

EFFECTS OF LASER ZONA THINNING ON PREGNANCY RATES IN PATIENTS WITH IMPLANTATION FAILURE

BAŞARISIZ İMPLANTASYON ÖYKÜSÜ OLAN HASTALARDA LAZERLE ZONA İNCELTME YÖNTEMİNİN GEBELİK ORANLARINA ETKİSİ

¹Ranan Gülhan Aktaş, ²Muhammed Emin Altunkaynak, ³Gülden Tunali, ³Naciye Arat, ³Kenan Sofuoğlu, ³Dr. Ebru Çöğendez, ³Tayfun Kutlu, ³Dr. Belgin Devranoglu, ⁴Handan Ankaralı

¹Maltepe Üniversitesi Tıp Fakültesi, Kanser ve Kök Hücre Araştırma Merkezi, İstanbul

²Ondokuz Mayıs Üniversitesi, Histoloji ve Embriyoloji AD, Samsun

³Zeynep Kamil Kadın ve Çocuk Hastalıkları Eğitim ve Araştırma Hastanesi, Yardımcı Üreme Teknikleri Merkezi, İstanbul

⁴Biyoistatistik AD, Tıp Fakültesi, Düzce Üniversitesi, Düzce

İletişim Ranan Gulhan Aktaş, Maltepe Üniversitesi Tıp Fakültesi, Kanser ve Kök Hücre Araştırma Merkezi, Marmara Eğitim Köyü, Başbüyük, İstanbul

Tel: 0 218 444 06 20 / e-mail: ranan.aktas@maltepe.edu.tr

ÖZET

Amaç

Embriyo transferlerinin başarısını arttırmak için uygulanan zona pellusidanın inceltme işlemi olan "assisted hatching" in başarısı halen tartışmalıdır. Bu çalışmada; daha önce başarısız embriyo transferi öyküsü olan hastalarda ikinci kez taze embriyo transfer edilmeden önce zona tabakasının inceltmesinin gebelik oranları üzerine değişiminin incelenmesi amaçlanmıştır. Çalışmada; aynı hastanın, aynı klinikte ve aynı koşullar altında yapılan ard arda iki tedavisinin sonuçları karşılaştırılmıştır.

Gereç ve Yöntem:

Kliniğe en az iki kez başvuran 188 çifte ait toplam 376 siklusun gebelik sonuçları değerlendirilmiştir. Hastalar aşağıdaki kriterlere göre gruplandırılmıştır: 1. Grup: 35 yaş ve üzeri, 2. Grup : Zona pellusidası kalın embriyolara sahip hastalar, 3. Grup: Birden fazla IVF-embriyo transfer denemesi olan ve başarısızlığın nedeni saptanamamış hastalar. Tüm bu hastaların embriyolarına ilk denemelerinde lazer uygulaması yapılmamış, ancak ikinci denemelerinde embriyoların zona pellusidaları lazer ile inceltmiştir. Tüm laboratuvar olanakları, kullanılan medyumlar, hasta ile ilgilenen klinisyen ve embriyologların bu birbirini takip eden iki tedavide aynı olmasına dikkat edilmiştir. 2. Ya da 3. Gün transfer uygulanan hastalar değerlendirmeye alınmıştır. Gebelik oranları transfer gününe göre istatistiksel olarak karşılaştırılmıştır.

Sonuçlar:

İleri yaş hastaları içeren grupta gerek 2. gerekse 3. Gün transfer yapılan hastalarda klinik gebelik oranları belirgin şekilde artmıştır. Zona pellusidası

kalın embriyoya sahip çiftlerde ve neden saptanamamış hastalarda ise 3. gün transferlerinin ardından gebelik oranlarında belirgin artış görülmüştür.

İstatistiksel sonuçlar; ardarda gelen iki siklus karşılaştırıldığında zona inceltme işleminin gebelik oranının arttırdığını göstermektedir. Bu sonuçlara göre; başarısız implantasyon öyküsü olan hastalarda embriyoların lazerle zona pellusidasının inceltmesi mutlaka düşünülmesi gereken bir yöntem olmalıdır.

Anahtar Kelimeler: İnfertilite; Embriyo; Lazer; Hatching; Gebelik

ABSTRACT

The effectiveness of assisted hatching to improve embryo transfer outcome remains controversial. The current study analyses the impact of zona thinning by laser on pregnancy rates after a failed, fresh, nondonor cycle. The study presents the comparative results of two consecutive treatments of the same patients, which were treated under same conditions in same clinic.

Materials and Methods:

Pregnancy results of 188 couples (376 cycles) were examined. These patients met one of the following criteria: 1st group: ≥ 35 years of age, 2nd group: Thick zona pellucida of the embryo, 3rd group: More than one IVF-embryo transfer attempts with no clear reason. Embryos of these patients did not have laser treatment during their first trial, while the zona of the embryos of the same patients were thinned by laser during their second try. All laboratory conditions, mediums as well as clinicians and embryologists were the same for the subseque

ent therapies. The pregnancy rates of each attempt were statistically examined according to the day of the transfer.

Results:

Clinical pregnancy rates increased significantly after hatching both on 2nd and 3rd day transfers on the group with advanced age. The rates were obviously higher after 3rd day transfers on the patients who had embryos with thick zona as well as at the patients who had several attempts before with no clarified reason.

Conclusions:

Statistical results show that zona thinning causes improved pregnancy rates in subsequent cycles. Laser treatment of embryos should definitely be considered for the patients with at least one prior implantation failure.

Keywords: Infertility; Embryo; Laser; Hatching; Pregnancy

INTRODUCTION

Hatching is a process, which allows embryo implantation at blastocyst stage. It is achieved through mechanical expansion and contraction of blastocyst and the thinning of zona pellucida. It is conceivable that defective embryo hatching plays a part in the mechanisms involved in the decrease of embryo implantation rates. Advancing age, thick zona pellucida might be the reasons of difficulty of hatching of the embryos. The cause of the recurrent implantation failures might also be the same reason. Zona thinning by laser assisted hatching might be helpful for all these patients. The effectiveness of assisted hatching to improve IVF-embryo transfer outcome remains controversial. Some studies reported that assisted hatching improved pregnancy and implantation rates in patients with a poor prognosis (1-5), while others found no significant improvements after assisted hatching of embryo (6-9). There is a large data analysis report by Seiff et al. (10) stating that there is insufficient evidence to determine any effect of assisted hatching on live birth rates. The increased multiple pregnancy rates are of concern although it is likely that with a policy of single embryo transfer this may be lowered. Another aspect, which remains unclear, is selection of clinical indications for assisted hatching. There are also researches focusing on the benefits of assisted hatching in good prognosis patients (11).

Because of all these controversial results, assisted hatching is still being discussed from different aspects. Some clinics prefer laser treatment and suggest that it improves implantation rates, while others do not recommend and claims that there is no additional effect of this application.

It has been decided to use laser for zona thinning

of embryos from all patients with poor prognosis in our clinic in 2007, as suggested previously (1-4, 12). Embryos of patients who had previous implantation failure, women with advanced age (≥ 35 years), or embryos with thick zona pellucida are being treated with laser since the clinic purchased laser equipment after this decision.

The study aimed to examine the pregnancy results of the patients who had been applied to the clinic at least twice. Patients, who had laser treated embryos during only their second try but not the first one, were chosen for statistical analysis. The indications, which were mentioned above, were the criteris to choose the embryos for laser treatment. First application of these patients was before laser application in the clinic. The pregnancy rates, with or without laser assisted hatching (LAH) of embryos during their two sequential therapies, were compared.

The aims of the study were;

(i) to find the effectiveness of LAH in women of advanced age (≥ 35 years).

(ii) to clarify if zona thinning helps to improve implantation rate of embryos which have thick zona and

iii) to examine the usefulness of this treatment on the patients which have recurrent implantation failure.

MATERIALS AND METHODS

Patients and selection criteria

Pregnancy results of 376 cycles of ICSI were examined in the study. All patients had been applied to the Assisted Reproductive Technologies Center in Zeynep Kamil Women and Children Diseases Hospital at least two times. Womens age changed between 30 and 40. Number of embryo transferred was one for all patients, All transfers were made either second or third day according to the decision of the clinician and the embryologist. Transfers were made on either 2nd or 3rd day according to the embryo quality on first day. If the embryo was sored as Z1 or Z2, the transfer was made on 3rd day. The embryos, which were classified as Z3 or Z4, were transferred on 2nd day. The quality of all embryos was Grade 1 during transfer day: The embryo was having blastomeres of equal size and there was no cytoplasmic fragment.

Laser treatment had not been applied during their first trial, since the center was not having laser appliances during that period. The patients in the study met one of the following criteria: 1st group: ≥ 35 years of age, 2nd group: Thick zona pellucida of the embryo (more than 20 μ m), 3rd group: More than one IVF-embryo transfer attempts with unknown failure reason. None of groups included the same patients. All women had received controlled long ovarian hyperstimulation protocol. Timing of hCG administration were adjusted according to the usual criteria of follicular maturation. Administration of hCG was performed when at least three follicules exceeded 17 mm in diameter and E2 levels per mature follicle

(≥ 17 mm in diameter) were higher than 250 pg/ml. Oocyte retrieval was performed approximately 36 hours after hCG administration by transvaginal ultrasound-guided aspiration.

Fertilization and embryo culture

Oocytes were rinsed and preserved in 3 ml. fertilization medium (G-IVF PLUS, Vitrolife, Göteborg, Sweden) until sperm preparation. Gradient method was used for sperm preparation. Then, sperms were suspended in the same fertilization medium (G-IVF PLUS, Vitrolife, Göteborg, Sweden) in the incubator until the application of intracytoplasmic sperm injection (ICSI). The study included the patients who had enough sperms for ICSI at normal morphology and motility according to WHO classification. ICSI was performed for all patients. After fertilization; zygotes were cultured in microdrops of 20 μ l of cleavage medium (G1 PLUS, Vitrolife, Göteborg, Sweden) at 37°C under %6 CO₂. Routine embryo evaluation was done by using Scott's scoring system after 16-18 hours following ICSI (13). Other inspections were made after 42-44 hours on 2nd day and after 64-68 hours on 3rd day. Embryos were graded according to the number of cells, the sizes of blastomeres, the presence of fragmentation on these days. Embryos of four blastomeres of similar size and no fragmentation graded as I. Those with three to five blastomeres of similar size, but having 20% or less fragmentation were considered as grade II. Embryos with more than 20% fragmentation without notification of the size and number of blastomeres were classified as grade III. Grade IV embryos were containing severe fragmentation with blastomeres at different size or morphology. Embryos with grade I and II were transferred on day 2 or 3. The patients who had transfers of the embryos with grade III and/or IV were excluded in the study. The same type embryo transfer catheter (Rocket Bulb Tip Embryo Transfer Catheter, United Kingdom). were used for the whole patients. From collection of oocytes to the transfer of embryos; same gynecologists, embryologists and technicians were involved. Laboratory conditions and equipments were also same for all specimens.

Zona thinning by laser

The patients, which met the criteria mentioned above and applied to the clinic second time, had zona thinning application. Partial assisted hatching were applied, since it is associated with higher implantation and pregnancy rates than total assisted hatching (13). Partial hatching was done just before embryo transfer. Zona pellucida was thinned using a 300mw laser with one microsecond pulse length (ZILOS-tk, Hamilton-Thorne Products, Beverly, USA). Laser type was 1480 nm, infrared solid state diode. The laser was fired three times on each embryo. Each exposure was initiated at one point and continued along the zona pellucida until 25-30% was dissolved using three adjacent pulses.

Statistics

The results of bHCG pregnancy tests were evaluated for each attempt of all patients. The results of the three groups, with or without laser application, were compared statistically by using Likelihood ratio chi-square test. PASW (SPSS ver 18) was used for all calculations. Statistically significant level was considered as $p \leq 0.05$.

RESULTS

Clinical pregnancy results of total 188 couples (376 cycles) were compared. The statistical results of each group were as follows:

Group 1:

The pregnancy results of 73 women (total 146 cycles), whose age between 35 and 40, were compared (Table 1).

Table 1. Clinical pregnancy rates of the patients with advanced age before and after laser assisted hatching.

	LAH(-)		LAH(+)	
	2nd day Transfer	3rd day Transfer	2nd day Transfer	3rd day Transfer
Clinical Pregnancy (+)	8 (26%)	11 (26%)	19 (46%)	17 (53%)
Clinical Pregnancy (-)	23 (74%)	31 (74%)	22 (54%)	15 (47%)
TOTAL PATIENT NUMBER	31 (100%)	42 (100%)	41 (100%)	32 (100%)

LAH: Laser Assisted Hatching

Totally, clinical pregnancy tests were positive in 19/73 patients (26%) and 36/73 (49%) patients, before and after LAH respectively. More patients were selected for embryo transfer on 2nd day during their second trials in this group. It was observed that clinical pregnancy rates were 26 % without laser treatment on second day transfers. This rate was raised to 46 % after zona thinning with laser. Pregnancy rate was the same (26%) on 3rd day transfers without laser thinning. The rate rised to 53% after zona thinning (Figure 1).

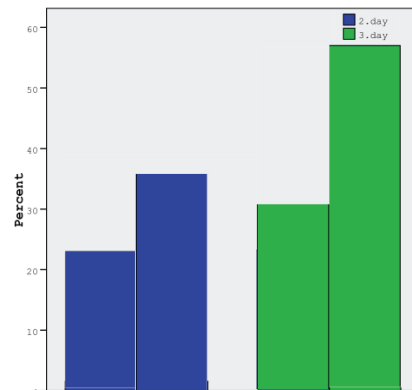


Figure 1. Effect of laser pregnancy rates of the patients with advanced age.

Group 2:

There were 62 couples who had embryos with thick zona pellucida (thickness was $\geq 20 \mu\text{m}$). The ages of women were less than 35 years old. Total 124 cycles were compared statistically (Table 2).

Table 2. Clinical pregnancy rates of the patients having embryos with zone before and after laser assisted hatching.

	LAH(-)		LAH(+)	
	2nd day Transfer	3rd day Transfer	2nd day Transfer	3rd day Transfer
Clinical Pregnancy (+)	8 (23%)	8 (31%)	10 (42%)	24 (63%)
Clinical Pregnancy (-)	27 (77%)	19 (69%)	14 (58%)	14 (37%)
TOTAL PATIENT NUMBER	35 (100%)	27 (100%)	24 (100%)	38 (100%)

LAH: Laser Assisted Hatching

Clinical pregnancy tests were positive in 16/62 patients (26 %) and 34/62 (55 %) patients, before and after LAH respectively. In contrast with the first group, 3rd day transfers increased on second trial. Clinical pregnancy rates were 23 % and 42% before and after zona thinning on second day transfers, respectively. These rates were raised from 31% to 63% on 3rd day transfers (Figure 2).

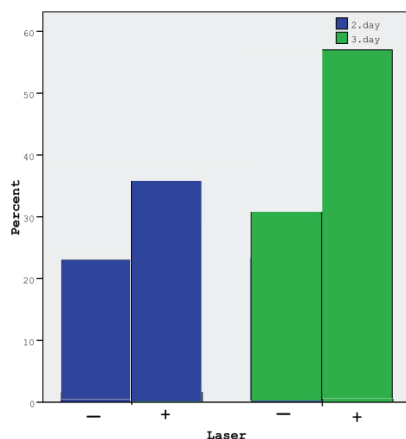


Figure 2. The rates of pragnancy.

Group 3:

53 couples had more than one trial with implantation failure and specific cause for infertility could not be identified for this last group. The age of patients was below 35 years old and the thicknes of zona was less than $20 \mu\text{m}$. Clinical pragnancy tests were positive in 14/53 patients (26 %) and 24/53 (45 %) patients, before and after LAH respectively. More patients could get embryo transfer on 3rd day during their second trial (Table 3).

Table 3. Clincical pregnancy rates of the patients having recurrent implanation failure before and after laser assisted hatching

	LAH (-)		LAH (+)	
	2nd day Transfer	3rd day Transfer	2nd day Transfer	3rd day Transfer
Clinical Pregnan-cy (+)	8 (23%)	6 (31%)	10 (36%)	14 (56%)
Clinical Pregnan-cy (-)	27 (77%)	12 (69%)	18 (64%)	11 (44%)
TOTAL PATIENT NUMBER	35 (100%)	18 (100%)	28 (100%)	25 (100%)

LAH: Laser Assisted Hatching

*No reason for infertility was clarified for these patients. The age of the women was below 35. The thickness of zona was not higher than 20 micrometer

When we compare 2nd day transfers, we noticed that pregnancy rates were changed from 23% to 36%. The results of pregnancy rates on third day were 31% and 56 % without and with treatment, comparatively (Figure 3).

Statistical examinations of the results demonstrate that:

- i) Pregnancy rates increased significantly both on 2nd and 3rd day transfers in women of 35 years or older ($P=0.012$ and $P=0.018$, respectively) after zona thinning.
- ii) Examination of the pregnancy rates after 2nd day transfers of embryos with thick zona did not rise significantly ($p=0.05$). However; the rates were obviously higher after 3rd day transfers ($p=0.01$).
- iii) The rise at pregnancy rates were statistically meaningful on 3rd day transfers ($p=0.074$) of the patients who had two or more trial after laser treatment; while no meaningful increase was observed after 2nd day transfers ($p=0.262$).
- iv) Comparison of pregnancy rates between these three groups after laser application was very similar. There was no significant difference between the pregnancy rates for these three groups ($p=0.68$).

DISCUSSION

Artificial disruption of zona pellucida by laser has been proposed as a method for improving the success of assisted conception. However, it is still being discussed if assisted hatching facilitates live birth and clinical pregnancy (14). In the past decade, zona manipulation has been offered to older women, following repeated implantation failure and high risk of zona hardening (15). There are also researches to determine its benefits in good prognosis patients (16). Recently, Das et al. (14) examined different trials and reported that there was a significant improvement in clinical pregnancy rate amongst wom-

en with a poor prognosis but no evidence of an improvement was reported amongst women with good prognosis.

LAH has been used in our clinic for all poor prognosis patients since December, 2007. The current study was designed to compare the pregnancy rates of these patients who had laser therapy during their second trial but not for the first one. Methodology in the study was designed as follows to decrease the risk of bias:

- Day of transfer was either second or third day for the same patient. If the patient had blastocyst transfer (s) on 5th or 6th days, their results were excluded from the study.
- Similar number of embryos with similar quality had been transferred for each patient.
- All patients with these conditions were included in the study to avoid allocation concealment.
- Sequences for each patient were similar as mentioned previously. All laboratory conditions, materials, equipments, physicians and embryologists were the same for two subsequent therapies for each patient.

The results of the study and their comparison with previous studies were summarized under subheadings as below:

1. Laser therapy on embryos of women, who are 35 years or older, might result with better pregnancy rates.

There are studies which give conflicting results about the benefits of assisted hatching on the patients with advanced age. Makrakis E et al (3) found that implantation rate was significantly higher in LAH group in women of advanced age. However, Hornq SG et al.'s (17) data failed to demonstrate any benefit of LAH in improving implantation or pregnancy rates. Women aged over 37 included in this study were allocated into 2 groups. In group I, embryos were cultured and transferred without LAH, whereas embryos of group II were examined and treated with LAH just before being transferred. Laser manipulations were different from the current study: The laser was aimed at the zona to create openings of about 20 micron in diameter : It has been reported that the size of the opening may affect the implantation rate (18). This might be the reason of the differences between the results of the studies.

Results of Peterson CG et al. (5) also suggest that zona thinning in the population aged $>$ or $=$ 38 years may have no impact on ICSI success rates. However, this conclusion is limited to a situation in which length of the laser zona thinning was $<$ or $=$ 20 micron and the laser was applied to four different positions.

There is a recent study by Valojerdi M et al. (19) They report that LAH improves the pregnancy and implantation rates in patients with frozen-thawed embryos but has no effect in patients with advanced female age or recurrent implantation failure. The zona pellucida of the selected embryos in the test group were opened about 40 μ m by

using an infrared optical laser system, whereas in the control group they were all intact.

Frydman et al. (8) did not show any beneficial effects of LAH in women aged \geq 37 years. Zona of embryos were thinned in their study, which is similar to our application. They divided patient into two groups. Embryos of first group patients had assisted hatching while the control group did not have. They concluded that other embryonic factors might affect the results.

2. Zona thinning improves the outcome on 3rd day transfers of embryos with thick zona pellucida .

Valojerdi et al. (19) reported that LAH improved the pregnancy and implantation rates in patients with frozen-thawed embryos but had no effect in patients with advanced female age or recurrent implantation failure. Laser was used to open the zona in that study. Another study states that the size of the opening may affect the implantation rate (18). In current study; zona pellucida has been thinned, not totally opened. There is one study which suggest that total removal of the zona pellucida is associated with higher pregnancy, implantation and delivery rates compared with partial opening for vitrified blastocyst transfer (18). They used acide thyrode solution, not laser therapy. The results were related with the vitrified blastocysts.

Ge et al. (20) reports that in the fresh embryo transfer cycles, laser for zona thinning has no impact on the rates of positive HCG whereas in frozen-thawed cycles, assisted hatching significantly increases the pregnancy rates.

Hageman et al. (9) examined the results of patients younger than 38 years with embryos with thick Zona Pellucida and could not find any improvement on pregnancy rates. They used acidified tyrode solution.

3. The statistical results of this study has demonstrated that LAH has benefits on patients who has more than one trial with unknown reason.

Dayal et al. (21) report that patients, with one prior implantation failure, benefit from hatching at the subsequent cycle. The data of Grace J et al. (22) suggest that the prognosis for treatment is better if assisted hatching is performed after failure despite optimal embryos compared with failure associated with suboptimal embryos and embryo quality is the most significant factor affecting outcome.

Germond et al. (7) discuss about how to select clinical indications for assisted hatching. They summarize that there are disparate results about the benefits of hatching. The most relevant conclusion obtained so far is that it has a beneficial effect in women with repeated failures of embryo implantation. They conclude that the place of hatching in clinical practice in comparison with other approaches has to be reevaluated.

Laser treatment does not have statistically meaningful effects on these two groups of patients if the embryo transfers were made on 2nd day. This result might be related with the lower success rates after

2nd day transfers without regarding the reason of infertility. The number of patients in these groups statistical analysis was enough, however it was low when compared the other groups.

In conclusion, comparison of the pregnancy results of patients before and after laser therapy shows that zona thinning increases positive results. Unfortunately, the present study was inadequate to detect differences in live birth rates since most of the patients were monitored in various clinics throughout the country after they got their hCG results. However, our data for the past ten years indicate that with a positive hCG test, the historical clinical pregnancy and live birth rates were 40% and 30% respectively. This is an indicator for the level of expected attrition from a positive hCG test to live birth.

In conclusion, it is noteworthy that current results strongly support the hypothesis that the application of laser for zona thinning on embryos might have beneficial effects on the patients who apply to the infertility clinics and had implantation failure before.

REFERENCES

1. Tucker MJ, Morton PC, Wright G, et al. Enhancement of outcome from intracytoplasmic sperm injection: does co-culture or assisted hatching improve implantation rates? *Hum. Reprod* 1996; 11: 2434-2437.
2. Magli MC, Gianaroli L, Ferraretti AP, Fortini D, Aicardi G, Montanaro N. Rescue of implantation potential in embryos with poor prognosis by assisted zona hatching. *Hum Reprod* 1998; 113: 1331-1335.
3. Makrakis E, Angeli I, Agapitou K, Pappas K, Dafereras A, Pantos K. Laser versus mechanical assisted hatching: a prospective study of clinical outcomes. *Fertil Steril* 2006; 86: 1596-1600.
4. Hsieh YY, Huang CC, Cheng TC, Chang CC, Tsai HD, Lee MS. Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. *Fertil Steril* 2002; 78: 179-182.
5. Petersen CG, Mauri AL, Baruffi RL, et al. Zona thinning with a noncontact diode laser in ICSI embryos from women of advanced age. *Assist. Reprod Genet* 2002; 19: 512-516.
6. Horng SG, Chang CL, Wu HM, et al. Laser-assisted hatching of embryos in women of advanced age after in vitro fertilization: a preliminary report. *Chang. Gung. Med. J.* 2002; 25: 531-537.
7. Germond M, Primi MP, Senn A. Hatching: how to select the clinical indications. *Ann N Y Acad Sci* 2004; 1034: 145-151.
8. Frydman N, Madoux S, Hesters L, et al. A randomized double-blind controlled study on the efficacy of laser zona pellucida thinning on live birth rates in cases of advanced female age. *Hum Reprod* 2006; 21: 2131-2135.
9. Hagemann AR, Lanzendorf SE, Jungheim ES, Chang AS, Ratts VS, Odem RR. A prospective, randomized, double-blinded study of assisted hatching in women younger than 38 years undergoing in vitro fertilization. *Fertil Steril* 2009; 93: 586-591.
10. Seif MM, Edi-Osagie EC, Farquhar C, Hooper L, Blake D, McGinlay P. Assisted hatching on assisted conception (IVF & ICSI). *Cochrane Database Syst Rev* 2006 25;CD001894. Review. Update in: *Cochrane Database Syst Rev* 2009; 2: CD001894.
11. Sagoskin AW, Levy MJ, Tucker M, Richter KS, Widra EA. Laser assisted hatching in good prognosis patients undergoing in vitro fertilization-embryo transfer: a randomized controlled trial. *Fertil Steril* 2007; 87: 283-287.
12. Petersen CG, Mauri AL, Baruffi RL, Pontes A, Franco Júnior JG. Implantation failures: success of assisted hatching with quarter-laser zona thinning. *Reprod. Biomed. Online.* 2005; 10: 224-229.
13. Scott L, Alvero R, Leondires M, Miller B. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Hum Reprod* 2000; 15: 2394-2403.
14. Das S, Blake D, Farquhar C, Seif MM. Assisted hatching on assisted conception (IVF and ICSI). *Cochrane Database Syst Rev* 2009; 15: CD001894.
15. Al-Nuaim LA and Jenkins JM. Assisted hatching in assisted reproduction. *BJOG* 2002; 109(8): 856-862.
16. Hiraoka K, Fuchiwaki M, Hiraoka K, et al. Zona pellucida removal and vitrified blastocyst transfer outcome: a preliminary study. *Reprod Biomed Online* 2007; 15: 68-75.
17. Horng SG, Chang CL, Wu HM, et al. Laser-assisted hatching of embryos in women of advanced age after in vitro fertilization: a preliminary report. *Chang Gung Med J* 2002; 25:531-537.
18. Hiraoka K, Fuchiwaki M, Hiraoka K, et al. Effect of the size of zona pellucida opening by laser assisted hatching on clinical outcome of frozen cleaved embryos that were cultured to blastocyst after thawing in women with multiple implantation failures of embryo transfer: a retrospective study. *J Assist Reprod Genet* 2008; 25: 129-135.
19. Valojerdi MR, Eftekhari-Yazdi P, Karimian L, Ashtiani SK. Effect of laser zona pellucida opening on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen-thawed embryos. *Fertil Steril* 2008; 90:84-91.
20. Ge HS, Zhou W, Zhang W, Lin JJ. Impact of assisted hatching on fresh and frozen-thawed embryo transfer cycles: a prospective, randomized study. *Reprod. Biomed. Online.* 2008;

- 16: 589-596.
21. Dayal MB, Dubey A, Frankfurter D, Peak D, Gindoff PR. Second cycle: to hatch or not to hatch? *Fertil Steril* 2007; 88:718-720.
 22. Grace J, Bolton V, Braude P, Khalaf Y. Assisted hatching is more effective when embryo quality was optimal in previous failed IVF/ICSI cycles. *Obstet Gynaecol* 2007; 27: 56-60.
 23. Ghobara TS, Cahill DJ, Ford WC, Collyer HM, Wilson PE, Al-Nuaim L, et al. Effects of assisted hatching method and age on implantation rates of IVF and ICSI. *Reprod Biomed Online* 2006; 13: 261-267.