

ORIGINAL ARTICLE

Laser assisted thinning *versus* drilling in cryopreserved embryo transfer cycles

Laser assisted thinning versus drilling in cryopreserved cycles

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ABSTRACT

Objective. Human embryo cryopreservation is a crucial aspect of Assisted Reproduction (AR). Nonetheless there is a potential concern regarding zonal hardening, which may have a negative impact on AR outcome. Assisted hatching (AH) has been employed to facilitate microdissection of the zona pellucida. Both laser assisted thinning and drilling techniques have been used to address this issue. However insufficient studies compared laser thinning versus opening technique. We aimed to study the effect of laser assisted hatching on cryopreserved embryos, comparing both laser assisted thinning and laser assisted drilling techniques and their effects on both implantation rate chemical and clinical pregnancies.

Patients and Methods. 300 women were randomized into 3 equal groups (thinning, drilling and control group) and prepared for transfer of frozen/thawed embryos. The thinning and opening techniques were performed on the day of embryo transfer while the zona of the control group was left intact.

Results. Both the chemical and clinical pregnancy rates were higher in laser-thinning group over the control group in all women enrolled in the study (p-value, 0.04, 0.031 respectively) and specifically above age of 35 years old (p-value, 0.003, 0.006 respectively) with a significant implantation rate (p-value 0.043) on addition. However, laser-drilling group showed no advantage over neither the control group or the thinning group.

Conclusions. Laser thinning technique for cryopreserved embryos increased both the chemical and clinical pregnancy rates in frozen embryo transfer especially above age of 35 years old and it seems to have the upper hand in relation to laser opening technique.

Key words

Cryopreservation; Zona pellucida; Laser assisted hatching; Laser assisted thinning; Laser assisted opening; Implantation rate and Pregnancy rate.

Introduction

Frozen embryo transfer (FET) has become a common practice in assisted reproductive techniques. Many techniques had been used in intracytoplasmic sperm injection (ICSI) to improve endometrial receptivity [1] but also the embryo quality has an important role in pregnancy occurrence. It has been found that embryo cryopreservation increases the cumulative pregnancy rate in ICSI cycles [2]. Zona pellucida (ZP) hardening as a consequence to cryopreservation can impair embryo hatching and in turn lowering the implantation rate [3]. The presence of fewer and smaller holes in the zona pellucida (ZP) of cryopreserved embryos compared to those found in fresh ZPs could alter the hatching process [4].

More than 30 years ago, Assisted Hatching (AH) had been described to improve implantation rate among certain groups as women with advanced age, previous implantation failures, cryopreserved embryos [5]. AH is a microsurgical technique conducted on embryos aiming to alter the hatching process. This procedure involves manipulating the zona pellucida (ZP) in various ways, such as creating holes, slots, or thinning it to different extents. Mechanical, chemical, or laser methods, including drilling, cutting, digesting, or melting the ZP, are employed to achieve these modifications. [6]. Laser AH (LAH) and specially 1.48 diode laser has an advantage over other AH techniques as the procedure do not physically touch the cells and in turn no traceable toxic effects on the living cells. LAH is a user-friendly technique in terms of handling [7].

Laser-assisted hatching (LAH) encompasses two techniques: drilling and thinning. In drilling LAH (D-LAH), a laser beam creates a 20-40 μm hole in the zona pellucida (ZP), facilitating direct contact between the embryo and the culture medium. This facilitates the hatching process once the embryos have developed [8]. However, D-LAH has a theoretical risk of harming blastomeres through exposing the embryo to antibiotics and nutrients in the media [9]. On the other hand, thinning LAH (T-LAH) involves reducing the thickness of a quarter to half of the ZP peripherally, minimizing the impact of heating on the blastomeres. The size of the ZP thinning area in LAH could be helpful in the outcomes of frozen-thawed embryo transfer (ET) during the cleavage stage [10,11].

Recently, there has been considerable interest in exploring the impact of LAH on the implantation rate of frozen/thawed embryo transfer (FET). While some studies have indicated an improvement in the clinical pregnancy rate in couples facing infertility and repeated implantation failure, there is currently insufficient evidence regarding the effectiveness of LAH in FET cycles.

We conducted a preliminary investigation to assess the impact of two techniques, LAH zona thinning and drilling, on thawed cryopreserved embryos. Our focus was to determine how these techniques influenced the implantation rate and clinical pregnancy outcomes.

Patients and Methods

Study design

A randomized prospective comparative study was conducted at the National Research Center and Walad we Bent fertility center. The study was held between March 2022 to December 2022. The study involved 300 women who were scheduled for the transfer of cryopreserved embryos. The participants were randomly assigned to three equal groups using sealed envelopes: a group undergoing laser-assisted thinning, a group undergoing laser opening, and a control group. Each group consisted of 100 women.

Patients and superovulation protocol

Women included in this study is between 22 to 40 years old in Age. All women should have a planned FET. A male factor infertility, tubal factor infertility and unexplained infertility were the indications for the ICSI procedure. All cryopreserved embryos were exposed to LAH. The exclusion criteria were women planning preimplantation genetic test cycle, women with any uterine malformations, Hydrosalpinx, endometriosis, previous pelvic surgery, and smokers within the last 6 months.

An antagonist fixed induction protocol during their ICSI cycles were used for all participants. On day 2 of the menstrual cycle, a starting dose between 150-450 IU/day of intramuscular follicle stimulating hormone (Gonapure, Minapharm, Egypt) were given to women in all groups. The dosage was adjusted based on factors such as age, weight, and ovarian response. Day 6 of the ovarian stimulation, a subcutaneous injection of Cetorelix 0.25mg/day (Cetrotide, Merck Serono, Germany) was administered daily. The women's progress was monitored using transvaginal ultrasound (TVUS) to determine the number of mature follicles >15 mm and serial measurements of estradiol (E2) levels till the day of trigger. When a number of two or more dominant follicles reached a size of 18-20 mm, a trigger with 10,000 IU human chorionic gonadotropin (HCG) was given to women intramuscularly followed by ovum pickup 34-36 hours later. Couples were informed about the possibility of cycle cancellation if there were fewer than three follicles with a diameter less than 14 mm after 8-9 days of gonadotropin therapy, or if the criteria for HCG administration were not met after an additional 4-5 treatment days.

ICSI procedure

Incubated semen samples were used for the ICSI procedure after performing semen analysis and sperm preparation. Each sperm was evaluated and examined before being injected in the oocyte. The sperm should be normal in terms of morphology and motility and then it will be immobilized in polyvinyl Pyrrolidone (PVP) (Irvine, USA). In a sterilized dish using holding pipette and injection needle, the immobilize sperm was injected inside the oocyte cytoplasm. the protocol of Van Steirteghem was followed in ICSI procedure [12]. After oocyte injection, it was

washed and placed in a culture dish containing global total media (Life Global, Europe). The media was covered with sterile, warmed, and equilibrated global oil (Life Global, Europe). The dish was kept at 37 °C, 6% CO₂ and a humidity at 90-95% until fertilization occurred. After 16-18 hours from sperm injection, fertilization is checked followed by another check for embryo quality 40-44 hours post-injection. Embryos were categorized into three groups: G1 (≥ 4 cells and $< 10\%$ fragmentation), G2 (≥ 4 cells or 10% - 20% fragmentation), or G3 (< 4 cells and $> 25\%$ fragmentation) [13]. Only embryos of good quality on day 3 (G1 or G2) were chosen for transfer or cryopreservation.

Cryopreservation and thawing procedures

Both freezing and thawing processes for the embryos followed established protocols [14]. Regarding freezing technique, First, the embryos were transferred from the culture media to 300 μL of equilibration solution (ES) (consisting of HEPES within Basic Culture Media, DMSO (7.5% v/v), and ethylene glycol (7.5% v/v) from Kitazato, Japan) for a duration of 9-15 minutes at room temperature. Secondly, embryos were transferred to 300 μL of vitrification solution 1 (VS 1) (containing HEPES within Basic Culture Media, DMSO (15% v/v), ethylene glycol (15% v/v), and sucrose (0.5 M) for 30 seconds. Thirdly, they were then transferred to 300 μL of vitrification solution 2 (VS 2) (consisting of HEPES within Basic Culture Media, DMSO (15% v/v), ethylene glycol (15% v/v), and sucrose (0.5 M) for an additional 30 seconds. Finally, the embryos were quickly collected in a minimal volume within 5-10 seconds and immediately preserved in liquid nitrogen using a cryo-device. **For thawing**, the embryos were removed from the cryopreservation device to a thawing solution (TS, Kitazato, Japan) (comprised of HEPES within Basic Culture Media with added 1 M sucrose) for 1 minute. As a next step, the embryos were then transferred to a diluting solution (DS, Kitazato, Japan) (containing HEPES within Basic Culture Media with added 0.5 M sucrose) for 3 minutes. Subsequently, the embryos were transferred to a washing solution (WS, consisting of HEPES within Basic Culture Media) for 5 minutes. Afterward, they were cultured overnight at 37 °C and 7% CO₂ [15].

LAH of embryos

Early in the morning of ET, the thawing process took place followed by LAH 2 hours before ET. The average thickness of ZP in all embryos of the same patient was calculated. The OCTAX NaviLase TM dynamic laser system developed by Jacques Cohen in Washington; USA was used for AH. This system utilized the OCTAX EyeWare™ software, which includes the OCTAX high-resolution Eye™ USB2 camera for imaging and archival purposes. The laser system was connected to an inverted microscope (Nikon eclipse Ti) and controlled by the Viewer imaging software, which incorporated digital laser targeting and archival capabilities (OCTAX NaviLase, USA). The laser settings were adjusted to produce pulses with a length of 0.450 ms to 0.650 ms, to perform thinning technique targeting one-fourth of the ZP's circumference. On the other hand, the laser was set to generate pulses with a length of 0.450 ms to 0.650 ms and create a hole with a diameter of 40 μm in the ZP as a drilling technique. Before being transferred, the embryos were cultured for a period of 2 hours [16].

A Preparation for FET

When the endometrium showed a trilaminar pattern (similar to the 3rd day of ovulation) with a thickness of 8-10 mm, we considered it ready for ET. Progynova (6 mg/day) was prescribed for women who did not have natural ovulation &/or irregular menstruation. These tablets were started between the 3rd to the 5th day of the menstrual cycle followed by adding Prontogest 200

mg/day vaginal suppositories from day 8 until the day of transfer. On day 11 to 15 from the start of menstruation, embryo transfer was done under ultrasound guide.

Embryo Transfer

Embryos were assessed after thawing and we chose the best three suitable embryos for ET to perform AH on them. We tried to select embryos with a cumulative embryo score of at least 12. The cumulative embryo score was calculated by multiplying the number of cells by the embryo grade (ranging from 1 to 4). According to the degree of fragmentation the embryo got a score. For example, a grade 4 embryo exhibited no fragmentation, while a grade 2 embryo had up to 50% fragmentation.

To document early pregnancy, a β -human chorionic gonadotropin (β HCG) level of 25 IU or higher was considered positive. A second sample was taken 48 hours later to confirm an increase in β HCG greater than 66%. At 7 weeks gestation, the presence of a fetal heartbeat established a clinical pregnancy. Luteal support was continued until 8 weeks of gestation, involving Prontogest 400 mg (vaginal suppository in the evening), Progynova 2 mg (three tablets per day), and prenatal vitamins.

Statistical Analysis

Data were coded and entered using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 25 for Microsoft Windows. Data were statistically described in terms of mean \pm standard deviation (SD) for quantitative variables and frequencies and percentages for categorical variables as age and BMI. Comparisons between groups were made using variance (ANOVA) analysis with multiple comparisons Tukey post hoc test in normally distributed quantitative variables. For comparing categorical data, Chi-square (χ^2) test was performed. P values less than 0.05 were considered statistically significant [17].

Results

According to the CONSORT guidelines, only 300 women were included in the study among 388 women who fulfilled the inclusion criteria (figure 2). They were divided into 3 groups; laser-assisted thinning (T-LAH) group, laser-assisted opening (D-LAH) group, and control (non-laser assisted hatching) group.

Regarding the patients' characteristics, there were no significant differences in age, BMI, and the number of transferred frozen embryos in women of T-LAH, D-LAH, and control groups ($P > 0.05$), as shown in table 1.

Regarding pregnancy outcomes (table 2), Implantation rates in T-LAH, D-LAH, and control groups were 14.73%, 10.94%, and 9.81%, respectively. The chemical and clinical pregnancy rates were in the T-LAH group (44% and 37%, respectively), the D-LAH group (35% and 26%, respectively) and the control group (30% and 23%, respectively). However, the only significant difference in chemical and clinical pregnancy rates was between the T-LAH and control groups ($P = 0.04$ and 0.031 , respectively) while no significance was found when we compared the D-LAH versus control group and also there were no advantage to D-LAH over T-LAH.

We then subdivided each group according to age into women aged 35 years or less and women above 35 years. The pregnancy outcomes in the subdivided groups were shown in Tables 3 and 4. Interestingly, there were no significant differences in the implantation rates, chemical

pregnancy rates, and clinical pregnancy rates between all women of 35 years or less in the three groups ($P > 0.05$). On the contrary, women above 35 years in the T-LAH versus control groups showed significant differences in implantation rate, chemical and clinical pregnancy rates ($P = 0.043$, 0.003 and 0.006 , respectively). When we compared the T-LAH versus D-LAH groups and also D-LAH versus the control group no significance were found.

Discussion

Challenges for embryo hatching due to the ZP hardening can have a negative impact on ICSI results [18]. A thicker ZP is associated with reduced embryo implantation [19]. AH has been shown to enhance implantation rates in assisted reproduction [20]. Laser-assisted hatching using either D-LAH or T-LAH has shown significant increases in implantation and clinical pregnancy rates [18, 21]. AH has shown to be particularly beneficial for certain groups of women as those aged 38 or older, women with at least two unsuccessful assisted reproduction cycles, or those who have poor-quality embryos. However, the existing literature provide insufficient data to determine the optimal AH technique for clinical practice as well as limited evidence regarding the impact of AH on outcomes beyond clinical pregnancy, such as miscarriage and live birth, particularly among patients with a poor prognosis.

According to the literature, the most commonly practiced technique for frozen/thawed embryos is D-LAH of the zona pellucida [22]. This approach may have certain technical disadvantages that could potentially decrease implantation rates. Alternatively, zona T-LAH is considered a less traumatic procedure [23] and also preserve the embryo integrity. It seems unlogic utilizing a more invasive method without established benefits. While some preliminary studies indicate assisted hatching through drilling may increase implantation in specific patient cohorts, the LAH protocol that yields optimal outcomes has yet to be elucidated.

In our study We assessed the effect of T-LAH and D-LAH on frozen/thawed embryos, focusing on the impact of LAH on both implantation and clinical pregnancy rate. Our data revealed that both chemical and clinical pregnancy rate showed to be significant when we compared T-LAH group with the control group on all women involved in the study and implantation rate found not to be significant. In women above the age of 35 years old, we found that implantation rate, chemical and clinical pregnancy rates were significant when compared T-LAH to the control group with the same age group. Also, our results even showed that there was no advantage for D-LAH on implantation rate, chemical and clinical pregnancy rate when compared with the T-LAH group as well as the control group.

In 2022, Wang and his research group found that Implantation rate and the clinical pregnancy were significantly higher in the T-LAH group (32.73%, 50.98%) versus the D-LAH group (29.09%, 43.95%) ($P < 0.05$ and $P < 0.01$, respectively [24]. There results were not agreeing with ours when we compare T-LAH group with the D-LAH group. Yet we found that D-LAH has no benefit upon implantation rate, chemical and clinical pregnancy when compared to T-LAH although T-LAH has a significant effect on chemical and clinical pregnancy when compared with the control group especially above age of 35 years old.

In 2020 a Chinese group [25] had a publication concluded that there were no significant differences between the two groups in clinical pregnancy and live birth rates but the embryo implantation rate of the D-LAH group (72.7%) was significantly higher than that of the T-LAH group (61.8%) which is on the contrary to our results. This difference in the results could be due to difference in patient selection criteria as our cases as well as the sample size.

A research group in 2022 compared the T-LAH to D-LAH and they concluded that there is no privilege to any of them on the other regarding chemical and clinical pregnancy. This was in agreement with our research when we compared the thinning and drilling group with all age groups but we found that thinning has an advantage over the control group above the age of 35 years old while Drilling did not show any significance [27]. Also, in agreement with our study Mostafa et.al study proved that both chemical and clinical pregnancy rate improved significantly after a quarter LAH for embryos of poor prognosis women in ICSI cycles ($P= 0.024$). Clinical pregnancy rate was 38.4% in quarter LAH group versus 20% in the control group with a statistical significance of $P=0.021$ [28].

A previous study in 2017 showed that LAH not only did not significantly improve the clinical pregnancy rates in cases undergoing ICSI procedure but also may decrease the live-birth rate in first-cycle, autologous frozen embryo transfer. They also recommend that LAH should be carefully considered, especially in patients 38 years old and older [26]. In this previous study they used the D-LAH only as a common practice in most IVF centers. In our present study drilling confirmed the previous results by being insignificant to clinical pregnancy rates but thinning as a new technique had showed to be better practice specially in women above 35 years old.

The greatest limitation in our work was the small sample size of the study. Most of the couples did not like to try any new unproven techniques of unsecured results as they do the assisted reproductive techniques on their own expenses. For this reason, we could not confirm that T-LAH is a better practice regarding efficacy. We are trying to design a bigger multicentric study with multiple limbs in order to have the chance to create evidence.

Conclusion

In our attempt to define the best technique of LAH, we found that T-LAH a safe practice of LAH with a better chemical and clinical pregnancy rates especially in older age groups. T-LAH could have an advantage over D-LAH as thinning mimics the physiology though allowing natural hatching and also decreases the risk of exposing the blastomeres to the media and nutrients. Prospective evaluations directly assessing short and long-term achievement of T-LAH versus D-LAH could help guide clinical practice by limiting iatrogenic harm and elucidating superior techniques.

COMPLIANCE ETHICAL STANDARDS

Authors contribution

T.N.: Conceptualization, Methodology

M.R: Formal Analysis

M.N.: laboratory procedures

E.A.: methodology, laboratory procedures

E.S.: Methodology, Data curation, Formal Analysis, validation, Writing – original draft, & editing.

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Disclosure of interests

All authors declare that they have no conflicts of interest or financial ties to disclose.

Ethical Statement:

The study was approved by the quality education assurance unit, faculty of medicine, Al-Azhar University, Egypt (REC number: 00000400). This was done following the ethical standards of the 1964 Helsinki Declaration and its later comparable ethical standards or amendments, as well as the ethical standards of the national and/or the institutional research committee. The maneuver was explained, and written consent was taken from all couples before starting the study.

Informed consent

Written informed consent was obtained from each patient before being enrolled in the present study.

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Table 1: Age and BMI of women enrolled in the study:

Groups	T-LAH (n=100)	D-LAH (n=100)	Control (n=100)	P-value		
				T-LAH vs D-LAH	T-LAH vs Control	D-LAH vs Control
Age	31.10 ± 3.69	31.87 ± 3.97	32.08 ± 4.12	0.1570	0.0780	0.7140
BMI	24.48 ± 2.89	24.87 ± 2.87	24.67 ± 2.73	0.3395	0.6332	0.6142

ANOVA & Tukey post hoc test was used for continuous variables; values are represented as mean±SD

NS: Statistically not significant (P>0.05)

Table 2: Pregnancy outcomes in all women of different groups:

	T-LAH Group (n=100)	D-LAH Group (n=100)	Control Group (n=100)	p-value (T-LAH vs. D-LAH)	p-value (T-LAH vs.Control)	p-value (D-LAH vs. Control)
Implantatio n Rate	42/285 (14.73%)	28/256 (10.94 %)	26/265 (9.81%)	0.189 (NS)	0.079 (NS)	0.673 (NS)
+ve Chemical Pregnancy	44 (44%)	35 (35%)	30 (30%)	0.193 (NS)	0.04 *	0.45 (NS)
+ve Clinical Pregnancy	37 (37%)	26 (26%)	23 (23%)	0.094 (NS)	0.031 *	0.622 (NS)

ANOVA & Tukey post hoc test was used for continuous variables; values are represented as mean±SD

Pearson Chi-square test was used for categorical variables; values are expressed as № (%)

*: Statistically significant (p<0.05) NS: Statistically not significant (P>0.05)

- Implantation rate is the total number of embryos implanted over the total number of embryos transferred

Table 3: Pregnancy outcomes in women ≤35 years:

	T-LAH Group (n=78)	D-LAH Group (n=79)	Control Group (n=76)	p-value (T-LAH vs. D-LAH)	p-value (T-LAH vs.Control)	p-value (D-LAH vs. Control)
Gestational Sacs	1.65 ± 0.48	1.63 ± 0.49	1.61 ± 0.49	0.961 (NS)	0.809 (NS)	0.933 (NS)
Implantatio n Rate	34/199 (17.08%)	26/204 (12.74%)	24/199 (12.06%)	0.221 (NS)	0.155(NS)	0.834(NS)
+ve Chemical Pregnancy	35 (44.9%)	31 (39.2%)	29 (38.2%)	0.475 (NS)	0.398 (NS)	0.89 (NS)
+ve Clinical Pregnancy	29 (37.2%)	23 (29.1%)	22 (28.9%)	0.283 (NS)	0.278 (NS)	0.982 (NS)

ANOVA & Tukey post hoc test was used for continuous variables; values are represented as mean±SD Pearson Chi-square test was used for categorical variables; values are expressed as No (%)

NS: Statistically not significant (P>0.05)

- Implantation rate is the total number of embryos implanted over the total number of embryos transferred

Table 4: Pregnancy outcomes in women >35 years:

	T-LAH Group (n=22)	D-LAH Group (n=21)	Control Group (n=24)	p-value (T-LAH vs. D-LAH)	p-value (T-LAH vs.Control)	p-value (D-LAH vs. Control)
Gestational Sacs	1.86 ± 0.35	1.71 ± 0.46	1.70 ± 0.46	0.494 (NS)	0.444 (NS)	0.963 (NS)
Implantatio n Rate	8/86 (9.30%)	3/52 (5.76%)	1/66 (1.52%)	0.457(NS)	0.043*	0.204 (NS)
+ve Chemical Pregnancy	9 (40.9%)	4 (19%)	1 (4.2%)	0.119 (NS)	0.003 *	0.113 (NS)
+ve Clinical Pregnancy	8 (36.4%)	3 (14.3%)	1 (4.2%)	0.097 (NS)	0.006 *	0.234 (NS)

ANOVA & Tukey post hoc test was used for continuous variables; values are represented as mean±SD

Pearson Chi-square test was used for categorical variables; values are expressed as N° (%)

*: Statistically significant (p<0.05) NS: Statistically not significant (P>0.05)

- Implantation rate is the total number of embryos implanted over the total number of embryos transferred

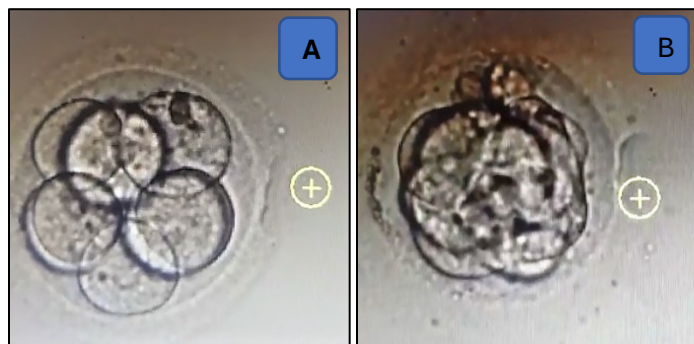


Figure (1): (A)Laser-assisted thinning (T-LAH) vs. (B) laser-assisted drilling (D-LAH) techniques

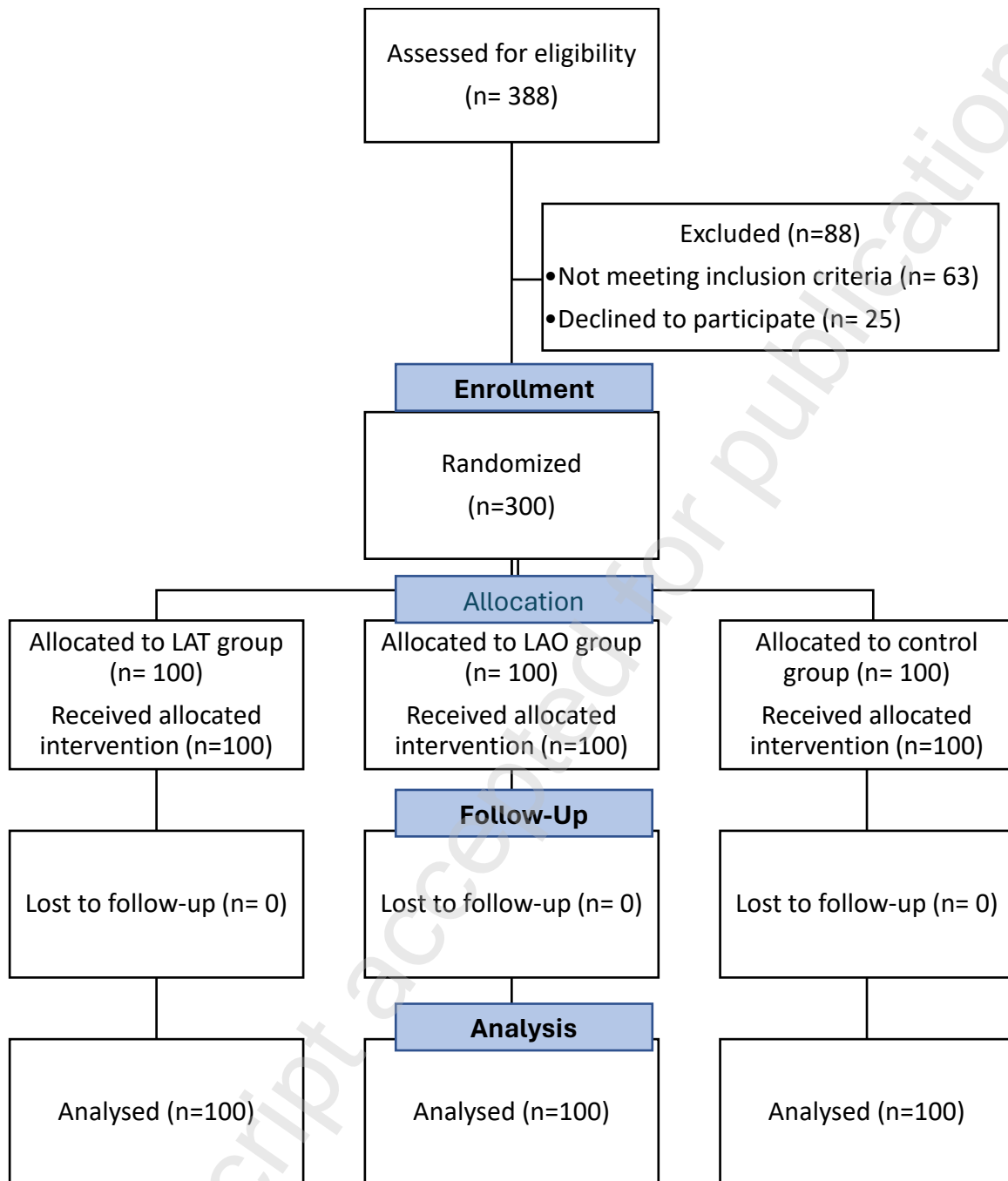


Figure (2) Flowchart of participants in the study