ORIGINAL RESEARCH REPORT

The Effect of Laser-Assisted Hatching on Pregnancy Outcomes of Vitrification Frozen Embryo Transfer

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ABSTRACT

Background: Zona Pellucida (ZP) thickness of less than 16 mm is better for embryo implantation inside the endometrium. Laser Assisted Hatching (LAH) is commonly used, especially in non-contact mode, using a 1.48-um infrared diode laser beam because of its short exposure time, accurate positioning, simple operation, indirect contact, safety, and effectiveness. **Objective**: This paper describes the potential of laser-assisted hatching in biochemical pregnancy outcomes in Frozen Embryo Thawed transfers. **Material and Method**: The total number of patients enrolled in this study was 141. Patients were prospectively treated during embryo transfers at Pusat Fertilitas Bocah Indonesia, Primaya Hospital at Tangerang, Indonesia, from December 2020 until December 2021. **Result**: There were no significant differences between the LAH and no-LAH groups regarding average age, infertility duration, infertility type, and etiology of infertility ($p>0.05$). In the same line, the blastocyst (0.76 ± 0.87; 1.25 ± 1.08) compared with cleavage (0.72 ± 0.84; 0.67 ± 0.98) ($p<0.534$ and $p< .032$). There was no significant difference, even though clinically, the proportions of live births, multiple pregnancies, biochemical pregnancies, and ongoing pregnancies in the LAH group were not exceptions to the outcomes of failures and miscarriages. **Conclusion**: LAH did not appear to increase the pregnancy rate in this study. However, the methodology seems to reduce bias in this study by considering methodology for selecting FET embryos based on the cryopreserved condition, embryo quality, and precise LAH.

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Highlights

1. LAH might effectively increase pregnancy outcomes for frozen-thawed embryo transfer.
2. Zona pellucida drilling might help increase pregnancy outcomes in FET.

BACKGROUND

Zona Pellucida (ZP) is an extracellular coat surrounding mammalian growing oocytes, ovulated oocytes, and early embryos. During the secondary follicle stage of oogenesis, the zona is secreted. The thickening of the ZP begins during the growth stage as the diameter of the oocytes increases. The zona thickness of less than 16 mm is better for embryo implantation inside the endometrium. It also protects the ovum during oviduct transit and the oocyte and embryo during the early stages of development from infection, as well as maintaining blastomere structure during preimplantation development (Le, et al., 2018; Nieschlag, et al., 2023). The structure of the ZP changes after fertilization, making it impossible for sperm to adhere to it. Before uterine implantation may occur, an enlarged blastocyst must hatch outside from the ZP, so if the thickness of the ZP is more than 16 mm, it is hard to implant. Proteolytic sublysis of the ZP by the embryo or the dam's endometrial lining and hydrostatic pressure created by fluid accumulation in the developing blastocoel cavity are two processes that allow ZP rupture and blastocyst escape (Jeong, et al., 2018).

In IVF-embryo transfer, abnormalities of the hatching process have been blamed for poor implantation. Assisted hatching (AH) was proposed to foster spontaneous hatching and improve embryo implantation (Hammadeh, et al., 2011).

Many studies have investigated the use of AH procedures to treat patients with poor-quality embryos and endometrium as a consequence of advanced maternal age (Le et al., 2018). Four methods used in the artificial rupture of ZP were mechanical partial ZP dissection, chemical ZP drilling using acid solution or enzymes, and laser micromanipulation (Gerri, et al., 2020) These methods aim to create holes in the ZP.

LAH is commonly used, especially in non-contact mode, using a 1.48-um infrared diode laser beam because of its short exposure time, accurate positioning, simple operation, indirect contact, safety, and effectiveness. Similar studies reported that efficacy of ZP dissection and implantation rate among embryos hatched by LAH, acid Tyrode's solution, and pronase enzymes (Wang, et al., 2022; Wei, et al., 2023). In other study Tannus, et al., (2019) showed no benefit of the ZP thinning in frozen-thawed embryo transfer.

OBJECTIVE

This article aimed to describe the potential of LAH in biochemical pregnancy outcomes in Frozen EmbryoThawed (FET) transfers.

MATERIAL AND METHOD

Patients' characteristics

The total number of patients enrolled in this study was 141. Patients were prospectively treated during embryo transfers at Pusat Fertilitas Bocah Indonesia, Primaya Hospital at Tangerang, Indonesia, from December 2020 until December 2021. This method was repeated every other day throughout this study.

All patients met the following inclusion criteria: ≥25 years of age with indications for IVF-embryo transfer: male factor, female factor, unexplained infertility, and mixed factor. Informed consent was obtained from all women, and this investigation received the approval of the Ethics Committee of Primaya Hospital in Tangerang City dated 26-03-2022.

Fertilization and embryo culture

All patients were treated antagonist short protocol for ovarian stimulation (Siristatidis, et al., 2022). Oocyte retrieval was performed by ultra-sound-guided follicle aspiration 36–38 hours after hCG administration. The oocytes underwent standard IVF and ICSI and were cultured in G-1 version 3.0 (Vitrolife, Kungsbacka, Sweden), supplemented with 10% recombinant human serum albumin (rHA, Vitrolife) for two days. On the day of embryo transfer (44–48 h after sperm insemination or injection), the embryos were scored according to the following Sydney and Gardner criteria for embryos (Blank, et al., 2020; Stigliani, et al., 2021). In some patients, when there were no more than two good-quality embryos, one or two extra poor-quality embryos were also selected for the transfer. However, the

patients with all poor-quality embryos had poor endometrial response or had a ZP thickness of >16 mm and were excluded from the study.

Embryos were randomly divided into two groups. In the control groups, the selected embryos were intact before the intrauterine transfer, whereas in the test groups, they were subjected to LAH. The embryo transfer was performed with a Kitazato catheter, whereas the physician was blinded to the control and test groups. The luteal phase was supported by intravaginal (Cyclogest 400, in two divided doses, for 800 mg/d; Actavis, Barnstaple, United Kingdom), starting the artificial cycle decided.

The patients were tested for serum ß-hCG 50mIU/ml assay 15 days after embryo transfer (Maheshwari, et al., 2019). If the biochemical pregnancy test was achieved, patients were followed with serial ultrasound to determine fetal viability. Clinical pregnancy was defined as the presence of a gestational sac on transvaginal ultrasound. When pregnancy occurred, luteal support was continued until 12 weeks gestation.

Freezing and thawing

The embryos were frozen using previously established protocols (ultra-rapid freezing). For thawing, the embryos were transferred from the culture media to the basal media (BM) containing HEPES (Quinn's SAGE, ART-1024) and supplemented with human serum albumin (20% v/v) (HAS, Vitrolife, 10064). The embryos were then transferred to the vitrification solution 1 (BM solution supplemented with DMSO (7.5% v/v) and ethylene glycol (7.5% v/v)) for 2–7 min. They were then transferred to the vitrification solution 2 (BM solution added DMSO (15% v/v), ethylene glycol (15% v/v), and sucrose (10% v/v)) for 30 seconds when shrank to 80%. They were then collected within 5–10 s in a minimal volume and transferred into a cryo device for immediate preservation in liquid nitrogen.

For thawing, the embryos were transferred from the cryo device to warming solution 1 (BM solution added 1 M sucrose) for 1 min and then to warming solution 2 (BM solution added 0.5 M sucrose) for 3 min. They were then transferred to basal medium for 5 min, washed using G2 solution and cultured 4 hours at 37 °C and 6% CO_2 (Allahbadia, et al., 2020; Korkmaz, et al., 2020).

Embryo selection and transfer

On the day of thawing, embryos were transferred. Embryos at both cleavage and blastocyst stages were assessed using a scoring system. Following thawing, the embryos were cultured for 4 to 6 hours at 37°C with 6% CO_2 before transplantation. Additionally, ultrasound scans were conducted to monitor endometrial thickening and receptivity in artificial cycles. The freeze-thawed embryos were transplanted on the third day of ovulation, specifically when the endometrium thickness was equal to or greater than 8 mm; hormone replacement treatment was administered for patients who did not experience natural ovulation. This included a daily dose of 2 to 4 mg of estradiol valerate starting from the third day of the menstrual cycle. Ultrasound-guided embryo transplantation was subsequently performed on days 18 to 19 (O'Shea, et al., 2016; Wang, et al., 2020).

Laser-assisted hatching procedure

The laser hatching procedure utilized a Nikon TE300 inverted microscope (Nikon, Tokyo, Japan) equipped with the Zona Infrared Laser Optical System (Hamilton-Thorne Research, Beverly, MA) employing a 1.48-mm infrared diode laser beam. Each embryo was placed on the microscope stage, and the laser was directed to the target area. The embryo was positioned to expose a Zona Pellucida (ZP) section to the laser beam. The laser was then activated and discharged multiple times, each lasting 0.5 ms, to create a single opening in the ZP, approximately 40 mm in size, called drilling. Zona thinning was made by putting a laser around the zona pellucida, estimated for 2 ms.

Data analysis

Group differences were tested using the t-test for numerical variables and the chi-square test for categorical variables using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, N.Y., USA). A $p < 0.05$ was considered significant.

RESULT

The basic parameters evaluated using both methods are shown in Table 1. There are no significant differences between the LAH and no-LAH groups regarding average age, infertility duration, infertility type, and etiology of infertility ($p>0.05$). In the same line, the blastocyst (0.76 ± 0.87; 1.25 ± 1.08) compared with cleavage (0.72 ± 0.84; 0.67 ± 0.98) ($p< 0.534$ and $p< 0.032$; Table 1).

Table 1. Characteristic patients used LAH and no LAH.

Characteristics	LAH (-)	LAH(+)	p-values
Total patients	25	116	
Woman's age (year, mean ± sd)	34 ± 4.08	33.01 ± 4.78	0.360[b]
Man's age (year, mean ± sd)	38.7 ± 7.6	35.3 ± 5.46	0.069[c]
Duration of primary Infertility (year, mean ± sd)	5.15 ± 2.8	5.51 ± 3.5	0.961[c]
Duration of secondary infertility (year, mean ± sd)	11 ± NA	6.4 ± 4.50	0.380[c]
Diagnosis of infertility (n,%)			
Female factors	16 (64)	67 (68.3)	0.565
Male factors	23 (92)	114 (98.3)	0.145[d]
Unexplained infertility	2 (8)	9 (7.8)	1.000[d]
Mixed factors	16 (64)	67 (57.8)	0.565
Embryo transfer			
Cleavage transfer n=69 (mean ± sd)	0.72 ± 0.84	0.67 ± 0.98	0.534[c]
Blastocyst transfer n=72 (mean ± sd)	0.76 ± 0.87	1.25 ± 1.08	0.032[c]

Legends: [a]chi square test; [b] independent t-test; [c]Mann Whitney; [d]Fisher

Table 2. Outcome characteristics with LAH and no LAH.

Characteristics	LAH(-)	LAH(+)	p-values
Total patients	25	116	
Miscarriage (n,%)	1 (4)	8 (6.9)	1.000[d]
Failed (n,%)	18 (72)	69 (59.5)	0.243
Live birth	3 (12)	25 (21.6)	0.408[d]
BO (Blighted Ovum)	2 (8)	1 (0.9)	0.081[d]
Multiple pregnancies	1 (4)	7 (6)	1.000[d]
Biochemical	0 (0)	6 (5.2)	0.591[d]
Clinical pregnancy	1 (4)	15 (12.9)	0.305[d]

Legends: [a]chi square test; [b]independent t-test; [c]Mann Whitney; [d]Fisher

The comparison of patient outcome characteristics in Table 2 reveals no significant difference, even though clinically, the proportions of live births, multiple pregnancies, biochemical pregnancies, and ongoing pregnancies in the LAH group are not exceptions to the outcomes of failures and miscarriages.

Table 3. Pregnancy outcome characteristics with LAH and no LAH.

Characteristic	LAH(-) (25)	LAH(+) (116)	p-value
Failed (n,%)	18 (72%)	69 (59,5%)	0,243[a]
Success (n,%)	7 (28%)	47 (40,5%)	

Legends: [a]chi square test; [b] independent t-test; [c]Mann Whitney; [d]Fisher

The comparison of patient outcome characteristics in Table 3 shows no significant difference (p=0.243), even though clinically, the proportion of successful outcomes (live birth, multiple pregnancy, biochemical, and ongoing) in LAH (40.5%) is more significant than in no LAH (28%) when compared to failed outcomes.

Table 4. Outcome characteristics with total single hole dan 1/4 jole.

Characteristics	Drilling	Thinning	p-values
Total patients	84	32	
Miscarriage (n,%)	7 (8.3)	1 (3.1)	0.442[d]
Failed (n,%)	53 (63.1)	16 (50)	0.199
Live birth	16 (19)	9 (28.1)	0.288
BO (Blighted Ovum)	0 (0)	1 (3.1)	0.276[d]
Multiple pregnancies	5 (6)	2 (6.3)	1.000[d]
Biochemical	3 (3.6)	3 (9.4)	0.345[d]
Clinical pregnancy	13 (15.5)	2 (6.3)	0.231[d]

Legends: [a]chi square test; [b] independent t-test; [c]Mann Whitney; [d]Fisher

The comparison of patient outcome characteristics in Table 4 shows no significant difference, even though clinically, the proportion of live births, multiple pregnancies, and ongoing outcomes in drilling>thinning is not exempted in both failed and abortion outcomes.

DISCUSSION

The zona pellucida is a layer of glycoproteins that envelop the oocyte, comprising outer and inner layers. The outer layer is characterized by being thick and easily dissolved, while the inner layer is more resistant to dissolution. Its primary function is to prevent polyspermy and the fertilization of the oocyte by sperm without an intact acrosome. Implantation, the initial process involving the embryo's contact with the endometrium in the uterus, is termed hatching in its earlier stages. As the embryo reaches the blastocyst stage, it breaks free from the zona pellucida and initiates hatching. Inadequate blastocyst hatching may result in implantation failure during in vitro fertilization (IVF) (Lu, et al., 2019; Liu, et al., 2020).

Our findings revealed that the group undergoing LAH (laser-assisted hatching) exhibited a higher biochemical pregnancy rate compared to those who received no LAH treatment. Still, there is no significant correlation between the two groups. This aligns with research conducted by Endo, et al., (2021) indicating a significantly greater rate of complete hatching in the AH (assisted hatching) treatment group compared to the untreated control group. The use of LAH remains a subject of debate and controversy in the medical literature. According to the most recent national summary of AH provided by Hall, et al., (2017), there was a significant increase in AH utilization in the USA, rising from 25,725 to 35,518 procedures between 2000 and 2010. This upward trend might be associated with guidelines published in 2008 by the American Society for Reproductive Medicine (ASRM), suggesting the potential clinical usefulness of AH for patients with a poor prognosis (Liu, et al., 2020). The laser-assisted system offers notable advantages over chemical, mechanical, or enzymatic methods. Firstly, LAH allows for a touch-free, precisely targeted delivery of laser light to the desired location with minimal embryo absorption. Secondly, laser technology in human reproductive medicine is cost-effective and easily adaptable to inverted microscopes. The American Society for Reproductive

Medicine Practice Committee, (2022) reported significant clinical outcomes on FET using LAH. In contrast, Lacey, et al., (2021) indicated that ZP digestion by enzymatic methods was unrelated to any benefit of FET outcome. These differences could be due to the difference in the type of LAH or cryopreserved conditions such as cryoprotectant, stage of cryopreserved embryo, or storage duration.

Inadequate hatching can be influenced by the embryos from infertile female patients aged 38 years and above, leading to a notable decline in developmental performance and a significant reduction in fertility potential. Similar to the previous study, it resulted in a decrease in the pregnancy rate. Recent research suggests that this decline might be attributed to uncharacterized zona hardening in embryos due to advanced maternal age, impacting the quality of embryos with poor morphology. This zona hardening may hinder the blastocyst from breaking free from the ZP. Although there is no molecular evidence indicating prematurely cleaved ZP2 or other molecular markers associated with zona hardening in these embryos, other studies suggest that using LAH was similar between treated and untreated groups (18.36% compared to 11.36% in non-hatched embryos) within this specific group of patients (Nagy, et al., 2019).

The embryo's quality is another significant factor that could disrupt hatching. Various intrinsic elements, such as basal FSH levels and diagnoses, the hormonal changes experienced by maturing oocytes during ovarian stimulation, or the controlled conditions within the in vitro culture, can create obstacles for successful hatching. The artificial environment of in vitro culture might negatively impact the quantity or quality of zona lysin, a substance produced by the trophectoderm crucial for blastocyst hatching. There could also exist inherent variations in lysin secretion, independent of assisted reproduction techniques, contributing to the incapacity of certain blastocysts to hatch effectively (Tannus, et al., 2019; Curfs, et al., 2023). Embryo fragmentation stands as a common abnormality in human embryos. This detrimental influence can be attributed to various mechanisms, including disrupted mitotic spindles, reduced cell count, diminished cell size, partial or complete loss of regulatory proteins, disturbance in cell communication and signaling, reduced number of mitochondria, irregular mitochondrial distribution, decreased ATP content, failure in standard compaction and blastulation processes, discrepancies in cell allocation, and difficulties in hatching (Cecchele, et al., 2022).

Sagoskin and colleagues conducted a prospective, randomized, controlled trial. They found that assisted hatching (AH) did not enhance clinical pregnancy outcomes in patients with a good prognosis when only transferring good-quality embryos (Tannus, et al., 2019; Curfs, et al., 2023). The assisted hatching at the cleavage stage might expose the embryo to free radicals, toxins, and immune cells, leading to potential DNA damage and hindering its development to the blastocyst stage in the uterus. There is a possibility that during the compaction process, the loosely connected cells in 2 or 3-day-old embryos could be trapped or lost through the artificially created holes in the zona pellucida (ZP). Conversely, blastocyst-stage embryos consist of larger, more tightly connected groups of cells, potentially making them more resilient to localized damage caused by AH than embryos at the 4 to 8-cell stage. Repeated manipulation of the same embryo, first through intracytoplasmic sperm injection (ICSI) and later through AH, could be another mechanism negatively impacting the implantation potential of the embryo. Previous research indicated that this dual manipulation resulted in a lower clinical pregnancy rate when compared to either a single manipulation or no manipulation (Zhang, et al., 2022).

Recent research findings indicated that miscarriage rates remained comparable, whether using fresh embryos or those that had undergone cryopreservation and subsequent thawing for transfer. However, variations in clinical pregnancy and live birth rates were attributed to differences in implantation rather than pregnancy loss (Roeca, et al., 2020; Insogna, et al., 2021).

Strength and limitations

Laser zona-thinning increases the implantation rate, presumably higher in biochemical and clinical pregnancy. However, the primary outcome measure showed statistically insignificant findings between LAH and non-LAH. The methodology of this study appears to reduce bias by choosing a FET embryo based on cryopreserved condition, embryo quality, and precise manipulation using LAH that allows non-contact manipulation and avoidance of any injury to the embryo that causes an increased number of biochemical, clinical, multiple pregnancies, live birth rates. Although these differences were not statistically significant, in studies in a larger population, specific methods for non-LAH are needed to obtain a more robust and reliable evaluation of the superiority of the LAH method. The limitation of this study was a small number of samples with low quantities.

CONCLUSION

Laser-assisted hatching does not appear to increase the pregnancy rate in this study. However, the methodology reduces bias in this study by considering the method for selecting FET embryos based on the cryopreserved condition, embryo quality, and precise LAH.

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Conflict of Interest
The authors have nothing to disclose.

Ethic consideration
The study has been approved by the Ethics Committee of Primaya Hospital in Tangerang City on 26-03-2022.

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None

Author Contribution
VL contributes to the conceptual and design, data analysis and interpretation, article drafting, and data curation. AR contributes to the conceptual and design, data analysis and interpretation, and article drafting. TD contributes to data analysis, interpretation, and critical revision of the article for important intellectual content. MDW contributes to data analysis and interpretation, critical revision of the article for important intellectual content, and final approval of the article.

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