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Routine Laser Assisted Hatching Results in Significantly Increased Clincal Pregnancies

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***Purpose*:** To elucidate the appropriateness of current indications for assisted hatching (AH) in cleavage stage human embryos and to confirm our preliminary findings that only young patients (about 67%) benefit from AH.
***Methods*:** Prior to transfer, 2 of 3 embryos selected for ET were subjected to laser assisted hatching (LAH). Control group consisted of patients matched by similar characteristics and protocol except LAH was not performed.
***Results*:** The clinical pregnancy rate in women ≤36 years was 64.9% (24/37) for embryos subjected to LAH but was significantly lower ($p = 0.029$) in the control (33.3%; 10/30). The implantation rate in women ≤36 years in the test group was 38.1% (40/105) that was significantly higher than that of the control group (17.5%, 14/80; $p = 0.0039$).
***Conclusions*:** LAH is beneficial for women ≤36 years but not for women ≤37 years, for embryos with thin zonae (≤16 μ) but not with thick zonae (≥17 μ), and for those with repeated failures (37–50%).

KEY WORDS: Assisted; hatching; increased; laser; pregnancies.

INTRODUCTION

Assisted hatching (AH) by mechanical, chemical, or laser manipulation is applied to enable implantation in situations where it is indicated, such as, age factor, high basal FSH, thick zona, or in those who have failed ART treatment on two previous attempts (1–3). Zona assisted hatching was introduced (1) to overcome the zona barrier in instances of suspected impairment to zona hatching due to either zona hardening or thickness, particularly in patients with repeated failures. Assisting hatching was performed mechanically or (PZD: partial zona dissection), chemically (2), or by use of laser technology (3).

There are three main factors affecting implantation in assisted reproduction procedures, namely (i) the quality of embryos transferred (4), (ii) quality of the endometrium (5,6), and (iii) factors associated with impairment of the zona hatching process (2) in particular zona hardening that could occur during in vitro culture (7).

In a preliminary investigation, undertaken between October 2000 through April 2001, laser assisted hatching (LAH) was performed on the embryos of 22 patients in whom AH was indicated. We observed that the use of LAH appeared to benefit young (≤36 years) women (about 67%; 4 of 6 clinically pregnant) but not older (≥37) women (12.5%; 2 of 16 clinically pregnant). The observation of higher pregnancy rate in younger women raises a number of questions, viz. (i) whether the zona pellucida is a paradoxical barrier to implantation? If so, (ii) whether this phenomenon is restricted to in vitro generated

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embryos attributed to zona hardening due to culture conditions, and/or (iii) whether it occurs in the general population contributing to lower fecundity in the human?

The present investigation attempts to answer question (i) above. A study was performed to determine whether the pregnancy rate can be increased if the zona barrier was compromised by LAH on day 2 embryos in patients selected at random irrespective of indication for AH. Only embryos generated by intracytoplasmic sperm injection (ICSI) were employed in the study.

PATIENTS AND METHODS

Patients

A total of 107 patients took part in this study. This investigation commenced in June 2001 till November 2001 at the Assisted Conception Unit, Women's Hospital, Hamad Medical Corporation, Doha, Qatar. Only embryos generated by ICSI were employed in the study. There were two main groups, the control and the test groups. These two groups were further subdivided by age to a total of four subgroups, namely those $\leq$36 years (control, $n = 30$; test, $n = 37$) and those $\geq$37 (control, $n = 20$; test, $n = 20$). Only two of three embryos transferred to patients in the test group were subjected to LAH to minimize the possibility of monozygotic twinning. Embryos of patients in the control group was not subjected to LAH but all other procedures were identical to that of the test group.

Methods

Human Spermatozoa Preparation. Husbands of female patients produced semen by masturbation. Spermatozoa were prepared for insemination by the standard swim-up technique or by differential centrifugation using Puresperm™. Final sperm suspension was made in culture medium (Medi-Cult™, Medi-Cult, Denmark or IVF-50™ Vitrolife, Sweden). The spermatozoa preparation was incubated under a gaseous phase of 5% CO_2 in air at 37°C until use.

Ovarian Stimulation and Oocyte Retrieval. Ovarian stimulation was performed following down-regulation as previously described (8).

Intracytoplasmic Sperm Injection (ICSI). ICSI was performed by standard methods.

Culture Procedures. Following ICSI, the injected oocytes were washed in culture medium (Medi-Cult™ or IVF-20™) and then transferred into 1.5–2.0 μL of ultra microdroplets of culture medium (ultra microenvironment culture) (8) held under mineral oil in the culture dish. The injected oocytes were incubated overnight in an atmosphere of 5% carbon dioxide in air at 37°C in an incubator saturated with humidity.

Determination of Day 2 Cleaved Embryo Quality. The quality of human day 2 embryos were determined as described previously (8). Since most healthy day 2 human embryos generally attain the 4-cell stage, an average blastomere score of 4 and above was considered excellent in a range of 2–6. The embryos were graded according to a scale of 1–4, where the numerical 1 denoted *poor quality*, and 4, *excellent quality*. Embryos were graded collectively by three laboratory personnel with the use of a television monitor. Excess embryos following embryo transfer of grade 3 and above were cryopreserved.

Laser Assisted Hatching (LAH). Assisted hatching was performed on day 2 cleavage-stage embryos using the nontouch Saturn™ Laser System of the Integra Model obtained from Research Instruments, U.K. The quality of the day 2 embryos were assessed by three personnel with the use of a TV-monitor. Three embryos were selected for transfer for individual patients. All patients in the test group received three embryos of which only two were subjected to LAH to minimize the incidence of monozygotic twinning. A Falcon Plastics ICSI dish (cat no. 1006, Becton Dickinson, U.S.A.) was used for the LAH procedure. Two embryos were placed individually in 60–80 μL microdroplet of Hepes buffered embryo handling medium (Gamete™-20, ref. No. 12006, Vitrolife, Goteborg, Sweden) overlaid with mineral oil. The cleaved embryo was positioned and held firmly using an ICSI holding pipette such that the point at which laser was applied had the largest available perivitelline space (PVS) of the embryo. A single opening was drilled of approximately 8–10 μ at the inner part of the zona and about 15–17 μ at the outer part of the zona using laser pulse length of 25 ms. If the zona was resistant to laser pulses of 25 ms and no perforation occurs, a laser pulse of larger length such as 125 ms was used. If the zona was still resistant to perforation with laser pulses of 125 ms, then lasers of 750 ms pulse length was applied.

Color of Zona Pellucida. Color of zona pellucida is scored in a range of 4–1, where 4 is scored for *normal or light coloration* and 1 for *poor or very dark coloration*.

Table I. Effect of Laser Assisted Hatching on Clinical Pregnancy and Implantation Rates

Treatment	% Fertilization rate	Average blastomere number of embryos transferred	Average embryo grade % Clinical of embryos transferred	pregnancy rate	% Implantation rate
Control women ≤36 years	75.4 (135/179)	3.93	3.68	33.3 (10/30)	17.5 (14/80)
Test women ≤36 years	75.8 (222/293)	4.15	3.58	64.9 (24/37)	38.1 (40/105)
t-test	$p = 1.000$	$p = 0.2767$	$p = 0.0260$	$p = 0.0203$	$p = 0.0039$
Control women ≥37 years	69.4 (84/121)	3.41	3.73	20.0 (4/20)	9.3 (5/54)
Test women ≥37 years	79.2 (76/98)	3.98	3.30	15.0 (3/20)	6.9 (4/58)
t-test	$p = 0.2322$	$p = 0.3861$	$p = 0.0007$	$p = 1.00$	$p = 0.911$

RESULT

Twenty-four (64.9%) of 37 women achieved clinical pregnancy in the test group of women ≤36 years of age. In these patients, LAH was performed immediately prior to transfer (Table I). Of the 24 patients positive for fetal heart, 13 (35.1%) were singletons, 6 (16.2%) were twins, and 5 (13.5%) were triplets. In the control group of the same age group, 10 (33.3%) of 30 patients became clinically pregnant. The lower pregnancy rate in the control group was statistically significant ($p = 0.029$).

The implantation rate was 38.1% (40/105) in the test group of women ≤36 years was statistically higher than that obtained in the control group of the same age group (17.5%; 14/80; $p = 0.0039$).

In the older age group of women ≥37 years of age, the clinical prenancy rate after LAH was 15% (3/20) that was statistically similar ($p = 1.000$) to the 20% (4/20) clinical pregnancy rate observed in the control group.

The implantation rate was 6.9% (4/58) in the test group of women ≥37 years was statistically similar to that obtained in the control group of the same age group (9.3%; 5/54; $p = 0.911$). Table II shows the effect of age, thickness of zona, colour of zona, or the length of laser applied to perforate the zona, on pregnancy in patients whose embryos were subjected to laser perforation immediately prior to transfer.

Age of the patient has significant effect on pregnancy and implantation after LAH (Table II). Younger women ≤36 years were more successful in achieving clinical pregnancies (64.9%; 24/37 vs. 15.0%; 3/20; $p = 0.0009$) and higher implantation (33.3%; 35/105 vs. 6.9%; 4/58) rates, respectively, compared to women 37 years or older after LAH.

The thickness of zona also has significant effect on pregnancy (Table II). Significantly more pregnancies (69.2%; 18/26) were obtained after the transfer of embryos with thin zonae (≤16 μ) compared to those with thicker zonae (≥17 μ; 25.0%; 3/12; $p = 0.0279$) after LAH. The colour of the zona, length of laser beam, and number of ART treatment attempts appear to have an effect but the difference were not statistically significant (Table II).

DISCUSSION

AH by mechanical, chemical, or laser manipulation has been conventionally employed to enable implantation in situations where it is indicated. Controversy over the usefulness of the AH is well documented. Contrary to previous reports, the present study shows that AH, particularly LAH, does not appear to benefit patients in whom it is indicated, rather, it

Table II. Relationship Between the Clinical Pregnancy Rate and Age of Patient, Thickness of Zona, Colour of Zona, Length of Laser Pulse Bombardment, and Number of ART Attempts

Description	Age of patient	Thickness of zona (μ)	Colour of zona	Length of laser beam (ms)	Number of ART attempts
Cutoff points	≤36 years ≥37 years	≤16 μ ≥17 μ	1–2 3–4	≤249 ≥250	≤1–2 ≥3
% Clinical Pregnancy	64.9 (24/37) 15.0 (3/20)	69.2 (18/26) 25.0 (3/12)	20.0 (1/5) 62.2 (23/37)	60.0 (21/35) 28.6 (2/7)	52.6 (20/38) 36.8 (7/19)
t-test	$p = 0.0009$	$p = 0.0279$[a]	$p = 0.1913$[b]	$p = 0.2095$[b]	$p = 0.3986$

[a] Patients in test group of all ages; data available for 38 cases only for analysis.
[b] Patients in test group of all ages; data available for 42 cases only for analysis.

appears to significantly increase the pregnancy rate in young women where AH is not indicated. The present study demonstrates that LAH need be performed on a routine basis for increasing the pregnancy rate in younger women because clinical pregnancy rates of about 65% and implantation rates of about 38% could be achieved. However, the incidence of twin and triplet pregnancies are increased, which could be attributed to LAH. The increased rate of multiple pregnancies is cause for concern. Stipulations are now in place to transfer no more than two embryos in women $\leq$36 years after LAH in our facility.

The very high pregnancy and implantation rates obtained following LAH in young women in the present study are similar to that reported for blastocyst transfers (9). A similar observation has also been made for day 2 transfers of human embryos generated in the continuous ultra microdroplet (cUME) culture procedure (10). With LAH there is no need to culture embryos to the blastocyst stage to increase the pregnancy rate in young women. Besides, extended culture in vitro may cause zona hardening (7), therefore, it may be appropriate to culture the embryo up to day 2 or 3 and then transfer following LAH. Such a measure may yield pregnancy rates similar to that of blastocyst transfers.

LAH is of no benefit in women $\geq$37 years. The pregnancy and implantation rates after LAH in the older women is lower than in the control group but this difference is statistically similar. Therefore, LAH need not be performed on embryos of older women. However, the decision to perform or not to perform LAH on older women should be made on the merits of individual case. LAH can be performed in repeated failures in the older women as there may be no other recourse in such situations. The present study has demonstrated that LAH could be useful in some instances of repeated failures in women of all age groups following ART treatment.

The thickness of zona appear to have significant effect on pregnancy rate after LAH. The pregnancy rate was significantly lower (25%) in embryos with zonae $\geq$17 μ in thickness compared to those with zonae $\leq$16 μ (69%) thickness irrespective of the age factor. It is thus obvious that LAH of embryos with thick zonae may not improve the pregnancy rate. Conventional wisdom dictate that AH be performed for embryos with thick zonae. On the contrary, however, the present findings demonstrates that AH is of no benefit in such embryos. It is also possible that the zona drilling protocol employed in the present study require a modification that could enhance the implantation of embryos with thick zonae. This remain to be elucidated.

The length of the laser beam applied to drill the zona also appears to influence the pregnancy rate but there is no statistical difference between the two groups, viz. even though the pregnancy rates were 60 and 29% for beam lengths of $\leq$249 and $\geq$250 μ. This, however, could be due to the small sample size. With a larger sample size the difference would have been significant. It is, however, apparent that beam lengths of $\geq$250 μ could harm the embryo. It is also possible that higher beam lengths have to be used to drill the zona when it is thick or hard. The low pregnancy rate associated with high beam lengths could be due to increased zona thickness and rigidity. Even thin zonae can resist laser drilling due to high rigidity. In such instances higher laser beam lengths have to be applied to drill into the hard zonae.

LAH is useful in women with repeated failures especially if they have embryos with thin zonae. About 37% of all patients with (3–4) repeated ART treatment failures will conceive with LAH. In patients who have failed on five ART treatment attempts, the clinical pregnancy rate was 50% (5/10) after LAH. Hence it is worthwhile performing LAH in these patients.

LAH is thought to be less traumatic than chemical or mechanical AH (3,11,12). The main problem with mechanical and chemical AH procedures is the inability to standardize and reproduce openings of identical dimensions in the zona, which may affect pregnancy outcome. In the LAH procedure openings of identical dimensions can be easily obtained or reproduced. Furthermore, mechanical and chemical methods could damage the blastomeres. LAH is a nontouch technique (3) and is known to produce no thermal or mutagenic side effects (11). Furthermore, LAH is a rapid technique that can be completed in minutes.

Most countries allow the transfer of more than two embryos, particularly if legislation does not exist, to ensure implantation and pregnancy. The high pregnancy and implantation rates of 65 and 38%, respectively, obtained in the present study suggest that the number of embryos transferred could be reduced to two instead of three embryos in young women if LAH is performed. By transferring two embryos after LAH, the incidence of multiple births could be reduced although the increased incidence of monozygotic twinning cannot be ruled out.

The zona is known to be a barrier for pathogens. In the present study LAH was not performed on

embryos of couples when either one or both are carriers of hepatitis. This is to ensure that embryos are protected. Hepatitis carriers appear to reproduce normally in the general population but subfertile/infertile hepatitis carriers seeking assisted reproduction treatment invariably experience difficulty in getting pregnant if ICSI was employed to achieve fertilization. We do not have sufficient data to prove this point and this remain to be elucidated.

In conclusion, routine LAH is extremely beneficial for increasing the pregnancy rate in young women of $\leq$36 years. It is also useful in patients with repeated ART treatment failures. The present findings suggest that the zona of the in vitro generated embryo is a barrier to implantation. This barrier has to be compromised to increase pregnancy rates. The present study has not attempted to determine whether in vitro culture conditions can be implicated for zona hardness. This remains to be elucidated. It is also not clear whether a similar phenomenon occurs in vivo. The less than optimal reproductive performance in the human may, in part, be due to the zona barrier, if a similar situation were to exist in vivo. The main outcome of this study is the revelation that excellent clinical pregnancy rates can be achieved if LAH is preformed on a routine basis in all women $\leq$36 years of age.

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