

Comparison of embryo morphokinetics following intracytoplasmic sperm injection in smoker and non-smoker couples: Are the results different?

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Abstract

Objective: To assess early embryo development via time-lapse in smokers and non-smokers.

Methods: The retrospective study was conducted at Novafertil IVF centers in Konya, Turkey and comprised oocytes of both smoker and non-smoker couples subjected to in vitro fertilisation / intracytoplasmic sperm injection from 2012 to 2015. Age, basal follicle-stimulating hormone, number of stimulation days, amount of gonadotropin used, number of metaphase II oocytes, number of embryos transferred and pregnancy, abortus and clinical pregnancy rates were noted. The embryos were observed for 72 hours in the time-lapse monitoring system. SPSS 22 was used for data analysis.

Results: Of the 257 couples, 132(51.4%) were non-smokers and 125(48.6%) were smokers. A total of 1,414 oocytes were collected from non-smokers and 1,280 oocytes from smokers. There was no significant difference in the age of patients and number of stimulation days between the smoker and non-smoker groups ($p>0.05$). The number of oocytes, fertilised oocytes, transferred embryos and metaphase II oocytes was significantly less in the smoker group ($p<0.05$). The rate of pregnancy and ongoing pregnancy was also lower in the smoker group ($p<0.05$). A difference was observed in time of pronuclei appearance, t8, t9+ cleavage times in time-lapse in the smoker and non-smoker groups ($p<0.05$). A prolongation was observed in time to pronuclear fading and t2 cleavage times in time-lapse in the non-smoker group ($p<0.05$). Some chromosomal number and structural defects were identified in pre-implantation genetic in some embryos with prolongation in time-lapse cleavage time in the smoker and non-smoker groups.

Conclusion: The negative impacts of smoking were not observed at each cleavage phase of embryo development in time-lapse.

Keywords: Embryo, Time-lapse, EmbryoScope, Smoke, Sperm, ICSI, PGT. (JPMA 67: 1552; 2017)

Introduction

The number of in vitro fertilisation (IVF)/intracytoplasmic sperm injection (ICSI) is increasing every single day. In the official registries of the World Health Organisation (WHO), it is stated that 170 million couples are waiting for IVF/ICSI according to unofficial records.¹ The number of sperms and structural defects, the age of the woman, oocyte reserves, the medicines used, genetic problems, environmental factors and the IVF/ICSI protocols may impact the take-home baby rates, including normal pregnancy. Particularly the negative impacts of smoking on the baby are well known.² There are more than 4,000 chemical components in the smoke of cigarettes. Although it is not known accurately which of these chemicals is harmful on the developing baby, it is

believed that especially nicotine and carbon monoxide have a negative impact on the pregnancy outcomes.^{3,4} It was demonstrated that it had a negative impact also on female ovaries, uterus and foetus during pregnancy.³⁻⁵ It was designated with animal studies that it leads to negative impacts on blastocysts and leads to implantation deficiency in IVF-ICSI.⁶ It was also indicated that smoking reduces aneusomy level in sperms and gives rise to low embryonic implantation rates, spontaneous abortus and foetal losses.^{4,5} The impacts of smoking and other harmful factors on embryonic development were reported mostly in animal experiments until today.^{7,8} One of the methods developed in recent years for following up early embryonic development is time-lapse (Embryoscope©) monitoring system. This is a special incubator system which contains an internal camera and takes pictures of and records each embryo at an interval of 10-20 minutes. Through this system, the embryos may be continuously monitored until the transfer day before they are taken out. It is possible to select the embryos with the highest potential of achieving pregnancy upon monitoring the early and late morphological

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characteristics of embryos as well as the rate and timing of embryonic development.⁹ Although they are not evidence-based, there are also case reports in literature, demonstrating that the findings obtained with time-lapse are in line with pre-implantation genetic diagnoses in the recent evaluation of embryos in genetic diseases.¹⁰ The current study was planned to assess early embryo morphokinetics (first 72 hours), pregnancy, miscarriage and clinical pregnancies in smoker and non-smoker couples via time-lapse following IVF-ICSI.

Patients and Methods

The retrospective study was conducted at Novafertil IVF centers in Konya/Turkey and comprised oocytes of both smoker and non-smoker couples subjected to IVF/ICSI between 2012 and 2015, and consequently followed up only with time-lapse and whose embryos underwent a pre-implantation genetic (PGT) trial. The participants were briefed about the procedures, embryoscopic follow-up and were informed that such follow-ups will be used for research purposes in the future. Informed consent was obtained from all participants. Permission was obtained the ethics committee on non-interventional clinical trials of the Necmettin Erbakan University, Meram School of Medicine, Konya, Turkey. According to the WHO definition,¹ females and males who smoke at least once a day or more on a regular basis for a minimum period of 1 year were regarded as active smokers. The records in the time-lapse imaging system of embryos of the patients were screened. The PGTs of 98 embryos obtained from ten smoking patients and 121 embryos obtained from fifteen non-smokers were available and were assessed.

Daily gonadotropin stimulation was started on the second or the third day of either a spontaneous or an induced menstrual cycle; the starting dose was determined (ranging from 75 to 300 IU) according to age, body mass index (BMI), follicular phase serum follicle-stimulating hormone (FSH) level, antral follicle count (AFC) and previous history of ovarian response if there had been a treatment. Gonadotropin-releasing hormone (GnRH) antagonist injections at a dose of 0.25 mg/day were started either on the sixth day of stimulation or when the leading follicle reached 14mm. Gonadotropin dosage was adjusted according to ovarian response on day five. Pelvic ultrasound and endocrine monitoring were performed thereafter. Injections were continued until ≥ 3 follicles reached ≥ 17 mm diameter; human chorionic gonadotropin (hCG) trigger which consisted of subcutaneous (sc) injection of recombinant human chorionic gonadotropin (rhCG)

(Ovitrelle, Merck Serono, Turkey) or intramuscular urinary human chorionic gonadotrophin (uhCG) (Pregnyl MSD, Turkey) was administered. Transvaginal oocyte retrieval was performed 35-36 hours after the hCG trigger.

ICSI was applied on metaphase II oocytes. The embryos were inserted into the time-lapse (Embryoslide: Unisense Fertilitech®) system with a specific culture dish. The system follows up early embryonic development with three gasses.¹⁰ The embryos were followed up in a setting with a low oxygen pressure of 37°C (5% O₂, 6% CO₂). Vitrolife was used sequentially and G1v5 media was used after ICSI while G2v5 media was used after the 3rd day in the embryonic cultures. The times from fertilisation to the following events were analysed: when two pronuclei were visible (2PN), pronuclear fading (PNF) occurred; when both pronuclei disappeared, the first cleavage, when the zygote divides into two cells (t₂) was observed; and the cleavage giving rise to 3 to 9 cells was observed for the first time (t₃ to t₉, respectively).¹⁰ The selection of the embryos to be transferred was made according to time-lapse morphokinetics. Top quality embryo was defined as 8 even and regular cells, with less than 20% fragmentation. Two embryos were transferred in total. The pregnancy test was performed 15 days after oocyte collection. Following the positive result of the pregnancy test, clinical pregnancy was verified upon observing the 18th day ultrasonographic gestational sac and foetal pulses.

The average and standard deviation and the lowest, highest, median, ratio and frequency values were used in the descriptive statistics of the data. The distribution of the variables was checked with the Kolmogorov-Smirnov test. The independent sampling t-test and Mann-Whitney U test were used in the analyses of quantitative data. Chi-square test was used in the analysis of qualitative data. SPSS 22 was used for data analysis. While selecting groups' and couples' numbers, the same demographic characteristics were taken into account.

Results

Of the 257 couples, 132(51.4%) were non-smokers and 125(48.6%) were smokers. Moreover, 1,414 oocytes were collected from non-smoker couples while 1,280 oocytes were collected from smoker couples. No difference was observed in either group in terms of age, basal FSH, and amount of gonadotropin used. There was no significant difference ($p > 0.05$) in the age of patients and number of stimulation days between the smoking and non-

Table-1: Age, hormone and oocyte number of the patients. Pregnancy, clinical pregnancy and miscarriage rates.

	Non-Smokers n=132		Smoker n=125		p			
	Aver.±s.s./n-%	Med(Min-Max)	Aver.±s.s./n-%	Med(Min-Max)				
Age	29,8±2,4	31	24-38	32,2±5,0	32	21-40	0,126	m
No.of oocytes	12,5±4,3	11	7-27	10,2±6,0	9	1-23	0,000	m
No.of fertilised oocytes	5,8±2,4	6	1-13	5,2±3,5	4	1-14	0,010	m
No.of transferred oocytes	2,0±0,2	2	1-2	1,8±0,4	2	1-2	0,000	m
No.of metaphase II oocytes	8,4±3,3	8,0	3,0-19,0	7,4±4,4	6,0	1,0-16,0	0,005	m
No.of stimulation days	10,0±1,2	10,0	7,0-12,0	10,6±1,8	10,0	8,0-15,0	0,131	m
Pregnancy	Yes	48	36,4%	70	56,0%		0,002	χ ²
	No	84	63,6%	55	44,0%			
Abortion	28	33,3%		35	63,6%		0,000	χ ²
Clinic Pregnancy	56	66,7%		20	36,4%			

m Mann-Whitney U test / χ² Ki-kare test.

Table-2: Early embryonic development times of the embryos of smoker and non-smoker groups.

	No.of Embryos in Nonsmoker n=1561		No.of Embryo in smokers n=1486		p			
	Aver.±s.s.	Med(Min-Max)	Aver.±s.s.	Med(Min-Max)				
tPB2	5,5±2,0	5,0	2,6-16,8	5,2±1,4	5,1	2,4-10,0	0,205	m
tPNa	10,3±3,8	9,6	5,7-46,9	9,7±3,0	8,7	5,6-22,0	0,000	m
tPNf	26,8±5,9	25,9	20,5-65,3	26,8±4,1	26,1	19,9-38,0	0,148	m
t2	34,1±7,7	32,8	23,8-68,7	35,0±8,2	33,1	24,3-69,3	0,413	m
t3	40,0±5,9	39,1	26,8-68,0	40,6±6,2	39,8	27,3-68,0	0,365	m
t4	41,8±5,8	40,7	30,2-66,0	42,2±6,3	41,4	32,9-68,3	0,107	m
t5	51,0±7,1	51,2	30,2-68,7	50,4±8,5	50,9	32,9-68,3	0,365	m
t6	54,8±6,2	54,0	38,0-68,7	54,8±7,9	54,7	40,6-68,6	0,938	m
t7	57,8±5,8	57,5	42,8-68,9	59,3±7,8	58,1	45,4-69,3	0,652	m
t8	62,7±5,3	64,0	50,8-70,0	63,4±6,0	66,3	46,1-69,3	0,005	m
t9+	65,0±3,8	67,2	58,1-72,2	68,3±1,5	68,8	65,3-70,0	0,000	m

m Mann-whitney u test

PN: Pronuclei

tPB2: Time for appearance of second polar body

tPNa: Pronuclei appearance

tPNf: Time to pronuclear fading

t2: First cleavage (2-cell stage),

t3: Second cleavage (3-cell stage),

t4: 4-cell stage

t5: 5-cell stage

t6: 6-cell stage

t8: 8-cell stage

t3-t2: Second cell cycle duration

t4-t3: Asynchrony period (3-cell stage duration)

t9+: Greater than nine cells.

smoking groups. The number of oocytes, number of fertilised oocytes, number of embryos transferred, number of oocytes in metaphase II was significantly lower ($p<0.05$) in the smoking group compared to the non-smoking group. The rate of pregnancy was significantly lower ($p<0.05$) in the smoking group compared to the non-smoking group. The rate of clinical pregnancy rate was significantly lower ($p<0.05$) in the smoking group compared to the non-smoking group.

The pregnancy rate was 84(63.6%) in the non-smoker group, while it was 55(44%) in the smoker group. Of them, the clinical pregnancy rate was 56(66.7%) in the non-smoker group and 20(36.4%) in the smoker group (Table-1).

For the time of pronuclei appearance (tPNa), t8, t9+ cleavage times in time-lapse in the smoker and non-smoker groups, no significant difference ($p>0.05$) was

observed in the time for appearance of second polar body (tPB2), time to pronuclear fading (tPNf), t2, t3, t4, t5, t6, t7 cleavage times. Moreover, a prolongation was observed in the cleavage times also in the tPNf and t2 phases in time-lapse among non-smokers. Some numerical and structural defects, such as 45, X/46, XY/47 and XXY, were observed in the five-band pre-implantation genetic screening in 42(3.3%) out of 98(7.6%) embryos of 10(8%) patients in the smoker group during the retrospective screening. These embryos were observed in the late cleavage group in time-lapse. In addition to the same chromosomal structural and numeric defects at PGT in 41(2.9%) out of 121(8.6%) embryos of 15(11.4%) patients in the non-smoker group, 8(0.57%) embryos were detected in translocation. It was observed that these embryos were present especially in the embryo groups with prolongation in the tPNf and t2 phase (Table-2).

Discussion

A higher dose hormone therapy is required in smoker women; as their blood oestrogen levels are lower and their oocytes are less, the possibility to cancel the ICSI therapy increases, the non-fertilisation risk of the oocytes collected is higher and possibility of the embryo to be implanted after transfer is lower.¹¹ Studies have shown that tobacco thickens zona pellucida, leads to gene damage in gametes and embryos and ovaries are suppressed in the production of glucocorticoids and mineralocorticoids.¹² Decrease is observed in the number of sperms and their motility and formal while an increase is seen in formal and functional abnormalities in smoker males. Second-hand smokers are also under a similar risk and smoking reduces their sperm quality.^{13,14}

A statistical difference was observed in the number of oocytes between the smoker and non-smoker group in our study. In fact, it was observed that while the number of oocytes was at least seven (number of oocytes= 7-20), this number dropped to as low as one in the smoker group (number of oocytes= 1-23).

The biggest problem in IVF treatments is to be able to detect from which embryo the pregnancy rate will be highest. Today, once the oocytes are fertilised, they continue to develop within an incubator with a fixed temperature, oxygen and carbon dioxide amount. In the conventional follow-up, these embryos are removed from the incubator 2-3 times and assessed by embryologists until the transfer is made and the embryos which are believed to be the best are transferred on the transfer day. During embryo

cleavage, it is believed that the embryos which are split irregularly or split rapidly in the embryo is less and that these characteristics may be better designated via time-lapse.¹⁰ It was observed in multi-centred studies that the rate of pregnancy was very low in embryos in which the 2nd cleavage (tPB2) occurred 5 hours before. This period was calculated as more than nine hours in hour group in time-lapse in the embryos of both smoker and non-smoker groups. It is estimated that the embryo probably cannot sufficiently copy its deoxyribonucleic acid (DNA) in case of rapid cleavage.¹⁵ Time-lapse entered into our practice for this reason.^{9,15} It was believed that this early embryo morphokinetic dysfunction could be one of the factors negatively impacting the clinical pregnancy rates. The conventional embryo morphological evaluations are conducted 44 hours after ICSI. Time-lapse currently appears to be significant for the transfer of embryos in the early evaluation of embryo morphokinetics in between.⁹ It may be considered to add embryoscope as a determinant in the decision phases in order to increase success in implantation until the transfer phase after ICSI.¹⁵ However, today, it is necessary to clarify certain details for the use of embryo monitoring systems in all embryology laboratories. Although it has demonstrated a beneficial effect in scientific studies, currently it is still not proven that is more advantageous to the conventional embryo evaluation methods in terms of pregnancy rates in comparative scientific studies. Another topic to be clarified about the monitoring systems is to what degree the cleavage phases are impacted by external factors (such as the stimulation protocol administered for the patient, the details of the embryo culture in laboratory the culture fluids used and temperature differences) and therefore, if they are impacted, in what direction will these factors change the ideal time intervals in the cleavage phases. Otherwise, it will not be possible to standardise these time intervals worldwide and, in relation with the timing recommended, the new scoring system will deviate in different laboratories operating with different techniques. As this gives rise to different results at each clinic, it generates challenges in reaching a consensus.

While observing the impact of smoking on early embryonic development in this study, it is not stated definitively thin time-lapse is the precise indication in embryo selection. The contribution on time-lapse to pregnancy rates is not yet known.

However, our study produced different results. While it was reported in the previous studies⁹ that smoking had

an impact on almost each phase of embryonic development in time-lapse, no significant ($p>0.05$) difference was observed in our study in the cleavage times of tPNa, t8, t9+ in time-lapse in the smoker and non-smoker groups. Interestingly, a prolongation was observed in the cleavage times at phases tPNf and t2 of time-lapse among non-smokers. Due to the prolongation in the same interval in the smoker group, no statistical difference appeared to be present in the average values.

We believe that embryoscopic studies should be supported with PGTs. As it's not clear whether smoking or another toxic agent or genetic defects have an impact on the embryo; we don't think that they may have a significant impact in differentiating the cleavage differences in time-lapse. We believe that it would be subjective to specify that there is a prolongation only in the cleavage times and to claim that this is associated with the targeted agent. In fact, numerical and structural defects such as 45, X/46, XY/47 and XXY were observed in the study in the five-band chromosomal assessment in 42 out of 98 embryos of only ten patients with PGT in the smoker group. It was observed that these embryos underwent late cleavage in time-lapse and that chromosomal structure and numerical defects and translocations such as 45, X/46, XY/47 and XXY were detected in 41 out of 120 embryos of fifteen patients with prolongation in the tPNf and t2 phases in time-lapse in the non-smoker group. Therefore, we believe that the statistical comparisons comprising PGT results in the background are more supportive in time-lapse comparisons in patients in the smoker and non-smoker groups or in any topic. It is known that PGT is more objective in the assessment of the embryonic structure.¹⁵ We don't think that only the prolongation in cleavage time in time-lapse may directly reflect the negative impact on the embryo. This may be a subjective finding, but may give rise to an objective error. We support the belief that time-lapse will help especially in embryo selection and raise the chance of pregnancy. No one can deny the fact that smoking is harmful for human babies. However, the phase in which it is harmful in baby development is not fully known. Considering the results of our study, it would not be realistic to stipulate that smoking has a negative impact only by looking at time-lapse, because the studies conducted do not comprise the analysis of the genetic structure of embryos on a routine basis. Maybe, it will be necessary to evaluate many embryotoxic impacts via PGT.

Furthermore, we believe that different follow-up

protocols will give rise to different embryo development issues in the concentrations on early embryo development in the long term and thin time-lapse will contribute to child take-home rates in IVF-ICSI applications. We recommend that smoking should be stopped at least two months prior to IVF treatment. Although smoking for a long period leaves an irreversible impact of the ovaries, quitting smoking before treatment may reduce the negative impact on treatment results.^{16,17}

We believe that there is a need for more studies with standardised time-lapse criteria and long-term follow-ups applied under the same conditions on patients with the same demographic characteristics. We believe in the efforts of technological developments to establish in vivo pregnancy conditions. However, we also believe the findings of our study were preliminary.

Conclusion

It was observed both clinically and statistically that smoking had a negative impact on only the low number of oocytes, clinical pregnancy and low rates. However, in the time-lapse morphokinetic monitoring, performed on early embryonic development and currently believed to be the most objective method, it was detected that it did not impact negatively all phases of embryo cleavage. However, it should be supported with the results such as those obtained in our study that it may be necessary to interpret the outcomes of time-lapse with PGT. It was observed that the embryo is much more resistant than estimated to the toxic effects of smoking and that the embryo did not permit any phase of embryonic development to be impacted, especially in the smoking groups.

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References

1. Tasoglu S, Safaee H, Zhang X, Kingsley JL, Catalano PN, Gürkan UA, et al. Exhaustion of racing sperm in nature-mimicking microfluidic channels during sorting. *Small* 2013; 9: 3374-84.
2. Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, et al. Effects of cigarette smoking on reproduction. *Hum Reprod Update* 2011; 17: 76C95.
3. Freour T, Masson D, Mirallie S, Jean M, Bach K, Dejoie T, et al. Active smoking compromises IVF outcome and affects ovarian reserve. *Reprod Biomed Online* 2008; 16: 96-102.
4. Soares SR, Simon C, Remohí J, Pellicer A. Cigarette smoking affects uterine receptiveness. *Hum Reprod* 2007; 22: 543-7.
5. Nabet C, Lelong N, Ancel PY, Saurel-Cubizolles MJ, Kaminski M.

- Smoking during pregnancy according to obstetric complications and parity: results of the EUROPOP study. *Eur J Epidemiol* 2007; 22: 715-21.
6. Dessolle L, Fréour T, Ravel C, Jean M, Colombel A, Daraï E, et al. Predictive factors of healthy term birth after single blastocyst transfer. *Hum Reprod* 2011; 26: 1220-6.
 7. Hassa H, Gürer F, Tanir HM, Kaya M, Gündüz NB, Sariboyaci AE, et al. Effect of cigarette smoke and alpha-tocopherol (vitamin E) on fertilization, cleavage, and embryo development rates in mice: an experimental in vitro fertilization mice model study. *Eur J Obstet Gynecol Reprod Biol* 2007; 135: 177-82.
 8. Huang J, Okuka M, McLean M, Keefe DL, Liu L. Effects of cigarette smoke on fertilization and embryo development in vivo. *Fertil Steril* 2009; 92: 1456-65.
 9. Thomas Fr, Pharm D, Lionel D, Lammers J, Barri P. Comparison of embryo morphokinetics after in vitro fertilization-intracytoplasmic sperm injection in smoking and non-smoking women. *Fertil Steril* 2013; 99: 1944-50.
 10. Gürbüz AS, Salvarci A, Özcimen N, Zamani AG. Early Morphokinetic Monitoring of Embryos after Intracytoplasmic Sperm Injection with Fresh Ejaculate Sperm in Nonmosaic Klinefelter Syndrome: A Different Presentation. *Case Rep Genet* 2015; 2015: 827656.
 11. Gruber I, Just A, Birner M, Losch A. Effect of a woman's smoking status on oocyte, zygote, and day 3 pre-embryo quality in in vitro fertilization and embryo transfer program. *Fertil Steril* 2008; 90: 1249-52.
 12. Zenzes MT. Smoking and reproduction: gene damage to human gametes and embryos. *Hum Reprod Update* 2000; 6: 122-31.
 13. Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S, et al. Effects of cigarette smoking on reproduction. *Hum Reprod Update* 2011; 17: 76C95.
 14. Waylen AL, Metwally M, Jones GL, Wilkinson AJ, Ledger WL. Effects of cigarette smoking upon clinical outcomes of assisted reproduction: a meta-analysis. *Hum Reprod Update* 2009; 15: 31-44.
 15. Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011; 26: 2658-71.
 16. Freour T, Masson D, Mirallie S, Jean M, Bach K, Dejoie T, et al. Active smoking compromises IVF outcome and affects ovarian reserve. *Reprod Biomed Online* 2008; 16: 96-102.
 17. Ben-Haroush A, Ashkenazi J, Sapir O, Pinkas H, Fisch B, Farhi J. High-quality embryos maintain high pregnancy rates in passive smokers but not in active smokers. *Reprod Biomed Online* 2011; 22: 44-9.
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