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*Research Article*

# The Consequence of Short Insemination Strategy on Sperm Biological Characteristics, Embryo Morphokinetics, and Clinical Outcomes in the IVF Program

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Overnight coincubation of the cumulus–oocyte complexes (COCs) with spermatozoa may be accompanied by overproduction of reactive oxygen species (ROS) and possible determinant effects on sperm cellular status in the in vitro fertilization (IVF) program. The COCs from 100 cases were divided into two groups of short (2 hr) and long (18 hr) inseminations. The malondialdehyde (MDA) level as by-product of ROS was assessed in the insemination medium. The sperm DNA integrity, the mitochondrial membrane potential (MMP), and acrosome reaction (AR) were evaluated using sperm chromatin dispersion (SCD), fluorescence stain of JC-1, and double staining, respectively. Normally, fertilized oocytes ($n = 525$) were assessed via time-lapse monitoring (TLM) for assessment of the time of fading (tPNF), 2 until 8 timing (t2-t8), s1 (t2-tPNF), s2 (t4-t3), s3 (t8-t5), and duration of cell cycles. Finally, the best embryos were transferred, and clinical outcomes were assessed. Higher rates of MDA concentration, DNA fragmentation, and AR and low rate of MMP were noticed in long compared to short insemination groups ($p \leq 0.0001$). The morphokinetic parameters showed five-cell stage (t5) and cell cycle 3 (cc3) that were significantly different between the groups ($p = 0.04$ and $p = 0.03$, respectively). A high level of ROS observed in the long insemination group might have a detrimental effect on sperm status. Although, similar embryo quality and clinical outcomes were noticed in two insemination groups, but the trend was toward the short insemination.

## 1. Introduction

In clinic settings, the standard in vitro fertilization (IVF) technique utilizes incubation of oocytes with spermatozoa in culture media during the night [1]. However, it has been suggested that long-term coincubation of oocytes with spermatozoa (16–26 hr) is correlated with excessive production of reactive oxygen species (ROS) [2]. Since the majority of oocytes are fertilized within 1 hr following insemination, there may be a beneficial effect in reducing the gamete contact, defined as short or brief coincubation [3].

Some studies declared that short coincubation is associated with significantly better clinical outcomes than long insemination [4, 5]. However, others reported no significant differences in the rates of normal fertilization or good quality embryo development [6, 7]. Therefore, the beneficial effects of this reduction time are still a debatable issue, and an in-depth study on the effects of short insemination on sperm cell function and embryo development is warranted.

Pathological effects of sperm cells that are associated with long insemination at the cellular level include mitochondrial defects, DNA damage, and premature acrosome reaction (AR) [8]. Impairment of mitochondrial function has also been associated with declining sperm motility over time [9]. Furthermore, high mitochondrial membrane potential (MMP) and DNA integrity in spermatozoa have been related

to higher progressive motility and fertilization rates after IVF [8, 10]. DNA integrity can be evaluated via sperm chromatin dispersion (SCD) test, and AR could be used to evaluate the embryo development in vitro [10].

Recently, time-lapse monitoring (TLM) has been recognized as an advanced device for the investigation of embryo morphokinetic in a stable culture environment [11]. TLM allows researchers to interpret the sequence of postfertilization events, identification and record the appearance and fading of the PNs, fragmentation, and blastocoel collapse [12].

The main purpose of this study was twofold: first, to survey the relevance between production of ROS (MDA) in insemination protocols and essential indicators of sperm fertility potential, including MMP, DNA integrity, and AR and, second, to assess the role of insemination time on the morphokinetics of embryo development. We propose that a comprehensive evaluation of embryo development and selection according to TLM algorithms may provide valuable information about insemination protocols in the IVF cycles.

## 2. Materials and Methods

*2.1. Participants.* This study included 100 couples that were enrolled to Yazd Reproductive Sciences Institute for IVF from July 2019 to March 2020. Nineteen cases, including total fertilization failure or with poor quality embryos, were excluded from the study. The inclusion criteria were female factors with age ≤38 years and a normal semen analysis from men without diabetes or smokers.

*2.2. Semen Analysis and Oocyte Preparation.* Semen were incubated at 37°C for 15 min; after that, sperm concentration, motility, viability, and morphology were determined based on the World Health Organization (WHO) 2010 criteria. The sperm samples were prepared via the direct swim-up technique as before study [13]. The motile sperm concentration to the recommended range was 5–20 million sperm per mL [14].

*2.3. Ovarian Hyperstimulation.* Gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide®; Merck Serono, Darmstadt, Germany) with recombinant follicle-stimulating hormone (FSH) (150–225 IU/day, rFSH; Gonal-F®; Merck Serono, Switzerland) was used for first stage of ovarian stimulation. When the diameter of at least one follicle reached 18 mm, recombinant human chorionic gonadotropin (rhCG) (Ovitrelle®; Merck Serono, Germany) was used for final maturation. The cumulus–oocyte complexes (COCs) were collected in a fertilization medium (Life Global®, Brussels, Belgium). After 3 hr, the COCs were randomly divided into short and long insemination groups.

*2.4. The Short and Long Insemination Procedure.* The insemination procedure was done by adding 100,000 progressively motile sperm cells in 1 mL of the fertilization medium (Life Global®). The insemination droplet was 100 $\mu$L and four expanded COCs were transferred to each droplet [1]. In the short group, COCs were transferred to a new medium without the presence of sperm after 2 hr. In the long or conventional group, the COCs remained in the fertilization medium for 16–18 hr. After this period, zygotes were individually transferred into media in pre-equilibrated Primo Vision™ culture dishes (Vitrolife, Sweden) at 37°C in 6% $O_2$, and the insemination droplets containing spermatozoa were collected from both groups.

*2.5. Time-Lapse Monitoring System.* TLM was recorded every 10 min in seven focal planes for 3 days. Individual morphokinetic variables included the pronuclei fading (tPNf) and formation of two cells (t2), three cells (t3), four cells (t4), five cells (t5), six cells (t6), seven cells (t7), and eight cells (t8). Also, calculated morphokinetic variables were evaluated according to s1 (t2-tPNF), s2 (t4–t3), s3 (t8–t5), and duration of the second cell cycle (cc2a = t3–t2, cc2b = t4–t2) and third cell cycle (cc3a = t5–t4, cc3b = t6–t4, cc3c = t7–t4, cc3d = t8–t4). The rate of embryo arrest and cleavage anomalies was calculated, including uneven blastomere size at two-cell stage, multinucleation in blastomere at two- and four-cell stage, direct cleavage (DC), reverse cleavage (RC), and atypical phenotypes as presence of fragmentation and vacuoles [15].

*2.6. Malondialdehyde (MDA) Assay.* The insemination medium was individually collected from short and long groups. Samples were centrifuged for 7 min at 2,500 rpm, and the supernatant was kept at −20°C. After warming, the samples were assessed using an MDA kit (ZB-MDA-96, ZellBio GmbH, Germany). According to instructions, 50 $\mu$L standard and samples with Reagent 4 were added to test tube. After adding almost 1 mL of chromogen solution (prepared according to the protocol), the solution was heated for 1 hr in a boiling water bath. Finally, the tubes were centrifuged at 4,000 rpm for 10 min. Absorbance of the supernatant was read on a spectrophotometer at 535 nm (Microplate Reader, BioTek, USA).

*2.7. Mitochondrial Potential Evaluation.* For evaluating sperm mitochondrial potential, a fluorescent cationic dye, 5, 5′, 6, 6′-tetrachloro-1-1′,3,3′-tetraethyl-benzami-dazolocarbocyanin iodide (JC-1), was used. In this assay, 20 $\mu$L of sperm suspension was incubated with 20 $\mu$L of JC-1 solution (concentration of 50 times diluted) for 30 min at 37°C. The cell suspension was centrifuged at 400 g for 5 min at room temperature (RT) in dark. After supernatant removal, the spermatozoa were observed using fluorescence microscopy (Olympus Co., Tokyo, Japan) at 1,000x magnification. If the cells had healthy mitochondria, JC-1 formed complexes seen intense red fluorescence known as J-aggregates. In cells with low MMP, JC-1 remained in a monomeric form and emitted green fluorescence [16].

*2.8. Sperm Chromatin Dispersion (SCD) Assay.* The rate of sperm fragmentation was evaluated by the Halosperm kit (SDFA kit, Tehran, Iran). Briefly, 20 $\mu$L of the sample and low-melting agarose mixture were added on the precoated glass slide. A coverslip was placed on the sample and then gently removed after 5 min at 4°C. Denaturing solution (for 7 min) and lysing solution (for 15 min) were poured on slides at RT. Subsequently, the slides were washed with distilled water, followed by dehydrating with ethanol solutions of 70%, 90%, and 100%, each for 2 min. Finally, the slides

were stained with solutions C, D, and E for 75 s, 3, and 2 min, respectively. A minimum of 200 spermatozoa were analyzed by bright-field microscopy. Spermatozoa were considered with minimal DNA fragmentation, if large or medium halos formed, while fragmented DNA was associated with small or no halos [17].

*2.9. Acrosome Reaction Staining.* Sperm suspension was fixed 1 : 1 with 3% glutaraldehyde (SERVA, Heidelberg, Germany) in 0.1 M cacodylate buffer. The glutaraldehyde was then removed by centrifugation at 1,000 *g* for 3 min twice. Aliquots of spermatozoa were transferred on slides and dried at RT. The spermatozoa were stained by 0.8% of Bismarck brown Y (B-2759, Sigma-Aldrich) for 10 min at 40°C, followed by washing with distilled water. Finally, they were incubated in 0.8% rose bengal 0.1 M Tris (R-3877, Sigma-Aldrich) for 40 min at RT, washed in distilled water, dehydrated in alcohol gradients (50%, 70%, 96%), and mounted with a coverslip [18]. Following staining, the intact acrosome showed a pink acrosomal region, while the white acrosomal region was a sign of degeneration.

*2.10. Fertilization Evaluation and Embryo Morphology.* For both groups, the oocytes remained in fertilization medium for 16–18 hr. Afterward, fertilization assessment was followed by the appearance of two pronuclei (2 PN). If more than two pronuclei were seen, they were considered as polyspermy. Normally formed embryos were allowed to develop and scored as A and B (top quality) or C and D (poor quality) [19].

*2.11. Morphokinetics and Embryo Selection for Transfer.* Embryo selection was performed according to the TLM algorithm. Embryos with direct cleavage, uneven blastomeres at the two-cell stage, and multinucleation at the four-cell stage were deselected (grade E). Based on t5, s2 (t4–t3), and cc2 (t3–t2) scores, the remaining embryos were divided from A to D grades. If t5 was between 48.8 and 56.6 hr and s2 occurred less than 0.76 hr, the embryo was categorized as grade A. If the embryo was in the correct range of t5, but s2 was out of range, the embryo was grade B. If the embryo was out of range t5, but s2 was less than 0.76 hr, the embryo was considered as grade C, and if both were out of two times, grade D was recorded. Positive or negative grades were given based on cc2 time points. The best TLM scored embryos were then either selected for transfer or vitrified.

*2.12. Clinical Outcomes.* Biochemical pregnancy is determined if the bhCG test is positive, 2 weeks after transfer. Clinical pregnancy was approved by the presence of gestational sacs with fetal heartbeat detected by transvaginal ultrasound examination 6 weeks posttransfer. The number of patients who achieving a delivery after 30 weeks was calculated for live birth rate [20].

*2.13. Data Analysis.* Normal distribution was assessed with a Shapiro–Wilk test. Data are shown as mean ± standard deviation (SD) for normal numerical variables, median ± interquartile range (IQR) for nonnormal, and percentage for categorical variables. Sperm parameters were compared by paired-samples *t*-test for parametric and Wilcoxon paired test for nonparametric data between two groups. Due to the large sample size of embryo number, morphokinetic variable was considered as a normal distribution; however, the independent-samples *t*-test was used for comparing morphokinetic embryos. Also, differences between proportions outcomes were computed using $\chi^2$ test. Odds ratio (OR) with two-sided 95% confidence interval (CI) was estimated for dichotomous outcomes using a random-effects model. *p*-value <0.05 was considered as statistically significant. The Statistical Package for Social Science software 22 was used for analysis (SPSS Inc., Chicago, IL, USA). Graphs were drawn by the Graph Pad Prism 8 software (Graph Pad Software, Inc., La Jolla, CA, USA).

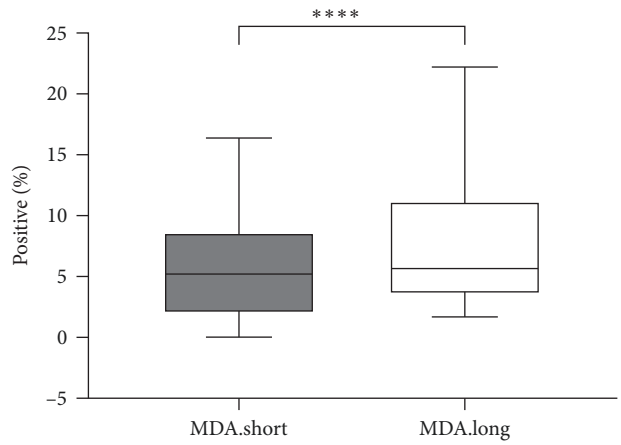


FIGURE 1: Comparison of MDA concentration between two groups (****$p \leq 0.0001$). Data were presented with median ± interquartile range (IQR). Data were compared using Wilcoxon paired test.

## 3. Results

The mean female and male ages were 30.3 ± 4.5 and 34.09 ± 5.2 years, respectively. The mean of semen parameters of concentration, progressive motility, nonprogressive motility, and immotile spermatozoa were 79.01 ± 30.5 million/mL, 49.33 ± 9.6%, 10.61 ± 2.41%, and 40.04 ± 9.61%.

*3.1. Comparison of Sperm Status between Two Groups.* The lipid peroxidation levels in culture media showed a higher rate of MDA concentration in long as compared to short insemination groups (6.16; IQR: 4.2–11.11 vs. 5.25; IQR: ±2.8–8.68, respectively) ($p \leq 0.0001$) (Figure 1). Also, the mitochondrial activity was shown a higher significant difference in the rates of $\Delta\Psi$m in short than long insemination groups (82; IQR: 74–90 vs. 76; IQR: 70.5–85.5) ($p \leq 0.0001$) (Figure 2).

In addition, a significant difference in the rates of sperm DNA fragmentation between the two groups was noted. The rate of DF was lower in short insemination (13.82 ± 0.57) compared to long insemination (16.77 ± 0.63) groups ($p \leq 0.0001$) (Figure 3). There were also significant differences in the rates of sperm AR between the groups ($p \leq 0.0001$). Figure 4 shows that the rate of AR was lower in the short

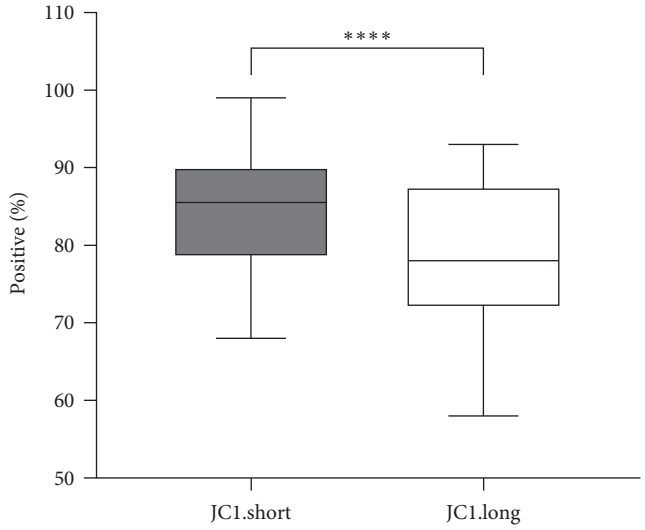


Figure 2: Comparison of mitochondrial membrane potential ($\Delta\Psi$m) concentration between two groups (****$p \leq 0.0001$). Data were presented as median ± interquartile range (IQR). Data were analyzed using Wilcoxon paired test.

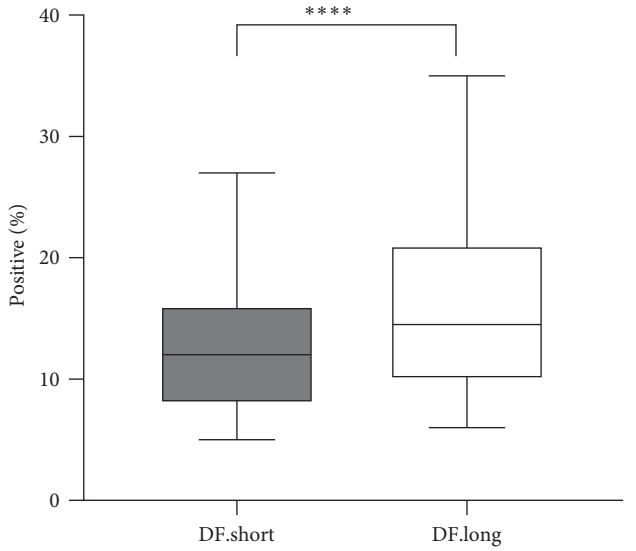


Figure 3: Comparison of sperm DNA fragmentation (DF) between two groups (****$p \leq 0.0001$). Data were presented as mean ± SD. Data were analyzed using paired-samples *t*-test.

(26.16 ± 0.57) compared to long insemination groups (31.91 ± 0.61).

*3.2. Comparison of Fertilization, Embryo Quality, and Clinical Outcomes.* Eight hundred and forty-five mature oocytes (MII) resulted from a total of 932 collected COCs, with 404 MII oocytes in the short and 441 MII oocytes in the long insemination groups. Three hundred and forty-three MII oocytes (77.29%) were normally fertilized, and 316 (76.53%) embryos were derived in short insemination group. Wilcoxon test showed that the rate of fertilization was similar between the groups ($p = 0.82$). Also, there were no significant differences regarding polyspermy rates between the

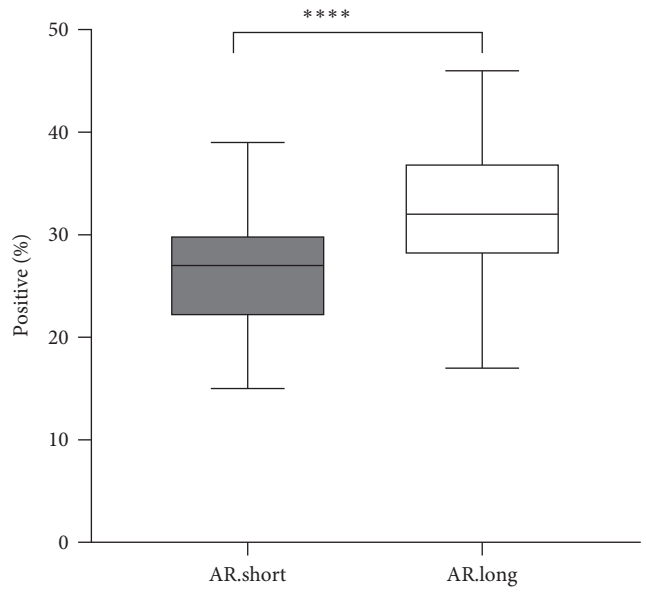


Figure 4: Comparison of acrosome reaction (AR) between two groups (****$p \leq 0.0001$). Data were presented as mean ± SD. Data were analyzed using paired-samples *t*-test.

short and long groups ($p = 0.97$). A tendency of higher rate of good quality embryos was achieved in short compared to long insemination groups; however, it was not significant ($p = 0.12$). Also, the rate of clinical outcomes was similar between the groups (Table 1).

*3.3. Individual and Calculated Morphokinetics Variables.* TLM showed that all individual time points were slightly earlier in short insemination group (Table 2). However, t5 was significantly different between the groups ($p = 0.04$). Of all the calculated TLM variables, only cc3a was significant in short group ($p = 0.03$; Table 3). There were no significant differences between the groups in the percent of fragmentation ($p = 0.46$), uneven cleavage (in two-cell stage) ($p = 0.81$), multinucleation in two- ($p = 0.40$) and four-cell stages ($p = 0.65$), DC ($p = 0.67$), RC ($p = 0.86$), and vacuoles ($p = 0.39$).

## 4. Discussion

The results of present study revealed a close association between high level of MDA, which increased due to high ROS level, with mitochondrial functionality, AR, and DFI levels in long insemination group. In conventional IVF, probable sources of ROS in culture media are different and could product from the oocytes, cumulus cell mass, and the spermatozoa used for insemination [4]. Despite many benefits of ROS, if it is produced in excess can be detrimental for both spermatozoa [21] and oocyte maturation [22]. Although the morphokinetic parameters have no significant differences, except t5 and cc3a, individual morphokinetics timing, including tPNf and t2–t8, slightly occurred later in embryos generated from long insemination group.

The present data showed that the culture media which oocytes were removed 2 hr after insemination had very low

Table 1: Laboratory parameters and clinical outcomes between two groups.

| Variables | Short insemination group | Long insemination group | $p$-value | OR (95% CI) |
|---|---|---|---|---|
| High-quality embryo (%) | 65.58 (162/247) | 58.99 (164/278) | 0.12 | 1.32 (0.92–1.88) |
| Chemical rate (%) | 48.57 (17/35) | 30.76 (12/39) | 0.11 | 0.47 (0.18–1.21) |
| Clinical pregnancy (%) | 34.28 (12/35) | 23.07 (9/39) | 0.28 | 0.57 (0.2–1.59) |
| Live birth rate (%) | 25.71 (9/35) | 17.94 (7/39) | 0.41 | 0.63 (0.2–1.92) |

Values are presented as $n$ and percentage. Data were compared using the $\chi^2$ test. Odds ratio with two-sided 95% CI was estimated for dichotomous outcomes. $p$-value <0.05 was considered as statistically significant. All of them were not significant.

Table 2: Comparison of morphokinetics "individual" variables between two groups.

| Time-lapse parameters | Short insemination ($n = 247$) | Long insemination ($n = 278$) | $p$-value |
|---|---|---|---|
| TPNF | $24.44 \pm 3.53$ | $24.74 \pm 5.06$ | 0.44 |
| t2 | $27.37 \pm 4.34$ | $27.66 \pm 4.97$ | 0.49 |
| t3 | $36.94 \pm 6.11$ | $37.35 \pm 6.2$ | 0.46 |
| t4 | $39 \pm 6.32$ | $39.66 \pm 6.69$ | 0.27 |
| t5 | $48.85 \pm 8.02$ | $50.29 \pm 7.36$ | 0.04* |
| t6 | $52.24 \pm 6.98$ | $52.65 \pm 6.16$ | 0.51 |
| t7 | $53.76 \pm 5.54$ | $54.29 \pm 5.59$ | 0.37 |
| t8 | $56.73 \pm 5.63$ | $57.02 \pm 5.43$ | 0.66 |

Results are presented as mean ± SD for normal numerical variables. TPNf, PN fading; t2, first cleavage (two-cell stage); t3, three-cell stage; t4, four-cell stage; t5, five-cell stage; t6, six-cell stage; t7, seven-cell stage; t8, eight-cell stage. *$p$-value <0.05 was considered as statistically significant. Data were compared using independent-samples $t$-test.

Table 3: Comparison of morphokinetics "calculated" variables between two groups.

| Time-lapse parameters | Short insemination ($n = 247$) | Long insemination ($n = 278$) | $p$-value |
|---|---|---|---|
| CC2a | $10.12 \pm 4.85$ | $10.02 \pm 4.67$ | 0.82 |
| CC2b | $12.04 \pm 5.28$ | $12.25 \pm 5.04$ | 0.66 |
| CC3a | $10.79 \pm 5.85$ | $11.90 \pm 4.8$ | 0.03* |
| CC3b | $14.44 \pm 4.91$ | $14.64 \pm 3.85$ | 0.63 |
| CC3c | $16.93 \pm 5.75$ | $16.78 \pm 4.14$ | 0.78 |
| CC3d | $19.66 \pm 8.03$ | $18.62 \pm 9.30$ | 0.32 |
| S1 | $3.15 \pm 2.50$ | $3.29 \pm 2.70$ | 0.55 |
| S2 | $2.21 \pm 4.10$ | $2.36 \pm 4.51$ | 0.71 |
| S3 | $9.85 \pm 7.01$ | $8.84 \pm 6.54$ | 0.21 |

Results are presented as mean ± SD for normal numerical variables. cc2a, t3–t2; cc2b, t4–t2; cc3a, t5–t4; cc3b, t6–t4; cc3c, t7–t4; cc3d, t8–t4; s1, synchronization of cell divisions of first cell cycle (t2-tPNf); s2, synchronization of cell divisions of second cell cycle (t4–t3); s3, synchronization of cell divisions of third cell cycle (t8–t5). *$p$-value <0.05 was considered as statistically significant. Data were compared using independent-samples $t$-test.

concentrations of MDA as compared with 16 hr of incubation. Compared to the long insemination group, the short insemination group had an overall better condition at the cellular level in terms of sperm DNA status, acrosome integrity, and MMP, which may be associated with the level of ROS production. Previous studies revealed that sperm cells have polyunsaturated fatty acids (PUFAs) in their membranes. This feature leads them to be sensitive to oxygen-induced damage mediated by lipid peroxidation (LPO). Thus, ROS caused to rapid loss of intracellular ATP and also decreased sperm viability; also, sperm capacitation and AR were damaged [23, 24]. In vitro experiments demonstrated that mitochondrial functionality is essential to preserve sperm fertilizing capacity [10, 25].

Sperm chromatin integrity is another valuable index of fertility potential [26, 27]. Moreover, spermatozoa lack antioxidants and DNA repair systems, and it is not able to repair the DNA damage caused to ROS [28]. Based on our results, it seems that high level of ROS can cause increased DNA damage and premature AR in long incubation protocols.

Data showed that the fertilization rates between our short and overnight coincubation of gametes were no difference. Others have reported similar finding in the short and long inseminations [29]. For example, Liu et al. [19] divided the sibling COCs into two groups of long (20 hr) and short inseminations (5 hr) and showed no differences in the fertilization and abnormal polyspermy. However, Barraud-Lange et al. [30] reported that the fertilization rate was lower in the short insemination group (1 hr) compared to the standard insemination one (18 hr) statistically (64.9% and 70.1%). This difference could be because oocytes cocultured

with cumulus cells maturated dramatically. In the long group, the immature oocytes have the opportunity to reach maturity and may explain for higher 2 PN rate in this group [30].

It has been postulated that different toxic compounds, such as ammonia and ROS produced by low-quality embryos, have deleterious effects on quality of developed embryos in vitro. IVF techniques, in particular, collection, manipulation, and culture of gametes, may generate ROS with a possible role in damage to the sperm and oocyte quality and, also, embryos structure [31]. This may be due to the presence of a large number of sperm cells in the insemination environment. In our study, both methods provided no significant differences in the number of high-quality embryos. However, we observed slight increase in the rate of good embryo quality in the short insemination. This data are similar to previous studies [19, 30]. The data of this study showed that there was a dramatic difference in the sperm status between short and long insemination groups that may had the detrimental effect on embryo culture condition.

Regarding TLM, Nikolova et al. [32] determined the influence of sperm abnormalities on embryo morphokinetic variables and clinical outcome of conventional IVF. They reported that time of tPNf and time for two cells were closely related to sperm parameters such as motility, morphology, and DNA fragmentation [32]. According to this study, the sperm cells were in normal range and mentioned time points were similar between the groups. On the other hand, these results could be related to our attention to assessment of early developmental stage of embryos based on laboratory policy. If this evaluation was monitored to the blastocyst stage, more variations could be observed between the groups.

TLM markers can affect the clinical outcomes, with both cleavage patterns, including too fast or too slow, leading to development problems in human embryos. In our study, all embryo developmental events were in similar ranges in both groups. Consequently, rapid embryo development of t3, t4, and t5 is beneficial for implantation, but only t4 might influence the euploid rate of blastocysts. It proved that some time points, such as t5, t8, s1, s2, and cc2, were different between the good- and poor-quality embryos. There was a correlation with higher implantation and pregnancy outcomes [33–35]. Among these variables, t5 plays an important role in prediction of implantation in ART cycles [36]. In present study, t5 was significantly faster in short group and t5 ($48.85 \pm 8.02$ vs. $50.29 \pm 7.29$) and cc3a ($10.79 \pm 5.85$ vs. $11.90 \pm 4.80$) were in optimal range and significant between short and long insemination groups.

In addition, there were no significant differences in the rate of our clinical outcomes. We observed slight increase in clinical outcomes in short group. Despite some controversies, several studies observed superior conditions in embryo parameters that included blastocyst formation, pregnancy, and implantation rates in short insemination compared with conventional IVF. In contrary to meta-analysis [5] that improved clinical outcomes in brief insemination group, others found no significant differences in clinical outcomes between the two insemination groups. Evaluation of 320 infertile women for cycles undergoing IVF showed that the LBR between the short and standard incubation groups had the same data. The possible reasons of these differences may be related to several factors, including patient age. Also, various sperm concentrations (0.7–3.0 million motile sperm/mL) that were used in the various studies had different outcomes [37].

On the other hand, there were different methodology design and sample size in previous studies. Short incubation duration was reported from 1 to 6 hr. Then, there is no common opinion on the optical time of exposure in short insemination to get the best result [37]. Limitation of sample size could influence our clinical outcomes. For controlling the heterogeneity of female patient characteristics, matured COCs of each patient were divided to either the short or conventional insemination group. In the results, both groups were identical in terms of factors, such as etiology and the duration of the infertility, age, and ovarian stimulation protocol. Using the sibling oocytes in our study declined the biases in patient characteristics and laboratory conditions.

## 5. Conclusion

Excessive production of ROS in long-incubation period possibly led to the dysfunction of mitochondria and insufficient production of energy, resulting in decreased MMP. Also, increased sperm DNA fragmentation and premature AR are other by-products of long incubation of sperm–oocyte in IVF. Therefore, shortening the time of gametes incubation improves the biological characteristics of sperm fertilization capacity. Our data found that polyspermy, fertilization rate, embryo quality, and clinical outcomes had no difference when insemination period was reduced in short insemination compared with standard IVF conditions. TLM revealed that t5 and cc3a were main factors for prediction of implantation that was faster in short group. For the first time, revising specific parameters, this study showed that conventional IVF could have negative effects on cellular and morphokinetic events.

## Data Availability

The data underlying this article cannot be shared publicly due to privacy of patient. The data will be shared on reasonable request via corresponding author.

## Ethical Approval

The Ethics Committee approved this study by IR.SSU.RSI.REC.1397.012.

## Consent

All participants signed informed consent forms.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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