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The Effect of Assisted Laser Hatching On the Clinical Pregnancy Rates, in Fresh and Frozen Embryo Transfer, in Infertile Women in Jalandhar, Punjab

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***Abstract*: Post embryo transfer, in an IVF cycle, the transferred embryo has to expand and perforate the Zone Pellucida (ZP), so as to implant and lead to a successful pregnancy outcome. The zone pellucida is a glycoprotein sphere covering the blastocyst. The lysis of zona pellucida is called Zone Hatching. Sometimes the embryo is not able to hatch out from the zona pellucida leading to failed implantation. Assisted laser hatching may be a novel method to improve the implantation rates in patients undergoing Fresh or Frozen Embryo Transfer cycle. The aim of this study was to determine if Assisted Laser Hatching of the transferred embryos on Day # embryos, before embryo transfer could improve the clinical pregnancy rates in 100 infertile patients, undergoing Fresh or Frozen Embryo Transfer Cycle. We noted that Assisted Laser Hatching did not favour the outcome of occurrence of biochemical and clinical pregnancy (LAH v/s no LAH: 52% v/s 64%). Also the occurrence of multiple gestations (LAH v/s no LAH: 94.2% v/s 95.3%) was not affected by AH. In fact, the proportions of those who experienced adverse events were significantly higher (25 %) in the Laser Hatching Group.**

***Keywords*: Age of patients, Clinical outcome of laser assisted hatching, Fresh embryo transfer, Frozen embryo, Frozen embryo transfer, Laser assisted hatching.**

1. Introduction

The zona pellucida is the thick outer protective coat that covers the mammalion eggs and embryos. It is the communicating link between the oocytes, eggs & embryos. [13] It forms a protective outer sheath of the eggs & embryos. Also, during fertilization, when the sperm head comes in contact with the zona pellucida, the plasma membrane fuses with the acrosome membrane of spermatozoa, & disintegrates. This leads to release of acrosin from acrosomal sac, allowing sperm head to penetrate the zona & enter into ooplasm a process called "acrosome reaction". The main function of the Zona Pellucida post fertilization is to protect the embryo. It helps the blastomeres to migrate through the reproductive tract, by maintaining the structure of the embryo. The ZP also is protective to the embryo against the hostile intrauterine milieu.

After the "Acrosome Reaction", some biochemical changes are observed post fertilization, which prevent polyspermic fertilization. Squealas of Zona Pellucida post fertilization.

2. Squealas of Zona Pellucida Post Fertilization

In humans, the Zona Pellucida degenerates five days after fertilization. It is replaced by underlying trophoblastic layer. The degeneration of the ZP occurs as a result of embryonic & uterine functions.

The blastocyst contains blastomeres, which erode the zona layer by enzymatic reactions & puncture it & squeeze out; so as to implant into the endometrial layers of uterus for nourishment.

After fertilization, the Zona becomes hard to prevent polyspermia. This also has a protective influence on the embryo, as it passes through the reproductive tract. Also in In-Vitro-Fertilization, there is additional hardening of Zona due to the influence of culture media. Such hardening of Zona Pellucida, amy interfere with the embryo implantation in the uterus.

3. What is Assisted Hatching?

Assisted hatching is a technology which is used to create a hole in the ZP with the help of certain chemical or instruments or Lasers, before Embryo Transfer, which facilities hatching of the embryo in uterus. Sometimes embryo does not hatch out from ZP spontaneously, because of which implantation may not occur. In such cases, AH (Assisted Hatching) may be recommended to overcome these problems. However, AH may not improve the rates of live birth, through it is suggested to be useful in aged women or couples to enhance chance of getting pregnant. Thus, Assisted Hatching may be indicated in couples undergoing IVF under the following circumstances,

1. 2 failed IVF cycles.
2. Poor Embryo Quality
3. Older Women (> 36 years of age)
4. In the Frozen Thaw Embryo Transfer

4. Assisted Hatching (AH)

Ah was first introduced in IVF by Mater & Cohen (1989) and

Cohen at al: [3] Several techniques have been developed such as Zonal dissection, drilling and thinning, Use of Acid Tyrode or other Acidic Solutions. Lasers are the latest techniques which are used for Assisted Hatching. Many studies have been performed with lasers in AH; but studies are difficult to correlate due to different study design, patient selection criteria & the techniques of AH. [3]

Benefits of Assisted Hatching:

The clinical pregnancy rates & long term effects on live births after IVF with Fresh or Frozen Embryo Assisted Hatching should be ascertained. These studies could improve the implantation results in infertile couples; with hatching difficulties. Also, such studies could help us find out the cause of implantation failure. Based on these consequences, a cross-sectional study was conducted among 100 infertile patients who underwent Frozen ET & Laser Hatching.

Methods: Usually, in an IVF cycle, either fresh or frozen embryo transfer, AH can be conducted when the embryos are at 6 to 8 cell stage at day 3 after insemination, or at the blastocyst stage of Day 5 or Day 6; after insemination. [1] If the hatching is performed in the Zona of Early-Cleavage IVF embryos, there could be loss of embryonic cells through the breaches in the ZP; due to the uterine contractions & movement of the hatched embryos in the endometrial cavity. Hence, AH could be done when the blastomeres are compact & become adherent to each other.

Tools used for Assisted Hatching

1. Micropipettes which are mounted on micromanipulators are used [5]. The procedure has to be quick & less time consuming so as to reduce toxicity to the embryos. AH is usually performed in microdrops of HEPES buffered medium covered with oil, under an inverted microscope stage at 37 ^{0}C. The perforation created in the ZP should be small enough to prevent loss of the blastomeres, but sufficient to assist the embryo during hatching. The adequate size of the hole could be 30-40 µm. But in the case of Frozen Embryos, when the zona is more hardened, better results are observed when > 50% of the ZP is perforated [1]. The manipulated embryos have to be cultured for 30 minutes prior to transfer into the uterus. The transfer should be least traumatic to avoid the damage to the manipulated embryos [50]. Treatment of four days starting from the day of oocyte pickup, with broad spectrum antibiotics and corticosteroids (Prednisone – 16mg daily) has been advocated by Cohen et al. This could avoid infection & immune cell invasion of the manipulated embryos [1].
2. *Partial ZP Dissection:* [2] It is a mechanical procedure of assisted hatching. The denuded embryo is held with a holding pipette & the ZP is pierced with a micro -needle from 1o'clock to 11 o' clock position. After this, the embryo is released from the holding pipette. The part of the ZP between the two points on the zona pellucida of the embryo are rubbed with the holding pipette so that a slit is made in the ZP The embryo is the washed in the fresh culture medium & then after a culture period of 30 minutes, is transferred in the uterus.
3. *Assisted hatching with acid tyrode solution:* It is considered that increased volume of the perivitelline space in zygotes & embryos could allow safe use of Acid Tyrode solution in human embryos for drilling. However, the use of acidic solutions for AH may be harmful for the blastomeres adjacent to the drilled portion of the ZP [1].
4. *Laser assisted hatching:* The use of lasers was first advocated by Tadir et al. [1] For this, it is necessary that the lasers are accurately controlled and produces opening of ZP efficiently without thermal effects. There is also fear of creating mutations on the manipulated embryos. Lasers induce photoablation of the ZP. Lasers may be Contact Lasers or Non-Contact Lasers. Non-contact lasers may be considered less damaging as they allow objective delivered accessibility of Laser Light to the embryo. Laser beam is propagated through water. This avoids UV absorption peak of DNA; so no mutagenic effect is expected. The study by Neev et al.; using non-contact holmium – tyttrium-scandium-gallium-garnet Laser (2.1µm wavelength) showed less embryo toxic effects & also improved blastocyst hatching. Some studies have shown higher implantation rates of Laser ZP drilled embryos than those of mechanically treated embryos.
5. *ZP Thinning:* The purpose of ZP thinning is to thin the ZP without lysis or perforating it. This can reduce the risk of blastomeres loss & embryonic infection is minimized [1]. Efficacy of laser for zona thinning at cleavage stage has been advocated to have beneficial effects on implantation & pregnancy rates, as advocated by authors Eg. Antinori et al. However, time lapse studies in mouse model suggest that Laser ZP thinning procedures may fail to facilitate hatching & also induce abnormal forms of hatching.
6. *Conclusions:* Several studies have been performed to demonstrated the utility of AH; in patients, with poor prognosis, advanced-aged patients, patients with increased FSH; patients with previous implantation failures, or patients with embryos with thick ZP; AH has been used on fresh embryos & also to frozen thawed embryos & vitrified blastocysts. Removal of necrotic blastomeres from a partially damaged cryopreserved-thawed embryos may help to boost their development potential [1]. The study designs and groups of patients described in the AH studies, are variable, & hence it is difficult to come to a definitive conclusion about the outcomes of AH. The Cochrane review of this era concludes that the available data do not show any positive effect of AH on live birth rates. Thus is could be concluded that performing Assisted Hatching does not increase pregnancy & implantation rates in patients in their first IVF attempt.
    - Patients with history of previous implantation failures could be benefitted by AH.
    - Clear evidence is that AH increases multiple

pregnancy rates [2].

- There is insufficient data to recommend AH as a routine technique in patients undergoing assisted reproductive techniques [5].

5. Scope of Assisted Hatching

Assisted hatching is claimed by to be associated with an increased chance of achieving clinical pregnancy. The assisted hatching procedure may cause specific complications independent of the IVF procedure itself; including lethal damage to individual blastomeres with reduction of embryo viability. In addition, artificial manipulation of the ZP has been associated with an increased risk of monozygotic (MZ) twin pregnancy. [21]

A cross sectional study was conducted among the 200 infertile patients who underwent Frozen ET & 50% of these underwent Laser Hatching.

Study Setting:

Jalandhar is a district consisting of five tehsils/subdivisions viz. Jalandhar-I, Jalandhar II, Nakodar, Phillaur and Shahkot and five sub-tehsils, viz. Adampur, Bhogpur, Kartarpur, Goryan and Nurmahal. The district is divided into 11 development blocks, viz, Jalandhar East, Jalandhar West, Bhogpur, Adampur, Nakodar, Shahkot, Phillaur, Nurmahal, Lohian, Rurka Kalan and Mehatpur [22].

It totally consists of 21.9 lakh population with 10.5 lakhs being females. The district has 21 hospitals, 28 primary health centres, 124 dispensaries, 6 CHCs/hospitals, 4 Unani institutions and 8 homeopathic. There are 2792 doctors and 14 family planning centres. “Demography.” Demography, Jalandhar Web Portal, India, jalandhar.nic.in/demography.

There are nearly fifteen centres enrolled as Assisted Reproductive Technology (ART) Clinics under ‘National Registry of ART Clinics and Banks’ [46] under ICMR in Jalandhar and our study setting is one of the NABH accredited advanced fertility centre with state of art equipment. (ICMR.

Table 1
Assisted hatching results reported by different authors (1992-1999) [2]

First author, year increase	Study Method	Population	Randomized	Pregnancy rate
Cohen,1992 (8)	AT	Normal FSH ≥15µm ZP	Yes Yes	Yes, NS Yes, Sig.
Tucker,1993 (73)	AT ZP Thinning	All IVF	Yes	No
Olivennes,1994 (96)	PZD	Impl. Failures Day3,FSH>15mIU/mL	No (no control) Yes	- Yes, Sig.
Obruca, 1994 (52)	Er:YAG laser	Impl. Failures	No	Yes, Sig.
Tucker, 1994 (97)	AT, CC	Age ≥38 years, impl. failure	Yes(Control:AT)	Yes, Sig.
Schoolcraft,1994 (47)	AT	Elevated FSH, age ≥39 years Impl. Failures	No	Yes, Sig.
Schoolcraft, 1995 (98)	AT	Age ≥40 years	No, retrosp.	Yes, Sig.
Stein, 1995 (99)	PZD	≥3 Impl. Failures Age ≥38 years	Yes	Yes, Sig.
Hellebaut,1996 (75)	PZD	First Cycle	Yes	No
Antinori, 1996 (54)	UV laser	Impl. Failures	No	Yes, Sig.
Check, 1996 (100)	AT	Frozen ET	No	Yes, NS
Antinori, 1996 (66)	Er:YAG laser	First Cycle	Yes	Yes, Sig.
Tucker, 1996 (101)	AT	ICSI, Age ≥35 years	No	Yes, Sig.
Bider, 1997 (102)	AT	Age ≥38 years	No	No
Chao, 1997 (103)	PZD	Impl. Failures	Yes	Yes, IVF No, TET
Hurst, 1998 (104)	AT	First Cycle	Yes	No
Magli, 1998(105)	AT	Age ≥38 years ≥3 Impl. Failures Both	No Yes	Yes, Sig. No
Lanzendorf,1998(106)	AT	Age ≥36 years	Yes	No
Meldrum,1998 (107)	AT Er: YAG laser	Age ≥35 years First Cycle	No Yes	Yes, NS Yes (?)
Antinori, 1999 (67)	PZD	≥6 Impl. Failures	Yes	Yes (?)
Edirisinghe, 1999 (108)		Age ≥38 years, ZP≥15 ≥1µmImpl. Failures	No	No
Baruffi, 1999 (62)	Diode Laser, ZP Thinning	Age ≥37 years, first cycle	Yes	No
Viega, 1999 (88)	Diode Laser	Impl. Failures, CC	Yes	Yes, NS
		First Cycle	Yes	No (unpubl.)
Cieslak, 1999(38)	3D-PZD	All IVf	Yes (control: conv. PZD)	Yes, NS
Alikani, 1999 (109)	PZD+frag.removal	≥6% frag.	No, retrosp.	Yes, Sig.
Nakayama, 1999 (110)	Piezomicromanipulator	≥2 Impl. Failures	Yes	Yes, Sig.

Abbreviations: AT, acid Tyrode’s; blast., blastomere; CC, co-culture; conv.,conventional; Er:YAG, erbium:yttrium-aluminum-garnet; ET, embryo transfer: frag., fragmented: FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; impl. implantation; IVF, in vitro fertilization; NS, not significant; PZD, partial zona pellucida dissection; retrosp., retrospective; sig., significant; TET, thawed embryo transfer; unpubl., unpublished; ZP, zona pellucida

Table 2
Assisted hatching results reported by different authors (2000-2010) [2]

First author, year increase	Study Method	Population	Randomized	Pregnancy rate
Mansour,2000 (111)	ZP removal,AT	First Cycle Age ≥40 years, ≥2 impl. failures	Yes Yes	No Yes, Sig.
Mantoudis,2001 (112)	Diode laser Total AH, partial AH, ZP	Poor responders aged ≥38 years, ≥2 impl.failures, frozen ET	No	Yes, Sig. (for ZP thinning)
Malter, 2001 (113)	Diode laser versus AT	All IVF/ICSI	Yes (control: AT)	No
Balaban, 2002 (79)	PZD AT Pronase thinning	All IVF/ICSI	No, retrosp.	No No No
Rienzi, 2002 (22)	Diode laser	Frozen ET	Yes	Yes, Sig.
Hsieh,2002 (114)	Diode laser versus AT	Age ≥38 years	Yes	Yes, Sig.(for laser)
Milki, 2002 (115)	AH D+3 versus CC	Age 40-43 years	No, retrosp.	No
Vanderzwalmen, 2003(33)	PZD blastocysts	Frozen (vitrif.) ET	No, retrosp.	Yes, Sig.
Gabrielsen, 2004 (116)	AT	Frozen ET	Yes	No
Petersen,2005 (76)	Diode laser, ZP thinning	≥2 impl. failures	Yes	Yes, NS
Ng, 2005 (81)	Diode laser, ZP thinning	Frozen ET	Yes	No
Nadir,2005 (91)	Diode laser, ZP thinning	Endometriosis	Yes	No
Frydman, 2006 (117)	Diode laser, ZP thinning	Age ≥37 years	Yes	No
Balaban, 2006 (118)	Diode laser	Frozen ET	Yes	Yes, Sig.
Sifer, 2006 (80)	Pronase, ZP thinning	Frozen ET	Yes	No
Petersen,2006 (82)	Diode laser, ZP thinning	Frozen ET From OHSS IVF cycles	Yes	Yes, NS
Sagoskin, 2007 (119)	Diode laser	Good prognosis	Yes	No
Hiraoka, 2007 (41)	Diode laser versus AT Total ZP removal versus partial drilling, Blastocyst AH	≥2 impl. failures Frozen ET	No, retrosp.	Yes, Sig.(for total removal)
Hiraoka, 2008 (40)	Diode laser ZP drilling ET 50% versus 40 µm	≥2 impl. failures Frozen ET	No, retrosp.	Yes, Sig.(for 50%)
Hiraoka, 2009 (42)	Diode laser, ZP thinning D+3 50% cersus 25%	Frozen ET	Yes	Yes, Sig.(for 50%)
Valojerdi, 2010 (83)	Diode laser, ZP thinning D+3 vitrified	Frozen ET	Yes	No.(sig. decrease)
Fang, 2010 (84)	Mechanical expansion D+3	Frozen ET	Yes	Yes, Sig.

Abbreviations: AT, acid Tyrode's; blast., blastomere; CC, co-culture; conv.,conventional; Er:YAG, erbium:yttrium-aluminum-garnet; ET, embryo transfer: frag., fragmented: FSH, follicle-stimulating hormone; ICSI, intracytoplasmic sperm injection; impl. implantation; IVF, in vitro fertilization; NS, not significant; PZD, partial zona pellucida dissection; retrosp., retrospective; sig., significant; TET, thawed embryo transfer; unpubl., unpublished; ZP, zona pellucida

List of Enrolled Assisted Reproductive Technology (ART) Clinics Under National Registry of ART Clinics and Banks in India.

ICMR, New Delhi, 2018, pp. 1-51,

https://www.icmr.nic.in/sites/default/files/whats_new/New-list-of-approved-ART-Clinics-14-09-2018.pdf.

Fig. 1. Map of Jalandhar district depicting study setting with marked study area
Source: "Demography." Demography, Jalandhar Web Portal, India, jalandhar.nic.in/demography

Study place:

The study was conducted at Babies World IVF centre, Chhabra Hospital & Maternity Home, Jalandhar, Punjab. It is an advanced fertility centre with state of art equipment with NABH Accredited IVF lab, bearing number ISO 9001:2015. It is run by a team of experts & dedicated professionals, namely infertility consultants, andrologist, microbiologist, counsellors, programme coordinator, ART Specialist, Gynaecologist & technical staff.

It is a sterile well equipped lab with hepafilter room, Co2, incubator, olympus microscope with narshige micromanipulator, CASA, Laser hatching system and has an IVF theatre, which deals only with ovum pick-up andembryo transfer which reduces the risk of the embryos being exposed to the detrimental fluctuations in environment. It is backed by ultrasound, hormonal assays andendoscopic facilities. Endoscopic Procedures are done in a separate general operation theatre. The andrology lab is located within the IVF unit and can deal with all types of male infertility. Sperm banking facilities are also available thus offering all aspects of infertility treatment under one roof. A viewing gallery is provided to

enable patients and doctors to observe the actual IVF/ICSI/IUI procedures. It also has semen collection room equipped with audio-visual facilities provided for the male partner for semen collection. On an average more than 100 cycles of IVFs are conducted in a year.

"Infrastructure,
Babiesworldivfcentre.com,
www.babiesworldivf.com/infrastructure.)

Study Period: This study was conducted for a period of three years from February 1st 2017 to January 31st 2020).

Study Design: It is a randomized control trial

Sampling: Purposive sampling was adopted

Sample size: 200 cases.

Inclusion Criteria:

All the study subjects who underwent IVF treatment for infertility at Babies World IVF centre, Chhabra Hospital & Maternity Home, Jalandhar, Punjab between the study period and all those woman who underwent long agonist protocol or short antagonist protocol, with ejaculated or frozen sperms origin with D3 /D4 transfer in the fresh embryo transfer group & vitrified D4 embryo transfer (ET) in the frozen cycle group, euthyroid, had both ovaries, normal prolactin levels and even self and donor cycles were included in the study.

Exclusion Criteria:

Patients with noted history of major medical illnesses, uterine anomalies or adnexal pathology were excluded. Those with history of cycles cancelled prior to oocyte retrieval were also excluded.

Study subjects: After considering inclusion and exclusion criteria, 200 patients who underwent IVF (in-vitro fertilization)/ ICSI (Intra-cytoplasmic sperm injection) at Babies World IVF centre, Chhabra Hospital & Maternity Home, Jalandhar, Punjab between the study period were considered for the study.

A. *Ethical issues and ethical committee clearance [Annexure-I]*

The ethical approval was taken by the Institutional Ethics Committee of Babies World IVF centre, Chhabra Hospital & Maternity Home, Jalandhar, Punjab.

B. *Informed Consent [Annexure-2]*

Patients were explained about the study procedure and importance of the study in their own language of understanding and written informed consent was taken from them.

Procedure of data collection:

It was a randomized controlled trial conducted among 200 patients who underwent IVF/ICSI at Babies World IVF centre, Chhabra Hospital & Maternity Home, Jalandhar, Punjab between the study period who full-filled the inclusion and exclusion criteria during the study period from February 1st 2017 to January 31st 2020 were included. Based on the inclusion and exclusion criteria, a total of 200 patients were enrolled after obtaining written informed consent from patients and ethical committee clearance from the institutional ethical committee board of which 50% underwent Assisted Laser hatching.

First patient was randomly allocated among two arms using a coin toss with heads falling to group A undergoing Fresh embryo transfer and tails falling to group B, receiving Frozen (vitrified) embryo transfer. After the selection of first patient, next following patients were allocated systematically to the next consecutive arms alternately between group A without AH and group B with AH. Laser hatching on the embryos and its further effect on pregnancy rates would be studied.

Data collection:

All information pertaining to the patients viz., of name, age, gender, address, relevant past and present medical history, height, weight, other examination findings were obtained under three headings:

i) S*ocio-demographic data:* Name, age, gender, address.
ii) *History:* Details on presenting complaints, history of previous treatment received, family history, marital history, menstrual history, previous conception and also data related to risk factors for poor ovarian response (POR) viz., history of chronic smoking, drinking, previous ovarian surgery, previous chemotherapy [10].
iii) *General Physical Examination (GPE) and Systemic Examinations:* Routine physical examination and anthropometry measurements, along with specific gynaecological examinations were conducted. BMI in kg/m^2 was calculated and categorized according to WHO Asia-Pacific guidelines.
iv) *Investigations:* Findings of transvaginal scans, laparoscopic findings, tubal evaluation, along with baseline hormone profile: serum FSH (follicle stimulating hormone), LH (luteinizing hormone) and AMH levels along with the parameters of semen analysis of the male partner were recorded.

Method of estimation of AMH levels: Generation 2 ELISA kit with sensitivity being 0.025 ng/ml, and intra- and inter-assay variation of the assay being 7% AMH levels were measured

Controlled ovarian stimulation, ovarian response & Trigger:

Both Standard Antagonist (flexible) protocol and Long agonist protocols were followed among patients based on the indication for the patient and standard clinical practice adhered to in our hospital.

Long agonist protocol: Following was the treatment protocol which was started from Day 21 of the menstrual cycle:

- Inj. Leupride acetate 0.5 mg (Sun Pharmaceutical Ind Ltd, Mumbai) was given subcutaneously once daily till down regulation was achieved or day 2 of menstrual cycle with serum estradiol <50 pg/mL, endometrial thickness <5 mm, with no cyst in the ovaries and Serum Leutinising hormone <2.0 IU/L.
- After the down regulation was achieved, the dose of inj. Leupride was reduced to 0.2 mg daily and recombinant FSH i.e., inj. Gonal-f, Merck Serono Specialities Pvt.

Ltd., Italy was started with a starting dose of 75–150 IU. After 4 days of FSH stimulation, the dose was adjusted depending on the ovarian response, evaluated by transvaginal scan (using 7.5 MHz vaginal probe, Voluson 730 pro, GE Healthcare, Milwaukee, Winconsin USA) and serum estradiol levels.

In the later days of stimulation, human menopausal gonadotropin Gynogen HP, Unisankyo, Mumbai, India) was added on an individual basis at the discretion of the treating doctor. Monitoring of the follicular growth was done by serial ultrasonography and the doses of FSH and HMG were adjusted based on serum estradiol levels and dynamics of ovarian follicular growth.

Standard Antagonist (flexible) protocol:

- After the basal Day 2 Transvaginal scan (TVS) for Antral follicle count, the patient was administered an initial dose of 225-375 IU human menopausal gonadotropin (HMG) or Follicle Stimulating Hormone (FSH purified or recombinant). The doses were adjusted based on the response to gonadotropin as assessed by serum E2 levels and sonographic monitoring of follicular growth till it reached 18-20 mm in size starting from the Day 2 of the cycle.
- Once the follicles reached 13-14mm, Injection Cetorelix 250 µg subcutaneously was added in the morning time.
- The ovarian response was monitored by serial trans-vaginal scans for increasing size of the follicles and serum E2 levels on days 7, 9 and 11.

Trigger:

- When three or more follicles of size 18 mm or more were seen, final oocyte maturation trigger was given with inj. hCG Injection Ovitrelle 250 µg*2 subcutaneously, or with inj. Leupride acetate 0.5 mg (Sun Pharmaceutical Ind Ltd, Mumbai).

Oocyte Retrieval:

- Under sedation, oocyte retrieval was performed trans-vaginallyusing a 35 cm 17G oocyte aspiration needle 34–36 hr after the trigger. For most of them it was done at 36 hrs but for those with poor reserve it was scheduled at 34 hrs.

Fertilization:

- The retrieved Eggs were incubated at 37◦C in HEPES, later denuded and their quality was assessed by embryologist.
- The oocytes were classified based on nuclear maturation grading into metaphase II (mature) or non-metaphase II categories. The latter category included oocytes at the metaphase I and prophase I stage. The oocytes that did not develop to metaphase II after 8 hours of incubation were discarded.
- ICSI dishes were prepared and performed by a senior embryologist.
- Fertilization was defined as the formation of zygotes with two pronuclei after 16–18 hours(normal fertilization) and later the zygotes were evaluated using the Z-score system.
- The embryos were cultured using Fert culture media at 37°C, 5% CO2in the Benchtop incubator.
- On day 2 (44–46 h after insemination or sperm injection) and on day 3 (66–72 h insemination or sperm injection), cleavage embryos were assessed by embryologist and were graded according to the criteria described by Veeck.

Frozen embryo transfer:

- The frozen embryos, frozen at 6 cell stage by Rapid freeze protocol such as vitrification were transferred for frozen embryo transfer group following the preparation of the endometrium and/or down regulation.

All patients were prescribed 600 mg of micronized progesterone for 2 weeks as luteal phase support. On the day of hCG administration, serum estradiol, LH and progesterone levels were measured

Positive Pregnancy:

- Serum β-hCG> 50 mIU/l was considered as positive. Clinical pregnancy was defined as a viable intrauterine pregnancy (positive cardiac activity) on transvaginal scan performed at 6 weeks.

Kaur H, Krishna D, Shetty N, Krishnan S, Srinivas MS, Rao KA. A prospective study of GnRH long agonist versus flexible GnRH antagonist protocol in PCOS: Indian experience. Journal of human reproductive sciences. 2012 May;5(2):181. [27]

Study outcomes:

- The primary outcome of the study was clinical pregnancy rate.

Operational Definitions:

- Fertilization rate was calculated based on the number of mature oocytes fertilized into zygote with two pro-nuclei [16].
- Top quality embryos grading was based on Cleavage Stage Embryo scoring described by Veeck as mentioned below:

Grades Description:

- Grade 1: Embryo with blastomeres of equal size, no cytoplasmic fragments.
- Grade 2: Embryo with blastomeres of equal size, minor cytoplasmic fragments or blebs.
- Grade 3: Embryo with blastomeres of distinctly equal size, none or few cytoplasmic fragments.
- Grade 4: Embryo with blastomeres of equal or unequal size, significant cytoplasmic fragmentation.
- Grade 5: Embryo with blastomeres of any size, severe or complete fragmentation [15].

C. Anthropometric Measurements

1) Measurement of Height

The height was measured with stadiometer. Subjects were made to stand without footwear on the flat surface, with evenly distributed weight on both feet and heels together, and the head positioned so that the line of vision is perpendicular to the body (Frankfurt line). [12] The arms were hung freely by the sides,

the head, back, buttocks, and heels in contact with the vertical board. The individual was asked to inhale deeply and maintain a fully erect position. The movable headboard was brought onto the topmost point on the head with sufficient pressure to compress the hair. The height was recorded to the nearest 0.5 cm [17].

2) Measurement of weight

The weight was measured in kilograms using standardized bathroom weighing machine with person standing erect on centre of the platform, with the body weight evenly distributed between both the feet with light clothing and looking straight. The weight was recorded to the nearest 0.5 kg.

3) Body Mass Index (BMI)

It is also called as Quetelet's index used to assess obesity and computed by weight (kg) divided by height in meters square [53].

$$BMI = \frac{\text{Weight in Kilograms}}{\text{Height in meter}^2}$$

Table 3
Classification of BMI -WHO Asia-Pacific guidelines

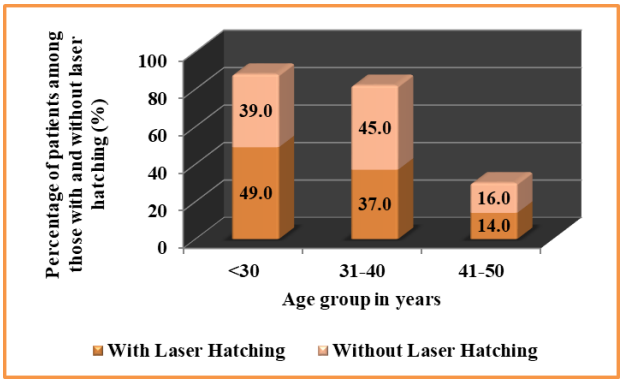
BMI	Category
<18.5	Underweight
18.5 - 22.9	Normal range
23 - 24.9	Overweight
25 - 29.9	Obese – I
≥ 30	Obese – II

Table 4
Age-group distribution among two groups with or without laser hatching

Age group in years	Laser hatching		χ^2 – value (*P*-value)
	Yes n (%)	No n (%)	
≤30	49 (49.0)	39 (39.0)	2.05 (0.36)
31-40	37 (37.0)	45 (45.0)	
41-50	14 (14.0)	16 (16.0)	
Total	**100 (50.0)**	**100.0 (50.0)**	

Most of them in the group who has undergone laser hatching i.e., 49.0% of them were younger than 30 years followed by 37.0% were in the age group of 31-40 years and 14.0% were in the age group of 41-50 years. However, in the other group i.e., who has not undergone laser hatching, most of them i.e., 45.0% were in the age group of 31-40 years followed by 39.0% were in the age group less than 30 years and least were in the age group of 41-50 years. There was no significant difference among the groups (P>0.05).

In the current study most of them i.e., 49.0% of them were younger than 30 years among those with laser hatch group and in the other group i.e., without laser hatch group, most of them i.e., 45.0% were in the age group of 31-40 years. The mean ages of the study participants among the groups with and with no laser hatch groups were 32.23±6.36 years and 33.57±6.16 years respectively. The participants ranged from a minimum of 22 years to a maximum of 48 years in the fresh embryo group and in the other group it ranged from 24 years to 48 years.



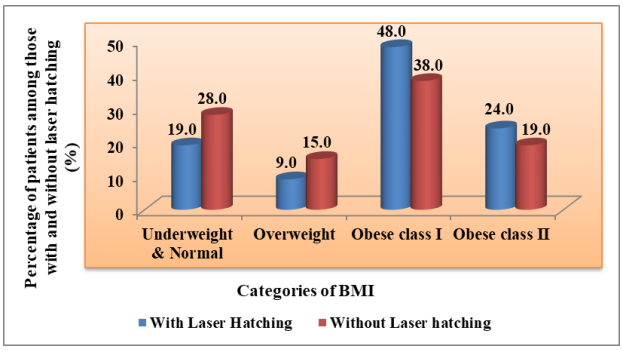
Graph 1. Distribution of age groups among both with and without laser hatch group

Table 5
Distribution of study subjects based on BMI classification among those who underwent laser hatching and those who did not undergo laser hatching

BMI (kg/m^2)	Laser hatching		χ^2 – value (*P*-value)
	Yes n (%)	No n (%)	
Underweight & Normal	19 (19.0)	28 (28.0)	4.97 (0.17)
Overweight	09 (9.0)	15 (15.0)	
Obese class I	48 (48.0)	38 (38.0)	
Obese class II	24 (24.0)	19 (19.0)	
Total	**100 (50.0)**	**100.0 (50.0)**	

* indicates statistically significant association at P<0.05

In the laser hatching group, most of them belonged to obese class I (48.0%) followed by obese class II (24.0%), underweight and normal (19.0%) and overweight (9.0%). In the other group, most of them belonged to obese class I (38.0%), followed by underweight and normal (38.0%), obese class II (19.0%) and overweight (15.0%). The difference of proportions among the laser hatching group versus without were not statistically significant (P>0.05).



Graph 2. BMI-wise categories among the groups which underwent laser hatch versus no laser hatch

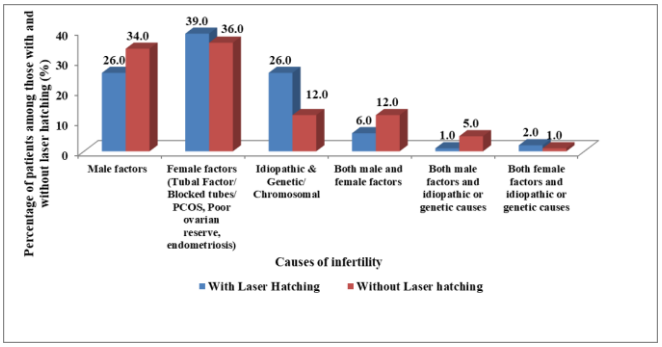
Forty-eight percent (48.0%) of those who underwent laser hatch thirty-eight percent (38.0%) of those who did not undergo laser hatch belonged to obese class I which formed the majority. The mean height, weight and BMI in laser hatch group was 1.60±0.05 m, 69.12±9.84 kg and 26.87±3.69kg/m^2 and in the other group it was 1.59±0.05 m, 65.89±10.65 kg and 26.07±4.69 kg/m^2. The mean height (t = -1.72, *P*= 0.09, 95%

CI [-0.025 to 0.0017]) and BMI (t = -1.33, *P*= 0.19, 95% CI [-1.97 to 0.38]) did not vary significantly among the groups. However, the mean weight was significantly higher among those who underwent laser hatching compared to the other group which did not undergo laser hatching (t = -2.23, *P*= 0.03, 95% CI [-6.09 to -0.37]).

Table 6
Distribution of study subjects based on causes of infertility among those who underwent laser hatching and those who did not undergo laser hatching

Causes of infertility	Laser hatching	
	Yes n (%)	No n (%)
Male factors	26 (26.0)	34 (34.0)
Female factors (Tubal Factor/ Blocked tubes/ PCOS, Poor ovarian reserve, endometriosis)	39 (39.0)	36 (36.0)
Idiopathic &Genetic/ Chromosomal	26 (26.0)	12 (12.0)
Both male and female factors	06 (6.0)	12 (12.0)
Both male factors and idiopathic or genetic causes	01 (1.0)	5 (5.0)
Both female factors and idiopathic or genetic causes	02 (2.0)	1 (1.0)
Total	**100 (50.0)**	**100 (50.0)**

Among both the groups with and without laser hatching, female factors (39.0% - with laser hatching; 36.0% - with no laser hatching) were the most commonly elicited cause of infertility followed by male factors (26.0% among those with laser hatching and 34.0%among those with no laser hatching), idiopathic/ chromosomal group (26.0% - with laser hatching; 12.0% - with no laser hatching). Both male and female factors as a cause of fertility were present among 6.0% of those who underwent laser hatching and 12.0% of those who have not undergone laser hatching. Both male and idiopathic factors were present among 1.0% of those who underwent laser hatching and 5.0% of those who have not undergone laser hatching and both female factors and idiopathic or chromosomal/ genetic causes were reported among 2.0% of those who underwent laser hatching and 1.0% of those who have not undergone laser hatching.



Graph 3. Percentage of study subjects with different causes of infertility among the groups which underwent laser hatch versus no laser hatch

Among both groups with and without laser hatching, nearly equal proportion had female factors of infertility 39.0% and 36.0% respectively and higher proportion of those without laser hatching had male factors (34.0%), both male and female factors (12.0%) and both male and idiopathic factors or genetic causes (5.0%). However, higher proportions of those who underwent laser hatching had idiopathic and genetic or chromosomal causes and both female factors and idiopathic or genetic causes.

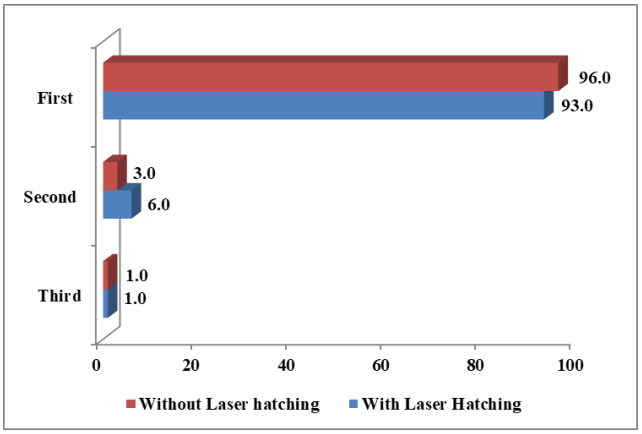
The mean duration of infertility was 8.00±2.68 years and it ranged from a minimum of 4 years to a maximum of 18 years. It was 8.01±2.41 years in those who did not undergo laser hatching and 7.99±2.94 years in those who underwent laser hatching and there was no significant difference among the duration of infertility among both groups (t – value [95% C.I]**:** 0.05 [-0.73 to 0.77]; *P*=0.96).

Table 7
Distribution of study subjects based on order of planned pregnancy among those who underwent laser hatching and those who did not undergo laser hatching

Order of planned pregnancy	Laser hatching		*P*-value§
	Yes n (%)	No n (%)	
First	93 (93.0)	96 (96.0)	0.54
Second and Third	07 (7.0)	04 (4.0)	
Total	**100 (50.0)**	**100 (50.0)**	

§Fisher's Exact test

The proportion of patients with first order of planned pregnancy was relatively higher in those who did not undergo laser hatching (96.0%) group compared to those who underwent laser hatching (93.0%). However, there was no significant difference among the groups.



Graph 4. Percentage of study subjects with different order of planned pregnancy among the groups which underwent laser hatch versus no laser hatch

Majority of the study subjects in both laser hatch and no laser hatch group had first order of pregnancy (93.0% v/s 96.0%) followed by second (6.0% v/s 3.0%) and third (1.0% in each).

The table 8, shows the average serum levels of different hormones viz., leutinizing hormone, follicle stimulating hormone, estrogen and anti-mullerian hormones among the two different groups of different type of embryo transfers. The levels of LH (Fresh v/s frozen: 7.9 v/s 7.66 IU/L; t=0.91, *P*>0.05), FSH (Fresh v/s frozen: 10.21 v/s 9.86 IU/L; t=1.14, *P*>0.05), estrogen (Fresh v/s frozen: 22.71 v/s 20.91 ng/ml;

t=1.94, *P*=0.05) and AMH (Fresh v/s frozen: 1.32 v/s 1.76 ng/ml; U=4452.5, *P*>0.05) did not vary significantly among the two different type of embryo transfer groups.

Table 8
Average levels of different hormones at the baseline among different groups which underwent laser hatch versus no laser hatch

Hormonal Levels	Laser hatching (Mean±SD)		t – value [95% C.I]/ U-value	*P*-value
	Yes (n = 100)	No (n=100)		
LH (IU/L)	7.72±1.87	7.85±1.86	0.49 [-0.39 to 0.65]	0.63
FSH (IU/L)	9.91±1.91	10.15±2.43	0.78 [-0.37 to 0.85]	0.44
Estrogen (ng/ml)	21.03±5.08	22.59±7.75	1.68 [-0.27 to 3.39]	0.09
AMH¥(ng/ml)	1.76 (0-7)	1.27 (0-6)	4498.5	0.22

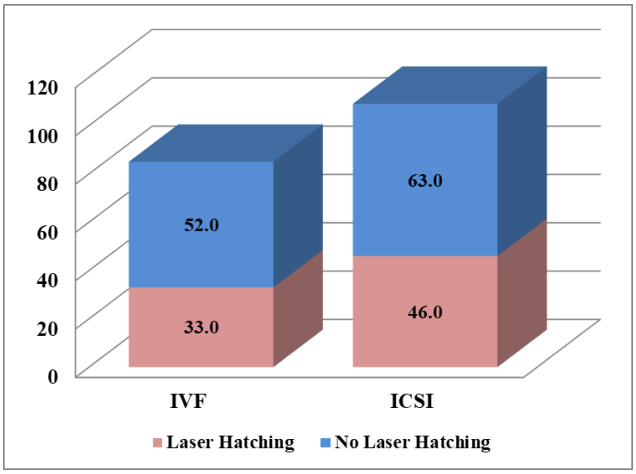
LH - Leutinizing Hormone; FSH - Follicle Stimulating Hormone; AMH - Anti-Mullerian Hormone
¥- Median values with range and Mann Whitney U test applied as test of significance

Table 9
Comparison of modes or part of treatment among different groups which underwent laser hatch versus no laser hatch

Mode/ part of fertility treatment	Laser hatching		χ^2 – value (*P*-value)
	Yes n (%)	No n (%)	
IVF	50 (33.0)	35 (52.0)	4.60 (0.03)*
ICSI	46 (46.0)	63 (63.0)	8.89 (0.003)*

* indicates statistically significant association at P<0.05

The proportions of those study subjects who underwent IVF (33.0%)and ICSI (46.0%) as a part of treatment were significantly lesser in those who underwent laser hatching compared to those who did not undergo laser hatching (P<0.05).



Graph 5. Percentage of study subjects with different modes/part of infertility treatment among different groups which underwent laser hatch versus no laser hatch

Among those who underwent IVF and ICSI, only 33.0% and 46.0% of them underwent laser hatching respectively.

Table 10
Average levels of estrogen, progesterone, leutinizing hormone (LH) and endometrial thickness on day one of USG among different groups which underwent laser hatch versus no laser hatch

Hormonal Levels	Laser hatching (Mean±SD)		t – value [95% C.I]/ U-value	*P*-value
	Yes (n = 100)	No (n=100)		
Estrogen (ng/ml)	21.14±5.92	22.39±9.88	1.08 [-1.02 to 3.52]	0.28
Progesterone (ng/ml)¥	1.2	0.9	4405.5	0.14
Leutinizing Hormone (LH) (IU/mL)	7.71±1.87	8.16±1.83	1.70 [-0.07 to 0.96]	0.09
Endometrial thickness (mm)	9.75±1.17	9.75±1.16	0.0 [-0.32 to 0.32]	1.00

¥- Median values with range and Mann Whitney U test applied as test of significance

The above table shows the average serum levels of different hormones viz., estrogen, progesterone, leutinizing hormone along with the endometrial thickness among the two different groups of those who underwent laser hatch and those who did not undergo laser hatch. The estrogen levels (Laser hatch v/s no laser hatch: 21.14 v/s 22.39 ng/ml; t=1.08, *P*>0.05), progesterone levels (Laser hatch v/s no laser hatch: 1.2 v/s 0.9 ng/ml; U=4405.5, *P*>0.05), leutinizing hormone (Laser hatch v/s no laser hatch: 7.71 v/s 8.16 IU/ml; t=1.70, *P*>0.05) and the endometrial thickness (Laser hatch v/s no laser hatch: 9.75mm in both; t=0.0, *P*>0.05) did not vary among the two groups of those who underwent laser hatch and no laser hatching.

Table 11
Average number of oocytes, embryos available and fertilized along with the fertilization rate among different groups which underwent laser hatch versus no laser hatch

Particulars	Laser hatching (Median with range)		U-value	*P*-value
	Yes (n = 100)	No (n=100)		
Number of oocytes	7 [3 - 21]	7 [2 – 24]	4699.0	0.46
Number of embryos available	7 [2 - 21]	8 [2 – 29]	4470.0	0.19
Number of embryos fertilized	5 [2 – 18]	6 [2 – 23]	4319.0	0.09
Number of embryos transferred	3 [2 – 3]	3 [2 - 4]	4945.5	0.80
Fertilization rate (%)	80 [36 – 100]	80 [30 – 100]	4573.0	0.29

The number of oocytes (Laser assisted hatch v/s no laser assisted hatch: 7 v/s 7), number of embryos available (Laser assisted hatch v/s no laser assisted hatch: 7 v/s 8), transferred (Laser assisted hatch v/s no laser assisted hatch: 3 v/s 3), and fertilized (Laser assisted hatch v/s no laser assisted hatch: 5 v/s 6), did not show any significant difference among different groups which underwent laser hatch versus no laser hatch (*P*>0.05). Similarly, the fertilization rate also did not vary significantly across the groups (fresh v/s frozen: 80 v/s 80) (*P*>0.05).

Though lesser proportions i.e., 52/100, 52.0% were positive for biochemical pregnancy and clinical pregnancy among those who underwent laser assisted hatching compared to the other group who did not undergo laser assisted hatching i.e., 64/100, 64.0%, there was no significant association of occurrence of pregnancy with laser assisted hatching (P>0.05).

The proportions of those who experienced adverse events were significantly higher among those who underwent laser assisted hatching (25.0%) however none of them experienced any adverse event among those who did not undergo laser hatching (P<0.05).

Among the positive pregnancies higher proportions i.e., 5.8% had more than one foetuses among those who underwent laser hatching compared to those who did not undergo laser hatching (4.7%). However, there was no significant association of number of foetuses with the laser hatching (P>0.05).

Table 13
Association of occurrence of any adverse event (miscarriage and ectopic pregnancies) with the laser hatching

Adverse events during pregnancy	Laser hatching		*P*-value¥
	Yes (n=52) n (%)	No (n=64) n (%)	
Yes	13 (25.0)	00 (0.0)	<0.005*
No	39 (75.0)	64 (100.0)	

¥ - Fisher's exact test applied
* - indicates statistically significant association at P<0.05

Clinical outcomes among those who underwent laser assisted hatching v/s no laser hatching:

Both the groups with and without laser hatching were also comparable in terms of mean age (32.23 v/s 33.57 years), BMI (26.87 v/s 26.07kg/m^2), duration of infertility (8 years in each), oocytes available (7 in each), embryos available (7 v/s 8) and transferred (3 in each). Female tubal factors constituted more among both the groups who underwent laser assisted hatch (39.0%) and no laser hatching (36.0%).

Table 14
Association of study subjects based on number of fetuses with the laser hatching

Number of foetuses	Laser hatching		*P*-value¥
	Yes n (%)	No n (%)	
One	49 (94.2)	61 (95.3)	1.00
More than one	03 (5.8)	03 (4.7)	

¥ - Fisher's exact test applied

We noted that laser assisted hatch did not favour the outcome of occurrence of biochemical and clinical pregnancy (LAH v/s no LAH: 52.0% v/s 64.0%) and also occurrence of multiple gestations (LAH v/s no LAH: 94.2% v/s 95.3%). In fact, the proportions of those who experienced adverse events were significantly higher (25.0%) in the LAH group.

6. Recommendations and Conclusion

Laser assisted hatching additionally did not affect the occurrence of pregnancy. But selective laser hatch for elderly age group might favourably influence the pregnancy rates.

7. Future Scope

Assisted Hatching may have a potential in improving ART results in selected patients. Hence; more studies with adequate methodological quality, preferably multicentric trials with appropriate design and follow-up, should be undertaken to investigate the efficacy of AH at different stages of embryo development, & also in different groups of infertile women, undergoing frozen embryo transfers; Also women in older age group with high FSH levels, women with thickened ZPs or poor quality embryos, may be studied pertaining the results of Laser Assisted Hatching. Large randomized studies comparing assisted hatching methods in different age groups of women; with regard to implantation & live birth rates are needed. The Laser Equipment is expensive. The 1.48 µm diode infrared Laser System for ZP during offers low risk, is quick & relatively simple to perform & may be considered to be the most suitable method for AH in the IVF Laboratory.

8. Limitations of the Work

Though observing the effect of laser assisted hatching group was a part of this study, sampling of the groups could not be achieved while selecting the study groups. Hence the effect of selection bias cannot be ruled out in interpreting the same.

Long term follow up for maternal and neonatal outcomes could not be conducted as it was beyond the scope of the current objectives of this study.

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