Original Article

Clinical Pregnancy After Elimination of Embryo Fragments Before Fresh Cleavage-stage Embryo Transfer

Luis H. Sordia-Hernandez; M.D. Ph.D., Felipe A. Morales-Martinez; M.D., Lorna M. Frazer-Moreira; M.D., Lilith Villarreal-Pineda; Q.F.B., María Ofelia Sordia-Piñeyro; M.D., Otto H. Valdez-Martinez; M.D.

Department of Gynecology and Obstetrics, Dr. Jose Eleuterio Gonzalez Hospital, School of Medicine, Nuevo Leon Free University, Monterrey, Mexico

Received June 2020; Revised and accepted September 2020

Abstract

Objective: To determine if the elimination of fragments in cleavage-stage embryos, before fresh transfer, improves pregnancy rates in *in vitro* fertilization cycles.

Materials and methods: This is a Prospective observational case-control study carried out at a University Reproductive Center. We included Twenty-six infertile patients divided into two groups. Group one: 13 patients with embryos classified as grade B and C (embryos with fragments) according to the Hill classification, and Group two: 13 patients with grade A embryos (embryos with no fragments). Embryo Defragmentation was performed in embryos of group one 65 to 68 hours after conventional fertilization. Fresh embryo transfer was made after two hours post fragments removal. Reproductive results were evaluated and compared between both groups.

Results: The total number of clinical pregnancies was nine. In group one there were 5 (38.5 %); in group two, there were 4 (30.8%). The difference was not statistically significant (p = 0.68). Two abortions were reported in the study, both in group one; were fragment elimination was performed. This represents an abortion rate of 40% in patients who got pregnant in this group. These patients had twice the probability of suffering an abortion (OR 2.1; 95% CI 1.4-3.37). Ongoing pregnancies were similar in both groups.

Conclusion: Removal of fragments in freshly transferred day three embryos could be an alternative to increase clinical pregnancy and ongoing pregnancy rates in patients who have only poor-quality embryos. Despite the relationship with a higher abortion rate, this strategy could represent a real alternative for this type of patient.

Keywords: Embryo Fragment Removal; Fresh Embryo Transfer; Pregnancy

Introduction

The degree of embryo fragmentation is one of the criteria most commonly used to define embryo quality and to select an embryo that will be transferred in assisted reproduction cycles (1). The generation of small cytoplasmic fragments is a common characteristic in the development of human embryos. These fragments are formed mainly by mitochondrial tissue.

The greater the number of fragments the lower the quality of the embry; also, the presence of an elevated number of fragments is related to a lower implantation rate. This reduction is attributable to a reduction in the cytoplasmic space available for normal cell division or to a reduction of cell intercommunications. It has also been proposed that cytoplasmic fragmentation induces apoptosis and limits the rate of blastomere division (2-5).

The formation of fragments has been related to a variety of factors. Among these, inadequate embryo

Correspondence:
Dr. Luis H. Sordia-Hernandez
Email: luissordia@telmexmail.com

culture, poor egg and/or sperm quality, advanced maternal age, apoptosis, and oxidative stress in embryos (6). The impact of moderate fragmentation on the results of assisted reproduction techniques is controversial. However, recent studies debate whether the elimination of these fragments equals or improves the pregnancy rate by cycle in comparison to embryos originally of better quality (7).

The objective of this study is to determine if the elimination of fragments in cleavage-stage embryos before fresh transfer improves pregnancy rates in *in vitro* fertilization cycles.

Materials and methods

This was a prospective observational case-control study performed in the Reproductive Medicine Center of the Universidad Autónoma de Nuevo Leon University Hospital in Monterrey, Mexico from May to November 2019. After prior approval from the Ethics Committee of our hospital (registration no. GI19-00004) and obtention of written informed consent, a total of 26 patients were included with an age range of 18 to 42 years and treated with *in vitro* fertilization of their eggs in their first cycle of treatment with fresh transfer. The indications for *in vitro* fertilization could be due to an ovary, tube, male or metabolic factor, or unexplained infertility.

A formula of the estimation of the mean in two populations was used to determine the sample size with a confidence of 95% and a power of 80% obtaining a sample size of 13 patients for each group.

Group one (cases) consisted of 13 patients with embryos classified as grade B and C according to the Hill classification (8,9); in other words, with unequal blastomeres in size and a fragmentation percentage of up to 10% for embryos classified as grade B and from 10% to 50% for grade C embryos. Group two (Controls) consisted of 13 patients with grade A embryos; in other words, with uniform blastomeres, and without fragmentation.

Stimulation protocol and egg recovery: Recombinant FSH (Gonal-F Merck Serono) in an antagonist protocol was used for controlled ovarian stimulation. The initial dose was individualized for each patient depending on age and ovarian reserve. After a baseline echograph to rule out the presence of residual follicles in the first three days of the cycle, ovarian stimulation was started no later than the third day of the cycle. Monitoring was carried out with serial transvaginal echography that was started five days after stimulation; for this, a General Electric Logic P5 ultrasound was used. Serum estradiol levels were also monitored. On day 6 of stimulation, the dose of recombinant FSH was adjusted (only if necessary). This same day, application of the GnRH antagonist (Cetrotide 0.25mg, Merck Serono) 0.25 mg per day was started. These two drugs were continued until the day recombinant human chorionic gonadotropin (HCGr) (Ovidrel 250 µg, Merck Serono) was applied. Administration of HCGr was carried out when at least three follicles reached a minimum diameter of 18 mm. Follicular aspiration was performed under endovenous sedation, 35 hours after the application of HCGr. Support of the luteal phase was performed with micronized progesterone administered vaginally, 200 mg every 8 hours. This was started from the day of follicular aspiration and continued up to 12 weeks of gestation if pregnancy was achieved. If the pregnancy test was negative, the application of progesterone was discontinued.

Embryo fertilization: The insemination of ovules was carried out 4-5 hours after their recovery. Insemination was performed in a Petri dish with a mineral oil culture medium. The previously capacitated spermatozoids were added to the wells containing the culture medium with a final concentration between 100,000 and 250,000 spermatozoids per milliliter. The cumulus-oocyte complexes were then added to the fertilization wells. Fertilization was evaluated later at 17 hours. After fertilization was confirmed, the embryos were transferred to G-TL Vitrolife culture medium. All embryos were cultured in a sequential medium using a global culture medium (G-IVF vitrolife, G-TL vitrolife).

Embryo classification: Approximately 48 hours after insemination, the number of cells and embryo morphology were evaluated and graded according to the Hill scale (9) in grade A embryos: blastomeres of the same size, without fragmentation; grade B: with uneven blastomeres, fragments <10%; grade C: unequal blastomeres with fragmentation ≤ 50% and grade D: embryos with uneven blastomeres and significant fragmentation ≥50%, in addition to the presence of large dark granules in the cytoplasm. After this morphological assessment, the study groups were integrated. Group one included all the patients that had only grade B or C embryos. These were included consecutively until the number of cases required for this study was reached. Group two included all patients with two Grade A embryos for transfer. Patients were also consecutively included until the number calculated for the study was reached.

Embryo defragmentation: Defragmentation was

performed 65 to 68 hours after conventional fertilization. Plates 60 mm in diameter with a drop of Human Tubal Fluid (HTF) medium buffered with HEPES were used. The entire plate was coated with 12 mL of mineral oil. The whole process was carried out on a 400x inverted microscope with a micromanipulation system. The embryo was incised creating a hole with a maximum diameter of 30µm in the zona pellucida. For this, a 1480 nm infrared diode laser was used. For fragment extraction, each embryo was fixed with a holding micropipette (internal diameter: 120-150 µm); subsequently, fragments were aspirated gently in each of the embryos, using a 10-12 µm micropipette. To access all the fragments, the embryo was rotated without releasing it from the holding pipette.

Embryo transfer: Embryo transfer was performed between 66 and 70 hours after fertilization. In cases where embryo defragmentation was performed, transfer was performed two hours after the procedure. In all cases, two embryos were transferred. In all of the patients of the control group two grade A embryos were transferred.

Reproduction results: Chorionic gonadotropin hormone quantification was performed 14 days after embryo transfer; biochemical pregnancies were considered if beta-HGC levels were reported above 25 IU. Two weeks later, transvaginal ultrasound was performed to confirm clinical pregnancy, which was defined by the presence of a gestational sac and at least one embryo with a heartbeat. Six weeks later, a pelvic ultrasound was done to confirm ongoing pregnancies, which were defined as pregnancies with an intrauterine fetus with a heartbeat. If a gestational loss occurred, data of the event and how it was resolved was obtained. Biochemical pregnancies, clinical pregnancies, ongoing pregnancies, and gestational losses were documented

Data analysis: Data analysis was carried out with SPSS IBM Statistics v 24.0 and in Microsoft Excel for Mac version 16.31. The results of quantitative variables are expressed in central tendency measures (mean, standard deviation) and percentages. To contrast variables, ANOVA, the chi-square test, and Fisher´s exact test were used according to the type of variable analyzed. Considering an alpha error of 5%, a p-value < 0.05 was considered statistically significant with a statistical power of 95%. Numerical, nominal, and ordinal measurement scales were used.

Results

A total of 26 patients, 13 in each group, were included. No statistically significant difference was found between both groups regarding age, body mass index, personal medical history, type of infertility (primary or secondary), as well as the cause of infertility. The baseline characteristics of the patients included in the study are summarized in table 1.

The controlled ovary stimulation protocol was the same for both groups. A statistically significant difference was found only in the total dose of FSH used (2896 ± 718 IU vs 2325 ± 626 IU; $p = 0.04$). No significant differences were found between the groups concerning the total number of recovered oocytes, the number of mature oocytes, the number of inseminated oocytes, the number of normal fertilizations, as well as between the number of embryos with a division on day 2 and 3 after fertilization. Finally, the number of atretic oocytes was greater in group one; however, this difference was not statistically significant ($p = 0.08$). These results are shown in table 2.

The total number of clinical pregnancies was nine. In group one there were 5 representing 38.5 %; in group two, there were 4 (30.8%). The difference was not statistically significant ($p = 0.68$).

Two abortions were reported in the study, both in group one; that is, in those in which fragment elimination was performed. This represents an abortion rate of 40% in patients who got pregnant in this group. These patients had twice the probability of suffering an abortion (OR 2.1; 95% CI 1.4-3.37).

Finally, there were 3 ongoing pregnancies for group one (23%) and 4 for group two (30.8%). This difference was not statistically significant ($p = 0.65$). These results are shown in table 3. No relationship was found between age and the degree of initial fragmentation (p = 0.8) in an independent analysis of possible covariates Similarly, the days of ovarian stimulation did not show a significant relationship with the degree of fragmentation (p = 0.15).

The obtained results showed that there was no statistically significant difference regarding the effect of SDM on anxiety between the control and intervention groups immediately after the counseling session (P=0.46). However, the collected data showed that the mean value of the anxiety scores (16.52 ± 3.06) was higher among women in the intervention group than that reported for the control group (13.80 ± 3.55) before receiving the results of maternal serum biochemical markers (P < 0.001).

Table 1: Characteristics of the patients studied

Characteristic	Cases (n=13)	Control (n=13)	P value
Age, mean ± SD	35.2 ± 5.2	35 ± 3.7	0.9
BMI, mean ± SD	30.26 ± 6.9	26.1 ± 4.95	0.09
Classification according to BMI			0.7
Normal	4 (30.8)	7 (53.8)	
Overweight	3 (23.1)	3 (23.1)	
Class I obesity	3 (23.1)	2 (15.4)	
Class II obesity	2 (15.4)	1 (7.7)	
Class III obesity	1 (7.7)	0	
Comorbidities			0.42
DM2	2 (14.3)	1 (7.14)	
HT	1 (7.14)	0	
Hypothyroidism	2 (14.3)	0	
SLE	0	1 (7.14)	
RA	0	1 (7.14)	
Multiple sclerosis	0	1 (7.14)	
None	9 (64.3)	9 (64.3)	
Type of infertility			1
Primary	8 (61.5)	8 (61.5)	
Secondary	5 (38.5)	5 (38.5)	
Infertility factor			0.16
Ovarian	7 (41.2)	4 (23.5)	
Tubal	1 (5.9)	5 (29.4)	
Male	4 (23.5)	3 (17.6)	
Metabolic	5 (29.4)	2 (11.8)	
Inflammatory	0	2 (11.8)	
Unexplainable	0	1 (5.9)	

DM2: Type 2 diabetes mellitus; HT: Hypertension; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis. Data are expressed as participants N (%), unless otherwise indicated.

The Hosmer-Lemeshow goodness of fit statistic test (X^2 < 0.001; df = 2; Sig=1) showed that the model fit the data well. The results of logistic regression indicated that the educational level of the women was a significant predictor of the anxiety score before receiving the results of the blood tests. According to the obtained data, it was demonstrated that the women with a university level of education were 10.60 times more likely to have higher anxiety scores than the subjects with lower educational levels (OR = 10.60; 95% CI: 2.07-54.24; P = 0.005).

Table 2: Assisted reproduction technique protocol performed during the study

Description	Cases (n=13)	Control (n=13)	P value
Days of ovarian stimulation, mean ± SD	9.46 ± 0.96	10.15 ± 1.2	0.12
Total FSH dose (mg), mean ± SD	2325 ± 626	2896 ± 718	0.04
Total LH dose (mg), mean ± SD	161.54 ± 307.5	276.92 ± 374.2	0.39
Total number of oocytes recovered	10.5 ± 5	11.5 ± 6.9	0.67
MII oocytes	7.08 ± 3.6	7.4 ± 5.2	0.86
MI oocytes	2 ± 2.45	3 ± 3.67	0.42
Intermediate	1.08 ± 1.8	1.15 ± 1.67	0.91
Atresic	0.38 ± 0.77	0	0.08
Number of oocytes inseminated	7 ± 3.6	6.9 ± 4	0.92
Normal fertilizations	5.1 ± 2	5.5 ± 3.5	0.78
Late fertilizations	0.39 ± 2	0.16 ± 0.37	0.43
Embryos divided in Day 2	5.7 ± 2	5.4 ± 3	0.71
Embryos divided in Day 3	5 ± 2	5 ± 3	0.94

Data are expressed as means ± standard deviation unless otherwise indicated.

Table 3: Reproduction results

	Cases (n=13)	Control (n=13)	P value
Biochemical pregnancy	5 (38.5)	4 (30.8)	0.68
Clinical pregnancy	5 (38.5)	4 (30.8)	0.68
Ongoing pregnancy	3 (23.0)	4 (30.8)	0.65
Abortion	2/5 (40)	0/4 (0)	0.44

Results are expressed in N (%).

Discussion

The results of this study show that the elimination of intercellular fragments before fresh transfer of embryos in the cleavage stage, initially classified as low-quality embryos (grade B and C), improves reproductive results and are comparable to those obtained in cycles with good quality embryo transfer (grade A) in patients treated with *in vitro* fertilization.

In the present study we tried to compare the reproductive results of patients who had only fragmented embryos (grade B or grade C) versus the results of patients to whom only good quality embryos were transferred, that is, with uniform blastomeres and no fragments. We did not compare the results with patients with fragmented embryos who have not undergone defragmentation because embryos of that quality are generally not transferred due to the poor reproductive results observed and previously reported (10,11). Furthermore, the transfer of highly fragmented embryos could be ethically questioned due to the possible association with fetal malformations (12).

We included grade B embryos even though these could be more likely to implant than grade C embryos, however, they also had fragments and were also removed.

Blastomere fragmentation is an important biomarker in relation to the adverse results of pregnancy. It has been proposed that cytoplasmic fragmentation induces apoptosis and limits the rate of blastomere cleavage. This fragmentation could represent an effort of the embryos to discard defective blastomeres that could impede correct embryo development (7). Other theories involve cell apoptosis and chromosome abnormalities (6); however, no consensus exists to explain the origin of the fragments found in developing embryos.

Embryo fragmentation has been related to several factors, including inadequate culture conditions, poor gamete quality, advanced age, chromosome abnormalities, an abnormal cell cycle, apoptosis, and oxidative stress in embryos (4,5). It is important to mention that in the present study no relationship was found between age and the degree of fragmentation. There was also no significant difference between the degree of fragmentation and the number of oocytes recovered, the number of mature oocytes, the number of inseminated oocytes, the number of normal fertilizations, as well as between the number of embryos with a division on day 2 and 3 post-fertilization.

Several studies have demonstrated a lower implantation and pregnancy rate when transferring fragmented embryos; even a higher percentage of fragmentation is directly related to adverse reproductive outcomes (5). Check et al (8). reported a clinical pregnancy and live birth rate of 10% in embryos with a fragmentation percentage greater than 25%, in comparison to rates of 22.2% in embryos without fragmentation (9).

Alkani et al. (7) in 1999 reported that the removal of embryo fragments lessened the deleterious effects of the fragments on blastomeres but the pregnancy rate achieved was only 6%. On the other hand, Halvaei et al. (10) reported that the removal of fragments and thick granules in low-grade embryos improved pregnancy rates in patients with implantation failure. We found in this study that the pregnancy rate was even higher, although certainly not statistically significant, in the group of patients who received embryos undergoing defragmentation.

Cosmetic micromanipulation (defragmentation) in embryos in cleavage stage with moderate fragmentation (<50%) improves reproductive results compared to those obtained in embryos without fragmentation. Similar data were reported by Halvaei et al. (2) in a prospective study that included only embryos with a fragmentation of 10-50%. Seok-Gi et al. (5) reported a notable improvement in clinical pregnancy rates (43.7%), ongoing pregnancy (36.8%), and implantation (25.8%) in a group of embryos that underwent defragmentation in the cleavage stage. These results were significantly better than the corresponding rates when compared with a group of embryos with the same degree of fragmentation transferred without defragmentation (28.8%, 22.1%, and 14.0%, respectively; $p < 0.05$) (5). In our study, the control group was composed of patients who received only good-quality embryos.

Eftekhari-Yazdi et al. (11) reported a study that evaluated the potential *in vitro* development of embryos subjected to defragmentation and assisted eclosion 50-56 hours after a spermatozoid injection. They concluded that defragmentation has a positive effect on fragmented embryos and produces a better

quality of blastocysts; however, in this study, the embryos were not transferred, therefore, their capacity to implant and produce a live birth is unknown, but this supports the theory that fragment removal can improve the viability and the development potential of low-quality embryos by improving cell-to-cell interactions and preventing the release of possibly harmful substances by the fragments, which can lead to degeneration or lysis of adjacent blastomeres (7).

A consensus has not been reached to determine to what degree of fragmentation the removal of fragments can improve clinical results. Different authors have established <10%, <35%, and <50% as cut-off points. Alikani et al. (7) retrospectively followed homogeneous transfers of fragmented embryos and reported that implantation was significantly reduced after embryo transfer with >15% fragmentation, despite the removal of fragments before embryo transfer. However, this theory has been discarded by multiple authors who have found benefits in embryos with a degree of fragmentation of up to 50 (2, 5, 10). In this study, we included embryos with a fragmentation percentage of ≤ 50%, finding that the reproductive results were comparable between both groups. The reason for including only embryos with up to 50% fragmentation in this study was because embryos with a fragmentation greater than 50% have been related to a higher rate of fetal malformations, perhaps related to apoptosis and chromosome abnormalities (12). Even the decision to transfer them has been questioned, and in many cases, informed consent would have to be considered by the future parents. This is one of the reasons for trying to improve pregnancy rates by removing fragments in embryos that would otherwise have to be discarded. Therefore, defragmentation represents an alternative for those couples that only have poor quality embryos available for transfer, based on the number of fragments.

In this study, we found a higher rate of abortions in the group of patients who had defragmented embryos transferred. The relationship between abortions and the number of fragments in embryos is controversial. We could infer that these embryos are genetically abnormal, maybe with chromosome abnormalities. These abnormalities have been previously documented (13-15). Another possibility is that the embryos suffer important structural damage when fragment aspiration is performed. However, the small number of cases included in this study and the lack of a chromosomal study of the aborted tissues of conception prevent us from having an accurate explanation of the reason for the greater number of abortions in this group.

Conclusion

The results of this study allow us to conclude that the removal of fragments in freshly transferred day three embryos could be an alternative to increase clinical pregnancy and ongoing pregnancy rates in patients who have only poor quality embryos. Despite the relationship with a higher abortion rate, this strategy could represent a real alternative for this type of patient. However, the sample size of this study makes the reader take this conclusion with caution. Controlled clinical trials that involve a greater number of cases to confirm these findings are needed.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

We thank Sergio Lozano-Rodriguez, M.D. for his help in translating and editing the manuscript. GI19-00004 by Ethic Committee of Universidad Autónoma de Nuevo León.
***Funding*:** Universidad Autónoma de Nuevo León

References

1. Halvaei I, Khalili MA, Nottola SA. A novel method for transmission electron microscopy study of cytoplasmic fragments from preimplantation human embryos. Microscopy Research and Technique 2016; 79: 459-62.
2. Halvaei I, Khalili M, Esfandiari N,Safari S,Talebi AR,Miglietta S, et al. Ultrastructure of cytoplasmic fragments in human cleavage stage embryos. J Assist Reprod Genet 2016; 33: 1677–84.
3. Safari S, Khalili MA, Barekati Z, Halvaei I, Anvari M, Nottola SA. Cosmetic micromanipulation of vitrified-warmed cleavage stage embryos does not improve ART outcomes: An ultrastructural study of fragments. Reprod Biol 2017; 17: 210-7.
4. Keltz MD, Skorupski JC, Bradley K, Stein D. Predictors of embryo fragmentation and outcome after fragment removal in invitro fertilization. Fertil Steril 2006; 86: 321-4.
5. Kim SG, Kim YY, Park JY, Kwak SJ, Yoo CS, Park IH, et al. Early fragment removal on in vitro fertilization day 2 significantly improves the subsequent development and clinical outcomes of fragmented human embryos.

Clin Exp Reprod Med 2018; 45: 122-8.
6. Gurgan T, Demirol A, Sari T, Benkhalifa M. Effect of embryo fragment removal of day 3 embryos in recurrent implantation failure. Fertil Steril 2005; 84:S284.
7. Alikani M, Cohen J, Tomkin G, Garrisi GJ, Mack C, Scott RT. Human embryo fragmentation an vitro and its implications for pregnancy and implantation. Fertil Steril 1999; 71: 836–42.
8. Check JH, Summer-Chse D, Juan W,Barci H, Brittingham D. The importance of Blastomere Number, Fragmentation and Symmetry as Determined by Evaluation of Outcome of Single Embryo Transfer (ET). Fertili Steril 2005; 83: S28.
9. Hill GA, Freeman M, Bastias MC, Rogers BJ,Herbert CM,Osteen KG,et al. The influence of oocyte maturity and embryo Quality on Pregnancy Rate in a Program for in Vitro Fertilization-Embryo Transfer. Fertil Steril 1989; 52: 801-6.
10. Halvaei I, Ghazali S, Nottola SA, Khalili MA. Cleavage-stage embryo micromanipulation in the clinical setting. Syst Biol Reprod Med 2018; 64: 157-168.
11. Eftekhari-Yazdi P, Rezazadeh valojerdi M, Kazemi Ashtiani S,Baghaban Eslaminejad MR,Karimani L. Effect of fragment removal on blastocyst formation and quality of human embryos. Rep BioMed Online 2006; 13: 823-32.
12. Ebner T, Yaman C, Moser M, Sommergruber M,Polz W,Tews G. Embryo fragmentation in vitro and its impact on treatment and pregnancy outcome. Fertil Steril 2001; 76: 281-5.
13. Plachot M, Junca AM, Mandelbaum J, de Grouchy J, Salat-Baroux J, Cohen J. Chromosome investigations in early life. II. Human preimplantation embryos. Hum Reprod 1987; 2: 29 –35.
14. Munne´ S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphol- ogy, developmental rates, and maternal age are correlated with chro- mosome abnormalities Fertil Steril 1995; 64: 382–91.
15. Munne´ S, Cohen J. Chromosomal abnormalities in human embryos. Hum Reprod Update 1998; 4: 842–55.

Citation: Sordia-Hernandez LH, Morales-Martinez FA, Frazer-Moreira LM, Villarreal-Pineda L, Sordia-Piñeyro MO, Valdez-Martinez OH. **Clinical Pregnancy After Elimination of Embryo Fragments Before Fresh Cleavage-stage Embryo Transfer**. J Fam Reprod Health 2020; 14(3): 198-204.