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Efficacy of Laser-Assisted Hatching on Pregnancy and Live-Birth Rates of Human Frozen-Thawed Embryos

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Abstract:

Unexplained repeated *in-vitro* fertilization (IVF) failure is a major problem that faces both genders. The present study aims to estimate the influence of the laser-assisted hatching (LAH) technique on implantation, clinical pregnancy and live-birth rates using human cryopreserved–thawed embryos produced by intracytoplasmic sperm injection (ICSI). **Materials and methods:** A total of 208 subjects was divided randomly into 100 subjects in the control group (Non-LAH), in which the uterine frozen embryo transfer (FET) was done directly; and 108 subjects in LAH group, in which laser shots were applied on day-3 frozen-thawed embryos before uterine transfer. All groups were further subdivided into three female age categories (22-30; 31-35 & 36-40 years). Number of mature, fertilized oocytes, fertilization, clinical pregnancy and live-birth rates were determined according to age distribution. **Results:** Both pregnancy (37.0 %) and live-birth (57.5 %) rates in LAH group were insignificantly lower than Non-LAH control group having pregnancy and live-birth rates of (45.0 % and 73.3 %, respectively). LAH caused a mild increase in clinical outcomes in females aged over 36 years, but in contrast, no improvement was observed in younger subjects. **Conclusion:** Our experimental study did not support using LAH as a routine strategy in frozen-thawed embryo transfer cycles.

Keywords: *In-vitro* fertilization; ICSI; Laser-assisted hatching; Frozen embryos; Pregnancy

1. Introduction

Laser is an accurate technique for microsurgical handling with human embryos because its energy can be effortlessly concentrated on the target spot producing a controlled and precise opening in the zona pellucida [1-2]. This precise laser-assisted microdissection could be safely repeated without affecting further progress of the embryos. Which makes laser-assisted hatching (LAH) effective and safe due to many benefits that are addressed in any other hatching techniques such as chemical and mechanical hatching. These benefits are concluded as short

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exposure time, simple process, accurate positioning, indirect contact, safety and effectiveness [3-4].

The technique has to be carried out in aseptic conditions with no need for more micromanipulation using an IR diode laser. It can accurately open zonas of highly expanded blastocysts with no detectable damage [5].

Assisted hatching (AH) is used in vitrification cycles for its effective role in enhancing both pregnancy and implantation rates, in which embryos with thicker zone pellucida are thought to most benefit. Although various methods for AH are well known such as mechanical, chemical and laser, however, laser-assisted hatching (LAH) has the advantage of being able to minimize the exposure of embryos outside the incubator [4]. But if this could really improve further pregnancy and implantation processes is still representing a question mark for scientists. Application of LAH should then be carefully considered and prospective studies should be carried out to clarify its benefits and negative effects [6-7].

Therefore, this study aimed to investigate the feasibility of LAH use as a routine technique in the IVF protocol in order to enhance pregnancy and uterus implantation rates, especially in women suffered from repeated failure of IVF.

1. Materials & Methods

2.1. Materials

For embryo cultivation, Falcon 1006 dishes and culture media were supplied from **(**Falcon, Franklin Lakes, NJ, USA) and (Global Total LGGT, Life Global, USA), respectively.

For LAH, zona laser hatching system (OCTAX LASER SHOT®) mounted to IVF workstation and inverted microscope (OLYMPUS® 1× 71) using infrared diode laser class (1480 nm) with power in focus 100 m Wand irradiation time 0.1 ms steps in a computer-controlled non-contact mode.

2.2. Subject couples

All methods were performed in accordance with the relevant guidelines and regulations. In addition, informed consent was obtained from all subjects included in the study. Two-hundred-and-eight candidates with female age range (22-40 years) were recruited from couples undergoing assisted reproductive techniques (ART) programs. All couples underwent intracytoplasmic sperm injection (ICSI) due to either male factor or unexplained infertility over 2 years before initiation of the study.

2.3. Experimental design

Envelop sealing on day of transfer of embryos was used to randomly divide subject couples into two groups. Group 1 (Non-LAH group) consisted of 100 couples who underwent ICSI without laser-assisted hatching, in which frozen embryo transfer (FET) was done without any further interference. Group 2 (LAH group) consisted of 108 couples who underwent ICSI with a laser-assisted hatching step before FET. The two groups were further subdivided according to female age into three categories; 22 to 30; 31 to 35 & 36 to 40 years old [8].

2.4. Ovarian stimulation and embryo preparation using intracytoplasmic sperm injection (ICSI)

Controlled ovarian stimulation (COS), including regulation and desensitization, was designed specifically for each participating female in order to retrieve adequate number of oocytes needed to perform ICSI according to [6]. Resulted embryos were then washed, 20-µl microdrop of culture media was added, covered by oil, and incubation was done on a Falcon 1006 dish at 37 ºC and 6 % CO_2 under humidified conditions prepared for transfer [9-10].

2.5. Embryo selection and cryopreservation

At culture day-3, embryo quality was assessed for selection, and hence, cryopreservation of only good (8 cells; fragmentation between 10–20 %) and excellent (≥ 8 cells; fragmentation ≤ 10 %) quality embryos. Poor quality embryos (< 8 cells; fragmentation > 20 %) were excluded as embryo quality affects profoundly its survival rate [11-12].

Cryopreservation was carried out following the laboratory standard protocol. Briefly, selected embryos were frozen using the vitrification procedure consisted of two main steps using equilibration and vitrification solutions, with each having different concentration of the cryoprotectant. Finally, vitrified embryos were frozen under aseptic conditions on a sterilized disposable cryotop, a carrier system used for vitrified embryos. Each cryotop is used for only one vitrification cycle. Cryopreserved embryos could be stored for at least 2 months.

2.6. Laser-assisted hatching (LAH) and uterine frozen embryo transfer (FET)

Excellent quality cryopreserved embryos were thawed following the laboratory standard protocol before their transfer into the uterus. However, Group 2 thawed embryos were first exposed to LAH technique at 1-2 h before being transferred into the uterus. Laser was focused to breach embryo zona pellucid at one-fourth to one-eighth of its surface apart from blastomeres to keep them intact. Zona required two successive laser shots to be completely

breached. This was followed by incubating the laser-breached embryo with a fresh drop of the culture media and for 2 h, during which further embryo development was monitored before its uterine transfer [13-14].

2.7. Pregnancy and implantation rates determination

Positive pregnancy was determined after day-15 from uterine transfer of embryo using quantitative beta-human chorionic gonadotropin (βHCG) assay. From week 4 to 6, a transvaginal ultrasound was done to reveal the development of intrauterine gestational sac and fetal pulsations and to define the first outcome of the study, i.e., clinical pregnancy rate (CPR) [9]. For the calculation of the second study outcome, i.e., the implantation rate (IR), the equation below was used:

$$IR = \text{No. of gestational sacs at week} - 4/\text{Total No. of transferred embryos}$$

2.8. Statistical analysis

Data analysis was done using IBM statistical package for the social sciences (IBM® SPSS®) software version 23. Duncan's test was utilized to study similarity in the variables among the age categories in each group, i.e., LAH and Non-LAH (control). Independent t-test has been used to determine statistical differences among continuous variables within LAH and Non-LAH cases. Determination of association of the studied ordinal variables within LAH and Non-LAH cases was done using Mann-Whitney test. The p values of < 0.05 were considered significant.

2. Results

3.1. Age distribution and pregnancy rate in Non-LAH and LAH groups

This study included 208 subjects divided randomly into: Non-LAH group (Non-laser-assisted hatching; negative control group) consisted of 100 subjects and LAH group (laser-assisted hatching group) consisted of 108 subjects. Mean age was found to be similar in the two groups: 28.48 ± 0.40 years in control group and 29.74 ± 0.45 years in LAH group (Table 1). Our data revealed that using laser-assistance (LAH group) resulted in a clinical pregnancy rate of 37.0 % and live-birth rate of 57.5 %, which was insignificantly lower than control group (Non-LAH group) with clinical pregnancy and live-birth rates of 45 % and 73.3 %, respectively (Table 2). In both groups, clinical pregnancy and live-birth rates were insignificantly associated with age distribution (Table 3). However, LAH group showed a significant elevation in both participating female age and fertilization rate, as compared to the Non-LAH group (Table 1).

Table 1. Age distribution, number of mature and fertilized oocytes as well as fertilization rates in Non-LAH and LAH cases

Parameter	Non-LAH (N= 100)	LAH (N=108)	p-value
Age (years)	28.48 ± 0.40	29.74 ± 0.45*	0.04
Number of mature oocytes	13.91 ± 0.51	13.48 ± 0.53	0.56
Number of fertilized oocytes	10.54 ± 0.42	10.86 ± 0.45	0.61
Fertilization rate	77.14 ± 1.66	81.42 ± 1.37*	0.04

P<0.05: significant difference, according to independent t-test.

LAH: Laser-assisted hatching; Non-LAH: Non-laser-assisted hatching.

Table 2. Clinical pregnancy and live-birth rates per oocyte collected from Non-LAH and LAH cases

	Non-LAH (N= 100)	LAH (N= 108)	p-value
Clinical pregnancy rate	45/100 (45.0 %)	40/108 (37.0 %)	0.244
Live-birth rate	33/45 (73.3 %)	23/40 (57.5 %)	0.127

P>0.05: insignificant difference, according to Mann-Whitney test.

LAH: Laser-assisted hatching; Non-LAH: Non-laser-assisted hatching.

Table 3. Clinical pregnancy and live-birth rates in Non-LAH and LAH cases, according to age group (22-30; 31-35 & 36-40 years)

Parameter	Age (Years)	Non-LAH (N=100)	LAH (N=108)	p-value
Clinical pregnancy rate	22-30	32/67 (47.8 %)	24/61 (39.3 %)	0.340
	31-35	12/30 (40.0 %)	11/32 (34.4 %)	0.649
	36-40	1/3 (33.3 %)	5/15 (33.3 %)	1.000
Live-birth rate	22-30	23/32 (71.9 %)	15/24 (62.5 %)	0.461
	31-35	10/12 (83.3 %)	6/11 (54.5 %)	0.260
	36-40	0/1 (0.00 %)	2/5 (40.0 %)	0.667

P>0.05: insignificant difference, according to Mann-Whitney test.

LAH: Laser-assisted hatching; Non-LAH: Non-laser-assisted hatching.

3.2. Comparison between female age categories

Table 4 shows a subgroup analysis comparing the two groups according to current female age category (i.e., 22-30; 31-35 & 36-40 years old). Number of mature and fertilized oocytes as well as fertilization rate were found to be similar among the different age categories of Non-LAH group. While, in LAH group the number of mature oocytes at 22-30 years age category was significantly greater than that at 36-40 years age subgroup. Also, in LAH group, fertilization rate in female older age category (36-40 years) was reported to be significantly higher than that at younger age subgroup (31-35 years).

Table 4. Number of mature, fertilized oocytes and fertilization rates in Non-LAH and LAH cases, according to female age subgroup (22-30; 31-35 & 36-40 years)

Parameter	Age (years)	Non-LAH (N=100)	LAH (N=108)
Number of mature oocytes	22-30	14.21 ± 0.65^{a}	14.54 ± 0.80^{b}
	31-35	13.37 ± 0.87^{a}	12.63 ± 0.71^{ab}
	36-40	12.67 ± 2.91^{a}	11.00 ± 1.01^{a}
Number of fertilized oocytes	22-30	10.75 ± 0.56^{a}	11.82 ± 0.68^{a}
	31-35	10.30 ± 0.62^{a}	9.69 ± 0.64^{a}
	36-40	8.33 ± 1.20^{a}	9.47 ± 0.82^{a}
Fertilization rate	22-30	76.19 ± 2.04^{a}	$82.18 \pm 1.87^{ab*}$
	31-35	80.11 ± 3.05^{a}	76.98 ± 2.41^{a}
	36-40	68.53 ± 6.47^{a}	$87.79 \pm 2.88^{b*}$

In the same column, mean values marked with same superscript letters are insignificantly different ($p>0.05$), whereas those marked with different letters are significantly different ($p<0.05$), according to Duncan's test.

*: significant difference ($p<0.05$) as compared to the Non-LAH group.

LAH: Laser-assisted hatching; Non-LAH: Non-laser-assisted hatching.

3.3. Comparison between pregnancy rate and live-birth rate

The live-birth and pregnancy rates in both LAH and Non-LAH groups were compared; they suggested a dissimilar estimation. Clinical pregnancy rate of Non-LAH was insignificantly greater than in LAH group (Fig. 1). Moreover, the live-birth rate of LAH was insignificantly lower than Non-LAH group (Fig. 2).

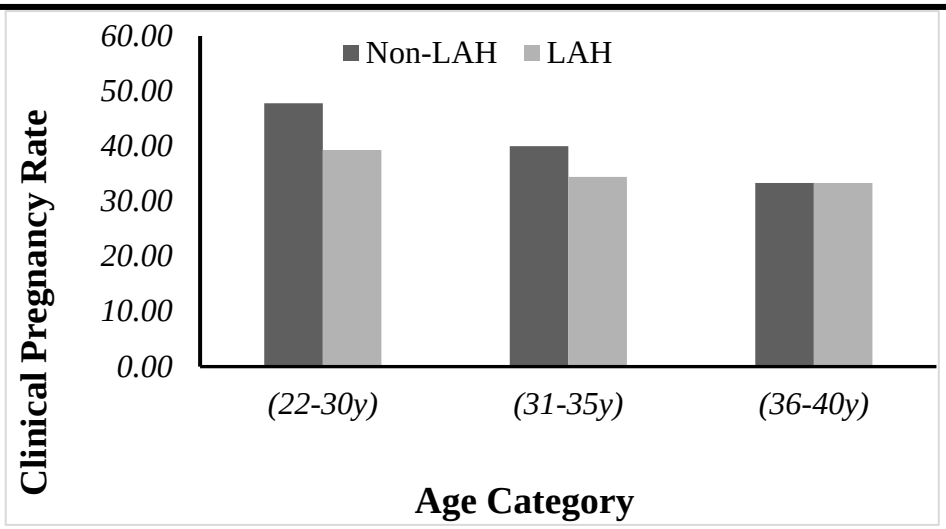

Fig. 1: Clinical pregnancy rate in Non-LAH and LAH cases, according to age group (22-30; 31-35 & 36-39 years)

LAH: Laser-assisted hatching; Non-LAH: Non-laser-assisted hatching.

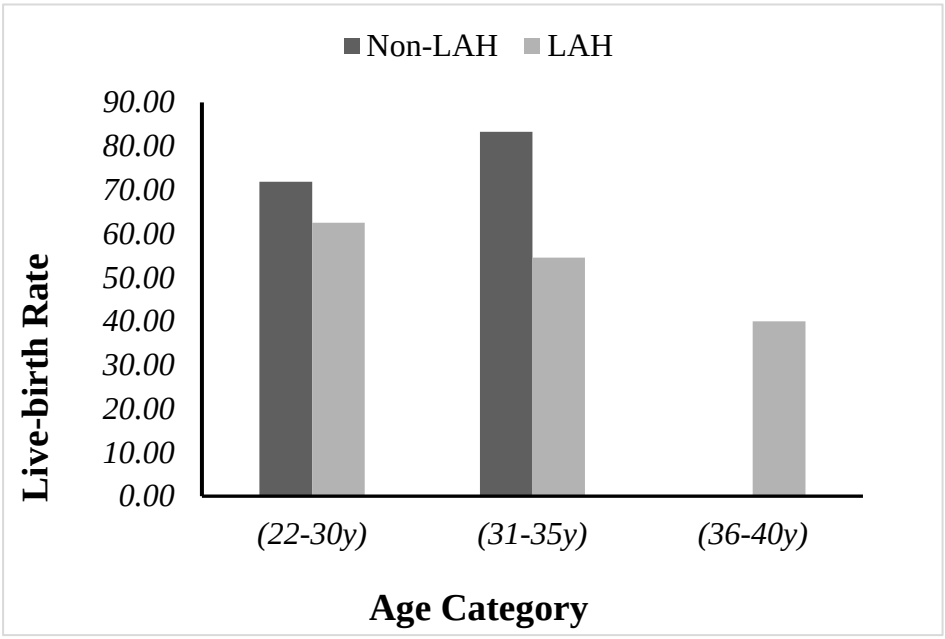

Fig. 2: Live-birth rate in Non-LAH and LAH cases, according to age group (22-30; 31-35 & 36-39 years)

LAH: Laser-assisted hatching; Non-LAH: Non-laser-assisted hatching.

3. Discussion

The subject population in this study is quite homogenous as they all underwent intracytoplasmic sperm injection (ICSI) and either good (8 cells; fragmentation between 10–20 %) or excellent (≥ 8 cells; fragmentation ≤ 10 %) quality embryo selection and cryopreservation in a controlled laboratory environment. The subjects and embryos characteristics in the laser-assisted hatching (LAH) and Non-LAH (control) groups were similar and they also had same inclusion and exclusion criteria.

The exposure to laser shots did not cause any harmful effects to embryos of LAH group and no embryos had to be excluded, in which all examined embryos did maintain excellent and good quality. This indicated the safety of LAH technique and accuracy of our work.

Although data collected from our study showed higher implantation and pregnancy rates in the control (Non-LAH) group than in LAH group, however, by comparing different female age subgroups, subjects from LAH group between 36 and 40 years old showed better live-birth rate than younger age categories, which might go with the idea of performing laser-assisted hatching in this age group. According to [13], age is thought to be a primary factor of infertility, and hence, is prognostic of live-birth rate applying known reproduction assisting techniques. Females above 35 years old are more probably to receive LAH than younger age females, though, throughout the years, females undergoing ICSI using LAH have decreased live-birth rate compared with those without LAH.

The study [14] also supported that women above 36 years have better implantation and pregnancy rates using LAH. It stated that with aging hardening of zone pellucida could be increased. There are numerous studies that revealed the valuable impact of LAH in increasing live-birth rates in older females who suffered from prior unsuccessful implantation. However, they indicated that, according to many recent researches, neither pregnancy nor live-birth rates could be enhanced in females with advanced maternal age using LAH technique.

In contrast to the studies [15-16] that could statistically confirm increased implantation and clinical pregnancy rates following application of LAH on cryopreserved embryos, other studies [17-19] did support our results as they could

not introduce any evidence-based conclusion that LAH technique has any advantage in enhancing cryopreserved embryos implantation, pregnancy and eventually live-birth rates.

Assisted hatching showed a significant increase in pregnancy rate in subjects who underwent ICSI for the first time [20]. However, other studies [21] denied that non-selective assisted hatching can exert such significant effect. Despite these conflicting findings, several embryologists recommended application of assisted hatching in specific cases indicating hardening of the zona pellucida (e.g., frozen-thawed embryos, repeated implantation failure and advanced maternal age).

LAH-induced thinning of zona pellucida could be a possible explanation for LAH less successful influence reported by our results. Which goes in line with the studies [19-20] which stated that the implantation rate could be affected by reducing zona pellucida size caused by LAH application.

The zona pellucida, a 13-15 μm thick acellular matrix composed of glycoproteins, carbohydrates, and proteins peculiar to the zona pellucida, surrounds the human oocyte and early embryo. The zona pellucida is bilayered, with a thick outside and a thin but durable inner. During the development of fertilization and pre-implantation, the zona pellucida is structural and functional. It is involved in the binding of sperm, the stimulation of acrosome reactions, and the fusing of eggs [24-25]. The discovery that fertilized embryos with artificial gaps in their zona pellucida appear to have high implantation rates and that divided embryos with an excellent prognosis for implantation have lessened the thickness of the zona led to the clinical introduction of assisted hatching. In addition, other studies reported that at the early stage of development some embryos showed dissimilarity in zona thickness and that zona thinning is an active development [26-27].

According to their age, the female subjects in this study were separated into three subgroups (from 22 to 30, from 31 to 35 and from 36 to 39 years). The ovaries produce Anti-Mullerian Hormone (AMH). As a result, AMH levels normally decrease as people get older. Laser-assisted hatching (LAH) technique still a controversial issue among researchers and embryologists in different IVF laboratories. Some of the recent researches suggested LAH as a must in repeated

failure cases and in advanced maternal age, however; other researches recommended this technique for all frozen-warmed embryos [28-29].

This study suggests that the extent of the zona pellucida thinning may influence the results, which could explain why laser-assisted hatching is not as successful. The extent of the zona pellucida thinning area caused by LAH might impact the implantation rate.

4. Conclusion

Laser-assisted hatching (LAH) caused a mild enhancement in pregnancy rate in female subjects aged over 36 years showed after uterine transfer of day-3 frozen-thawed embryos. However, it did not exert any improvement in females aged below 36 years. Hence, our data did not support using LAH as a routine strategy before cycles of uterine transfer of frozen-thawed embryos.

5. Recommendations

Further researches have to be conducted in order to increase pregnancy and live-birth rates by overcoming the problem of the thickness of the frozen-thawed embryos' zona pellucida. Also, additional studies are needed to evaluate the safety of the hatching procedure on the embryos in a genetic manner.

6. Clinical significance

Unless for indicated cases with hard zona pellucida, LAH could not be recommended to be used as a routine strategy in IVF laboratory protocol, as it did not enhance either pregnancy or live-birth rates in females under 36 years old, in comparison to control (Non-LAH) group.

7. Ethics approval

The study was approved by the Ethical Committee, Quality Education Assurance Unit, Al-Azhar Faculty of Medicine, Al-Azhar University, Nasr City, Cairo, Egypt under Registration Number:

"Bio._11Med.Research._Laser-Assisted.Hatching.Pregnancy.Live.Birth.Rates.Frozen-Warmed.Embryos._0000011". All methods were performed in accordance with the relevant guidelines and regulations. In addition, informed consent was obtained from all subjects included in the study.

Author Declarations

Competing interests

The authors have no relevant financial or Non-financial interests to disclose.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable

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Data/code availability

The authors confirm that the data supporting the findings of this study are available within the article.

Authors' Contributions

According to the relevant CRediT roles, authors' individual contributions are listed as follows: **Shorouk El-Toukhy:** Methodology, investigation, formal analysis, data curation, visualization, and writing - original draft. **Mohamed Ali El-Desouky:** Conceptualization, methodology, investigation, validation, writing - review & editing, and supervision. **Ragaa Taha Mansour:** Conceptualization, methodology, investigation, validation, writing - review & editing, and supervision. **Mariam Abdur-Rahman**: Conceptualization, methodology, investigation, validation, writing - review & editing, visualization, and supervision.

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الملخص العربى

فعالية خروج الجنين من جداره بمساعدة الليزر في معدلات الحمل و الإنجاب للأجنة المجمدة المدفئة

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2.قسم الكيمياء، كلية العلوم، جامعة القاهرة، الجيزة، مصر

لقد اصبح الحقن المجهري للبويضات عن طريق الحيوان المنوي للزوج من وسائل الاخصاب المساعد الرائده حول العالم، فمنذ بدأ الحقن المجهري اصبح يستخدم بزياده منتظمه حول العالم حيث ان نتائجه من حيث نسبه الحمل و نسبه ولاده اطفال احياء ترتفع بوضوح مع تقدم السنين و هذا بسبب التحسن المستمر في مختلف طرق تنشيط التبويض، الاخصاب، اوساط التحضين و نقل الاجنة.

ان تصلب المنطقه الشفافه للجنين و فشل حدوث الفقس يمكن اعبارهما من اهم اسباب فشل زرع الاجنه بالرحم لعمليات الحقن المجهري. قدمت عمليات الفقس المساعد كوسيله للمساعده علي حدوث زرع للاجنة في ارحام حالات الحقن المجهري حيث تعد تقنية الفقس المساعد بالليزر اكثر طرق الفقس المساعد انتشارا و نموذجية، ولكن تظل نسبة حدوث الحمل للاجنة المدفئة بعد التجميد المساعدة على الفقس بالليزر محل جدل.

الهدف من الدراسة هو تقييم كفاءة عمليات الفقس المساعد باستخدام الليزر للاجنة المدفئة بعد التجميد من حيث نسبة الحمل و نسبة ولاده اطفال احياء في عمليات الحقن المجهري.

تم خضوع 208 اجمالي حالة لعملية الحقن المجهري تم استشاراتهم و وقعوا علي اقرارات لاجراء الدراسة و تم تقسيمهم لمجموعتين؛ مجموعة (108 سيدة) تم استخدام الليزر للمساعده على فقس الاجنة و المجموعة الضابطة (100 سيدة) بدون مساعده للاجنه على الفقس.

كان المتوسط العمري متقارب جدا حيث بلغ 29.74±45. في المجموعه المستخدم بها الليزر للمساعده علي الفقس بينما بلغ 28.48±40 للاخرى الضابطة. بلغت نسبه حدوث الحمل الاكلينيكي للمجموعة الضابطة التي لم يتم مساعده الفقس بها بالليزر (45%) بزيادة غير معتبرة عن المجموعة الأخري التي بلغت نسبة الحمل بها (37%).

كما قلت نسبة ولادة الاطفال الأحياء للمجموعة التى تم مساعدتها علي الفقس بنسبة غير معتبرة حيث بلغت (57.5%) بينما كانت في المجموعه الضابطة (73.3%).

كان عدد البويضات الناضجة و نسبة التخصيب متطابقين بين مختلف الفئات العمرية للمجموعة التي لم تخضع لفقس مساعد للأجنة بالليزر في حين كانت نسبة الاخصاب اعلي بزيادة معتبرة للفئة العمرية بين (22-30) عاما عنها للفئه (36-39) عاما كما ان نسبة الاخصاب للفئة السابقة اعلى بزيادة معتبرة عن الفئة العمريه (31-35) عاما.

الاستنتاج: مما سبق ذكره فان المعلومات الناتجة عن هذه الدراسة أوضحت ان استخدام الليزر في عمليات الفقس المساعد للاجنة المدفئة بعد تجميدها في اليوم الثالث لعملية الحقن المجهري لم يحسن نسبه حدوث الحمل للفئه العمريه اقل من 36 عاما بينما حدث تحسن طفيف للفئه العمريه الاكبر من 36 عام. و بالتالي فان نتائجنا لا تدعم الاستخدام الروتيني لمساعدة الاجنة المدفئة بعد تجميدها بالليزر قبل زرعها برحم الام.