pregnant (11/69) with a utilization rate of 37%. (Table 2, Fig. 4). The *t*-value (the significance of the difference between utilization rates of the R and C groups) was 3.98. The *p*-value was < 0.001. The value of χ^2 square test for comparison of R group patients pregnancy rates and C group pregnancy rates (18%, 69 patients) showed the following results: the χ^2 -square was 6.9064. The *p*-value was 0.0086. Significant at $p < 0.05$ ²⁷⁻³¹.

Table 2
Average pregnancy and utilization rates calculated for R and C groups sub-groups (> 35 year) of patients.²⁷⁻³¹. The significance of the difference between pregnancy and utilization rates were calculated. The *p*-values were due to the size of the examined sets and considered statistically significant if $p \leq 0.05$.

	Utilization rate	p value	Pregnancy rate	p value
> 35 year R sub-group	53%	< 0.001	34%	< 0.05
> 35 year C sub -group	37%	-	18%	-

Discussion

In this study the research team demonstrated that the angle between a PB and MS can be used as valuable indicator of oocyte maturation and the selecting the appropriate time for oocyte fertilization. MS is a microtubular structure involved in chromosome segregation, and therefore it is crucial in the sequence of events leading to the correct completion of meiosis and subsequent fertilization.

The research team compared the average oocytes utilization rates and pregnancy rates of IVF patients using visualized MS (R group) and control patients treated with standard IVF without MS visualization (C group). In R group polarization microscopy evaluation of MS was done 3–4 hrs after OPU followed by evaluation of all the oocytes MS by polarization microscopy which took between 3 to 5 minutes. It is important to emphasize, that MS evaluation is an easy method to apply during the IVF process. In our research, we proceeded with ICSI in R group patients according to the value of the angle α between MS and PB. The oocytes with the $\alpha \leq 30^\circ$ could be according to ^{2,16-18} insufficiently mature (Telophase I). Therefore, the patients (53) who had more than 40% of oocytes with $\alpha \leq 30^\circ$ were fertilized by ICSI after 4 hours post MS evaluation. The rest of R group patients with higher probability to have fully mature oocytes were fertilized by ICSI typically within 2 hours after MS evaluation.

As a result of this research we also discovered that in the part of the oocytes where MS and PB were in close proximity, the angle between MS and PB began to typically increased after two hours.

The average oocytes utilization rate of R group was 54%. The average oocyte utilization rate of C group was 38%. The fact that in the R group the number of high-quality embryos was significantly higher compared to the C group had a positive effect on the probability of patient pregnancy³¹. The patients from the R group consequently had a higher pregnancy rate of 38% during one cycle of IVF, while conversely, 27% of the patients from the C group became clinically pregnant. Significantly, in group of 79 women > 35 years old, 34% became pregnant when a was evaluated and ICSI time adjusted to achieve the full oocyte maturation, compared to only 18% clinically pregnant in the control group.

According to^{27–30} the IVF pregnancy rate in one cycle is estimated to be 25–30% and this rate can be affected by several factors. Apart from the age of women, the quality of oocytes and sperm, an extremely important factor is the oocytes maturation state. This is especially significant, for females > 35 years old where there is an increased probability of significant changes in the dynamics of the oocyte maturation process^{19,22,23}.

Conclusions

The aim of this research was to identify a novel method of assessment of oocyte maturation as an indicator of oocyte readiness for fertilization. By incorporating the microscopy evaluation of oocytes we increased the probability of obtaining a high-quality embryo and significantly improve clinical pregnancy rates. Our study delivers the following findings: 1) The angle between the meiotic spindle and PB can serve as a marker for the improved timing of oocyte fertilization using ICSI. This simple and non-invasive modification of the IVF process that adds MS evaluation in polarization light can improve pregnancy and utilization rates. 2) MS evaluation based timing of oocyte fertilization resulted in a significant increase in embryo quality and positively increased the probability of a patient achieving pregnancy in future IVF steps. 3) The evaluation of MS using an optical microscope with a polarizing filter is an easy non-invasive method, that does not increase stress on oocytes being evaluated, and it does not negatively affect pregnancy and utilization rates. We believe that the above research in this study has the potential to be used in additional randomized clinical studies and to become a standard method for identifying oocyte maturation status prior to fertilization by ISCI.

Methods

Oocytes and sperm

The oocytes evaluated in this study were collected from patients between March and November 2020 in the Centre of assisted reproduction (CAR), Department of Obstetrics and Gynecology of the First Faculty of Medicine, Charles University and General Teaching Hospital, Prague 2, Czech Republic. All subjects participating in the study provided informed consent. The study was conducted in accordance with the Declaration of Helsinki. All the experimental protocols were approved by a Department of Obstetrics and Gynecology of the First Faculty of Medicine, General University Hospital, Prague and by Ethic Committee of the General University Hospital, Prague (the list is enclosed). During this research we recorded the

following two datasets: 1) Oocyte utilization rates - the number of high - quality embryos per oocyte with a detected PB ^{32,33}. 2) Patients clinical pregnancy rate – the detection of an embryonic sac during a transvaginal scan ≥ 5 weeks after embryo transfer after one cycle of IVF.

The sampled population were all patients of CAR. Patients were randomly divided into two groups before IVF/oocyte OPU: retrospective (R) and control (C). The selection criteria ensured that the results would not be affected by their number and quality. Key criteria for inclusion were, planned ICSI and a spermogram containing at least 1 mil/ml of progressively motile spermatozoa measured during previous spermogram evaluation. In both groups (R,C) oocytes with microscopically observable PB were taken into consideration. In the C group the status of the meiotic spindle was not determined before ICSI and ICSI was performed 6-8 hrs after OPU.

In the R group, after denudation at 3-4 hours after Ovum Pick-Up (OPU) the research team examined each oocyte using an optical microscope with a polarization filter in order to determine the angle between meiotic spindle and PB (α) and assessed the oocyte maturation using the standard method. MS evaluation lasted on average 3-5 minutes. R group patients with oocytes having $\alpha > 30^\circ$ (thus a higher probability to be fully mature) were fertilized (ICSI) after a shorter time after the polarization microscopy control (typically a maximum of 2 hours). In R group 69 patients were identified with oocytes having $\alpha \leq 30^\circ$ that were considered as insufficiently mature in Telophase I, and ICSI for these patients was performed 4 hours after the polarization microscopy evaluation.

We evaluated the pregnancy rates and average oocytes utilization rates (number of high-quality embryo per oocyte with detected PB) in stratified patients. Each pregnancy rate was calculated as the ratio of numbers of pregnant patients to all patients belonging to a specific group. Average oocytes utilization rates were calculated as the number of high-quality embryos ^{32,33} divided by the number of collected oocytes with microscopically detected polar bodies averaged over the number of patients from specific groups.

The cumulus oophorus complexes were cultivated in 4-well plate (NUNC) using Sage Fertilization medium [™], Origio, Denmark Sage 1-Step[™], Origio, Denmark under paraffin oil (OVOIL [™], Vitrolife, Sweden) et 37.0° C and 6% CO₂.

The oocytes were denuded (HYASE-10X[™], Vitrolife, Sweden,) after pick-up and the and the maturation stage was examined. GV stage oocyte were not included. M1 were matured similarly to cumulus oophorus. ICSI was performed to standard protocol using ICSI/holding micropipettes (#002-5-30/#001-120-30, Microtech IVF, Czech Republic), polyvinylpyrrolidone (ICSI[™], Vitrolife, Sweden), and Eppendorf (Hamburg, Germany) micromanipulation system equipped with thermoplate (Tokaohit, Japan).

We cultivated embryos for 2-5 days. Embryos were cultivated in 4-well plate (NUNC) using Sage 1-Step[™], Origio, Denmark under paraffin oil (OVOIL [™], Vitrolife, Sweden) et 37.0° C and 6% CO₂.

For both groups, high quality embryos were used for subsequent transfer or freezing defined as cleavage embryos - gr.1-3 with a blastocyst with good expansion of blastocoel cavity and the integrity of both the inner cell mass and trophectoderm cells. Blastocysts with embryoblast and trophoblast C were excluded in blastocysts^{32,33}.

The R group comprised of 124 patients between 22 and 42 years old (mean age 35 years). 77 patients from R group were >35 years old, 68 patients from R group were older than 35 years and younger than 40 years. The total number of oocytes with evaluated meiotic spindles collected from this group was 540. Evaluation of meiotic spindle was carried out 3-4 hrs after OPU. Based on the visualisation of MS in polarized light, patients in the study were divided into four sub-groups according to meiotic spindles and PB positions: 1) Patients with more than 40% oocytes with $\alpha \leq 30^\circ$; 2) Patients with 50% or more of oocytes with $\alpha = 45^\circ (\pm 15^\circ)$; 3) Patients with at least 10% oocytes $\alpha > 60^\circ$; 4) Patients with some oocytes immature or with a poorly visible MS. For the majority cases the oocytes of a single patient belonged to several groups.

The quality of sperm was also monitored so that correlation between sperm quality and utilization and pregnancy rates could be evaluated: (N) normospermia (46%); (A) asthenozoospermia (10%); (O) oligospermia (10%); (OA) oligoasthenozoospermia (4%), (OTA) Oligo-astheno-teratozoospermia (30%).

Optical microscopy

Using an optical microscope³⁶⁻³⁸ with a Nikon CEE GmbH polarizing filter, the research team could effectively image the mutual position of PB and the dividing spindle in oocytes that were collected from patients under standard conditions within the IVF cycle.

Statistical evaluation of the data

To quantify the statistical significance of the collected data, we calculated the p values as; the rates of obtaining test results that were at least as extreme as the results actually observed during the test assuming that the null hypothesis was correct (i.e., the rate that we falsely rejected the null hypothesis). This means that even though the results may show effect in terms of numbers, we assume that this is caused by random variations and that this difference is therefore not a real, statistical one. The results were controlled by using the MaxStat Pro 3.6 program and Pearson Calculator³⁹. Patients average utilization rates were calculated as average over ratios of number of quality embryos and the number of oocytes collected from each patient.

The t-value (signal to noise ratio where the signal is the difference between the sample mean and the denominator, the noise, is the standard error of the mean) as significance of the difference between the compared groups of patients was calculated using Pearson Calculator. The t-values (normal distribution) and z-value (binary distribution) were calculated for all the compared group of patients according to their

pregnancy rates, utilization rates and spindles visualisation. We also used Pearson's c square test for comparison of R group pregnancy rates and expected pregnancy rates - according to results obtained in ²⁷⁻³⁰ IVF pregnancy rates observed in one cycle of IVF. Pearson's chi-squared test was used to determine whether there is a statistically significant difference between the expected values and the observed values. The correlation between sperm quality, patient's age and both utilization rate and probability of patient's clinical pregnancy were also calculated.

Declarations

Author Contributions:

Olga Teplá, Zinovij Topurko, Martina Moosova and Eva Fajmonova - data curation,

Karel Rezabek - methodology,

Jaromir Mašata, Katerina Komrskova and Simona Jirsová- formal analysis,

Irena Kratochvilova investigation and writing—original draft preparation.

All authors have read and agreed to the published version of the manuscript.

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Figures

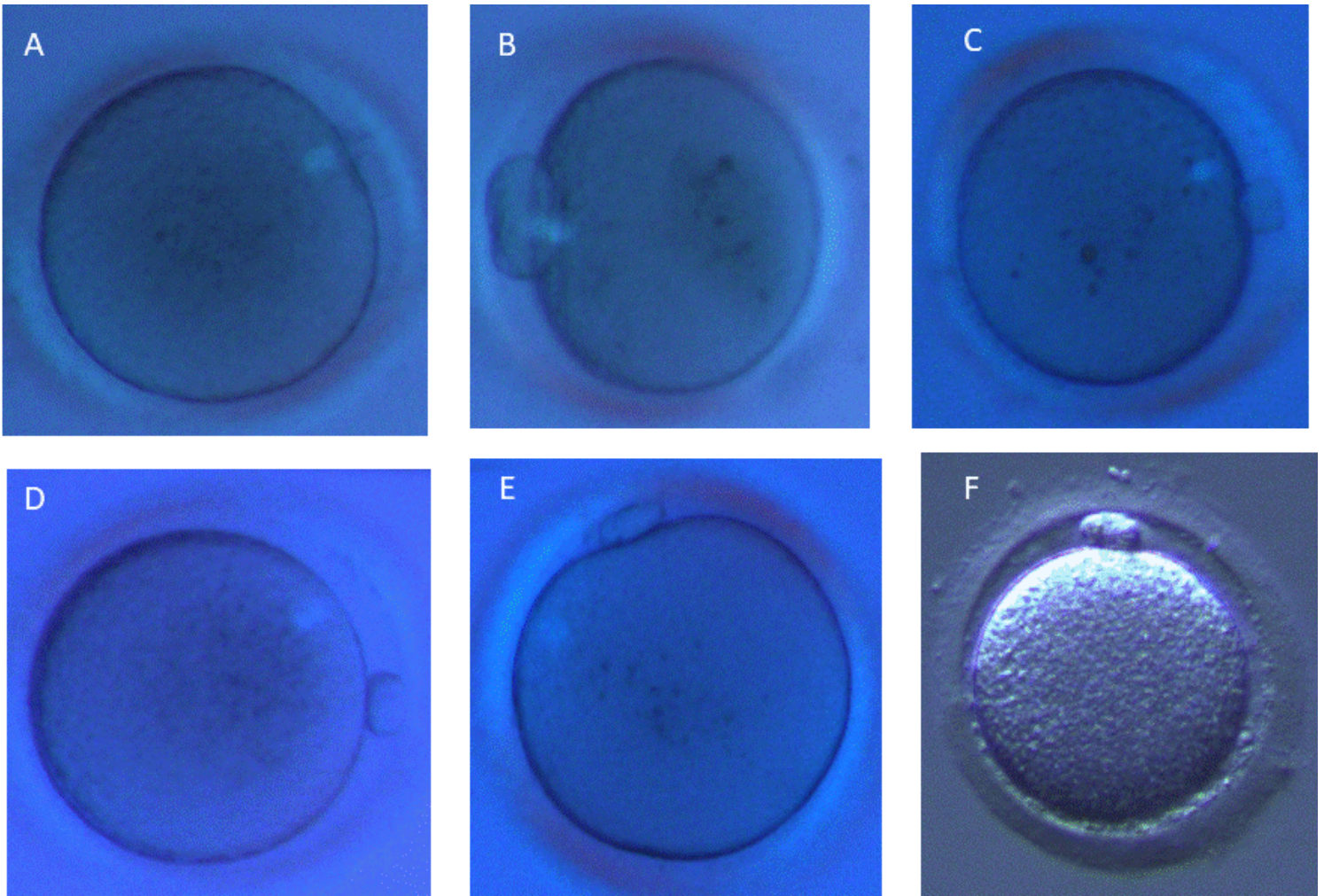


Figure 1

The example of birefringent MS of human oocytes (standard size) visualised by polarization microscopy
A-E, F: oocyte visualised by standard optical microscope.

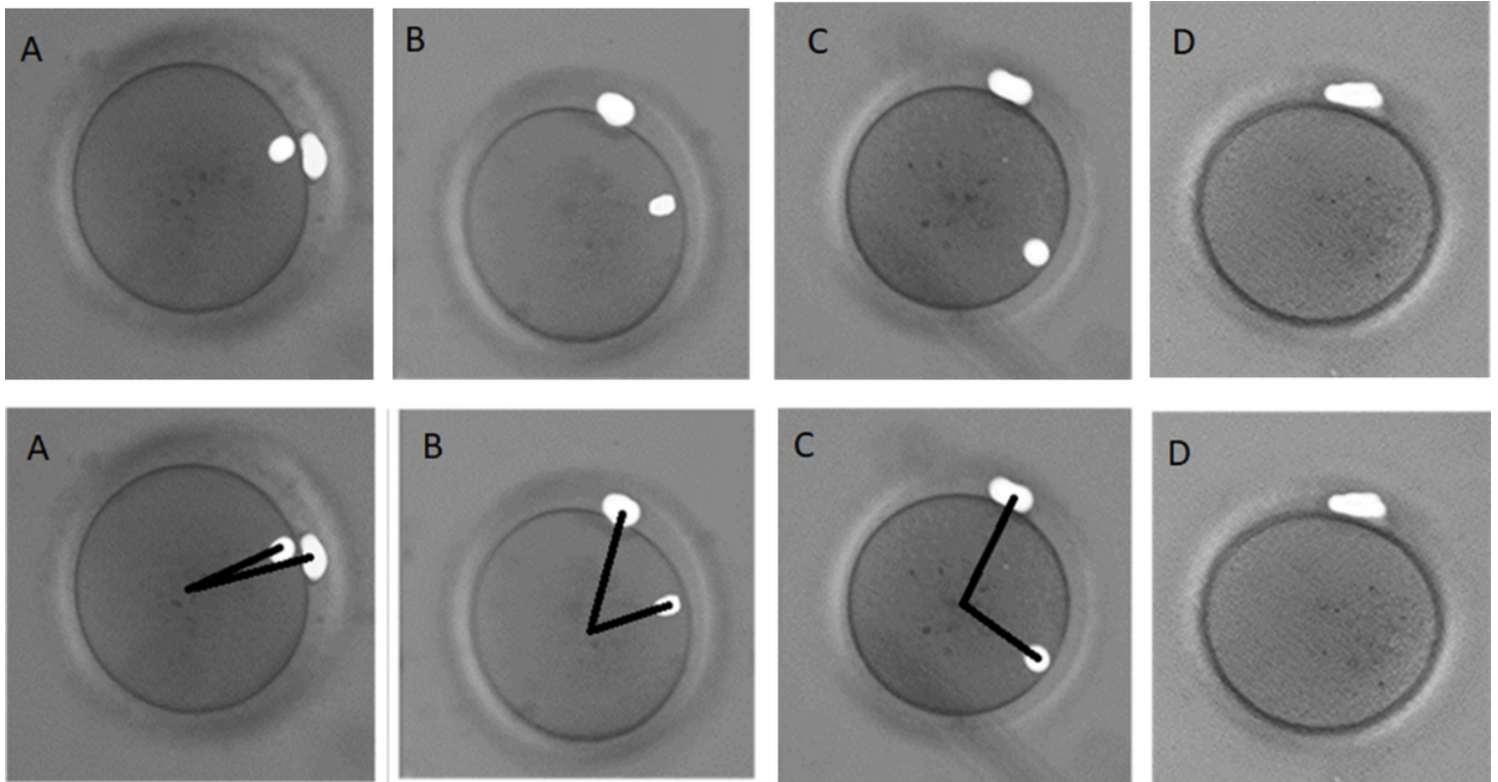


Figure 2

Visualisation of MS in polarized light and angles α between MS and PB angles α . Top: the birefringent MS obtained by polarization microscopy with white highlighted PB and MS. Bottom: the birefringent MS obtained by polarization microscopy with α angles between MS and PB illustrated in black. A (first category): $\alpha \leq 30^\circ$, B (second category): $\alpha = 45^\circ \pm 15^\circ$, C (third category): $\alpha > 60^\circ$, D (fourth category): immature or invisible MS.

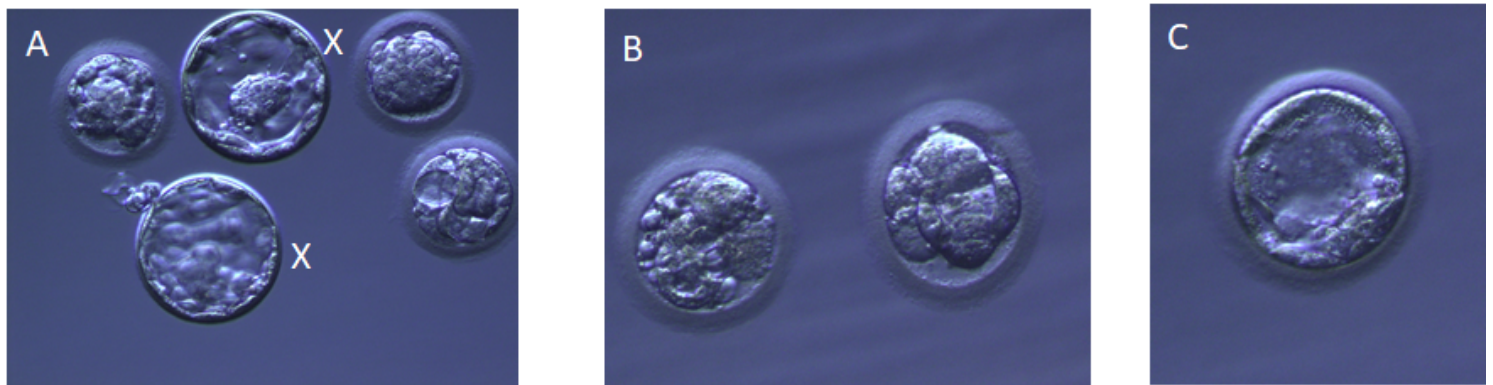


Figure 3

Oocytes after ICSI: development of embryos. A) Blastocysts developed (after ICSI) from category 2 oocytes. Those marked with an X are the most advanced blastocysts. B), C) No blastocyst developed after ICSI from oocytes belonging to categories 3,4. Representative micrographs are shown.

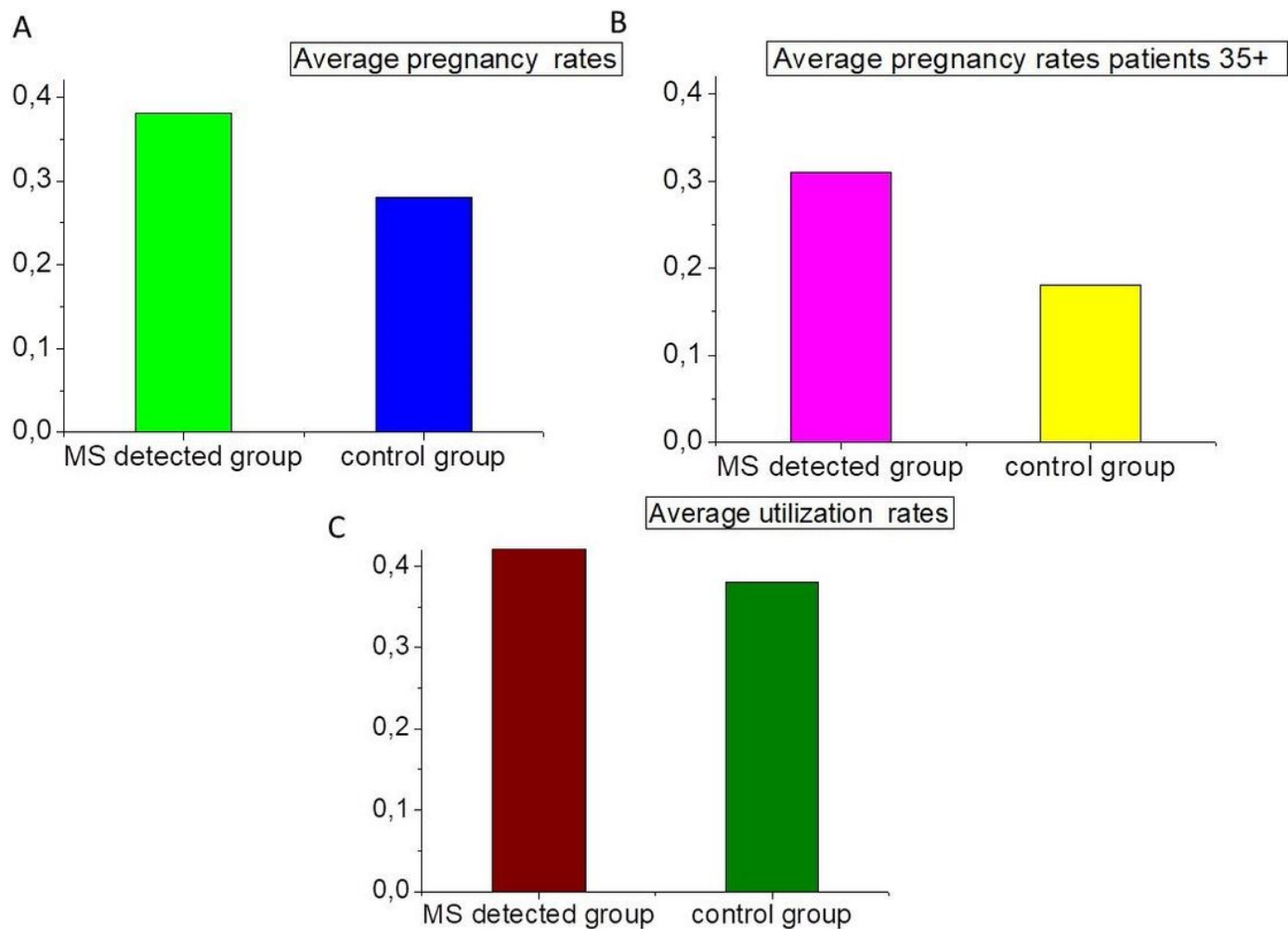


Figure 4

Average pregnancy and utilization rates calculated for R and C groups of patients. A: Average pregnancy rates calculated for all patients in R and C groups B: Average pregnancy rates calculated for > 35 year old patients in R and C groups. C: Average oocytes utilization rates calculated for all embryos and oocytes of patients in R and C groups. Each pregnancy rate was calculated as the ratio of number of pregnant patients to all patients belonging to a specific group. Average oocytes utilization rate was calculated as the number of high-quality embryos divided by the number of OPU oocytes.

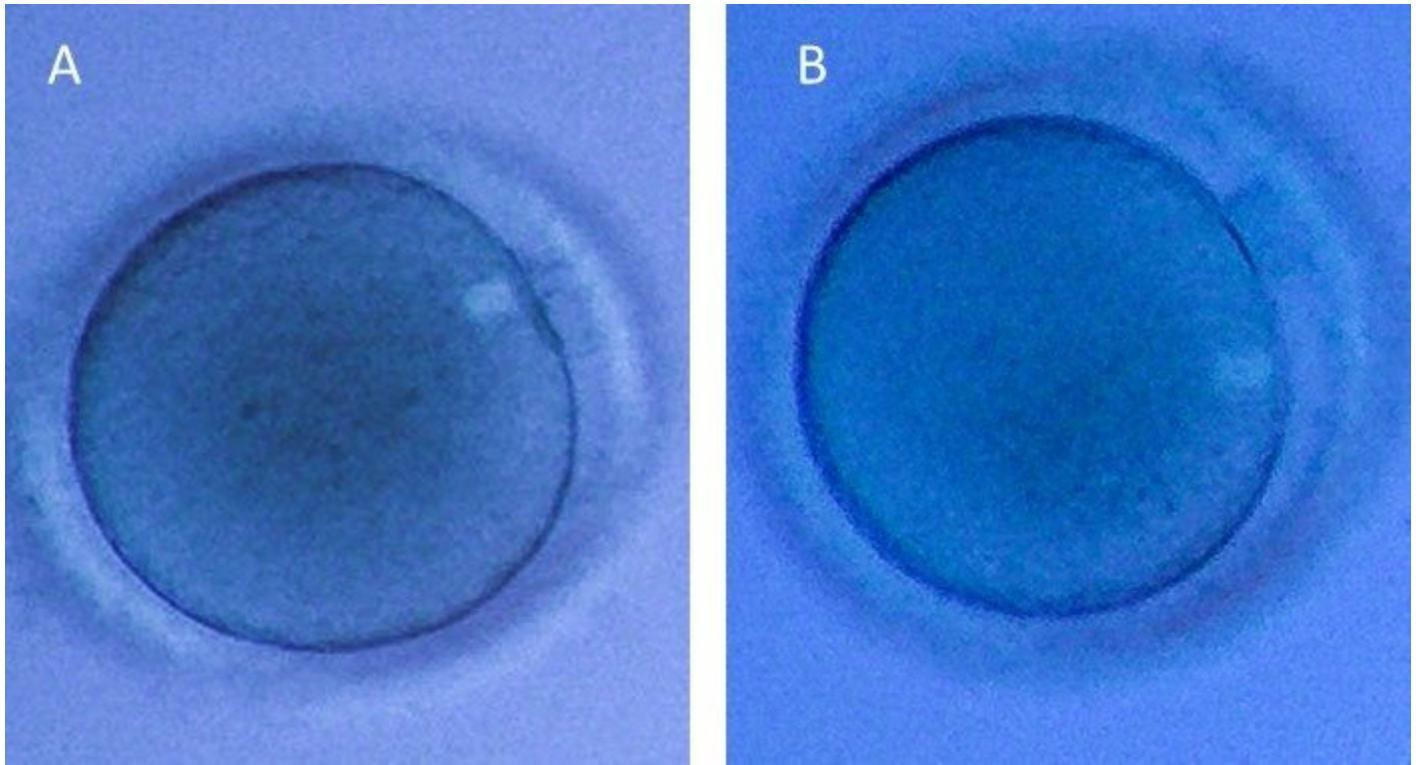


Figure 5

Images of oocytes obtained by polarization microscopy. Oocytes with MS and PB in close proximity (A), after two hours the α angle began to increase (B).

Supplementary Files

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