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STUDY TO IMPROVING EMBRYO YIELD PREGNANCY ON THE EFFECT OF ASSISTED HATCHING (LAH) IN VITRO FERTILIZATION

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person except that which appears in the citations an acknowledgements. Nor does it contain material, which to a substantial extent I have submitted for the qualification for any other degree of another university or other institution of higher learning.

Name: Tam An Nguyen Thi Signed:

ABBREVIATION

AH: Assisted hatching

CI: Confidence interval

CPR: Clinical pregnancy rate

DR: Delivery rate

FET: Frozen embryo tranfer

FSH: Follicle Stimulating Hormone

ICSI: Intracytoplasmic sperm injection

GnRH: Gonadotropin releasing hormone

GV: Germinal vesicle

IVM: in vitro maturation

IR: Implantation rate

IVF: Invitro Fertilization

LAH: Laser Assisted hatching

LBR: Live birth rate

LH: Luteinizing hormone

PESA: Percutaneous Epididymal Sperm Aspiration

PGD: Preimplantation genetic diagnosis

PVS: Perivitelline space

TE: Trophectoderm

ZP: Zona pellucida

WHO: World health organization

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1. ABSTRACT

Laser assisted hatching (LAH) technique is based on the hypothesis that making an artificial hole on zona pellucida (ZP) can help embryo to easily hatching out of ZP. This technique has been shown to increase implantation and pregnancy rates in women of advanced age, in women with recurrent implantation failure and following the transfer of frozen–thawed embryos. Assisted hatching (AH) by mechanical, chemical, or laser manipulation is applied to enable implantation in situations where it is decreased, such as, aging, high level of basal FSH, zona hardening, or in all ART treatments were implantation fail on two previous attempts. Zona assisted hatching was introduced to overcome the zona barrier in instances of suspected impairment to zona hatching due to either zona hardening or thickness, particularly in patients with repeated failures.

This study aimed to assess the effect of LAH in both fresh and frozen thawed embryos before to be transferred into a recipient uterus. Within a 18-month period a total of 220 embryo transfer cycles (110 LAH and 110 control) were performed using fresh embryos (fresh transfer) and 220 embryo transfer cycles (110 LAH and 110 control) using frozen-thawed embryos (frozen-transfer). The mean of embryos transferred in each cycle was 2.6±1.6 in fresh-transfer and 2.4±1.2 in thawed-transfer cycles. Implantation rate in fresh-transfer cycles was 41.8% vs 23.6% respectively in LAH and control groups. In frozen-transfer cycle, implantation rate in the LAH group was 50.98 % and in the control group 38.1%.

The rate of clinical pregnancies in fresh-transfer was 34.5% vs 18.1% in LAH and control respectively, while in the thawed-transfer was 40.9% in LAH and 30.9% in control. The percentage of early miscarriages related to biochemical pregnancies in fresh-transfer cycles was 10.5% in LAH and 10% in control while and in frozen-thawed cycles the percentage of early miscarriages related to clinical pregnancies was; 8.89% and 8.82% respectively in LAH and control groups. This result was equal or higher than other researchs about embryos transfer with or without LAH and we conclude that LAH improves outcome of fresh or frozen-thawed embryo transfer practices.

2. INTRODUCTION

2. 1. The development and implantation of human embryos

The human embryo enters the uterine cavity approximately 4 to 5 days post fertilization. After passing down the fallopian tube or an embryo transfer catheter, the embryo is moved within the uterine lumen by rhythmic myometrial contractions until it can physically attach itself to the endometrial epithelium. It hatches from the zona pellucida within 1 to 2 days after entering the cavity thereby exposing the trophoblastic cells of the trophectoderm to the uterine epithelium. Implantation occurs 6 or 7 days after fertilization [52]. During implantation and placentation, a human embryo must attach itself to the uterus under conditions of shear stress. The embryo is rolling about within a mucus rich environment between the opposing surfaces of the endometrial walls of the uterus. This interactive process is a complex series of events that can be divided into three distinct steps: apposition, attachment and invasion [34].

Embryo proper at the beginning of the third week following initial contact between the two gametes, a faint midline structure, the primitive streak, appears in the primary ectoderm. All subsequent embryonic and fetal tissue will develop from this structure [13]. The appearance of the primitive streak is the point at which the body plan begins to become established and signals the commencement of gastrulation. From a biological perspective the appearance of the primitive streak is the first developmental time point at which a multi-cellular structure is formed that will uniquely develop into the new individual encoded by the new genome [57].

2.1.1. The development and implantation of in vivo human embryo

How far embryo development in vivo is near to development in vitro. Historically, embryos cultured in vitro lag behind their in vivo developed counterparts. However, with the development of sequential media based on the premise of meeting the changing requirements of the embryo and minimizing trauma coupled with use of reduced oxygen concentrations in the gas phase, in vivo rates of embryo development can now be attained in vitro in the mouse. The one proviso is that each laboratory must have sufficient quality systems in place to ensure the optimum operation of a given culture system. Such advances in culture systems represent a significant development for the laboratory, for now there exists a means of producing blastocysts at the same time and with the same cell number and allocation to the ICM as embryos developed in the female tract [52, 86].

The fertilisation process commences with the sperm initiating the penetration of the zona pellucida. The oocyte then completes the meiotic process and extrudes the second polar body, which contains one chromatid from each chromosome pair. Two pronuclei become visible 9 - 12 hours after the initial contact between the sperm and oocyte. One pronucleus contains the haploid paternally inherited genome while the other contains the haploid maternally inherited genome. Microtubules in the ooplasm draw the two pronuclei together. As long as the pronuclear membranes are intact, the mixing of the genetic material contributed by the sperm and oocyte cannot occur [84].

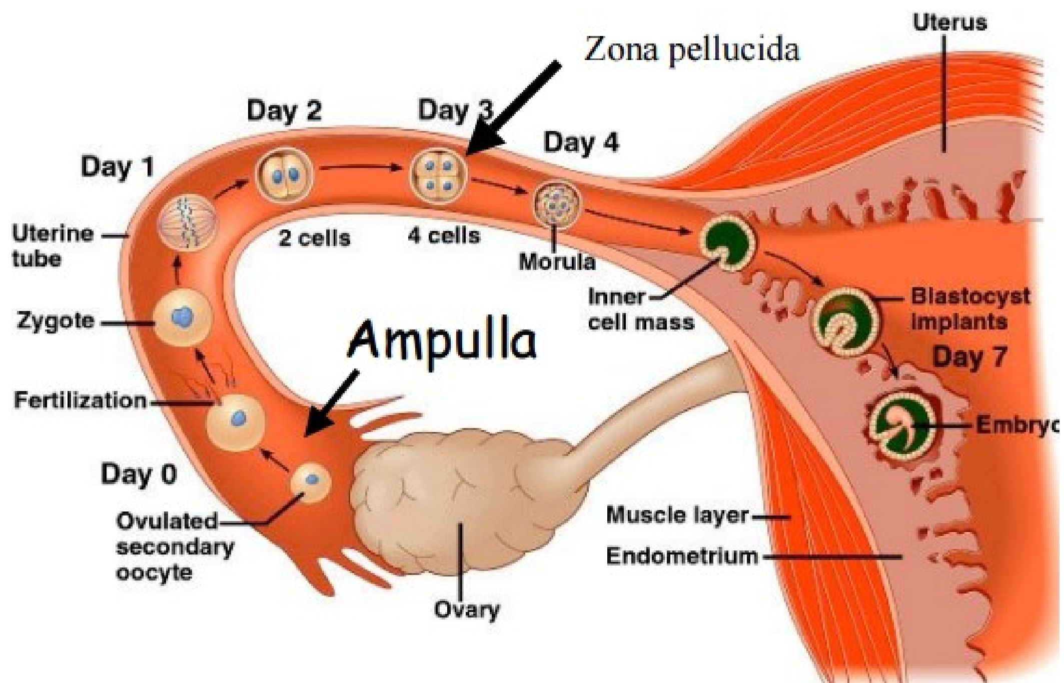


Figure 1. The first week of human embryonic development . Diameter of the uterine tube 1-2 cm; diameter of the embryo 0.12 mm

Before fertilisation the sperm cells contain the male genetic contribution to the new genome that will be produced at the completion of fertilisation. Sperm cells are produced during the process of meiosis, which occurs in the testis during spermatogenesis [70]. At the completion of meiosis each sperm contains a haploid genome (one chromatid from each chromosome pair). In order to deliver this genetic material into the oocyte, which contains the female genetic contribution, the sperm must penetrate the zona pellucida and the oocyte membrane.

In contrast, the oocyte retains the full maternal chromosome content until ovulation when it initiates meiosis by extruding one chromosome from each chromosome pair to form the first polar body. No conjugation of the two parental haploid genomes has occurred at this stage [57].

It has been estimated that up to 85% of produced embryos do not implant. The ability of an embryo to develop and implant primarily relates to the quality of

originating gametes and intrinsic characteristics of the embryo, such as its chromosomal constitution and the quality of its cytoplasm [47].

2.1.2. The development and implantation of in vitro human embryo

Implantation is the final frontier to embryogenesis and successful pregnancy. Over the past three decades, there have been tremendous advances in the understanding of human embryo development [124]. Since the advent of Invitro Fertilization, the embryo has been readily available to study outside the body. Indeed, the study has led to much advancement in embryonic stem cell derivation. Unfortunately, it is not so easy to evaluate the steps of implantation since the uterus cannot be accessed by most research tools [1].

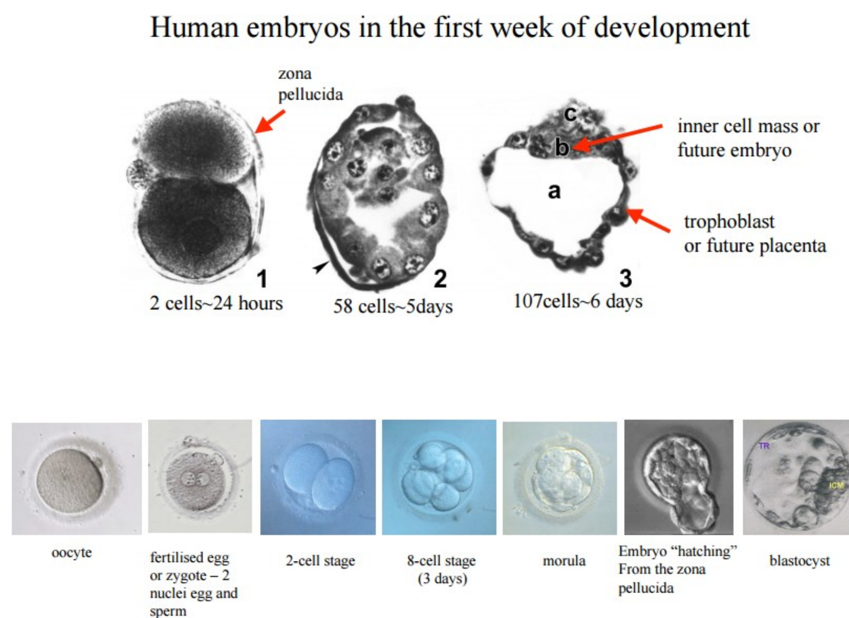


Figure 2. Human embryo in the first week of development

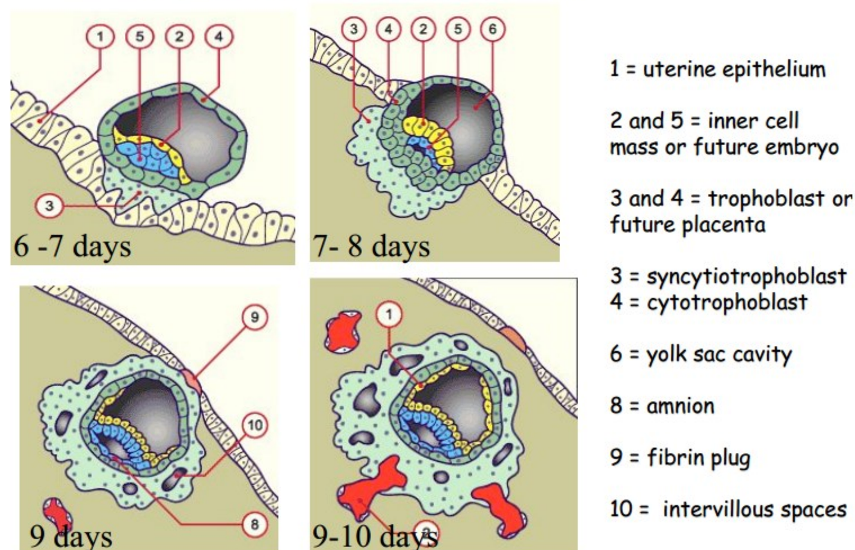


Figure 3. Human implantation

Fertilisation complete approximately 20 hours after the sperm has entered the oocyte the pronuclear membranes dissolve, allowing the haploid maternally and paternally inherited genomes to combine [108]. The alignment of the chromosomes from the maternally and paternally inherited genomes on the microtubular spindle occurs at 20-22 hours after sperm entry and is known as syngamy. However, as the pronuclear membranes have dissolved, the occurrence of syngamy cannot be visually confirmed on a live entity until the first mitotic division (cleavage) is initiated, which usually occurs 1 – 3 hours after syngamy. From syngamy until the first cleavage the entity is called the zygote. Cleavage stages Individual cells (blastomeres) cleave in two approximately every 18 hours [57].

2.1.3. Research of failure implantation

One of the factors that can cause embryo implantation to fail is the failure of zona rupture. Cohen et al (1990) suggested that zona hardening and subsequent failure of hatching could be frequent after IVF. This hypothesis was suggested by previous observations of the in-vitro hatching process following zona drilling of mouse and human embryos [3] . It is very frustrating to both a patient and her clinician to transfer a beautiful embryo into a prepared uterus only to have it fail to implant. This chapter will review the mechanisms of human embryo implantation and discuss some reasons why it fails to occur Hatching occurred earlier and embryos implanted more often after micromanipulation [68, 94].

It was suggested that many in-vitro fertilized embryos could be deficient in lysins necessary for hatching. So, if this hypothesis was correct, the implantation rate of embryos should be increased by micromanipulation to open the zona pellucida. Before beginning a clinical trial, several experiments were performed showing that the development of mouse embryos was not altered by such an invasive procedure, if the hole was large enough [7, 90].

At present, there are three methods that can be used to improve the hatching of day 3 embryos selected for transfer: the mechanical technique,partial zona dissection using glass microneedles; chemical assisted hatching, using acidic Tyrode's solution and laser-assisted hatching.

The World health Organization estimates that one in six couples experience some delay in conception and an increasing number require treatment by the assistedconception (AC) procedures of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The implantation rate of embryos resulting from in vitro fertilization cycles is generally less than 20%.

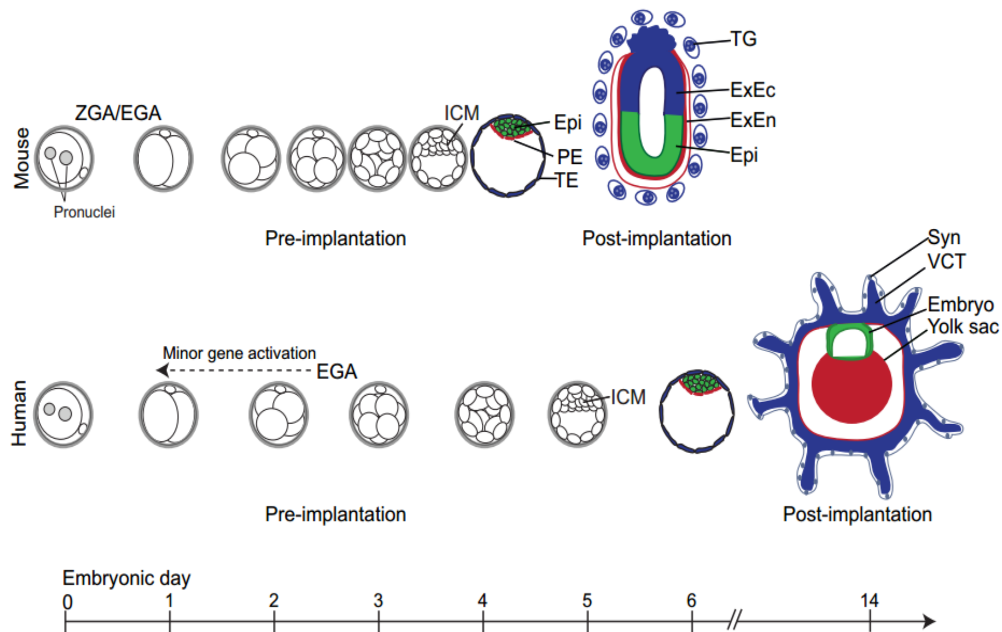


Figure 4. Human embryo early development

Recurrent implantation failure is today the major reason for women completing several IVF/intracytoplasmic sperm injection attempts without having achieved a child, and is probably also the explanation for many cases of unexplained infertility [53].

Recent research has indeed documented that both syndromes can be caused by the same embryonic chromosomal abnormalities and the same maternal endocrine, thrombophilic and immunological disturbances [10].

Consequently, many treatments attempting to normalize these abnormalities have been tested or are currently used in women with both recurrent implantation failure and recurrent miscarriage [119]. However, no treatment for the two syndromes is at the moment sufficiently documented to justify its routine use. In this review, an overview is given regarding present knowledge about causes that may be common for recurrent implantation failure and recurrent miscarriage, and suggestions are put forward for future research that may significantly improve understanding and treatment options for the syndromes [14].

2.2. Embryo frozen

Cryopreservation allows you another chance of achieving a pregnancy without having to go through another expensive and demanding cycle of IVF. It may also be done if embryo transfer is not advised or is not possible during the actual IVF cycle. [124]. Nationally, pregnancy rates are about 20 to 30% using frozen embryo transfer. Pregnancy rates vary from clinic to clinic, but we know that frozen embryo transfer is just as effective as fresh embryo transfers in creating a pregnancy. There is no increased rate of miscarriage or birth defects in these pregnancies compared to natural pregnancies [79, 109-112]. The long-term health of children resulting from cryopreservation is being reviewed. Good quality embryos have a high survival rate. Usually, more than 80% survive the freezing and thawing process. Usually, all normally fertilized embryos are cultured to the blastocyst stage (day 5 after egg retrieval) to allow us to select the highest quality embryos for transfer. Any good-quality blastocysts that develop, that are not transferred, will be frozen. [5, 26, 82].

Vitrification presumably requires high concentrations of cryoprotective additives and very high cooling rates to produce the glassy state. Vitrification of reproductive cells and tissues, Nowadays, has been achieved by suspending them in solutions containing 15–30 % permeating cryoprotectants plus 18 % saccharide and cooling very small samples at rates of >10,000 °C/min . The methods and protocols for cryopreservation – slow cooling or rapid cooling protocols –both satisfy the fundamental cryobiological principles for reduction of damage by ice crystal formation during cooling and warming [24].

Both methods include six principal steps: Initial exposure to the cryoprotectant (intracellular water has been removed by gradual dehydration). Cooling (slow/rapid) to subzero temperatures (−196 °C in storage). Thawing/warming by gradual rehydration [49, 92]. Dilution and removal of cryoprotectant agents and replacement of the cell lular and intracellular fluids at a precise rate. Recovery and return to a physiological environment [79]. The procedures to achieve vitrification have gone through a historical process of evolution, and the first reports of cryopreservation of tissue cells date to 1766, when Spallanzani published the first cryopreservation of sperm in snow

and the capture of mobile spermatozoa developed in 2010 and reported by W. Xing et al., is a method which has very high survival rate in the preservation of oocytes and ovarian tissue. This technique uses a metal surface, previously frozen at −180 °C by partial immersion in liquid nitrogen, which serves as a base for the cooling of micro-droplets containing vitrification solutions, ovarian tissue, or embryos, which creates sufficient space for vitrification of tissues, maximizes freezing rates, and avoids the gas phase caused by bubbles of liquid nitrogen [5].

What is embryo cryopreservation? During an assisted reproductive technology (ART) cycle, we advise that no more than 2 or 3 embryos be transferred to the uterus at one time. This is because transferring more embryos greatly increases your risk for giving birth to multiples, but does not improve your chances of becoming pregnant. But, more than 2 or 3 good quality embryos often result from an in vitro fertilization (IVF) cycle. These potentially viable embryos can be used later if they are frozen (cryopreserved). Embryo cryopreservation is the process of freezing the embryos that are not transferred [19].

2.2.1. Historical of embryo frozen

Since the publication of the first reports demonstrating the feasibility of using vitrification to cryopreserve human embryos, this method became increasingly popular among embryologists, because of its significant advantages with regards to cost and time requirements when compared to slow freezing. The main concern remains however, is the need to use high concentrations of cryoprotective solutions that might lead to osmotic shock, which can affect embryo survival [130]. The feasibility of using vitrification to cryopreserve human embryos has far been shown by several groups, however the current best available evidence does not allow for solid conclusion to be made with regards to the association of this technique with higher pregnancy rate. Therefore, we aimed to review our data and assess the efficacy of slow freezing technique of human embryos and compare it to vitrification, in terms of post-thaw survival and clinical pregnancy rates [25, 75, 132].

Cryopreservation allows the transfer of limited number of fresh embryos back to the uterus while saving the remaining ones for future use; thus maximizing the cumulative effectiveness of an IVF cycle [72, 128]. Additionally, cryopreservation makes the postponement of embryo transfer (ET) to a future cycle feasible, thus decreasing the incidence of ovarian hyperstimulation syndrome (OHSS) in high-risk patients, while maintaining the probability of pregnancy. Cryopreservation of human embryos from the 2-cell stage up to the morula stage is a safe procedure which has been carried out for the last 25 years [25].

Experience with blastocyst cryopreservation is still limited and pregnancy rates after the use of frozen, thawed blastocysts vary extremely. Vitrification has improved the success of embryo cryopreservation. However, this technique cannot yet be considered as a routine procedure [71]. Despite all of the advantages for infertile couples, cryopreservation of human embryos creates severe ethical problems, because of surplus frozen embryos which either have to be destroyed or perhaps used for research. Embryo adoption may provide a solution to solve imminent medical, ethical and social problems [79]. Currently, slow freezing is the most widely used method for cryopreserving human embryos. During this technique, embryos are exposed to combined controlled cooling rates with the use of low concentration of cryoprotectants. Alternatively, embryos can be cryopreserved by vitrification, a technique that combines ultra-rapid cooling with minimum volume along with use of high concentrations of cryoprotectants, allowing embryos to rapidly enter a glass-like state [51].

2.2.2. Some elements influent to the quality of embryo after frozen-thawing

Preservation of female genetics is currently done primarily by means of oocyte and embryo cryopreservation. The field has seen much progress during its four-decade history, progress driven predominantly by research in humans. It can also be done by preservation of ovarian tissue or entire ovary for transplantation, followed by oocyte harvesting or natural fertilization [100, 122, 123].

Two basic cryopreservation techniques rule the field, slow-rate freezing, the first to be developed and vitrification which in recent years, has gained a foothold. The slow-rate freezing method previously reported had low survival and pregnancy rates, along with the high cost of cryopreservation. Although there are some recent data indicating better survival rates, cryopreservation by the slow freezing method has started to discontinue [88, 98]. Vitrification of human embryos, especially at early stages, became a more popular alternative to the slow rate freezing method due to reported comparable clinical and laboratory outcomes [15, 66, 85]. In addition, vitrification is relatively simple, requires no expensive programmable freezing equipment, and uses a small amount of liquid nitrogen for freezing. Moreover, oocyte cryopreservation using vitrification has been proposed as a solution to maintain women's fertility by serving and freezing their oocytes at the optimal time [67]. The aim of this research is to compare slow freezing and vitrification in cryopreservation of oocytes, zygotes, embryos and blastocysts during the last twelve years. Therefore, due to a lot of controversies in this regard, we tried to achieve an exact idea about the subject and the best technique used [1, 79].

Blastocysts are preimplantation embryos that have successfully passed the critical step of genomic activation and so showed a high developmental potential. They have the advantage of containing numerous small cells; thus the loss of some cells during freezing and thawing is probably less harmful for further development of the embryo and vitrification may allow individual blastocysts to be cryopreserved at their optimal stage of development and expansion [87].

The formation of intracellular ice crystals is directly proportional to the volume of blastocoel which in turn affects the survival rate of the blastocyst. Vanderzwalmen et al, 2002 were able to report a survival rate of 58.5% from 167 vitrified blastocysts. After artificial reduction of the blastocoelic cavity by puncturing the blastocyst with a special pipette before vitrification [99].

Mautoudis et al, 2001 reported high survival rate (60%) and a pregnancy rate (27%) when laser assisted hatching was used to thinning the zona pellucida for a quartersegment.

Mukaida et al (2006) reported that an artificial shrinkage of the zona pelludida before blastocyst vitrification increased success of the technique. They reported a survival rate of 79% and a pregnancy rate of 36%. Nowadays, blastocyst vitrification has tended to be extremely successful with increased survival rates and clinical outcomes without puncturing or artificial shrinkage [63]. More attention has been focused on vitrification due to significantly higher survival and pregnancy rates [49]. Vitrification technique is achieved after the cryopreservation of blastocysts using cryotop, cryoloop , or the hemistraw. Moreover, Hong et al found high pregnancy (70.5%) and implantation (40.6%) rates with the use of a new vitrification technique. Cryopreservation of blastocyst stage was performed mainly by the slow-rate method that let acceptable outcomes. However, with advances in vitrification, the method of choice for cryopreserving blastocyst stage embryo is vitrification rather than the slow-rate technique [120] .

It was indicated that vitrification causes less cell apoptosis in blastocysts compared to slow freezing. More attention has been focused on vitrification due to significantly higher survival and pregnancy rates. Vitrification technique is achieved using cryotop, cryolop, or the hemistraw. Moreover,Hong et al (2009) found high pregnancy (70,5%) and implantation (40,6%) rates use of microscopic grides as vitrification devices. Cryopreservation of plastocyst stage has been performed mainly by the slow-rate method that let acceptable outcomes. However, with advances in vitrification, the method of choice for cryopreserving blastocyst stage embryo is vitrification rather than the slow-rate technique [79].

2. 3. Laser assisted hatching technique

The zona pellucida (ZP) of mammalian eggs and embryos is an acellular matrix composed of sulfated glycoproteins with different roles during fertilization and embryo development [5]. Three distinct glycoproteins have been described both in mice and in humans (ZP1, ZP2, and ZP3). Acrosome-reacted spermatozoa bind to ZP receptors, and biochemical changes have been observed after fertilization that are responsible for the prevention of polyspermic fertilization. The main function of the

ZP after fertilization is the protection of the embryo and the maintenance of its integrity [3, 69, 88].

It has been postulated that blastomeres may be weakly connected, and that the ZP is needed during the migration of embryos through the reproductive tract to maintain the embryo structure [13, 76]. Implantation has been observed after replacement of zona-free mouse morulae or blastocysts, while the transfer of zona-free precompacted embryos results in the adherence of transferred embryos to the oviductal walls or to one another. A possible protective role against hostile uterine factors has also been described [47, 74, 83, 84].

Degeneration of sheep eggs after a complete or partial ZP removal that could be ascribed to an immune response was described by Trounson and Moore Once in the uterus, the blastocysts must get out of the ZP (hatching) so that the trophectoderm cells can interact with endometrial cells and implantation can occur. The loss of the ZP in utero is the result of embryonic and uterine cooperation [98].

Zona hardening after zona reaction subsequent to fertilization occurs, and is evidenced by an increased resistance to dissolution by different chemical agents [12].

This physiological phenomenon is essential for polyspermy block and for embryo protection during transport through the reproductive tract. It has been postulated that additional ZP hardening may occur in both mice and humans as a consequence o in vitro culture [20] [2]. Hatching could be inhibited in some in vitro cultured human embryos owing to the inability of the blastocysts to escape from a thick or hardened ZP. Schiewe et al (2009) performed a study to characterize ZP hardening in unfertilized and abnormal embryos and to correlate it with culture duration, patient age, and ZP thickness [20].

Dispersion of ZP glycoproteins and the time needed for complete digestion after-chymotrypsin treatment were assessed The results obtained proved that zona hardening of fertilized eggs was increased, compared with inseminated unfertilized eggs [109]. Wide patient-to-patient variation in zona hardness was observed, but no correlation was established between zona hardness or thickness and patient age.

Furthermore, the data obtained did not support the concept that additional ZP hardening occurred during extended culture. Expansion and ZP thinning occur in mammalian during blastocoel expantion prior to hatching [114].

2.3.1. Laser assisted hatching techniques

When breaches are made in the ZP of early-cleavage IVF embryos, embryonic cell loss may occur through the zona because of uterine contractions after replacement of the embryos. It is advisable to manipulate embryos for AH after the adherence between blastomeres has increased, just before compaction Artificial opening of the ZP of blastocysts can also be performed to promote complete blastocyst hatching [14].

Embryos at the six to eightcell stage at day 3 after insemination, or at the blastocyst stage, at day 5 or 6 after insemination, can be manipulated with different methods for the performance of AH[22]. Microtools for AH can be made by means of a pipette puller and microforge, but are also commercially available. Micropipettes are mounted on micromanipulators [11, 95, 119].

It is important that the size of the hole created in the zona is large enough to avoid trapping of the embryo during hatching, but not so large that it permits blastomere loss [16]. Monozygotic twinning has been described as a consequence of AH. The adequate size of the hole seems to be 30–40 μm when AH is performed on day 3 embryos [21]. Nevertheless, AH applied to cryopreserved blastocysts seems to give better results when ≥50% of the ZP is opened or ZP is totally removed. Half of ZP thinning in early-stage vitrified embryos seems to be associated with higher pregnancy rates than quarter thinning [57, 96, 113].

Embryo transfer to the uterus has to be performed as atraumatically as possible to avoid damage of ZP-manipulated embryos. Treatment during four days, starting on the day of oocyte retrieval, with broad-spectrum antibiotics and corticosteroids (methyl prednisolone, 16 mg daily) has been postulated [23, 58]. These autor suggested that such treatment may be useful for patients whose embryos have been assisted-hatched, to avoid infection and immune cell invasion of the embryos Recently, several studies have recommended AH in women with a realatively poor

prognosis, including those with advanced maternal age ≥ 38, previous failed IVF cycles (≥ 2), poor embryo morphology, thickened zona pellucida or frozen-thawed embryos, but have not recommended AH in women with a relatively good prognosis [59, 95].

2.3.1.1. Assisted hatching for embryos by mechanical partial zona dissection (PZD)

There are several methods of assisted hatching including acidic Tyrode's solution, mechanical partial zona dissection (PZD) and laser. All the methods based on either a hole forming or a leak on the zona pellucida, or thinning it to facilitate the passage of the embryo [8, 25]. Although very few studies have compared these methods, in general laser drilling seems to be a less invasive and safer method compared to acidic Tyrode's solution [60, 93].

The embryo is held with a holding pipette, and the ZP is tangentially pierced with a microneedle from the 1 o'clock to the 11 o'clock position. The embryo is released from the holding pipette, and the part of the ZP between the two points is rubbed against the holding pipette until a slit is made in the zona [29]. The embryo is washed twice in a fresh culture medium and placed in the transfer dish. A 3D-PZD in the shape of a cross has been described (Kim et al, 2010). The procedure starts as conventional PZD and a second cut is made in the ZP under the fi rst slit. A crossshaped cut can be seen on the surface of the ZP [62, 103].

This method allows the creation of larger openings while permitting protection of the embryo by the ZP fl aps during embryo transfer. A new technique called "controlled zona dissection"has been recently described as a variation of PZDThe embryo is held at the 8 o'clock position by a bevelopened holding pipette, and a thin angled hatching needle with a blunted tip pierces the ZP at the 5 o'clock position. [30-32] [10] [65].

The hatching needle is inserted deeply into the holding pipette until the embryo is pushed to the angle of the hatching needle [52]. The curve of the needle is then pressed against the bottom of the dish to cut the pierced ZP. A large slit (two-thirds of

embryo's diameter) created by controlled zona dissection enhances signifi cantly the rate of complete in vitro hatching of blastocysts compared with the 3D-PZD [8].

A new method for mechanical AH, inspired by the natural expanding effects of blastocysts on the ZP, has been described (82) [33]. This mechanical AH method expands/ stretches the ZP by injecting hydrostatic pressure into the perivitelline space using an ICSI injection needle and culture medium, inducing a short time (≤30 seconds) ZP thinning.

These 2 reports showed that complete hatching after Laser partial zona thinning has been associated withhigher implantation and pregnancy rates than total laser AH, especially in women who suffer from recurrent implantation failure[35]. The enzymatic action of pronase to thin the ZP of human early-cleaving embryos yields similar benefits to other AH methods. Nevertheless, zona thinning for cryopreserved–thawedembryos, using pronase action or laser methodology, has failed to show improvement in the implantation rate [12, 36, 37].

Mechanically expansion of ZP of frozen– thawed day 3 human embryos with injected hydrostatic pressure has shown to increase implantation and clinical pregnancy rate when compared with control embryos. Several studies have been performed to demonstrate the usefulness and efficacy of AH in different groups of patients using the various methods described [38] [54]. Most of the studies have been done in patients with poor prognosis, including advanced-age patients patients with elevated concentrations of follicle-stimulating hormone (FSH), patients with previous implantation failures, or with embryos with thick ZP, some of them with contradictory results. One study included women with endometriosis, not showing improvement after AH [41] [16] [76].

Frozen blastocyst were generally thawd at 9.1.a.m and tranrates of LZD group compared with PZD or no-AH group are significantly higher in vitrified-thawed cycles as well as fresh cycles and LZD can completely over-come zona hardening [13]. Also, This report showed that the size of the ZP opening is important for implantation or complete hatching [65], [12] .

2.3.1.2. Assisted hatching for embryos by tyrode acid

It has been described that zona hardening and the increase in volume of the perivitelline space in zygotes and embryos allow an efficient and safe use of acid Tyrode's (AT) solution in human embryos for ZP drilling compared with oocytes [84]. Nevertheless, it has to be taken into account that the use of acidic solutions for AH may be detrimental for the blastomere adjacent to the drilled portion of the ZP.[42] [81]. Limiting embryo exposure to AT by adequate and quick manipulation is necessary to avoid harmful effects on embryo development. AT solution can be prepared in the laboratory based on the protocol of Hogan et al adjusted to a pH of 2.5, or can be purchased commercially [21, 114, 125].

Accumulation of AT solution in a single area must be avoided. Extracellular fragments can also be removed during the procedure. As soon as a hole is created in the ZP, suction is applied to avoid excess AT solution entering the perivitelline space [125]. [43] If the inner region of the ZP is difficult to breach, creation of the hole can be facilitated by pushing the AT micropipette against the ZP . It is necessary to rinse the embryo several times in fresh culture medium [28, 118].

2.3.1.3. Laser assisted hatching

The use of laser techniques in the fi eld of assisted reproduction for application in gametes or embryos was fi rst described by Tadir et al[44] [106]. For a fast and an efficient clinical use of laser systems in AH, it is important that the laser is accurately controlled and produces precise ZP openings without thermal or mutagenic effects. The application of a laser on the ZP for AH results in the photoablation of the ZP [32, 45].

Contact Lasers

The procedure is performed on a microscope slide, and the embryo is placed on a drop of the medium covered with paraffin oil. The embryo is held with a holding pipette, and the laser is delivered through a microscopic laser glass fiber, fitted to the manipulator by a pipette holder [46, 121], in direct contact with the ZP. Because each

laser pulse removes only small portions of the ZP, the fiber tip has to be continuously readjusted to guarantee that the laser is in close contact with the remaining zona [35].

The first use of a laser for ZP drilling was reported by Palanker et al [55] which used with an ArF excimer laser (UV region, to touch the ZP with the laser-delivering pipette (contact mode laser). The erbium:yttrium–aluminum– garnet laser (2940 nm radiation), also working in contact mode, has been used for ZP AH and thinning, and its safety and efficacy have been demonstrated in clinical practice. [36] No degenerative alterations were observed using light and scanning electron microscopy after ZP drilling with such a system. Antinori et al [30, 56, 89].

Non contact Lasers

Noncontact laser systems allow microscope objective–delivered accessibility of laser light to the target. Laserpropagation is made through water, and as it avoids the UV absorption peak of DNA, no mutagenic effect on the oocyte or embryo is expected. Blanchet et al, 2008 . first reported the use of a noncontact laser system (248 nm KrF excimer) for mouse ZP drilling described the use of a non contact laser holmium: yttrium scandium–gallium–garnet (Ho:YSGG) laser (2.1 μm wavelength) for AH in mice. The study showed a lack of embryotoxic effects as well as improved blastocyst hatching [35].

Similar results were reported by Schiewe et al. Rink et al. designed and introduced a noncontact infrared diode laser (1.48 μm) that delivers laser light through the microscope objective [39]. The drilling mechanism is explained by a thermal effect induced at the focal point by absorption of the laser energy by water and/or ZP macromolecules, leading to the thermolysis of the ZP matrix. Laser absorption by the culture dish and medium is minimal. The effect on the ZP is greatly localized, and the holes are cylindrical and precise. Exposure time can be minimized. The safety and usefulness of the system was demonstrated in mice and humans. Its use for polar body as well as blastomere and blastocyst biopsy has also been reported [40].

The system is compact and easily adapted to all kinds of microscopes. The size of the hole is related to the laser exposure time, and thus the system is simple, quick,

and easy to use [83]. Figure 14.1 shows an eight-cell embryo in which laser AH has been performed. Antinori et al. have reported the use of a compact, noncontact UV (337 nm wavelength) laser microbeam system to create holes in the ZP of human embryos [44] [45].

Zona Pellucida Thinning

The aim of ZP thinning is to thin the ZP without complete lysis and perforation. By not breaching the zona, the potential risk of blastomere loss and embryonic infection is minimized [118]. ZP thinning with AT has been described in mice and humans. It involves bidirectional thinning of a cross-shaped area of the ZP over about one-quarter of the embryo circumference [48].

Embryos are washed in fresh droplets of the medium and cultured before transfer. This methodology has proved useful for hatching enhancement in mice but not in humans, probably because of differences observed in both the morphologic and the biophysical characteristics of the ZP between the two species. [75]. The mouse ZP has a monolayer structure whereas the human ZP, as shown by electron microscopy, is composed of a less dense, easily digestible, thick outer layer and a more compact but resilient inner layer [118].

The use of laser technology for ZP thinning at the cleavage stage seems to be benefi cial for embryo implantation for certain authors. Antinori et al. demonstrated a signifi cant increase in implantation and pregnancy rate when 50% of the zona thickness from 2-day-old embryos was thinned to a length of 20 μm using a YAG contact laser [126]. Diode laser ZP thinning enhances the variation of zona thickness in human embryos, allows natural zona thinning, and increases signifi cantly the rate of blastocyst hatching.Acceptable clinical pregnancy rates were obtained after transfer of frozen–thawed blastocysts that underwentlaser-assisted thinning at the day 3 cleaving stage before freezing [55].

2.3.2. The state of art in embryo assisted hatching.

Why Perform Assisted Hatching.The ratio of lysin production to ZP thickness could determine whether the embryo will lyse the zona and undergo hatching. Embryos with thick zonae or those that present extensive fragmentation or cell death after freezing and thawing may benefit from AH [127, 126, 129, 131]. It has been demonstrated the usefulness of ZP thinning with acid Tyrode's to improve hatching in hatching-defective mouse embryos created by the destruction of one-quarter of the blastomeres [103]. Normal implantation rates in pseudopregnant female mice has been reported after the transfer of assisted-hatched embryos that had cell numbers reduced. The mechanism by which AH promotes embryo implantation remains unclear [75] The implantation window is the critical period when the endometrium reaches its ideal receptive state for implantatio [77, 80, 91, 113].

Precise synchronizationbetween the embryo and the endometrium is essential. In a randomized study, Liu et al. demonstrated that implantation occurred signifi cantly earlier in patients whose embryos were submitted to AH when compared with the control group, probably by allowing an earlier embryo– endometrium contact [78]. The presence of an artificial gap may alter the two-way transport of metabolites and growth factors across the ZP, permitting earlier exposure of the embryo to vital growth factors [80, 115-117, 131]. Significantly higher clinical pregnancy rates were observed if blastocysts that hatched close to the ICM were transferred (72%) as compared with those that herniated from the mural trophectoderm (51%). [97, 98, 101].

3. RESEARCH OBJECTIVES

The first major objective of this work is to evaluate the effectiveness ofl laser assisted hatching method before transfer of fresh embryos (group I, 220 cycles) and frozen embryos (group II, 220 cycles) day 3 or day 5.

The second major objective is to assess the influence of zona thinning on embryo storage after thinning the 50% of the zona pellucida in ¼ of the surface using a 1,48 micron wavelength infrared laser.

4. METHODOLOGY

This was a prospective, randomized, double blind study, conducted on IVF-ET patients from December 2013 to June 2015 at

Centre of Reproductive Endocrinology and Infertility, Thua Thien Hue, Viet Nam.

Two hundred patients were randomly assigned to two groups:

Transferred with fresh embryos after laser assisted zona thinning

Transferred with frozen/thawed embryos that had laser assisted zona thinning

Transferred with intact, not laser treated embryos

Zona thinning was performed on 110 embryos cycles using a non-contact 1,48 µm diode laser and the hatching rate in vitro was compared with 110 embryos cycles control embryos. Variation of zona thickness and degree of zona expansion was assessed. Scaning electron microscopy was performed on embryos entrapped during hatching to identify the site of hatching. There main methodologies include:

4.1. Ethical approval

The participants were explained about the research, its benefits an risk and asked to sign consent to participate.

Study protocols were approved by Hue University of Medicine and Pharmacy Institutional Review board.

4.2. Trials design

Embryos day 2 and day 3 embryos obtained from randomly were divided into 2 groups

+ Trial 1.Effect of laser assisted zona pellucida thinning on outcomes after transferring fresh embryos.

Fresh embryos were randomly assigned to two groups:

LAH: embryos that have had zona pellucida thinning before to be transferred in oder to assisted hatching process

Controls: Embryos that were transferred intact, without any manipulation for assisted hatching

+Trial 2: Effect of laser-assisted zona pellucida thinning and cryopreservation on outcomes after embryo transfer.

Fresh embryos were randomly assigned to two groups before freezing:

LAH-C: embryos that have had laser-assisted zona pellucida thinning before to be frozen, thawed and transferred

Controls: Embryos that were cryopreserved intact, without any manipulation for assisted hatching, thawed and transferred in recipient uterus.

4.3. Criteria for the selection of patients in the study sample

All patients to be transferred with fresh or frozen embyos, transferred embryos easily. In the frozen group were transferred embryos having good quality (class1). The participant to this trial agree these researches and were randomized into groups. The thickness of endometrium was ≥ 8mm and ≤ 14mm. The number of transferring embryos is equivalent, the transfer was made easily without blood.

Selection for patient donor to be transferred embryos

4.3.1. Selection of egg donor woman

Egg Donor Application and Screening

Egg donors must apply to our egg donation program, only the donor applicants who pass all these screening steps will be added to our egg donor program, and can be matched with a recipient couple for requirement of some testing with clinical coordinators and another with our physicians

Suppression and Ovarian Stimulation for the Egg Donor

During the ovarian stimulation phase, the egg donor uses daily injections of gonadotropin to stimulate her ovaries. In a natural cycle, only one egg matures; gonadotropins injections encourage more than one egg to mature for retrieval.

During ovarian stimulation, the egg donors are monitored closely through blood tests and ultrasound, ensuring that the ovaries are responding well and not going into hyperstimulation. This means that egg donors need to visit our center Reproductive Endocrinology and Infertility during this phase. These monitoring sessions are scheduled in early morning in order to avoid interfering with the donors' daily schedule of school and work.

Triggering Ovulation and Egg Retrieval for the Egg Donor

When the ultrasound imaging shows that the donor's eggs have sufficiently developed, the donor will be instructed to trigger ovulation with an injection of hCG. Two days later, her eggs are retrieved in a short in-office procedure, called egg retrieval. While the donor is asleep one of our physicians will use aspiration needle, guided by ultrasound, to transvaginally retrieve the eggs. The donor will be required to take the rest of the day off to recover.

4.3.2. Method about selection of donor sperm

Sperm Donor Application and Screening

Sperm donors must apply to our sperm donation program from sperm banking, only the donor applicants who pass all these screening steps will be added to our sperm donor program, and can be matched with a recipient couple for requirement of some testing

Process for sperm application

All donors should have tests for certain infections such as syphilis, hepatitis B, gonorrhea, chlamydia, streptococcal species and trichomonas. The donor's semen should also be checked for the presence of white blood cells which can indicate an infection within the reproductive tract.

Donors are excluded from a donor program if he or his sexual partner have experienced any of the following: a blood transfusion within one year, a history of homosexual activity, multiple sexual partners, a history of IV drug use, or a history of genital herpes.

4.3.3. Private Selection criteria

Trial 1: Fresh embryos were selected to be transferred at day 3 and day 5 after insemination

Trial 2: Frozen-thaws embryos were transferred at day 3

4.3.4. Exclusion criteria

Other than those patients that did not agree to participate in these researches were excluded from trials also

Patients transferring embryo ≥ 38 years old has failed IVF above 2 cycles

Statistical analysis

The analysis of the results was undertaken using Student’s *t*-test for numerical continuous variables and chi-square or Fisher exact test for categorical variables. *P* value of <0.05 was accepted as significant.

4.4. Researching methods

4.4.1. Method used to embryo culture and manipulation

First the woman must be stimulated with medications and have an egg retrieval procedure to obtain number of eggs for using Intra-cytoplasmic sperm injection (ICSI) by only sperm. Oocytes were picked up and incubated in CO_2 incubator for 2 hour

before implementing injected only sperm into oocyte (ICSI), embryos day 3 or day 5 were frozen by Kitazato medium Japan of equilibration solution (ES) and vitrification solution (VS). Assisted hatching by laser system of thinning embryo with 7-9µm of length and about 50%the circumference of embryo. The ICSI procedure was performed according to the technique described by Seif et al [118, 121] [106]. The oocytes are first observed for 16 to 18 hr during the procedure in order to determine the presence or absence of pronuclei. The morphological quality is then evaluated by the cumulative embryo score of Schlenker et al [105].

4.4.2. Characteristic measurements: determine zona pellucida thickness (ZT)

ZT was performed with a laser diode (Startun 5 active, Researcher Intrusment, United Kingdom) with 1,48 micron wavelength operating through an objective coupled to an Zeiss Axio Vert.A1 Inverted Photo Microscope. Using a high power ablation laser and a visible pilot laser transmitted through fiber optics, the RI Saturn 5™ laser system is the most accurate system on the market today.

At the time for transfer (day 3 or day 5), the embryos were placed in single drop culture of petri 30 containing G1 culture medium. Using the stage of the microscope and no micromanipulators, the embryo was then positioned in the optic field and the ZP was focused in the direction of the laser light [104].

ZP thinning was them performed by applying 12-15 ms irradiation, which is sufficient to thin the ZP between 7-9µm intacted. ZP thickness was measured with a scale present in the eyepiece of the microscope. After the ZT procedure, the embryos were transferred to G-IVF culture medium in order to avoid possible toxicity to the products due to the action of laser on the organic components of the ZP.

4.4.3. Method for freezing and thawing embryos

Freezing protocol

Embryos day 3 had good quality (G1 and G2), were frozen by vitrification method. We used frozen and thawed cultureby Kitazato, Japan. Equilibration: Using

pipette to move the embryos from culture medium droplet placed on the medium ES within 15 minutes, observed under a microscope stereoscopic to view the size of the original embryo. Using pasteur pipette to taken embyos from ES to VS medium. Sucking up and down to changed embryo position. After that, using the pasteur pipette to put embryos under surface of cryotop and put cryotop into liquid nitrogen quickly. This process was taken within 30 second, maximum with 01 minutes. Frozen embryos were put in stable culture and vitrification culture before puttingembryos inthe surface of cryotop and put cryotopinside nitrogen liquid quicly. Thaw kit by Kitazato, Japan was used for frozen embryo process. All tools and chemical substances were are set at room temperature, were implemented under the guidance of Kitazato, Japan. Fresh embryos were assisted hatching before transfering to the uterus.Thaw-embryos was seen as alive with the rate of 50% intact embryo or had at least 3 plastocysts alive or 1 cell divided after thawing (Rienzi, 2002). Embryo culture medium after thawing: G-2 TM Plus (Vitrolife, Gothenburg, Sweden) was used for thawed-embryos culture. Embryos day 3 was conducted overnight [102].

Thawing protocol:

Thawing solution was kept in CO2 incubator before using at leats 30 minutes. Put 0,5 ml TS (Thaw solution) at the first well. Put 150 µl DS (dilution solution) at the first drop, Put 150 µl WS1 (wash solution) at the second drop, Put 150 µl WS2 (wash solution) at thethird drop. Writing patient name at the thaw dishes, the name of culture in the dishes. Thawing: Taken the petri dishes has TS solution was prepared and put in the Stereo Microscope, cryotop after taken from nitrogen liquid was put immediately in the TS solution within 01 minutes. Put embryos to the DS solution with 03 minutes, put within 05 minutes in the WS1 solution. Rewash in the WS2 with 1 minutes. Put embryos to the G1 dish and put at the CO2 incubator has condition at temperature 37ºC. assess quality embryo after thawing. Embryos were kept inculture overnight and transferring in the next day [4].

4.4.4. Assisted hatching by thinning method

Embryos were placed in drop G2 (Vitrolife, Sweden). Using Saturn 5 active laser system, thinning assisted hatching with 30-40μm of length and about 50% of width of the membrane thickness. After that, embryos were soak in Embryo Glue (Vitrolife, Sweden) culture within 15-30 minutes before trasfering into the uterus [103].

4.5. Study Design

Clinical sample randomization in control group were calculated according to the World Health organization (WHO) guidelines

$$n = \frac{(Z_{1-\frac{\alpha}{2}}\sqrt{2PQ} + Z_{1-\beta}\sqrt{p_1(1-p_1) + p_2(1-p_2)})^2}{(p_1 - p_2)^2}$$

In which: n is number of patients enrolled in a study group

P = (p1 + p2) / 2; Q = 1-P, q1 = 1-p1, p2 q2 = 1- p2

α = level of statistical significance, the probability of the mistake type I

β = probability of type II mistake.

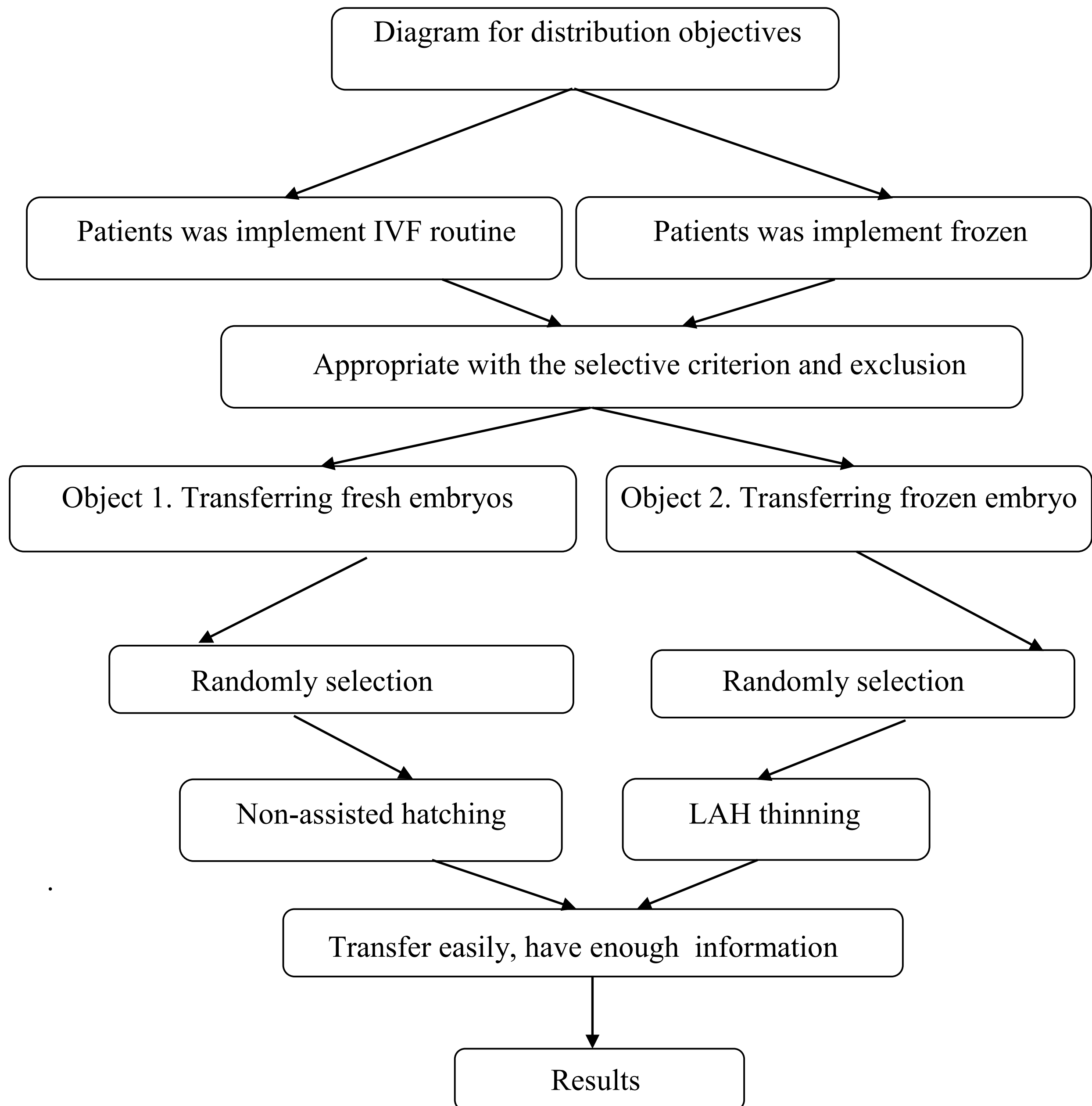
The study subjects were selected in 2 steps:

- Step 1: Patients which performed in vitro fertilization and were transferred fresh embryo were randomized into 2 groups: control group and thinning group.

- Step 2: Patients which performed in vitro fertilization and transfer the frozen embryo for patients who appropriate selection criteria and exclusion were randomized into 2 groups: control group and thinning group.

4.6. Diagram for distribution objectives

DIAGRAM FOR DISTRIBUTION OBJECTIVES



5. RESULTS AND DISCUSION

During the study period from the December in 2013 to the June in 2015, we have collected 220 fresh embryos cycles and 220 frozen embryos cycles. The cases that did not meet the selection criteria were excluded from the study. Chi square test was used to compare rates of matched pairs between fresh and frozen embryo groups. Data are expressed as percentages. Mean values were compared using Analysis of Variance (ANOVA) test and values were expressed as Mean ± Standard Deviation (Mean ± SD). Data compared results on 220 fresh embryo transfer cycles Comparison of results correlate with or without assisted hatching support for embryos. Data analysis for the research results are as follows: Research on fresh embryo transfer groups of 220 cycles with 2 groups: control group including 110 cycles. Group do not support the hatching, thinning AH group: 110 cycles.

5.1. Trial 1: Effect of laser assisted zona pellucida thinning on outcomes after transfer of fresh embryos

Table 1. Causes of infertility in patients included into the trial

Causes of infertility	LAH (n= 110)	No LAH (n=110)	P value
Mean female age (years)	32.3±1.5	31.4±2.1	NS
Duration of infertility (years)	4.8 ±0.5	6.3±0.5	NS
Basal FSH level (mIU/ml)	6.3±0.6	7.2±0.4	NS
Causes of infertility			
Male	24 (42.1%)	20 (35.1%)	
Tubal	12 (21.0%)	16 (28.0%)	
Mixed	15 (26.3%)	14(24.5%)	
Unexplained	6 (10.5%)	7 (12.2%)	

Table 1 resumes the causes of infertility in the studied couples. Male infertility is the higher cause of infertility with 42.1% and 35.15% in control and LAH group respectively and is probably due to alcohol and tobacco abuse. Tubal diseases are the 21% in the control and 28% in the LAH group. In higher rates are also mixed causes of infertility with 26.3% and 24.5% respectively in the control and LAH groups. Any of these data showed statistical difference between the experimental groups (p> 0.05 in each pair). Causes of female infertility may include: Ovulation disorders, uterine or cervical abnormalities, fallopian tube damage or blockage, endometriosis, primary ovarian insufficiency or pelvic adhesions. In endometriosis, for reasons that doctors don't entirely understand, tissue very similar to the endometrium begins to grow outside the uterus in various places that it shouldn't. It can appear in or on the ovaries, the fallopian tubes, the various structures that support the uterus, and the lining of the pelvic cavity. Fallopian tube damage or blockage is a common and painful disease that affects about 5.5 million women in North America and is one of the top three causes of infertility in women [116] .

Table 2. Infertility and age of patients enrolled in the trial

Infertility causes	Control	LAH	p
Infertility I	25.6 %	21.3%	NS
Infertility II	71.2 %	82.1 %	NS
Averaged infertility period (years)	7.3±2.0	6.4±4.2	NS
Avegaged age of patient (years)	39 ± 1.2	40 ± 2.2	NS

The results summarized in table 2 evidenced the higher proportion of patient suffering for secondary infertility (VSII) compared to primary infertility (VSI), percentage is 71.2% in the control group compared with LAH group 82.1 % . The infertility I is just 25.6 % in control group and 21.8 % in thinning group. The average duration of infertility is quite high, from 7.3±2.0 to 6.4±4.2 in the control group and

LAH group, and the average age of the mother's treatment is quite high, up to 39 ± 1.2 in the control group and 40 ± 2.2 in the thinning group.

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Table 3. Hormones concentration after 2 days from ovarian stimulation

Characteristics	Control	LAH	p
Endometrium day 2	3.7±0.8	2.2±1.5	NS
FSH day 2 (IU/L)	7.1±6.2	7.4±1.3	NS
LH day 2 (IU/L)	6.6±5.2	5.8±2.7	NS
Estradiol day 2 (pg/ml)	40.5±34.1	41.6±27.1	NS
Prolactin (mg/L)	30.7±2.512	34.6±2.431	NS
AMH (mg/ml)	4.6±3.2	4.1±2.5	NS

The table 3 resumes data on concentrations of plasma hormones after ovarian stimulation with HCG. Hormones plasma concentrations do not differ among LAH and controls. Plasma FSH concentration was 7.4±1.3 IU/L in LAH and 7.1±6.2 IU/L in control group LAH.

Estradiol concentration was 41.6±27 IU/L in LHA group and 40.5±34.1 IU/Lin control group and Prolatin was 34.6±2.4 IU/L and 30.7±2.5 respectively in LAH and controls respectively. AMH levels were 4.1±2.5 in LAH group and 4.6±3.2 in the control.

The thickness of zona pellucida do not differ statistically (Anova: P>0.05) between LAH and control groups relatively to the hage of donors. In Table 3 is showed the thickness of the zona pellucida related to three age class of donor woman: under 30 years; between 30 and 40 years and more than 40 years.

Table 4. The size of zona pellucida by age

Age class	Control (µm)	LAH thinning (µm)	p
Under 30 years	17.2 ±2.1	16.3±2.0	NS
30 – 40 years	16.5±2.3	15.7±2.8	NS
Above 40 years	15.7±2.1	15.6±2.0	NS

The statistical significance in LAH group the zona pellucida thickness ranged from 15.6±2.0 to 16.3±2.0 compared with the control were it ranged from 15.7± 2.1 to 17.2±2.1, without statistical difference between ages.

Appropriately powered, probably multicenter research is required to confirm the safest technique to use, and to identify patients that would benefit from assisted hatching before they have had repeated unsuccessful treatment cycles. The thickness of zona pellucida in two group is similar when compare with results of Hanna Balakier et al (2012): 16,1±1,97 and 16,7±2,02 in control and LAH group [61]. Balaban et al (2002) has the result of the most thin zona pellucida: in LAH group: 1,48±2,0 in control group: 12,1±1,9.Elasticity and thinning of the zona pellucida are essential for the hatching process, both of which can be adversely influenced by advancing maternal age and in vitroculture conditions [17, 89]. In vitroculture of embryos coupled with the stress induced by the freeze–thaw process may further lead to zona hardening in frozen–thawed embryo-transfer cycles [16, 84] Zona pellucida is dissolved in lysine, and quantitative or qualitative deficiencies in its secretion could result in hatching impairment [6].

Table 5. Parameters of spermatozoa used in embryo production in trial 1

Sperm parameters	Control (%)	LAH (%)	p
Abnormal concentration	3.7± 2.3	2.5±1.8	NS
Abnormal motility	14.8±10.2	15.6±8.7	NS
Abnormal morphology (%)	95.2	96.2	NS
Abnormal acrosome (%)	86,7	90,5	NS

Features sperm morphology in male patients such as concentration, motility, sperm and acrosome morphology and integrity are important characteristic affecting the quality of embryos. All these parameters did not show statistics differences between ejaculates used to produce LAH or control embryos (chi square test: P>0.05). An abnormal concentration of spermatozoa was evaluated in 2.5±1.8 of LAH group and in 3.7±2.3 of controls. Motility was impaired in 15.6±8.7 of LAH group ejaculates and in the 14.8±10.2% of controls. Abnormal head sperms and acrosomes were respectively 96.2% and 90.5% in LAH group and 95.2 and 86.7 in controls group (Table 5). In the table 5 are resumed data regarding oocytes recovered after hormonal treatment of donors and follilcles aspiration.

Some study indicate that sperm DNA damages in infertile males is significantly higher than fertile males and sperms with abnormal morphology and low levels of motility has more abnormal DNA damages than motile and normal sperms, Irvine et al evaluated the association between semen parameters and DNA integrity among a group of infertile patients and a group of normozoospermic donors. They showed that semen parameters, especially sperm concentration, were inversely correlated with the comet assay parameters. Sun and colleagues have demonstrated a significant negative correlation between semen parameters and DNA damage in sperm. However, the relationship was not found by Hughes et al (2008) who examined DNA fragmentation

rates in the spermatozoa of normozoospermic fertile donors and asthenozoospermic infertile patients with modified single-cell gel electrophoresis assay, possibly because of differences in composition of their study group. This is important as mature spermatozoa with DNA damage may exhibit lower functional potential and this may explain the patients'subfertility status [18, 107].

Table 6. Parameters of recovered oocytes used to embryo production in the trial 1

Characteristics of oocytes	Control	LAH	p
The average of occytes after aspiration	9.3±6.2	8.1±4.1	NS
The average recovered number of oocyte/cycle	8.2±2.5	7.2±1.2	NS
The number of GV	1.7±0.6	1.2±0.9	NS
The number of MI	0.6±0.3	0.1±0.5	NS
The number of MII	7.4±5.1	7.7±3.3	NS
lysed	1.1±0.3	1.2±0.6	NS
The rate of fertilized occytes (%)	86.7	89.2	NS

It have been aspirated 8.1±4.1 oocytes/cycle in the LAH group and 9.3±6.2 oocyte/cycle in the control group and the average recovered occytes is 7.2±1.2 and 8.2±2.5 respectively. In the LAH group were recovered 1.2±0.9 GV, 0.1±0.5 MI and 7.7±3.3 MII stage oocytes while in the control group it was been recovered 1.7±0.6 GV, 0.6±0.3 MI and 7.4±5.1 MII oocytes. Lysed oocytes were 1.2±0.6 and 1.1±0.3 respectively in LAH and control groups. After fertilization it was obtained 89.2 and 86.7 fertilized oocytes.

The table 7 summarize morphological data obtained visualizing oocytes under a microscope were it have been measured the granularity grade, the cytoplasmic darckness, the zona pellucida thickness, the grade of debris presence in the perivitelline space and the grade of perivitelline space (PVS).The characteristic of oocytes have no differeces between, Balaban et al (2002) has the transferred-embryos similar with our results: In control group: 3,4±0,7, in LAH group: 4,0±0,9. [8].

Table 7. Evaluation of oocytes morphological parameters

Characteristic of oocytes	Control %	LAH %	p
Quite a lot granularities (G1)	23.1±2.1	19.4±2.6	NS
Much granularities (G2)	45.3±2.3	35.6±2.7	<0.05
Too much granularities (G3)	31.6±1.4	45.0±1.3	<0.05
Cytoplasmic fairly dark (D1)	26.7±0.4	22.2±0.3	NS
Cytoplasmic dark (D2)	35.6±1.1	41.1±2.1	NS
Cytoplasmic overdark (D2)	37.7±1.4	36.7±2.1	NS
Zona pellucida fairly thick (Z1)	26.7±2.1	31.8±1.7	NS
Zona pellucida thick (Z2)	33.5±1.7	37.3±2.6	<0.05
Zona pellucida too thick (Z3)	19.8±2.5	20.9±1.6	<0.05
Quite a lot Debris (F1)	18.4±0.5	13.4±0.1	NS
Much debris (F2)	17.3±0.6	16.3±0.5	NS
Too much debris (F2)	4.3±2.1	10.3±1.5	NS
Quite large PVS (P1)	25.8±2.7	25.9±1.8	NS
Large PVS (P2)	55.3±3.5	47.6±2.7	NS
Too large PVS (P3)	18.9±0.5	26.5±0.4	NS

The oocytes with G2 granularity grade were 35.6±2.7 % and 45.3±2.3 % in LAH and control groups respectively, while G3 granularity grade were respectively 45.0±1.3 and 31.6±1.4. Most of oocytes in the two groups have large PVS 55.3±3.5

and 47.6±2.7. According to some researches, the appearance of debris in the perivitelline space (PVS) is not a severe pathology of oocytes but the reason for its occurrence is still not exactly clear [46]. Researchers showed the link between cytoplasm granularity grade and the premature exocytosis of secretory granules after treatment with high doses of gonadotropins during ICSI cycles [30]. The study group included 16 women aged 29 to 40 years (mean age 34±3,6 years) with 82 oocytes with debris in PVS. The control group was composed by 24 women aged 26 to 39 years (with a diagnosis of tubal peritoneal infertility, 34±3,1 years), and 147 oocytes. From these patients morphologically normal mature oocytes were obtained. Fertilization rate in both groups did not vary and amounted to 94,5±3,2% and 91,1±2,9%, respectively. This rate was significantly higher in the control group for 93±2,2% vs. 81,1±5,4% in the LAH group. PR was slightly higher in the control group, 34,4±1,1% vs. 30,5±3,1%. The investigation showed that the patient groups were not significantly different in the selected embryological criteria. This fact may suggest a limited impact of such diseases as debris in PVS on the further development of the embryo and the potential of its implantation in the uterus [87].

Table 8. Characteristic of embryos day 3 and day 5

Characteristic of embryos	Control	LAH thinning	p
The average embryos day 3	3.2±2.1	2.0 ±1.2	NS
The number G1embryos day 3	3.2±2.1	2.0±1.2	NS
The number G2 embryos day3	2.3±2.1	1.4±1.1	NS
The number G3 embryos day3	0.7±1.1	0.8±0.5	NS
The transferred embryo day 3	2.3±2.0	1.3±0.5	NS
The frozen embryo day 3	3.7±2.3	5.2±2.0	NS
The average embryos day 5	7.5±2.5	6.5±2.3	NS
The number G1embryos day 5	2.0±2.1	3.6±2.3	NS
The number G2 embryos day 5	2.2±0.6	2.3±1.2	NS
The number G3 embryos day 5	1.2±0.7	2.2±0.2	NS
The number G4 embryos day 5	0.7±1.2	0.3±0.7	NS
The transferred embryos day 5	1.5±0.7	2.0±1.1	NS
The frozen embryos day 5	4.5±3.2	5.7±2.0	NS

In Table 8 are resumed data of embryo produced and transferred at day 3 and day 5 from insemination. The mean of embryos obtained at day 3 (2,0±1,2 and 3,2±2,1 in LAH and control group respectively) and day 5 (6,5±2,3 and 7,5±2,5 respectively in LAH and control groups) did not present any significant difference. The embryos classified according to quality criteria did not show statistical

differences between the two experimental groups both at 3th and 5th day. The transferred embryos at day 3 and day 5 were 1,3±1,5 and 2,3 ± 2,0; 2,0 ± 1,1and 1,5 ± 0,7 in the LAH and control groups respectively.

Table 9. Outcome of fresh- transfer cycles

Characteristics	LAH thinning (%)	Control (%)	p
Cycles of thaw- transfer	110	110	NS
Total of transferred embryos	231	247	NS
Avegage transferred embryos	2.6±1.6	2.4±1.2	NS
Implantation rate/ transferred embryos (%)	46/231 (19.9%)	26/247 (10.5%)	<0.05
Implantation rate/cycles (%)	46/110 (41.8%)	26/110 (23.6%)	<0.05
Biochemical pregnancy (%)	42/110(38.1%)	22/110 (20%)	<0.05
Clinical pregnancy rate/cycles (%)	38/110 (34.5%)	20/110 (18.1%)	<0.05
Early miscarriages rate (%)	4/38 (10.5%)	2/10 (10%)	<0.05
Ongoing pregnancy rate (%)	34/110 (30.9%)	18/110 (16.3%)	<0.05
Singleton (%)	13/34 (38.2%)	10/18 (55.5%)	<0.05
Twin (%)	21/34 (47.0%)	8/18 (44.4%)	<0.05

In the trial a total of 220 embryo transfer cycles using frozen embryos were undertaken within a 18-month period. Implantation rate in the LAH group (46/110; 41,8%) was higher than in the control group (23,6%;26/110). The clinical pregnancy rate was statistically higher in the LAH group (18,1%) compared to the control (34,5%) group. The ongoing pregnancy rate was higher in the LAH group compared to the control group (16,3% vs 30,9 % respectively). Percentage of early miscarriages did not show statistical differences both for biochemical pregnancies (10% vs 10,5%

in LAH and control respectively) and early clinical miscarriages (8,8 % vs 11,7% in LAH and control respectively). These results was similar to other researchs in embryos transfer having or not LAH and improves outcome of fresh embryo transfer.

It has been suggested that some techniques of assisted hatching may be better than other techniques. In 2002, Hsiehe et al compared laser-assisted hatching to chemical-assisted hatching and reported a significant increase in pregnancy rate, implantation rate,and delivery rate (18). However, this work has not been repeated so far. Other workers have expressed various preferences regarding the various techniques used in assisted hatching on the basis of nonrandomized studies (19,20). [40], [65]. Yao-Yuan Hshieh has the result of LAH group with the average oocytes similar with our results: 5,24 compare with 5,51±2,7. The average transferred embryo is 3,86 compare with 3,62. However, the fertility rate in this research is lower than our result with 28,2% compare to 41,8% [58]; [64].

Bider et al (1997) has result for assisted hatching by using Tyrode under 312 transferred-fresh embryo cycles in the patients above 38 years old has the average r manternal age in drilling group 41,9±2,8; the average occytes 6,05; however the fertility rate is just 36,99%, the avegage transferred embryos 3,48±1,5. 274 cycles in control group has the average mantenal age 41,1±2,2, the average oocytes 3,74, the feritily rate is 37,98%, the avegage transferred embryos 2,58±1,1. The implantation rate and the pregnancy rate is quite low just in LAH group: PR: 8,9%, IR:3,75%, in control group: PR:5,1%, IR: 3,55%, p>0,05) [10]. The average of occytes in this research is quite low because of some reasons like that the techniques using in the laboratory or the culture system in CO_2 incubator .

Friedman et al reported a benefit of AH in patients with a poor prognosis such as those with two or more failed IVF cycles, poor embryo quality, and women >38 years of age. Petersen et al reported that for patients with repeated implantation failures, the implantation rate in those who received laser-thinned embryos was significantly higher (10.9%) than in those whose embryos were not laser-thinned (2.6%). This difference, however, was not seen in patients with only one previous

implantation failure. A recent systematic review by Carney et al examined the effectiveness of AH and concluded that the increased chance of achieving a clinical pregnancy by AH only just reached statistical significance. The data did not support an increase in live birth rate. In our study, LAH did not increase the pregnancy or live birth rates [35] ,[39].

5.2 Trial 2: Effect of laser assisted zona pellucida thinning on outcomes after transfer of frozen embryos.

This trial consists in 220 embryo transfer cycles using frozen embryos. 110 embryo were Laser manipulated for zona pellucida thinning (LAH group) while 110 were untreated (control group). Groups were selected homogeneously for patient and embryo characteristics. In the table 10 are showed data on infertility times and basal FSH levels in LAH and control groups

Table 10. Causes of infertility in patients included into the trial

Causes of infertility	LAH (n= 110)	No LAH (n=110)	p
Mean female age (years)	34.3±3.5	31.4±2.1	NS
Duration of infertility (years)	5.3 ±0.3	6.8±0.8	NS
Basal FSH level (mIU/ml)	7.13±0.5	6.2±0.1	NS
Causes of infertility			
Male	32 (49.2%)	36 (55.3)	
Tubal	13 (20%)	17 (26.1)	
Mixed	15 (23.0%)	14(21.5)	
Unexplained	5 (7.6%)	7 (10.7)	

In table 10 are resumed the causes of infertility in the couples used in this trial. As we can see in both LAH and control groups the causes of infertility derive mainly from males (49.2% and 55.3% in LAH and control groups respectively) and probably

caused by alcohol and smoke. The groups were also homogeneous for infertility causes that did not show significant differences between groups (p> 0.05).

In fact, in LAH and control group infertility due to tubal causes was respectively 20% vs 26.1%, mixed infertility was respectively 23.0% vs 21.5% and unexplained infertility was respectively 7.6% vs 10.7%. AH can be performed using different techniques such as partial zona dissection, acid Tyrode's treatment and the laser but the causes of infertility by some reasons as table 10. Some centres use AH for poor prognostic patients such as women with advanced age, poor-quality embryos, embryos with thick zona pellucida and previous implantation failures. Those reasons for infertility couple leading to the failures in results in terms of implantation and pregnancy rates [8]. The enhancement of the embryo–endometrium contact and synchronization may be one of the explanations (Liu et al., 1993). The artificial gap produced by hatching may also serve as a channel for the exchange of metabolites and growth factors to and from the endometrium [17].

LAH increase the clinical pregnancy rate as related to embryo number. Combined with ICSI, in table 10 the results showed that in cases where infertility of the couple was caused by male factor, the clinical pregnancy rate increased significantly. Thus the use of assistance should be considered according to the special circumstances of each couple.There are some limitations in this study that should be considered. First, this was a retrospective study, with a heterogeneous patient population. In addition, the numbers of patients in the subgroup that received only LAH was small[130].

Table 11. The infertility period time of patient

Group **Infertility causes**	**Control**	**LAH thinning**	**p**
Infertility I	24.8%	23.5%	NS
Infertility II	75.2%	73.1%	NS
Averaged infertility period	6.5±2.1	7.5±4.5	NS
Avegaged age of patient	40 ± 4.2	39 ± 2.1	NS

Results summarized in the table 11 evidence the higher proportion of patient suffering for secondary infertility (VSII) compared to primary infertility (VSI). VSII was 73,1 % in LAH group and 75.2% in the control group. Infertility I was 23.5 % in the LAH group and 24.8 % in the control one. Mean duration of infertility ranged from 7.5 ± 4.5 years in LAH to 6.5 ± 2.1 years in control. Patients were 39 ± 2.1 years old in LAH and 40 ± 4,2 years old in control.

In the table 12 are resumed data on zona pellucida thickness related to age of patients. Zona pellucida thickness did not shows differences between groups nor between age classes.

Table 12. The size of zona pellucida by age

Age class	Control (µm)	LAH thinning (µm)	p
Under 30 years	15.8 ±2.4	16.8±2.6	NS
30 – 40 years	16.9±2.5	15.1±2.1	NS
Above 40 years	15.6±2.7	14.8±2.2	NS

Embryo cryopreservation was an indispensable technique in assisted reproduction to plan successive embryo transfer but frozen-thawed embryo procedures can lead to hardening of the zona pellucida which can alter embryo implantation and decreases positive results of transfer cycles [43-45]. Our studies confirmed previous works where differences in the zona pellucida thickness in individuals of different ages were not evidenced [8, 9]. According to previous studies advanced maternal age is one of the options that influenced the chance for embryo hatching due to the hardening of ZP and that assisted hatching (AH) of the ZP in ART treatments may improve the implantation and pregnancy rates [27, 45, 46, 50, 62]. Assisted hatching still is a controversial issue in assisted reproduction according to patients population, operator experience, study design and the AH methods used [46]. Therefore, it is difficult to compare the results described for assisted hatching from different publications.

There are many investigations that shown the beneficial effects of AH on pregnancy and implantation rates in cases with advanced maternal age and previous implantation failures [45, 46, 50]. Besides very recent studies reported that the AH does not improve implantation and clinical pregnancy in patients less than 38 years of age . According to our study, laser assisted zona thinning of human embryo before transfer has beneficial effects on clinical outcome of the two different maternal age patient population; younger and older than 35 years. It has been suggested that, in vitro culture conditions may impair the mechanism of blastocyst hatching due to the absence of lysine and other molecules secreted in vivo from the natural surrounding tissue, or due to the zona's glucoprotein cross-links [43-46, 49].

Table 13. Parameters of spermatozoa used in embryo production in the trial 2

Sperm parameters	Control %	LAH %	p
Abnomal concentration	4.2± 3.6	3.5± 1.5	NS
Abnomal mobility	13.3± 10.1	15.2± 8.2	NS
Abnomal morphology	93.5	91.2	NS
Abnormal acrosome	90.2	89.3	NS

In the table 13 are summarized parameters of sperm used in embryo production procedures. Concentration, motility, sperm and acrosome morphology and integrity did not show statistical differences between ejaculates used to produce LAH or control embryos (P>0.05). An abnormal concentration of spermatozoa was evaluated in 3.5± 1.5% of the LAH group and in 4.2± 3.6% of control. Motility was impaired in 15.2± 8.2 of LAH group ejaculates and in the 13.3± 10.1% of controls. Abnormal head sperms and acrosomes were respectively 91.2% and 89.3% in LAH group and 93.5 and 90.2 in controls.

Table 14. Parameters of recovered oocytes used to embryo production in the trial 2

Characteristics of oocytes	Control	LAH	p
The average of occytes after Aspiration/cycle	9.5±6.0	8.3±3.1	NS
The average recovered occytes/cycle	8.3±4.0	7.1±3.6	NS
The number of GV	1.4±2.5	1.2±2.3	NS
The number of MI	0.3±0.8	0.2±0.4	NS
The number of MII	7.5±5.1	6.2±4.3	NS
lysed	1.0±0.02	1,5±0.04	NS
The rate of fertilized occytes (%)	82.6	87.3	NS

The intracytoplasmic sperm injection (ICSI) program for oocyte Recovery is offered as a means of establishing pregnancies from oocytes (eggs) recovered from the ovaries using ICSI, oocytes are injected with individual sperm from a stallion, and the resulting embryos are allowed to develop in the laboratory for approximately one week. Developed embryos are then shipped to a private embryo transfer facility for transfer to a recipient mare, as for standard embryo transfer [106].

It have been aspirated 7.1 ± 3.6 oocytes/cycle in the LAH group and 8.3 ± 4,0 oocyte/cycle in the control group. In the LAH group were recovered 1.2 ± 2.3 GV, 0.2 ± 0.4 MI and 6.2 ± 4,3 MII stage oocytes while in the control group it has been recovered 1.4 ± 2.5 GV, 0.3 ± 0.8 MI and 7.5 ± 5.1 MII oocytes. Lysed oocytes were 1.5±0.04 and 1.0±0.02respectively in LAH and control groups. After fertilization it was obtained 87.3 and 82.6 fertilized oocytes.

Table 15. Evaluation of oocyte morphological parameters

Characteristic of oocytes	Control %	LAH %	p
Quite a lot granularities (G1)	24.6±1.3	27.3±1.5	NS
Much granularities (G2)	46.2±2.4	36.2±2.8	NS
Too much granularities (G3)	30.3±1.4	32.4±2.5	NS
Cytoplasmic fairly dark (D1)	26.3±0.3	35.2±1.0	NS
Cytoplasmic dark (D2)	15.6±0.4	18.4±0.4	NS
Cytoplasmic overdark (D2)	20.3±1.2	25.6±1.4	NS
Zona pellucida fairly thick (Z1)	35.2±2.4	48.2±1.5	NS
Zona pellucida thick (Z2)	23.6±1.6	18.3±2.7	NS
Zona pellucida too thick (Z3)	30.3±0.6	26.7±0.8	NS
Quite a lot Debris (F1)	15.7±0.6	18.3±0.3	NS
Much debris (F2)	22.5±2.4	25.5±1.5	NS
Too much debris (F2)	7.3±1.4	8.2±2.5	NS
Quite large PVS (P1)	14.6±1.4	17.6±0.6	NS
Large PVS (P2)	41.6±1.7	32.3±0.4	NS
Too large PVS (P3)	35.6±0.1	42.6±1.2	NS

In the table 15 are presented the morphological parameters of the oocytes used in embryo production procedures. Data did not show statistical differences between LAH oocytes and controls. Oocytes with G2 granularity grade were 36.2±2.8 % and 46.2±2.4 % in LAH and control groups respectively, while G3 granularity grade were respectively 32.4±2.5 and 30.3±1.4.

Oocytes which presented large PVS were 32.3±0.4 LAH and 41.6±1.7 in control. Oocytes having PVS of P3 grade were 42.6±1.2 and 35.6±0.1 in LAH and control groups respectively. The absence of differences and homogeneity of oocytes of the two groups indicate a uniform impact of these diseases on the further embryo development and on its potential of implantation in the uterus [109].

Table 16. Characteristic of day 3 and day 5 embryos produced in the trial 2.

Characteristics	Control	LAH	p
Day 3 G1 embryos	3.2 ±2.1	2.0 ±1.2	NS
Day3 G2 embryos	2.3 ±2.1	1.4 ± 1.1	NS
Day3 G3 embryos	0.7 ± 1.1	0.8 ±0.5	NS
Day 3 transferred embryos	2.3 ± 2.0	1.3± 0.5	NS
Day 3 frozen embryos	3.7± 2.3	5.2 ± 2.0	NS
Average day 5 embryos	7.5 ±2.5	6.5± 2.3	NS
Day 5 G1embryos	2.0 ±2.1	3.6 ±2.3	NS
Day 5 G2 embryos	2.2 ±0.6	2.3 ± 1.2	NS
Day 5 G3 embryos	1.2 ±0.7	2.2 ±0.2	NS
Day 5 G4 embryos	0.7 ±1.2	0.3 ± 0.7	NS
Day 5transferred embryos	1.5 ± 0.7	2.0 ±1.1	NS
Day 5 frozen embryos	4.5 ± 3.2	5.7 ±2.0	NS

In the table 16 are resumed data of the embryos produced and transferred at day 3 and day 5 in the trial 2. The mean of embryos obtained at day 3 (2,0±1,2 and 3,2±2,1 in LAH and control respectively) and day 5 (6,5±2,3 and 7,5±2,5 respectively in LAH and control groups) did not present any significant difference. The embryos classified according to quality criteria did not show statistical differences between the two experimental groups both at 3th and 5th day. The transferred embryo at day 3 and day 5 was 1,3±1,5 and 2,3±2,0 in the LAH group and 1,5 ± 0,7 and 2,0 ±1,1 in the control group respectively.

Table 17. Outcome of thaw- transfer cycles

Characteristic	LAH drilling (%)	Control (%)	p
Cycles of thaw- transfer	110	110	NS
Total of transferred embryos	250	258	NS
Avegage transferred embryos	2.2±0,1	2.1±0.3	NS
Implantation rate/ transferred embryos (%)	56/250 (22.4%)	42/258 (16.2%)	<0.05
Implantation rate/cycles (%)	56/110 (50.9%)	42/110 (38.1%)	<0.05
Biochemical pregnancy (%)	48/110 (43.6%)	39/110 (35.4%)	<0.05
Clinical pregnancy rate/cycles (%)	45/110 (40.9%)	34/110 (30.9%)	<0.05
Early miscarriages rate (%)	4/45 (8.89%)	3/34 (8.82%)	NS
Ongoing pregnancy rate (%)	41/110 (37.2%)	31/110 (27.2%)	<0.05
Singleton (%)	17/41 (41.4%)	3/110 (5.2%)	NS
Twin (%)	24/41 (58.5%)	13/31 (41.9%)	NS

In this trial a total of 220 embryo transfer cycles using frozen embryos were undertaken within 18 months. These data showed that laser zona pellucida treatment to ameliorate hatching (LAH) improves outcome of frozen-thawed embryos after transfer into recipients. The implantation rate in LAH group (56/110; 50,9%) was higher than in control group (42/110; 38,1%). The clinical pregnancy rate was statistically higher in LAH group (45/110; 40,9%) compared to control (34/110; 30,9%). The ongoing pregnancy rate was higher in LAH group compared to control (37,2% vs 27,2% respectively). Percentages of early miscarriages for clinical pregnancies were similar between LAH (8,89%) and control group (8.82%). These results was similar to other

researchs in embryos transfer with frozen embryos having or not LAH and improves outcome of fresh embryo transfer [65],[40]. Some reviews suggest that the routinary use of assisted hatching is inappropriate in view of the lack of evidence of universal benefit and the potential risks. However, there are evidences of assisted hatching benefits in certain circumstances, such as in patients with poor pregnancy prognosis, including those with two or more failed IVF cycle, with poor embryo quality and in older women more than 38 year old. Higher clinical pregnancy and implantation rates have been observed after assisted hatching [58]; [64].

In the Table 18 are it have been compared our results and other published results about LAH treated embryos and untreated control. , design studies like part of our study, synthesized in the table found results of our study goes with the general trend of recent studies. In general, the majority of studies showed improved implantation and pregnancy rates after assisted hatching compared with the control group. Our results on frozen thawed embryo transfer evidence an increase of pregnancy rates after LAH compared to untreated embryos. There are many techniques in current use to assist hatching, which may differ in both efficacy and risks. Although the equipment may be expensive, the laser assisted technique would appear to have the lowest potential risk attached to it, and it is relatively simple to perform with consistency between operators [5]. Appropriately powered, probably multicentre research is required to confirm the safest technique to use, and to identify patients that would benefit from assisted hatching before they have had repeated unsuccessful treatment cycles. In this review there is extensive and focused revision of most researches and studies concerning with AH in the literature. Taking into consideration the most updated one, which deals with the mechanically expanding technique with emphasis on urgent need for trials to improve the potential of AH. Proper selection of the candidates for different AH, type of AH, number of patients, the implantation rate (%), pregnancy rates (%). There is a strong evidence supporting that laser AH is considered the best technique now as regard safety and efficacy [39, 47, 73].

Table 18. Comparison of AH treatment to control embryo transfer outcomes

Study	AH method	How to AH	pregnancy rate (%)			Implantation rate (%)		
			LAH	Control	p	LAH	Control	p
Our research, 2015	LAH	Thinning ¼ ZP	40,9	30,9	<0,05	22,4	16,2	<0,05
Amorocho et al, 2008	LAH	Thinning ¼ ZP	33,3	20,0	<0,05	23,2	11,5	0,04
Petersen et al, 2006	LAH	Thinning ¼ ZP	18,8	7,8	0,01	7,9	2,9	0,01
Zhang et al, 2009	LAH	Thinning 80µm	40,3	16,1	0,03	21,5	7,5	0,07
Ge HS et al, 2008	LAH	Thinning	25	14	<0,05	16,7	7,3	<0,05
Balaban et al, 2006	LAH	Thinning ¼ ZP	40,9	27,3	<0,05	20,1	9,9	<0,01
Sifer et al, 2006	pronase	Thinning all ZP	28	31	NS	17,8	15,2	NS
Ng.Ernet et al, 2005	TAH	Thinning ¼ ZP	12,5	15	NS	14,7	8,5	NS
Baratz et al, 2005	LAH	Thinning 1.5 times ZP	34,9	21,1	NS	14,7	8,5	NS
Mauri et al, 2001	LAH	Thinning ¼ ZP	17	17,6	NS	9,6	10,5	NS
Debrock et al, 2008	LAH	Thinning ¼ ZP	20	21,1	NS	13	15,9	NS
Friedman et al, 2010	LAH	drilling	22,5	42,5	0,03	17,8	28	0,005
Tanaka et al, 2006	LAH	drilling	19,1	16,7	0,03	51,6	37,5	<0,05
Gabrielsen et al, 2005	LAH	Drilling 30µm	17,6	11,1	NS	11,4	5,8	<0,05
Eva Johnson et al, 2001	LAH	Drilling 20µm	37	22	<0,05	15	12,9	NS
Fang Cong et al, 2010	mechanical	Drilling	37,7	20,3	<0,05	14	7,4	<0,05
Valojerdi et al, 2010	LAH	Drilling 20µm	31,2	11,1	0,01	12,8	4,2	0,000
Hiraoka et al, 2008	LAH	Drilling 40µm	43	17	0,01	27	10	0,01

6. CONCLUSION

Data of our study obtained after 220 embryo transfer cycles using fresh embryos and 220 embryo transfer cycles using frozen embryos aimed to determine if laser assisted zona pellucida thinning improve outcome of embryo transfer in woman that came from several year attempts to conceive a child, drive to the following conclusions:

1. Laser assisted hatching (LAH) improves outcome of fresh-transferred embryo which showed higher implantation rates, clinical pregnancy rates and ongoing rates compared to not treated embryos;
2. Laser assisted hatching (LAH) improves outcome of frozen-thawed embryos after transfer into recipients, LAH of frozen-thawed embryos evidenced after transfer into recipients higher implantation and pregnancy rates;
3. Our study showed that the embryo zona pellucida was unaffected by the age of donor woman and did not show differences in the mean size between different age donors;
4. Currently there are many techniques to assist blastocyst hatching, which may differ in both efficacy and risks. Although the equipment may be expensive, the laser assisted technique meet efficacy and safety being relative simple to perform by trained operators and evidencing a low potential risk for the manipulated embryo and it is relatively simple to perform with consistency of operators.

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9. APPLICATION FOR ETHICAL APPROVAL FOR IMPLEMENTATION FOR SCIENCIFIC RESEARCH