JOURNAL LA LIFESCI

VOL. 05, ISSUE 02 (144-152), 2024
DOI: 10.37899/journallalifesci.v5i2.1186

Success Rate of Frozen Embryo Transfer through Assisted Hatching Laser Method and Tyrode Acid Chemical Method in Day 3 and 5 Embryos

Prastuti Dwi Hartini[1,2], Lucy Lisa[2], Harini Nurcahya[1,3], Frizar Irmansyah[4], Deka Putri Gunarwati[2]

[1]Department of Biology, Faculty of Postgraduate Biology, National University.Jl.Harsono No.11, Ragunan, Pasar.Minggu, Jakarta 12550
[2]Jakarta IVF RS YPK Mandiri Jl.Gereja Theresia No.22,Gondangdia Menteng, Jakarta 10350
[3]National University, Jl.Sawo Manila, Pejaten, Pasar Minggu, Jakarta 12520
[4]RSIA Kemang Medical Care, Jl.Ampera Raya No.34, South Jakarta, DKI Jakarta 12550

**Corresponding Author: Prastuti Dwi Hartini*
Email: hprastutidwi@yahoo.co.id

Article Info

Article history:
Received 14 March 2024
Received in revised from 20 May 2024
Accepted 12 June 2024

Keywords:
Assisted Hatching
Vitro Fertilization
Frozen Embryo Transfer
Age
Zona Pellucida

Abstract

Repeated implantation failures in IVF, aging in women and vitrification at low temperatures cause the embryonic zona pellucida to harden.Technique assisted hatching (AH) laser and chemical methods of tyrode acid can help the process hatching in embryos in improving implantation. The purpose of this study is to determine the relationship between pregnancy success through AH laser method and chemical method of tyrode acid at the embryonic stage day to-3 (D3) and day to-5 (D5) on Frozen Embryo Transfer (FET). The study used a retrospective cohort with a population of 95 patients using AH laser method and 36 patients with tyrode acid chemistry. The results showed the incidence of pregnancy in laser treatment in 27 patients (28.4%) and 14 patients (38.9%) in chemical treatment. Data analyzed by statistical tests Chi-square with a 95% confidence degree and a p< value of 0.05. Exist The relationship between the incidence of pregnancy FET D3 and FET D5 laser treatment, but in chemical treatment no significant difference was found. Logistic regression modeling tests show the chemical method FET D5 has a greater chance of pregnancy of 0.277 than FET D3, will but insignificant with a p-value of >0.05, while the FET D5 laser stage method has a chance of 0.143 significantly higher than FET D3 with a p-value of <0.05.

Introduction

One of the goals of marriage is to expect the presence of children as the next generation in a family. *Infertility* is a condition in married couples who have had regular and adequate sexual relations for more than a year without using contraceptives, but have not obtained pregnancy or offspring (Anwar & Anwar, 2016). According to Wankhede et al. (2017), *Infertility* can be caused by factors that can trigger the occurrence of *Infertility* either from male, female, or even both.

Currently, the development of reproductive technology applications has grown rapidly and one of the developments in assisted reproductive technology carried out is *Assisted Reproductive Technology* (ART) / Assisted Reproduction Techniques (TRB) with TRB types that have a fairly high success rate, namely: *In Vitro Fertilization* (IVF) or better known as IVF. Program success *In vitro fertilization* (IVF) can also be affected by factors related to the patient's age and embryo quality. At the age of <35 years, fertility is very stable. Fertility then gradually declines after that. Fertility decreases sharply after the age of forty (Noni Nurhayati, 2017). The quality of the embryo also affects the success of implantation. Poor embryo quality has

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

greater implantation failure. One of the influencing factors is the chromosomal abnormality of the embryo and morphological assessment by looking at the size of the blastomere and the presence of fragmentation (Rizal, 2003).

Deterioration of embryo quality and hardening of the zona pellucida are constraints on embryo freezing techniques (Chi, 2002). The vitrification process at low temperatures causes the zona pellucida of the embryo to harden. This will hinder the process *hatching* in embryos so that embryonic growth is less than optimal (Hsieh et al., 2002). Therefore, it is necessary to carry out assistance techniques in overcoming the problem of hardening of the zona pellucida of embryos after vitrification by *assisted hatching* Such as using laser or chemical methods of tyrode acid with their respective advantages and disadvantages.

The purpose of this study is to Knowing the relationship between pregnancy success rates on *Frozen Embryo Transfer (FET)* through *Assisted Hatching* (AH) with laser method and chemical method of tyrode acid based on embryonic stage day 3 (D3) and day 5 (D5).

Methods

Materials and Tools

This study used samples of frozen embryos that were then thawed and came from legal married couples who carried out pregnancy programs with *assisted hatching* tyrode acid chemistry from May 2021 to April 2022 and laser from April 2022 to August 2023. The number of patients who participated in the FET program amounted to 131 patients, consisting of 36 patients with tyrode acid chemical AH and 95 patients with laser AH. The inclusion criteria in this study included data on individuals who transferred frozen embryos on day 3 and day 5 carried out chemical AH, tyrode acid and AH laser. The exclusion criteria in this study were patients with incomplete medical record data and patients who underwent pregnancy programs without using *assisted hatching.* The instruments used in this study are *micromanipulator microscopy* [Nikon,Eclipse Ti2], laser device [Lykos,Hamilton Thorne], *petridish* [Falcon], medium G.mops (+) [Vitrolife], *liquid paraffin* [Origio], tyrode acid medium [Origio], books and other stationery.

Method

This study was analytical using a retrospective cohort design that looked backwards using secondary data from the patient's medical record database *Frozen Embryo Transfer* (FET) via *Assisted Hatching* (AH) both on the 3rd day embryo and on the 5th day embryo by laser and chemical methods of tyrode acid. The data obtained was processed using the SPSS program version 2.0 squared kai test *(Chi-square)* To analyze the comparative data of two independent groups and multiple logistic regression tests to see the relationship of several independent variables with one or more dependent variables.

Assisted Hatching Laser Method

Procedure *assisted hatching* using this laser method refers to Hamilton Thorne's Lykos guidebook. Before it is done *assisted hatching* with laser for preparation of medium in Put in the incubator one day before. On the day of action are made drop droplets from the pre-warmed medium one day earlier. Take the embryos from the incubator 37°C, CO2 6% and O2 5%, then place the embryos in the drop. Set the target on the laser and perform thinning of the zona pellucida using the laser. Wash the embryos with a prepared medium and the embryos are ready to be used for the embryo transfer process.

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

Assisted Hatching Chemical Method with Tyrode Acid

Technique *assisted hatching* with chemical method tyrode acid pH 2.2 refers to (Cohen et al., 1990). This method is done by making a hole or resulting in thinning of the zone in the embryo. First, the embryo is ensured to be in the right position by holding it using Holding pipette at 9 o'clock position. At about the same time, an AH pipette with a diameter of 10 micro containing tyrode acid solution is oriented at the 3 o'clock position then brought closer to the zona pellucida and made a hole by spraying tyrode acid solution in a controlled manner on the surface of the zone. The embryos are then rinsed several times in G. mops (+) medium to remove excess tyrode acid and returned to culture media until the embryo transfer process will be carried out.

Determination of Pregnancy Success

The success of pregnancy is known by looking at the value of hormones from ß-HCG. The results of ß-HCG will be known two weeks after the embryo transfer process. ß-HCG Results >50 mIU/mL indicates the patient is successfully pregnant and ß-HCG <50 mIU/mL indicates that the pregnancy program has not been successful.

Results and Discussion

Analysis results the relationship between day 3 (D3) and day 5 (D5) FETs with pregnancy in the laser method (table 1) showed a significant relationship (P-Value = 0.0001) with OR value: 0.136 which means that stage FET D5 has a higher pregnancy success of 0.136 times than stage FET D3.

Table 1. Relationship Between *Frozen Embryo Transfer* (FET) Through *Assisted Hatching* (AH) D3/D5 with Pregnancy in Laser Method

Stage FET	Pregnancy				Total		OR (95% CI)	P-Value
	Pregnant		Not Pregnant					
	N	%	N	%	N	%		
D3	7	12.5	49	87.5	56	100	0.136 (0.049-0.373)	0.0001
D5	20	51.3	19	48.7	39	100		
Sum	**27**	**28.4**	**68**	**71.6**	**95**	**100**		

This is because embryo transfer at the blastocyst stage or day 5 can obtain a high implantation rate, possibly because the embryo has gone through a better selection process than the embryo on day 3, which is a natural selection process in the culture environment over a longer period of time and conventional morphological assessment that is more effective to describe the quality of the embryo. The blastocyst cells of day 5 have differentiated more clearly into *inner cell mass* and *trophectoderm* which can be assessed degree of expansion. Blastocysts will form inner cell masses / *inner cell mass* (ICM) and *trophectoderm* will develop into a placenta. Henceforth ICM will develop into epiblasts and hypoblasts. The epiblast will become the embryo and the hypoblast will become the extra membrane of the embryo (Puspitadewi, 2008). Culturing embryos until day 5 in the blastocyst stage is an effective method in selecting embryos because it has greater developmental potential in improving implantation and pregnancy. These results are consistent with Mangalraj research et al. (2009) which states that culturing embryos up to day 5 (D5) allows embryos with competent chromosomes to develop into the blastocyst stage and allows the selection of embryos that have the potential to continue development under the control of the embryonic genome. Thus, the selection of embryo transfer on the 5th day after activation *genomics* Endometrial synchronization will provide an

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

advantage for patients because it can increase the success of pregnancy compared to embryo transfer on day 3. In addition, the 5th day embryo is also used to select the most embryos *viable* and can lower the incidence of multiple pregnancies. This corresponds to Glujovsky & Farquhar (2016) which mentions embryo transfer on day 5 (D5) in the blastocyst stage is considered a more physiologically appropriate time for implantation and can improve synchronization between endometrium and embryonic development.

D5 embryos have physiological synchronization with the uterine endometrium and blastocyst embryo transfer, enabling fewer but high-quality embryo transfers, resulting in improved implantation rates and better pregnancy success. Similar results were obtained from research conducted by Eftekhar et al. (2020) which shows day 5 frozen embryo transfer at blastocyst stage can significantly improve pregnancy and implantation success compared to embryo transfer at division stage. The development of the embryo on day 5 at the blastocyst stage can also be assessed the expansion stage on the quality of the tropectoderm and cell mass which are important factors for the embryo to undergo implantation. However, the pregnancy and implantation rates from day 6 frozen blastocyst embryo transfer are significantly lower compared to day 5 frozen blastocyst transfer due to the slow rate of development of day 6 embryos into blastocysts (late blastocysts) and the increased likelihood of DNA damage causing aneuploidy and resulting in lower implantation rates.

5th day embryo transfer with application *assisted hatching* Laser methods on post-vitrified blastocysts seem to give better results when ≥50% of the zona pellucida (ZP) is opened or ZP is completely removed. Half of ZP depletion in early-stage vitrified embryos appears to be associated with higher pregnancy rates (Nakasuji et al., 2014). The use of lasers on *assisted hatching* also has accuracy on the target, the resulting hole is more precise, exposure time can be minimized, safety and usability of the system can be used as desired. Fast and efficient laser systems can be accurately controlled and produce precise zona zona pellucida openings without thermal or mutagenic effects. The use of lasers for thinning the zona pellucida in addition to having better safety also does not cause degeneration in embryos, besides that this system is also able to produce holes that are uniform and easily adapted to all types of microscopes. The size of the hole is related to the exposure time of the laser, it has a simple, fast and easy to use system.

Analysis results the relationship of day 3 (D3) and day 5 (D5) FETs with pregnancy on the Tyrode acid chemical method (table 2) showed that there was no significant association P-value = 0.173 (p-value >0.05) in the incidence of pregnancy between stage FET day D3 and stage FET day D5 in chemical treatment of Tyrode acid.

Table 2. Relationship *Frozen Embryo Transfer* (FET) Through *Assisted Hatching* (AH) D3/D5 with Pregnancy on Tyrode Acid Chemical Method

Stage FET	**Pregnancy**				**Total**		P-Value
	Pregnant		**Not Pregnant**				
	n	**%**	**N**	**%**	**N**	**%**	
D3	3	21.4	11	78.6	14	100	0.173
D5	11	50	11	50	22	100	
Sum	**14**	**38.9**	**22**	**61.1**	**36**	**100**	

Assisted hatching is a technique in overcoming several obstacles such as repeated IVF failure, poor embryo morphology, maternal age ≥ 38 years and hardening of the zona pellucida in the vitrification process. Assisted hatching (*assisted hatching*) can optimize the hatching time in embryos so as to produce optimal implantation in the endometrium to increase pregnancy rates

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

(Al-Nuaim et al., 2002). Pregnancy can occur when the embryo can exit the zona pellucida called *hatching,* So that the embryo can attach to the endometrial wall and implant in the process of pregnancy. If process *hatching* Failure to occur then the embryo cannot attach to the endometrium which means pregnancy will not occur. The ability of the embryo to exit the zona pellucida in the hatching process is influenced by the quality of the embryo. A good quality embryo will develop into an early blastocyst to then become a blastocyst with a larger size until it continues to the expanded blastocyst stage and is able to press the zona pellucida to become thinner so that in the end the embryo manages to get out of the zona pellucida. Thus, the process of selecting the best quality embryos plays an important role in determining the success of implantation and pregnancy.

Morphological assessment of embryo quality can be seen from the size of the blastomere and the presence of fragmentation. The assessment is a reference with uniform standards (excellent & good) to select embryos to be transferred based on SOPs in each clinic. The embryo assessment process plays an important role in providing information and insight to doctors to make clinical decisions that will be informed to patients before the implementation of embryo transfer in the hope of increasing pregnancy success rates. Embryos with good quality are described as having the same large blastomere size and no fragmentation (excellent) or less than 10% (good). Based on the results of research conducted by Hardarson et al. (2001) which shows that the size of the blastomere is not the same as having a lower developmental capacity in embryos compared to the same size of the blastomere. Unequal blastomere sizes also have higher aneuploidy/multinuclear rates, leading to low implantation rates and lower pregnancy success rates. While fragmentation in embryos is a phenomenon that often occurs in most embryos that come from the remains of cells that do not have a nucleus or are the result of the decomposition of one or more cells from the embryo itself and very poor oocyte quality can cause the embryo to become very fragmented. Research conducted by Alikani et al. (1999) states that the degree of fragmentation in embryos has a significant impact on implantation and pregnancy and the potential for fragmented embryos for implantation is partly determined by the distribution of fragments. In addition, a good quality embryo must exhibit endometrial synchronization of appropriate thickness and normal kinetic division, which occurs every 18 to 20 hours. The embryonic division stage starts from stage 2 of the cell to the morula which is characterized by blastomere begins to show increased attachment between cells undergoing a compaction process. Based on research conducted by Fu et al. (2009) showed that the early embryo division stage shows strong indicators of embryonic development potential with high implantation rates and pregnancy success rates compared to late embryo division.

The developmental stage of embryo division can determine the timing of embryo transfer which can be done on day 3 or day 5 depending on the number and quality of embryos obtained. If the number of embryos with good quality is less than five, in general, embryo transfer will be carried out on day 3. This is because only 80% of existing embryos can survive until day 5 so there is a risk of no embryos that can be transferred on day 5. Meanwhile, if more than five embryos are obtained with good quality, the embryo transfer process can be carried out on the 5th day. However, under certain conditions, such as obtaining many embryos but with poor quality, the doctor can determine the embryo transfer on day 3. Determination of embryo transfer time can be done on day 2 by looking at embryo development based on embryo quality level / embryo grade. approximately 48-54 hours after egg retrieval (OPU), embryo assessment is carried out based on its morphology (number and quality of cells or regularity of blastomeres), and presence or absence of fragmentation (0-100%), which follows an agreed scoring system. Oocytes that have been fertilized will divide into 2 to 8 cells. Embryos with blastomeres, which are the same size and no fragments (grade 1), embryos with unequal

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blastomeres, and less than 10% fragmentation (grade 2), embryos with 10-50% fragmentation or with non-viable blastomeres (grade 3) and embryos with fragmentation of >50%, or missing blastomeres, but still visible one blastomere (grade 4).

The quality of embryos can determine the number of embryos to be transferred. If more than 1 embryo is transferred, it can increase the number of twin pregnancies with the risk of pregnancy complications and premature birth (Klitzman, 2016). To reduce the risk of multiple pregnancies, pregnancy complications and the development of safer and more effective fertility treatments, it is necessary to pay attention to the number of embryos to be transferred. The number of embryos to be transferred is in accordance with the procedures applicable to each clinic and through a process of discussion between the embryologist and the clinician with the consent of the patient. Although there are calls to transfer a single embryo, the chances of success in this program are uncertain and less than 50%, so more than one embryo transfer can be performed in patients who fail in two or more IVF cycles or have an unfavorable prognosis according to their individual circumstances. The number of additional embryos to be transferred depends on the age and prognosis of the patient. The American Society of Reproductive Medicine (ASRM) states that patients with a good prognosis with euploid embryos, patients aged <35 years regardless of embryonic stage and patients aged 35-37 years providers should only transfer 1 embryo. For patients aged 38-40 years with untested embryos of division stage, no more than 3 embryos or 2 blastocysts should be transferred. As for patients aged 41-42 years, ASRM states that each embryo at the division stage should not be transferred more than 4 embryos or 3 blastocysts. Provisions for patients with the age of >43 years, there is not enough data to recommend a limit on the number of embryos to be transferred (Practice Committee of the ASRM, 2021).

Several factors can also affect embryonic growth so as to reduce the success of pregnancy such as the number of eggs obtained, the number of mature eggs and the quality of eggs and embryos, In addition, the implantation window is an important thing that must be considered which is an ideal receptive state for implantation between the embryo and endometrium (the condition of uterine acceptance of the embryo) in increasing the success rate of pregnancy (Mantoudis et al., 2001; Ng et al., 2005; Miyata et al., 2007).

Embryo implantation can be assisted by techniques *Assisted hatching* Tyrode acid chemical method which is a method to help the process *hatching* in D3 and D5 embryos in increasing the success rate of pregnancy. Although making holes in the zona pellucida may seem like a simple procedure, it is important to consider the potential dangers of this procedure as well as the inability to produce uniform, standardized holes and possible damage to the blastomere of the embryo (Grace et al., 2007; Miyata et al., 2010). There is concern that manipulation of zona pellucides with Tyrode acid solution has possible negative effects and requires the skill and experience of the operator.

The results of this study showed embryo transfer with *assisted hatching* Tyrode acid day 5 (D5) has a higher pregnancy success rate than day 3 (D3), but is not significantly different. This is likely due to Tyrode's acidic chemical solution with a pH of 2.2 and can spread to the walls of the zona pellucida so that it has a deeper eroding effect on both D3 and D5 embryos (Febretrisiana & Pamungkas, 2017).

The results of the analysis of pregnancy logistic regression modeling with laser and chemical methods of Tyrode acid (table 3) obtained the relationship between the stage of FET action and the success rate of pregnancy in the chemical method of Tyrode acid and laser method. The FET stage of day 5 embryos with the chemical method of Tyrode acid has a 0.277 greater chance of successful pregnancy than day 3 embryos. However, the p-value results show a value

ISSN 2721-1304 (Print), ISSN 2721-1207 (online)

of >0.05 so it is declared insignificant or meaningless. While in the laser method, the FET stage of the embryo day 5 has a 0.143 greater chance of pregnancy success than day 3. The p-value results show a value of <0.05 so it is declared significant or meaningful.

Although in this study the transfer method on the 5th day of embryonic development provided results in increasing the success of pregnancy through assisted hatching techniques both with the laser method and the Tyrode acid method, it is necessary to note the advantages and disadvantages of each method. In the assisted hatching procedure, Tyrode acid requires the ability and skill of the operator in making the same hole and standardized in the zona pellucida because the acidic nature of the Tyrode acid solution can damage the blastomere of the embryo. While the assisted hatching laser procedure has a system that is simple, fast and easy to use. Fast and efficient laser systems can be accurately controlled and produce precise zona zona pellucida openings without thermal or mutagenic effects. The use of lasers for thinning the zona pellucida in addition to having better safety also does not cause degeneration in embryos, besides that this system is also able to produce holes that are uniform and easily adapted to all types of microscopes. The use of lasers on *assisted hatching* also has accuracy on the target, the resulting holes are more precise, exposure time can be minimized, safety and usability of the system can be used as desired.

Table 3. Final Results of Logistic Regression Modeling between pregnancies with Laser Method treatment and Tyrode Acid Chemical Method

Method		**Sig (P-Value)**	**Exp(B) (OR/Opportunity)**	**95% C.I.for Exp(B)**	
				Lower	**Upper**
Chemical Method					
	Stage FET	0.102	0.277	.060	1.292
	Age Group	0,870	1.078	.438	2.651
	Laser Method				
	Stage FET	0.000	0.143	.051	.399
	Age Group	0.627	1.198	.578	2.486

Conclusion

In this study, we found that there was a significantly higher association on day 5 frozen embryo transfer pregnancy rates compared to day 3 with assisted hatching The laser method while the chemical treatment of tyrode acid is not significantly different. In logistic regression analysis, both tyrode acid chemical treatment and laser treatment have a higher day 5 embryo transfer pregnancy rate than day 3, but the chemical treatment shows a p-value of >0.05 so it is declared insignificant while in laser treatment the p-value <0.05 which means significant or meaningful.

For further research, supporting examinations are needed in certain cases before being carried out assisted hatching and embryo transfer by PGT-A examination (Preimplantation Genetic Testing for Aneuploidy) which is an examination of 46 chromosomes to find embryos that have normal chromosomes (euploid) to reduce the rate of miscarriage and failure in in vitro fertilization (IVF) or Frozen Embryo Transfer (FET).

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