

A randomized, prospective study comparing laser-assisted hatching and assisted hatching using acidified medium

The exposure of oocytes and embryos to the artificial conditions of *in vitro* culture may have negative effects on the embryo's ability to undergo normal hatching, resulting in low rates of implantation following *in vitro* fertilization (IVF) and embryo transfer (1). Increased maternal age, elevated basal FSH concentrations, and poor embryo and zona pellucida morphology may also decrease an embryo's ability to hatch and implant following transfer (2).

In 1989, Cohen and colleagues (3) reported a higher implantation rate in IVF patients transferred with embryos that had their zonae pellucida mechanically opened. Many IVF centers worldwide now perform the assisted hatching procedure for infertility patients in hopes of increasing each embryo's chances of successful hatching and implantation. Different technical procedures have been employed to create the zona opening, and include the use of various acidified solutions, the use of a thin microneedle to slice through the zona, or the creation of an opening with a laser beam.

The use of a laser for assisted hatching was initially developed to provide a precise and controlled method that could also be standardized between patients and operators (4). A multitude of studies have evaluated the effects of lasers on embryos from both human and animal models, and have demonstrated no adverse effects on embryo development *in vitro* (5–10). In addition, a number of clinical studies have presented data following the use of laser-assisted hatching in human infertility patients (11–15). However, it was not until recently that the US Food and Drug Administration (FDA) provided clearance for the clinical use of a laser to perform assisted hatching without

the IVF program first obtaining an investigational device exemption (IDE; 16).

Although extensively studied for safety and performance, there has not been a randomized comparison of laser-assisted hatching and assisted hatching with acidified medium in fresh IVF cycles to determine if the two methods differ in clinical outcomes. The objective of this study was, therefore, to compare rates of implantation and clinical and ongoing pregnancy between the two methods in a controlled study.

For this evaluation, we used the ZILOS-tk laser system, manufactured and marketed by Hamilton Thorne Research (Beverly, MA) for the laser-assisted hatching. This system provided a computer-controlled 1.48-micron infrared diode laser beam that could be directed through the objective of the microscope. These results were compared to the clinical outcomes of patients undergoing assisted hatching using acidified medium delivered through a micropipette controlled with micromanipulators. The results of consenting patients who could not be randomized into one of the two groups because of thin zonae pellucidae or a day 5 transfer were also evaluated.

MATERIALS AND METHODS

Patient Selection

This double-blinded prospective study was approved by the Washington University School of Medicine's institutional review board. At the time this study was initiated, the use of a laser for performing assisted hatching was not yet approved by the FDA so an IDE was obtained.

To be eligible for this study, patients had to meet one or more of the following criteria, which included that the age of the wife was >37 , the couple had failed to achieve a pregnancy following a previous IVF attempt in which embryo(s) were transferred, the couple had a previous IVF attempt in which only poor-quality embryos were available for transfer, or the couple had a previous IVF attempt in which abnormal zonae morphology was documented. Couples who consented to the study but whose data were not utilized included those with less than three mature eggs recovered at the time of the aspiration procedure, patients who failed to achieve any fertilization and patients who cryopreserved all of their embryos at the pronuclear stage of development. The number of patients consent-

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ing to the study represented approximately 37% of the eligible population.

On the day of embryo transfer, consenting couples who were candidates for assisted hatching were randomized to receive hatching using either the ZILOS-tk (laser) or the standard method with acidified Tyrode's medium delivered through a micropipette (acid). As our standard protocol, embryos with zonae measuring <13 micron thickness in one or more sites were not hatched but their clinical data was collected (thin) for this study. In addition, all patients with four or more embryos at the seven-cell stage or above were pushed to a day 5 transfer and their clinical data also collected (day 5). The randomization was blinded to the patients, physicians, nurses, and support staff. Patients were excluded from the study if less than three mature oocytes were recovered at the time of retrieval. Patients who did not achieve an ongoing pregnancy in their first study cycle were allowed to again participate in the study but in the other treatment group.

Ovarian Stimulation and Oocyte Retrieval Procedures

Ovarian stimulation was performed using gonadotropins in combination with a GnRH antagonist or GnRH agonist following established protocols. Serum E₂ was measured at baseline and after 3, 4, or 5 days of gonadotropin and then as needed until retrieval. Vaginal ultrasounds were performed concomitantly with estradiol levels. Human chorionic gonadotropin was administered when three or more follicles were at least 16 mm in largest diameter. Transvaginal follicular aspiration was performed 35 to 36 hours later (day 0).

Insemination and Embryo Culture

Sperm preparation and insemination, oocyte identification, and oocyte and embryo culture were performed using established protocols for in vitro fertilization and intracytoplasmic sperm injection. Single oocytes, cultured in 0.1-mL droplets of IVC-TWO (InVitroCare Inc., Frederick, MD) supplemented with 5% Human Serum Albumin Solution (HSA; Irvine Scientific, Santa Ana, CA) were inseminated with 10,000 to 20,000 motile sperm or injected with a single sperm. Inseminated oocytes were evaluated approximately 18 hours later for pronuclear formation, and the resulting embryos placed in 0.05-mL droplets of IVC-ONE growth medium (InVitroCare Inc.) supplemented with 10% Serum Substitute Supplement (SSS; Irvine Scientific). Embryo cleavage and morphology was evaluated again on the morning of day 3. Embryos remaining in culture for a day 5 transfer or for possible cryopreservation at the blastocyst stage were transferred to 0.05-mL droplets of IVC-THREE growth medium (InVitroCare Inc.) supplemented with 10% SSS (IVC-THREE + 10% SSS).

Assisted Hatching Procedures

Before either hatching procedure, the patient's embryos were evaluated to ensure that they were at the appropriate cell stage for hatching. To be hatched, embryos had to contain at least four blastomeres, not including blebs and/or fragments. All hatching procedures were performed on the morning of day 3 at least 1 hour before the scheduled embryo transfer. Immediately before hatching, the zonae thickness of each embryo was determined using the ZLTS computer's scale facility. All hatching patients (acid and laser groups) were treated with methyprednisolone (8 mg orally twice a day) starting on the day of oocyte retrieval and continuing for 5 days.

Assisted hatching with acidified medium Cycles randomized into the acid group underwent hatching using acidified medium delivered through a hatching micropipette. Narishige micromanipulators (Narishige USA Inc., Greenvale, NY) were used in conjunction with a heated microscope stage (Hoffman Surgical Equipment, Conshohocken, PA) on a Nikon Diaphot Phase Contrast Inverted Microscope (Nikon Inc., Garden City, NY). Egg-holding and assisted hatching micropipettes were obtained from Humagen Fertility Diagnostics, Inc. (Charlottesville, VA).

Before hatching, embryos were rinsed and placed in 0.1-mL droplets of Modified HTF Medium-HEPES (Irvine Scientific) supplemented with 10% SSS. The hatching procedure employed the release of Acidified Tyrode's Solution (Irvine Scientific) against the zona pellucida to create an opening of about 20 μ m in diameter as previously described (17). Following hatching, embryos were rinsed and moved to the embryo transfer dish containing 0.05-mL droplets of IVC-THREE + 10% SSS.

Laser-assisted hatching Cycles randomized into the laser group underwent hatching using the ZILOS-tk laser (Hamilton Thorne Research, Beverly, MA) mounted on a Nikon diaphot phase contrast inverted microscope equipped with a heated microscope stage. Before hatching, the embryos selected for transfer were rinsed and moved to the embryo transfer dish containing 0.05-mL droplets of IVC-THREE + 10% SSS. To perform the procedure, the embryo transfer dish was placed on the stage of the microscope and using the 40 \times laser-grade objective lens, the embryo positioned so a portion of the zona was in the path of the laser beam. The laser beam was activated using a pulse duration of 0.5 milliseconds and power of 300 mW and the laser fired to create a hole in the zona. If the hole did not breach the entire thickness of the zona, then it was enlarged by applying an additional laser pulse. Following hatching, the embryo transfer dish was removed from the stage of the microscope and returned to the incubator until the time of embryo transfer.

Embryo Transfer

Embryo transfers for the acid, laser, and thin study groups were performed on the morning of day 3. When multiple embryos were available, the Veeck criteria (18) were used to select the highest grade embryos for transfer. In general, two embryos were transferred to patients <35 years of age (yoa), three embryos transferred to patients 35 to 39 years of age, and ≥ 4 embryos in patients 40 years of age and older. These guidelines were modified for patients who had failed to conceive in two or more previous IVF cycles.

All patients with four or more embryos at the seven-cell stage or greater underwent a day 5 transfer. These embryos remained unhatched and were selected based on quality and appearance of the trophoblast and inner cell mass. For day 5 transfers, two embryos were transferred to patients <39 years of age and two to three embryos were transferred to patients 40 years of age and older. These guidelines were also modified for patients who had failed to conceive in two or more previous IVF cycles.

All transfers, regardless of day, were performed using a Wallace catheter (Irvine Scientific) with the patient in the dorsolithotomy position with a full bladder. Patients were instructed to remain resting on their backs for at least 30 minutes following the embryo transfer.

Progesterone in oil was administered on the day of retrieval (25 mg IM) and then daily (50 mg IM) for 8 weeks unless a negative beta-hCG was obtained. Patients ≥ 40 years of age were administered progesterone in oil (50 mg IM) on the day of retrieval and then daily (100 mg IM) for 8 weeks unless a negative beta-hCG was obtained.

All patients underwent an initial serum test for beta-hCG 12 to 14 days after embryo transfer. A clinical pregnancy was defined as the presence of fetal heart activity by ultrasound at 6 to 7 weeks. An ongoing pregnancy was defined as a pregnancy >21 weeks of gestation.

Statistical Analysis

Cycle demographics and treatment cycle variables were compared using either a Kruskal-Wallis test or a one-way analysis of variance. Proportions were analyzed with a 4×4 chi-squared test for independence followed by a 2×2 Fisher's exact test in which significance across all four groups had been identified. In all cases, $P < .05$ was considered statistically significant (GraphPad InStat version 5.1, GraphPad Software, San Diego CA).

RESULTS

Data was collected from a total of 134 patients undergoing 159 treatment cycles with 57 cycles randomized into the laser group and 54 randomized into the acid group. An additional 40 cycles met the criteria for a day 5 transfer while 8 cycles had no embryos hatched because all zonae measured <13 μL thick. Neither procedure used for assisted hatching, laser and acidified medium, resulted in visible damage to blastomeres or the embryos themselves.

Table 1 shows the demographic information for the cycles studied. Number of cycle attempts, the percentage of patients having intracytoplasmic sperm injection, zona thickness, and the percent distribution of primary diagnosis were not significantly different among the four groups.

TABLE 1

Cycle demographics.

Variables	Treatment			
	Laser (n = 57)	Acid (n = 54)	Day 5 (n = 40)	Thin (n = 8)
Age (y)	35.2 \pm 4.6 ^a	34.0 \pm 4.5	33.3 \pm 3.7 ^a	30.6 \pm 3.5
No. of attempts	1.9 \pm 0.2	1.6 \pm 0.1	1.4 \pm 0.1	2.0 \pm 0.3
Zona thickness	17.7 \pm 2.7	17.2 \pm 2.8	—	—
Patients with ICSI (%)	49.1	64.8	47.5	37.5
Primary diagnosis (% of total)				
Male	28.1	25.9	27.5	12.5
Tubal	31.6	14.8	25.0	37.5
Unexplained	17.5	18.5	27.5	0
Endometriosis	1.8	16.6	12.5	12.5
Diminished ovarian reserve	7.0	9.3	0	0
Ovulation dysfunction	14.0	13.0	2.5	37.5
Other	0	1.9	5.0	0

Note: Values are mean \pm SD. Values with the same superscript letters are statistically different.

^a $P = .016$.

Lanzendorf. Laser-assisted hatching. *Fertil Steril* 2007.

TABLE 2

Cycle treatment variables.

	Laser (n = 57)	Acid (n = 54)	Day 5 (n = 40)	Thin (n = 8)
No. of ampules	34.2 ± 14.7	30.8 ± 13.8	30.3 ± 11.4	26.1 ± 7.8
Peak estradiol (pg/mL)	1632 ± 782 ^g	1765 ± 673	2114 ± 818 ^g	16234 ± 393
No. of oocytes	9.2 ± 5.2 ^a	9.9 ± 4.1 ^b	15.0 ± 7.1 ^{a,b}	10.0 ± 3.3
No. of 2PN	5.6 ± 3.8 ^c	5.7 ± 3.0 ^d	10.2 ± 4.3 ^{c,d}	6.9 ± 3.4
No. of embryos transferred	2.5 ± 0.7 ^e	2.5 ± 0.7 ^f	2.1 ± 0.3 ^{e,f}	2.4 ± 0.5

Note: Values are mean ± SD. Groups with the same superscript are significantly different.

^{a,b,c,d,e} $P < .001$; ^f $P < .01$; ^g $P < .02$.

Lanzendorf. Laser-assisted hatching. Fertil Steril 2007.

However, the age of patients undergoing laser hatching was significantly higher than the age of patients that underwent a day 5 embryo transfer ($P < .016$). No difference was noted in the ages of patients in the acid, day 5 and thin groups.

Cycle treatment variables between the four groups are presented in Table 2. No difference was noted in the number of ampules of FSH and hMG used between the four groups. Peak estradiol was significantly lower in the laser group compared to patients receiving a day 5 transfer ($P < .02$), while no difference was noted in peak estradiol between the acid, day 5, and thin groups. As expected, the number of oocytes retrieved, the number of embryos produced, and the numbers of embryos transferred were significantly different in the laser ($P < .001$) and acid ($P < .01$) groups when compared to the day 5 group.

No significant differences were noted in clinical or ongoing pregnancy rates between the four groups (Table 3). The implantation rate for day 5 transfers was significantly greater than both the laser ($P < .0008$) and acid ($P < .0061$) treatment groups. No significant differences in singleton and multiple pregnancies were noted between the four groups.

A total of 21 patients completed two or more cycles in the study with 16 patients completing cycles in both the laser and acid groups. Two of these patients achieved a clinical pregnancy in both groups, eight patients failed to achieve a clinical pregnancy in either group, while three patients achieved a clinical pregnancy in the acid group and not in the laser group. Two patients achieved a clinical pregnancy in the laser group and not in the acid group. An additional patient failed to achieve a pregnancy in both the acid and thin groups but did achieve a clinical pregnancy in the laser group.

One patient who consented to the study was a day 5 transfer in both her cycles and she became pregnant in both. Four patients failed to achieve a clinical pregnancy following a day 5 transfer but, upon returning for another study cycle, one in the laser group and two of three in the acid group obtained a clinical pregnancy.

Of the 70 ongoing pregnancies that were established during this study, 68 patients have delivered and two pregnancies (one singleton in the laser group and one twin in the acid group) are ongoing. To date, a total of 91 children

TABLE 3

Pregnancy outcomes depending on treatment group.

Outcome	Treatment group			
	Laser	Acid	Day 5	Thin zonae
Clinical pregnancies (% ET)	26/57 (45.6)	25/54 (46.3)	27/40 (67.5)	5/8 (62.5)
Ongoing pregnancies (% ET)	22/57 (39.0)	20/54 (37.0)	24/40 (60.0)	4/8 (50.0)
Implantation (%)	37/145 (25.5) ^a	40/136 (29.4) ^b	40/83 (48.2) ^{a,b}	6/19 (32)
Plurality				
Singletons	17 (77.3)	11 (55.0)	16 (66.7)	3 (75.0)
Twins	3 (13.6)	7 (35.0)	8 (33.3)	1 (25.0)
Triplet	2 (9.1)	2 (10.0)	0	0

Note: Groups with same superscript are significantly different.

^a $P < .0008$; ^b $P < .0061$.

Lanzendorf. Laser-assisted hatching. Fertil Steril 2007.

have been born (48 males and 43 females) with 25, 29, 32, and 5 from the laser, acid, day 5, and thin groups, respectively. Pregnancy complications include one delivery of stillborn twins at 22 weeks in the laser group to a patient with a prior second trimester loss. One molar pregnancy occurred in a 26 year old patient in the acid group. There were no complications in either the day 5 or thin groups.

During the course of the study, two patients in the acid group were found to be pregnant with monozygotic twins. The first pregnancy resulted from the transfer of two embryos producing a dichorionic/triamniotic pregnancy with a singleton and diamniotic/monochorionic twins. Fetal demise of the diamniotic/monochorionic twins was documented at 16 3/7 weeks' gestation. The pregnancy progressed to 38 weeks with delivery of a healthy, singleton male.

The second pregnancy also resulted from the transfer of two embryos and was also a dichorionic/triamniotic pregnancy with a singleton and diamniotic/monochorionic twins. This pregnancy went to term with the delivery of three infants but with placenta abruption of the singleton that died at 18 weeks of age of kidney failure. The diamniotic/monochorionic twins, both males, are healthy.

DISCUSSION

Prospective, randomized studies have been performed to determine if assisted hatching improves implantation and/or pregnancy rates. Three studies that evaluated "broad" patient populations (patients >36 years of age, all patients, and good prognosis patients, respectively) found no benefit in assisted hatching (17, 19, 20). Three other studies reported significant increases in clinical pregnancy and implantation rates in patients with multiple IVF failures and/or patients >38 years of age (21–23). Regardless, most IVF programs in the United States offer assisted hatching as a clinical procedure to their infertile patients. Many centers employ the technique that utilizes acidified medium to create the opening in the zona. Others use the "slicing" technique to create the opening. More recently however, many IVF programs in the United States and abroad utilize a 1.48-micron infrared diode laser to produce the opening in the zona.

The use of lasers for assisted hatching has been discussed in the scientific literature since the early 1990s (24–26). Studies evaluating lasers for manipulation of oocytes and embryos first focused on equipment that required contact with the study material and used solutions that were found to be toxic to oocytes and embryos. In addition, the specialized micropipettes and/or fiber optics were difficult to produce and/or sterilize. Another problem encountered with these earlier lasers was the possible mutagenic effect on the oocytes and embryos because they used radiation with wavelengths close to the absorption peak of DNA.

More recent studies have demonstrated the benefits of the 1.48-micron infrared diode laser, including the fact that

the laser light can be directed up through the objective of a microscope so there is no contact between the equipment and the materials to be manipulated. In addition, the laser light has minimal absorption by the culture dish and medium so that it can pass through many different substances before reaching the zona pellucida. In addition, the emitted wavelength is far from the absorption peak of DNA, decreasing the likelihood of a mutagenic affect on the embryo. The precise position of the laser on the target, in this case the zona pellucida, is determined by the operator with the assistance of a computer allowing the operator to control very precisely the size of the opening so that adjacent cells remain unaffected.

In the mouse model, Germond and coworkers (5, 6) were able to demonstrate normal, live-born offspring that were fertile for four generations from embryos that had been zona dissected with the 1.48-micron infrared diode laser. Later, Montag and coinvestigators (13) found that hatching with the 1.48-micron diode laser significantly increased the number of mouse blastocysts developing to the hatching stage by day 5 compared to untreated controls. This same significant increase in hatching was seen in the bovine model using a very similar study design (7). These investigators also reported no damage to the embryos undergoing the laser-hatching procedure.

In the human model, Wong and colleagues (9) studied the effect of laser-assisted hatching on donated frozen-thawed embryos and reported no significant effect of the procedure on embryo development when compared to control embryos that were not hatched. The study also found a significant increase in the completion of in vitro hatching following the use of the laser compared to control embryos.

A potential problem with laser-assisted hatching is heating of embryo cells near the breach site in the zona pellucida. The local heating depends on the beam power and laser pulse duration. The ZILOS-tk laser system used in this study was designed to provide the intense, brief laser pulses specifically optimized for zona pellucida penetration with minimal local effects (8). In the present study, a laser power of 300 mW and pulse duration of 0.5 milliseconds was used to minimize heating of any blastomeres in the vicinity of the beam, while providing a clear drilled hole through the zona. When embryos had very thick zonae, it was often necessary to produce more than one pulse to provide an opening that breeched the entire zona. During the study, there were no instances of embryos and/or blastomeres damaged during assisted hatching with either the laser or the acidified medium.

The use of multiple short pulse durations is supported by the work of Tinney and colleagues (10). They altered the pulse duration, number of pulses, and beam intensity to determine the optimal setting for creating openings in the mouse zona pellucida. They found the 1.48-micron infrared diode laser to be safe, and significantly increased the hatching

rate with the most beneficial treatment being a lowered laser intensity with an increased number of laser pulses used.

Multiple clinical evaluations of the 1.48-micron infrared diode laser have been reported in the human. In 1997, Veiga and colleagues (27) reported its use for dissecting the zona pellucida of blastocysts for preimplantation genetic diagnosis (PGD). Boada and colleagues (28) also reported the use of this laser for PGD but using the procedure on cleavage stage human embryos. Jones and colleagues (29) found no detrimental effect on development when sibling embryos underwent embryo biopsy with a 1.48-micron infrared diode laser compared to those biopsied following hatching with acidified medium.

Although used for many years outside the United States to clinical perform assisted hatching, it was not until December of 2004 that the FDA changed the status of the 1.48-micron infrared diode laser from a Class III to a Class II device, allowing its use clinically. Before that approval, the 1.48-micron infrared diode laser could only be used in the United States for research purposes, or clinically after obtaining an IDE from the FDA. Citing the lack of delivery outcomes in the many articles previously published from IVF centers outside the United States, it was not until Hamilton Thorne appealed the classification of their ZILOS-tk into Class III that the infrared laser-hatching diode laser was reassigned to Class II. The study presented here, in fact, was initiated in an attempt to provide the FDA with delivery outcomes following laser assisted hatching in the United States.

A 2003 report has focused on the health of children born following laser-assisted hatching. Kanyo and Konc (14) reported no increase in the major congenital malformation rate or rate of chromosomal aberrations in 134 children born after laser assisted hatching. In 2004, Primi and co-investigators (15) discuss unpublished data on 62 children born following laser-assisted hatching, also with no increase in major congenital malformations or chromosomal abnormalities. In the present study, 25 healthy children have been born after laser-assisted hatching, and numerous more have been born outside of the study. Although the number of cases are small, there has been no evidence of an increase in genetic abnormalities in these children compared to those conceived following assisted hatching with acidified medium (acid) or those that were not hatched before embryo transfer (day 5 and thin).

Twenty-five percent of the cycles in the study fit the criteria for a day 5 transfer. These results were included for control purposes and to validate our selection criteria. As expected, the number of oocytes retrieved and the numbers of embryos produced in the two hatching groups were significantly lower than in the day 5 transfer group. In addition, the number of embryos transferred on day 5 was significantly lower compared to the laser and acid groups. What was unexpected was the finding that only the laser

group, and not the acid group, were significantly older than the day 5 patients. In addition, only the laser group had peak estradiol levels that were significantly lower than the day 5. It is possible that if the study had continued with larger numbers in each study group, a significant difference may have been found between the acid and the day 5 groups.

No significant differences were noted in implantation or clinical and ongoing pregnancies between the two test groups, laser and acid (Table 3). Although not significant, both the clinical and ongoing pregnancy rates were higher in the day 5 transfers. However, the implantation rate for day 5 transfers was significantly higher than the two test groups, supporting the practice of transferring fewer embryos on day 5.

The findings reported in this study are limited by both the small sample sizes within the study groups as well as the use of multiple cycles in some of the patients. We acknowledge that the small patient numbers provide limited power to detect small increases in both benefits as well as the detection of adverse effects of the laser. At the suggestion of our institutional review board, patients who did not achieve an ongoing pregnancy in their first study cycle were allowed to again participate in the study but in the other treatment group. This ensured that patients had the opportunity of any potential benefit that the study may have provided.

In this study, we report two incidences of monozygotic twins with both pregnancies established in the acid group. The risk of monozygotic twinning does appear to be increased following assisted reproductive technologies (30) with reported rates of 1.2% to 5%. This is compared to 0.42% of live births in the general population.

Although the actual cause of this increase remains unclear, theories include the assisted hatching procedure (31, 32), blastocysts transfers (33–35), or possibly the combination of events occurring during the IVF treatment cycle (36, 37). Our program also has an increased rate of monozygotic twinning (2.9% per live birth), but to date, no correlation has been found with the type of manipulation or day of transfer (unpublished data). The analysis of a larger number of IVF cycle outcomes are needed to determine if assisted reproductive procedures are causing an increase in monozygotic twinning.

In conclusion, we report the successful use of the diode laser for assisted hatching in our IVF program. No significant differences in clinical outcomes were observed compared to cycles in which assisted hatching was performed using acidified medium. The laser allows the performance of the procedure in a rapid and safe manner, with fewer dish changes and less time outside the incubators. In addition, micromanipulation equipment and micropipettes are not required for performing the procedure. The equipment can be set so that the same parameters are used between operators, decreasing the amount of intra-assay variability. (16).

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