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Comparing the IVM laboratory outcomes between stimulated IVF with unstimulated natural cycles

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Research Article

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Summary

Recently, more attention has been raised towards fertility preservation in women with cancer. One option is *in vitro* maturation (IVM) of the immature oocytes as there is not enough time for induction of an ovarian stimulation protocol. The aim was to compare the IVM laboratory outcomes between stimulated and unstimulated (natural) *in vitro* fertilization (IVF) cycles. In total, 234 immature oocytes collected from 15 cancer patients who underwent an IVM programme (natural IVM) and 23 IVF cycles with a controlled ovarian hyperstimulation protocol (stimulated IVM) were analyzed. The oocyte morphology, zona pellucida (ZP), and meiotic spindle presence were measured using PolScope technology. Also, the rates of oocyte maturation and fertilization were assessed in both groups. The IVM rate was higher in the stimulated cycle ($P < 0.05$), but the fertilization rate was insignificant in comparison with unstimulated cycles. There were no significant differences in the spindle visualization and ZP birefringence scoring between the groups ($P > 0.05$). The oocyte normal morphology was better in the stimulated cycle compared with the natural cycle ($P < 0.05$). In conclusion, IVM can be recommended for cancer patients as an alternative treatment when there is insufficient time for conventional IVF before chemotherapy initiation.

Introduction

In vitro fertilization (IVF) is an established procedure that results in more than 390,000 births annually (Fauser *et al.*, 2014). Controlled ovarian hyperstimulation (COH) allows a cohort of oocytes to mature, often with compromised quality (Rienzi *et al.*, 2012). However, some concerns associated with COH-IVF may increase the trend towards unstimulated IVF, as the first IVF pregnancy resulting from a natural cycle (Mak *et al.*, 2016; Allahbadia *et al.*, 2020). These concerns cover infertile couples, medical personnel, and society (Nargund *et al.*, 2001). However, low oocyte yield following natural IVF is addressed in the *in vitro* maturation (IVM) programme. In IVM, oocytes are retrieved when follicles reach 14 mm and then the immature oocytes are cultured in the laboratory (Mak *et al.*, 2016).

In an assisted reproduction programme, the collected oocytes are at different stages of nuclear maturation. Although, it has been reported that the immature oocytes have the competency to develop, they are usually discarded in IVF centres (Chian *et al.*, 2004; Reichman *et al.*, 2010), mostly because of concerns about congenital abnormalities that may occur during IVM culture (Cha *et al.*, 2005; Söderström-Anttila *et al.*, 2005; Shu-Chi *et al.*, 2006). These immature oocytes may be a final chance for a cancer patient in the goal of fertility preservation (Son *et al.*, 2019) or may be considered as a 'rescue' in patients who are at risk of an hyperovarian response (Vuong *et al.*, 2019).

At present, IVM programmes can be divided into two categories: stimulated and unstimulated cycles. In the unstimulated cycles, no external gonadotrophin is administrated, and this may be useful for avoiding ovarian hyperstimulation syndrome (OHSS) (Farsi *et al.*, 2013), especially in polycystic ovary syndrome (PCOS) patients who are at risk (Lim *et al.*, 2013).

There are some studies that have investigated the IVM outcomes between unstimulated and stimulated cycles (Li *et al.*, 2006; Tang-Pedersen *et al.*, 2012). The results of unstimulated IVF cycles are restricted despite various stimulated IVM studies. Many of them compared metaphase 2 (MII) oocytes from stimulated cycles with unstimulated IVM-derived ones (IVM and IVF groups) (Child *et al.*, 2002; Li *et al.*, 2006) or the outcomes from unstimulated cycles alone (Child *et al.*, 2001). But, there has been no study outcome comparing the IVM results from two stimulated and unstimulated cycles. Also, there have been some controversial reports

regarding oocyte quality parameters and derived embryo development (De Santis *et al.*, 2005; Rienzi *et al.*, 2011; Faramarzi *et al.*, 2019).

To the best of our knowledge, there has been no study that compared the IVM outcome between stimulated and unstimulated cycles. Therefore, in this study the maturation rate, fertilization rate, oocyte morphology, zona pellucida (ZP), and presence of meiotic spindle (MS) were compared between stimulated and unstimulated IVM cycles.

Materials and methods

The data were obtained from two different sources. One part (stimulated/IVF cycle) was approved by the Ethics Committee of Rafsanjan University of Medical Sciences, Rafsanjan, Iran and followed the Helsinki Declaration of 1975 (IR.RUMS.1399.241). The second part (unstimulated IVF/IVM cycle) included data from the medical records of the patients referred to Yazd Infertility Centre for ovarian cryopreservation. According to the Ethics Committee of Yazd Reproductive Sciences Institute, there was no need for access to medical records for research purposes.

Participants

The participants were as follows: (1) Candidates for stimulated/IVF cycles. These patients underwent COH, and were assigned for intracytoplasmic sperm injection (ICSI). The immature oocytes from the mentioned group were collected after denudation. All patients signed institutional consent for participation. (2) Candidates for fertility preservation. This group did not receive any drugs for ovarian stimulation. The immature oocytes from this group were collected from ovarian tissues, as described. Data collection was carried out from 2017 to 2019, while the participants underwent treatment at our infertility centre. Cancer patients were diagnosed with a retroperitoneal tumour or teratoma in the right ovary to ovarian adenocarcinoma, pelvic soft tissue sarcoma, cervicitis with squamous metaplasia and dysplastic change (cervical intraepithelial neoplasm), cervix squamous cell carcinoma or ovarian squamous adenocarcinoma. The only haematological cancer patient was a known case of acute lymphocyte leukaemia (ALL). If two ovaries were involved, oocytes retrieval was performed and the residual ovarian cortex was discarded.

Matching the groups

COH/IVM has no main contraindication compared with ovarian cryopreservation that has evidenced-based guidelines with exact indications and criteria (Backhus *et al.*, 2007; von Wolff *et al.*, 2011; Practice Committee of American Society for Reproductive Medicine, 2014). The only criterion that restricted our participants was age: being in pubertal age and <42–43 (Backhus *et al.*, 2007; Shi *et al.*, 2017). As all the patients that were referred for ovarian cryopreservation were under the age of 35, we also selected patients < 35 years in the COH/IVM group. As our cancer patients were fertile, the cases with severe male factor and with a history of IVF failure were excluded. In addition, the cases with tubal factor infertility and with unknown causes were considered as the control.

From May 2017 to August 2019, there were 117 immature oocytes [73 germinal vesicle (GV), and 44 metaphase I (MI)] that underwent IVM, also for comparison data from 117 oocytes were used from the data bank. This data bank was created during the preceding 2 years, and the aim was to compare the efficacy of different IVM media. The same numbers of GV and MI oocytes that were matured in the same maturation medium [G2 supplemented with 75 mIU/ml follicle-stimulating hormone (FSH) and 75 mIU/ml luteinizing hormone (LH)] were selected from those IVM samples.

COH/IVM

For the COH protocol, all patients were given the multiple dose GnRH-antagonist from the 2nd day of the menstrual cycle with rFSH (Cinnal-f, Cinnagen, Tehran, Iran) or Gonal-f (Merck, Serono S., Switzerland). Once the dominant follicle (13–14 mm) was detected using sonography, GnRH-antagonist (Cetrotide: Serono A, Geneva, Switzerland), 0.25mg/day was initiated up to the day of ovulation triggering. Recombinant human chorionic gonadotropin (hCG, PD preg: Pooyesh, Tehran, Iran) was administered for final maturation when at least one follicle reached a diameter of 18 mm, 36 h prior to oocyte retrieval. In this group, follicle aspiration was carried out at 14 ± 2 days of the menstrual cycle.

On the day of ovarian puncture, when ICSI was the treatment plan, oocyte denudation was performed and the immature oocytes were collected for IVM. These oocytes were cultured in homemade IVM medium (G2, Vitrolife, Gothenburg, Sweden), supplemented with 75 mIU/ml FSH and 75 mIU/ml LH; Ferring) at 37°C in an atmosphere of 5% CO_2. At 24 and 48 h post-IVM, the oocytes were screened under a stereo microscope (Olympus, Japan) for the presence of the first polar body (PB). ICSI was performed for all matured oocytes.

Natural cycle IVF/IVM

At any time of the menstruation cycle (luteal or follicular phase), by laparoscopic incision, nearly 3 × 1.5 cm^2 of the patient's ovarian tissue was transferred to our institute [in phosphate-buffered saline (PBS) with 5% HSA]. Under the laminar flow hood, using a 20-gauge needle, all detectable antral follicles were aspirated for oocyte rescue. In this group, follicle aspiration was done at any time of the menstrual cycle. The extracts were washed in Ham's F-10–HEPES medium and searched for the presence of GV oocytes under a dissecting microscope (Figure 1A, B). Morphologically, cumulus cells in the immature oocytes from natural cycles were more compacted in comparison with stimulated ones (with expander feature) (Figure 1C, D). Mild denudation was carried out until maturation status was distinguishable (Figure 2A–C). IVM was performed as described above and simultaneously the ovarian cortex was frozen for post-treatment fertility options. All patients with partners were included for fertilization comparison. In total, 117 immature oocytes were extracted from 15 patients, while ovarian cryopreservation was performed. After injection, if fertilization resulted in embryo formation, cryopreservation was initiated using Rapid Vit Cleave (Vitrolife, Goteborg, Sweden). In the natural cycle IVF/INM, it is common to administer hCG in midcycle. As the majority of the patients needed emergency surgery, there was no time for hCG administration. None of them had received chemotherapy before.

Oocyte morphological feature

After IVM, the corona cells were removed by mechanical denudation. All MII oocytes received a morphological score according to an MII oocyte morphological scoring system (MOMS)

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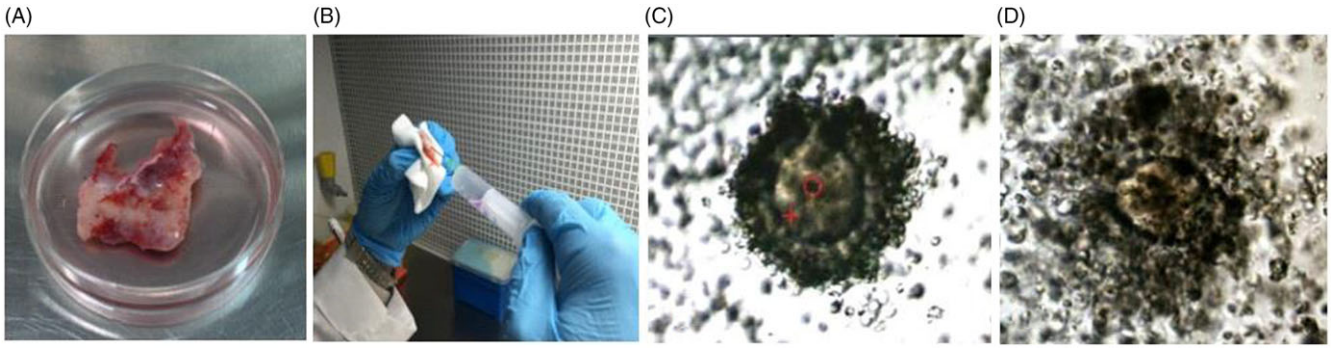

Figure 1. Oocyte extraction from ovarian tissue. (A) An ovarian biopsy. (B) Detectable follicles aspiration. (C) High dense GV oocytes from unstimulated cycle. (D) Low dense GV from the stimulated cycle, before mild denudation.

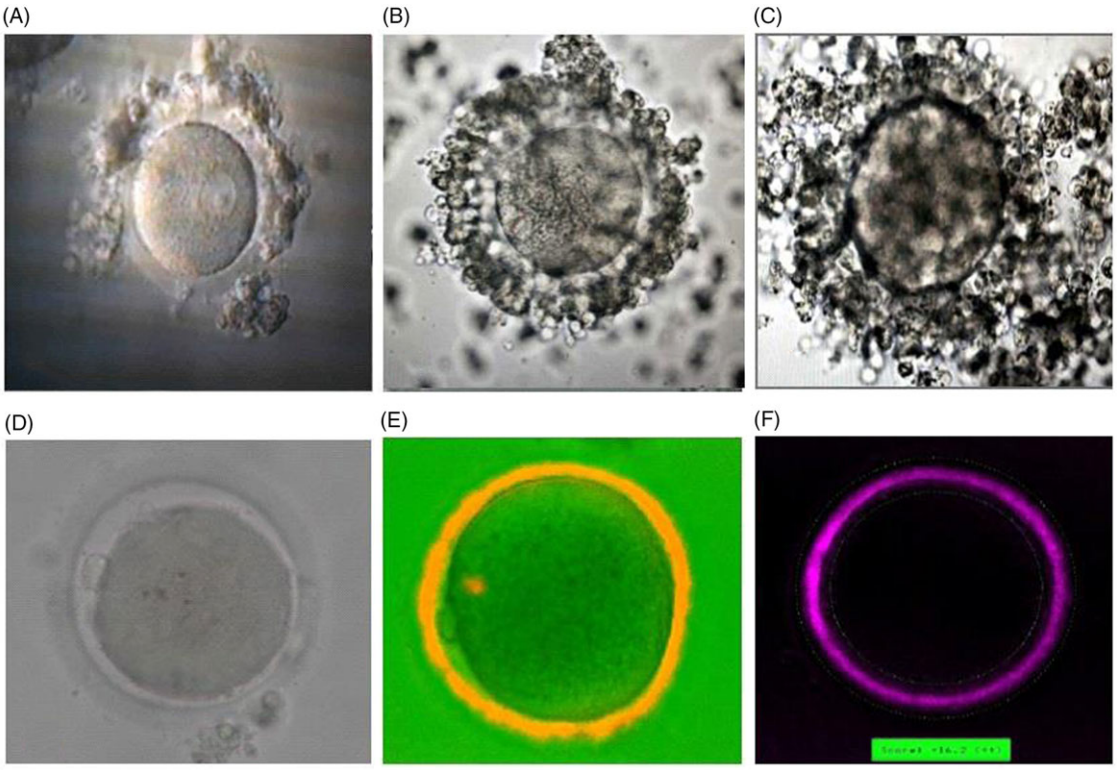

Figure 2. (A–C) GV oocytes at the start of IVM, after mild denudation, from both groups. (D–F) Oocyte morphology and structural assessment. (E) MS visualization. (F) Green ZP scoring.

(Rienzi *et al.*, 2008). The five points of this scoring system are organized under two general main parts of extracytoplasmic and cytoplasmic features. Abnormal I PB (×2), and large perivitelline space (×1.4) are categorized as extracytoplasmic; while, granular cytoplasm (×1.4), centrally located granulation (×2.7), and vacuoles (×2.1) were the components of the cytoplasmic feature. ZP and MS analysis was done under an inverted microscope (TE300; Nikon, Tokyo, Japan) equipped with a polarizing optical system (OCTAX PolarAIDE; Octax), in all matured oocytes (Figure 2D–F). This technique needed a droplet system (5 µl of buffered medium G-Mops-V1; Vitrolife) in a glass-bottomed dish (WillCo-Dish; Bellco Glass NJ, USA). The MII oocytes were loaded in these droplets and, after oocyte appearance, they were assessed for ZP birefringence and MS visualization. For ZP birefringence, the green colour was considered as high quality, yellow as moderate, and red as low quality (Figure 2E, F).

Intracytoplasmic sperm injection

Following morphology evaluation, MII oocytes were fertilized by ICSI procedure. Normal fertilization was confirmed by visualization of two distinct pronuclei and 2PB under an inverted microscope (Nikon Co, Japan) 16–18 h later. All developed embryos were vitrified for future use.

Statistics analysis

Data were analyzed using the statistical package for the Social Science version 20 (SPSS Inc, Chicago. IL, USA). Chi-squared test was run to show relationships between categorical variables (distribution of GV, MI or fertilization). For comparison of more than two categories of the dependent variable, multinomial logistic regression was used. In each table, the *P*-value was reported from chi-squared test, if it had two variables, but if it contained three and

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Table 1. Comparison of maturation and fertilization rates of rescued immature oocytes

Variable	Unstimulated	Stimulated
Female age	26.5 ± 4.8	24.4 ± 5.7
Immature oocyte	117	117
Maturation rate	64 (54.7)	81 (69.2)[a]
Fertilization rate	44 (37.6)	49 (41.9)
Spindle visualization	25 (39.1)	40 (49.4)

[a]Chi-squared test, the difference was significant.

Table 2. Maturation rate in two IVM groups

Group	Immature oocytes	Mature (%)	Immature	Degenerate
Unstimulated	117	64 (54.7)	40 (34.2)	13 (11.1)
Stimulated	117	81 (69.2)[b]	31 (26.5)	5 (4.3)[b]
P-value		0.03[a]		

[a]Multinomial logistic regression.
[b]Multinomial logistic regression also showed significantly higher degeneration rate in the unstimulated rather than stimulated group.

more, the *P*-value was calculated from running multinomial logistic regression (whenever the result from the chi-squared test was significant). A *P*-value < 0.05 was considered as statistically significant. Independent sample *T*-test was used to compare the mean of the quantitative variables.

Results

As all cancer patients were married, the matured oocytes were fertilized and embryos were vitrified at the day 2 stage. All zygotes from IVF cycles were discarded, as the centre fertility policy is to cancel IVM-derived embryo transfer. A mean of 7.8 oocytes was retrieved from each cancer patient. Here, 73% of the derived immature oocytes were GV and 37.6% were MI ($P < 0.001$) (Table 1). Also, 117 oocytes in each group were analyzed, and multinomial logistic regression analysis showed a significantly higher maturation rate and lower degeneration rates in stimulate in comparison with unstimulated cycles after IVM (Table 2).

Oocyte morphology

Before IVM, the quality of the oocytes was lower in the natural cycles. The cumulus cells were more compact with dark cytoplasm in comparison with immature oocytes from stimulated cycles (Figure 1C, D). After 48 h culture, oocyte denudation was difficult, and more pipetting and timing were needed for granulosa cell removal. According to MOMS, each MII was scored as 0 or 1 for five mentioned points (if all abnormalities were observed, the score would be 9.6). Finally, each oocyte had one morphology score. Data showed better oocyte morphology in stimulated in comparison with unstimulated cycles (Table 3). In addition, data showed that fertilized oocytes had a significantly better morphology score (Table 4; $P < 0.05$). As an indirect marker of oocyte quality, the ZP birefringence differences were insignificant between the groups (Table 5).

Discussion

Our analysis of 117 immature oocytes from the natural cycle showed a high power of reinitiating and completion of maturation, as well as the immature oocytes derived from COH. This is very promising for cancer survivors who desire fertility preservation, while the time is so limited. Although higher maturation rate was noted from IVM in the stimulated cycles, this was not accompanied by a higher fertilization rate in comparison with the natural cycle. This confirmed previous studies that one of the IVM limitations is asynchrony between the cytoplasm and nuclear maturation (Combelles *et al.*, 2002). Apparently, it is present in the immature oocytes derived from the stimulated cycles, while the aim is to retrieve more oocytes throughout ovarian hyperstimulation.

A higher rate of oocyte maturation in COH/IVM is more about extrinsic oocyte appearance and not an intrinsic event that involves organelle reorganization and storage of proteins, mRNAs and transcription factors that conduct overall maturation, fertilization and early embryogenesis processes (Ferreira *et al.*, 2009). Another study also mentioned that nuclear maturity is more accessible through *in vitro* culture, rather than cytoplasmic maturation (Combelles *et al.*, 2002). COH immature oocytes have undergone stimulation during the routine IVF protocol, and later in the culture medium they convert to the mature state, faster than natural cycle immature oocytes. This means more nuclear maturity and no cytoplasmic maturity, while this was not accompanied with a higher fertilization rate. This is in accordance with the previous studies that refer to the cytoplasmic maturation process as a key component of maturation, fertilization and early embryogenesis (Ferreira *et al.*, 2009; Lowther *et al.*, 2009; Trebichalská *et al.*, 2021). Cytoplasmic maturation consists of a variety of metabolic and structural events that guarantee normal fertilization, mitotic cell division, and accurate genetic and epigenetic pathways that lead to normal embryonic development (Trounson *et al.*, 2001). In an ultrastructural study on unfertilized human oocytes, it was found that cytoplasmic maturation determined the oocyte competency for normal fertilization and embryo development. Also, it showed that major cytoplasmic reorganization occurs before first PB extrusion, and the most prominent cytoplasmic event was organization of heterologous complexes composed of variable elements, such as endoplasmic reticulum and multiple mitochondria (Trebichalská *et al.*, 2021).

One reason for this asynchrony in nuclear and cytoplasmic maturation may be related to suboptimal culture medium (Walls and Hart, 2018). As maturation rate is higher but fertilization rates are the same in the one culture medium, ovarian stimulation might have exaggerated this asynchrony in maturation during the IVM process. In fact, a higher maturation rate in the COH immature oocytes may have been completed only in the nucleus (meiosis) and not paralleled in the cytoplasm, which is essential for oocyte activation and fertilization. Also, COH immature oocytes could not complete their maturity, despite receiving some stimulation and triggering hormones.

One of the limitations of this study was that we followed the IVM process until the zygote stage due to ethical issues, while the future development competency may be completely different in the two groups. One advance in the natural cycles IVM programme is biphasic IVM. In this new method, in addition to keeping intact physical contact between oocyte and surrounding cumulus cells, for paracrine signalling communication, it tries to maintain the oocyte (GV) in the meiotically arrested stage, and create an environment (in the pre-IVM step) that facilitates the

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Table 3. Mean score for oocytes morphology (%)

Group	Abnormal I polar body	Large perivitelline space	Granular cytoplasm	Centrally located granular area	Vacuoles	Mean total score
Natural cycle	31 (26.5)	37 (31.6)	33 (28.2)	29 (24.8)	22 (18.8)	2.4 ± 2.3
IVF cycle	26 (22.2)	40 (34.2)	27 (23.1)	23 (19.7)	19 (16.2)	2.11 ± 1.98
P-value	0.44	0.67	0.37	0.34	0.60	0.26[a]

[a]Independent sample *T*-test.

Table 4. Comparison between mean morphology score and fertilization rate

	Fertilized oocytes	Unfertilized oocytes
Number of oocytes	94(40.2)[b]	51(21.8)
Mean morphology score	1.8 ± 2.08[a]	2.5 ± 2.4
P-value	0.03[c]	

[a]Mean ± standard deviation (SD).
[b]Percentage.
[c]Independent sample *T*-test.

Table 5. ZP birefringence scoring in two groups of unstimulated and stimulated cycles

Zona pellucida colour	Unstimulated	Stimulated
Green	40 (62.5)	46 (56.8)
Yellow	14 (21.9)	20 (24.7)
Red	10 (15.6)	15 (18.5)
P-value	0.78[a]	

[a]Chi-squared test.

achievement of developmental competence for the oocyte over 24 h, while creating the conditions that mimic the post-LH surge follicular environment for initiation and progression of meiosis. This method assumed an increase in synchronous maturation and MII formation rates (De Vos *et al.*, 2021; Kirillova *et al.*, 2021).

Also, the results showed more viable oocytes in the immature COH during the 48 h culture. This result was, to some extent, predictable because of the low-quality features of the natural cycle oocytes. As mentioned above, pre-culture COC in the NC immature oocytes were more condensed and generally appeared darker in comparison with COH. This is the first time that IVM outcomes were compared between COH and NC immature oocytes. Previous studies concluded that immature oocyte quality is relevant to survival rates and clinical outcomes (Khalili *et al.*, 2013; Son *et al.*, 2019). However when the dark cytoplasm was analyzed as an individual point, it was found not to have predictive value for *in vitro* or *in vivo* parameters (Esfandiari *et al.*, 2006). Another study reported compromised embryo quality following the development of the dark cytoplasm oocytes (Ten *et al.*, 2007). In porcine oocytes, it showed that the cumulus complex feature is more related to cytoplasmic maturation rather than nucleus maturation (Alvarez *et al.*, 2009). So, lower quality at the time of retrieval can affect subsequent development.

In addition, more data analysis showed higher spindle visualization in the stimulated group, compared with the unstimulated group. Under normal conditions, there is synchrony between cytoplasmic and nuclear maturation. So, it is not inconceivable that spindle visualization was lower in the unstimulated cycle rather than the stimulated one. Whereas the oocytes in the natural cycles are not influenced by the extrinsic factor, so this synchrony is more preserved. LH mediates some changes in the oocyte nucleus in addition to the cytoplasm, such as oocyte meiosis resumption, GV breakdown, completion of metaphase I, and spindle formation and alignment (Coticchio *et al.*, 2004; Arroyo *et al.*, 2020). Whereas immature oocytes in the natural cycles, which may have been collected in the follicular phases (before LH surge), have not been influenced by the natural LH surge (mid natural cycle), but oocytes in the stimulated cycle undergo complete ovarian hyperstimulation. However, it must be mentioned that spindle visualization alone does not necessarily convey the information of chromosomal status (euploidy vs aneuploidy), We had to use noninvasive evaluation methods, when possible, as one part of the study's sample had treatment application, and cytoplasmic morphological evolutions are not enough evidence of the chromosomal status. Also, the patients must be aware of the fact that IVM may be accompanied by some risks (aneuploidy or imprinting), or be aware of preimplantation genetic diagnosis (PGD) or other chances for detecting these abnormalities.

Although spindle visualization may be influenced by ovarian stimulation hormones, the fertilization rate was not influenced. It was reported that the number of retrieved oocytes, fertilization rate, and the total number of frozen oocytes and embryos were the same in the luteal and follicular phases in the unstimulated IVM process (Mamam *et al.*, 2011). The mean total morphology score of the natural cycle was higher, which means lower oocyte quality, according to MOMS. The stimulated immature oocyte had received some stimulation drugs, however, this did not result in maturity (MII stage). This deficiency for complete maturation after undergoing stimulation may be one explanation for the same fertilization rate, despite higher maturation rate, in comparison with natural cycles. Stimulation protocols increase the incidence of full cumulus expansion and lose the attachment between COC and the follicular wall, subsequently resulting in easy oocyte aspiration (Trounson *et al.*, 1994). However it did not result in maturity, but stimulation drugs could improve oocyte quality. Data also showed that the fertilization rate was higher when morphology was better. In this study, COH immature oocytes had better morphology and higher fertilization rate; but the result was not significant for fertilization. Our study was the first study with low sample size that compared fertilization rates between COH and NC-IVF. However, more studies are needed to confirm our findings. Using the MOMS system for oocyte morphology determination, a higher fertilization rate was reported in which a better morphology score was recorded (Rienzi *et al.*, 2008).

Oocyte IVM programmes at this time are increasingly used in the ART laboratory, using commercially media that are produced especially for this purpose, with good outcomes (Fesahat *et al.*, 2017)

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for the patients for whom the immature oocytes may be the final chance for them. In addition, there are some studies that did not find any priorities for the commercial media, in both stimulated and unstimulated cycles (Moschini *et al.*, 2011; Pongsuthirak *et al.*, 2015). Some good outcomes have been reported for the non-commercial media, so in this study we used G2 medium as the base medium for *in vitro* maturation. The other reason for this selection was the limited expiry date of commercially media, plus they are costly and not routinely used, in comparison with the standard IVF media in the infertility centres. Also G2 is an available medium in any ART laboratory, and it must be mentioned that some family planning (FP) cases must be done, as soon as possible, without any delay! In this case a cleavage embryo medium can substitute, especially when the IVM programme is not routinely performed.

In conclusion, the data showed that IVM from natural cycles can perform as well as stimulated cycles. It is a promising approach for many patients, especially for FP in cancer patients who have no time for ovarian stimulation before chemotherapy. Also, it is an optional treatment for women who want to postpone childbearing, PCOS, or oocyte donor cycles.

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Conflict of interest. There is no any conflict of interest to be declared.

Ethical standards. The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008 and the authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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