Comparison of Embryonic Development in Cleavage Stage Mouse Embryo Biopsy Between Acid Tyrode's Solution and Laser Assisted Techniques

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Abstract

The study was carried out to determine the effectiveness and safety of the infrared 1.48 μm laser in cleavage stage mouse embryo biopsy, compared to the conventional acid Tyrode's solution. One hundred and thirty cryopreserved cleavage stage mouse embryos were included in the study. Fifty embryos were biopsied by acid Tyrode's solution. Forty-seven embryos were biopsied by the infrared 1.48 μm laser. Thirty-three embryos were incubated without biopsy as the control group. Thirteen of 50 embryos in the acid Tyrode's group and 16 of 47 in the laser assisted group became cavitating morulae on day 4, meanwhile 23 of 33 in the control group reached this stage. The blastocyst formation of acid Tyrode's, laser assisted and control group were 94.0, 97.8 and 100.0 per cent, respectively. The hatching rate of acid Tyrode's solution, laser assisted and control group were 78.7, 84.7 and 63.6 per cent, respectively. No significant difference in blastocyst formation and hatching rate was found. The percentage of grade 1 blastocysts in control group (96.9%) was significantly higher than those in acid Tyrode's solution (68.0%) and the laser assisted group (76.0%). There was no significant difference in the percentage of grade 1 blastocysts between the acid Tyrode's solution and the laser assisted group. In conclusion, the infrared 1.48 μm wavelength laser may be an alternative to acid Tyrode's solution in embryo biopsy.

Key word : Laser, Acid Tyrode's Solution, Embryo Biopsy, Preimplantation Genetic Diagnosis, Blastocyst

PHOPHONG P, DOSHI A, HARPER JC
J Med Assoc Thai 2001; 84: 1190-1198

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Preimplantation genetic diagnosis (PGD) has provided the opportunity of selection and transfer of unaffected embryos to couples with inherited diseases, leading to hopefully normal pregnancies and also avoiding termination of affected pregnancies[1]. This technique has offered an alternative to prenatal diagnosis (PND) for detecting single gene defects and aneuploidy as well as structural chromosomal abnormalities[2-4]. PGD consists of three important processes, which are *in vitro* fertilisation (IVF), isolation of genetic material from preconception oocytes or embryos at various stages of preimplantation development, and genetic diagnosis based on polymerase chain reaction (PCR) or fluorescent in situ hybridisation (FISH). With regard to the methods of zona pellucida opening in embryo biopsy for PGD, acid Tyrode's solution has been used by most centres because of its acceptable effectiveness and safety[5]. Recently, laser assisted opening of the zona pellucida by a non-contact infrared 1.48 μm diode laser has been introduced[6-8]. This technique has been introduced for polar body biopsy[9] as well as mouse and human blastocyst biopsy[10-12]. The efficiency of the procedure and blastocyst recovery rate have been encouraging. However, to date this technique has not been used clinically since more information is required to confirm its effectiveness and safety[5].

This study was carried out to determine the effectiveness and safety of laser assisted embryo biopsy in cleavage stage mouse embryos, compared to conventional embryo biopsy using acid Tyrode's solution.

MATERIAL AND METHOD

Cryopreserved-thawing of cleavage mouse embryos

Cryopreserved cleavage stage mouse embryos strain 3HI, the hybrid of C3H/HCH and 101/H, (the Mammalian Genetics Unit, Medical Research Council, Oxford) were used in this study. The cryopreserved straw containing the mouse embryos and cryoprotectant (1.5 M propyleneglycol) was held in the air for 40 seconds and in water at room temperature until the ice crystals disappeared. The contents of the straw were allowed to run out into a tissue culture dish (Falcon, USA) and incubated for 5 minutes at room temperature. The embryos were placed in medium M2 (Sigma, UK) for 5 minutes and rinsed in preequilibrated IVF medium (Medi-Cult, Denmark) covered by mineral oil (Sigma, UK). All embryos were eventually incubated under the culture condition of 37°C and 6 per cent carbon dioxide for 1 hour.

Evaluation and randomisation of thawed embryos

The thawed embryos and all blastomeres were evaluated and counted. Only the embryos containing more than or equal to 6 blastomeres were enrolled in the study. All enrolled embryos were randomly divided into three groups, which were

Table 1. Criteria used for grading the embryos on day 4 and 5 of embryonic development[13,14].

Categories	Descriptions
Degenerative	Embryo with degenerative blastomeres
Arrested	Embryo with the unchanged number of blastomeres
Morula	Embryo that increased the number of blastomeres so that it became morular shaped
Cavitating morula	Morula contained one or more cavities, which were initially small and eccentrically placed, gradually expanded to occupy most of the volume of the embryos. The cavities were possibly lined with trophectoderm or inner cell mass (ICM).
Blastocyst	The morula developed into blastocyst, where the blastocoelic cavity was largely lined by a single layer of trophectoderm and locally by the ICM.
	Grade 1 blastocyst: typical development, characterised by early cavitation resulting in the formation of as eccentric and then expanded cavity lined by a distinct ICM and trophectoderm
	Grade 2 blastocyst: the delay in the appearance of morphological differentiation of the two cell types of the blastocyst usually resulted in the typical blastocyst stage being reached a day or two after initial cavitation
	Grade 3 blastocyst: blastocyst that on the day of formation had several degenerative foci in the ICM and the cavity collapsed within 24 hours without expanding significantly or blastocysts that had a vacuolated appearance initially and then showed extensive degenerative foci on reaching the blastocyst stage

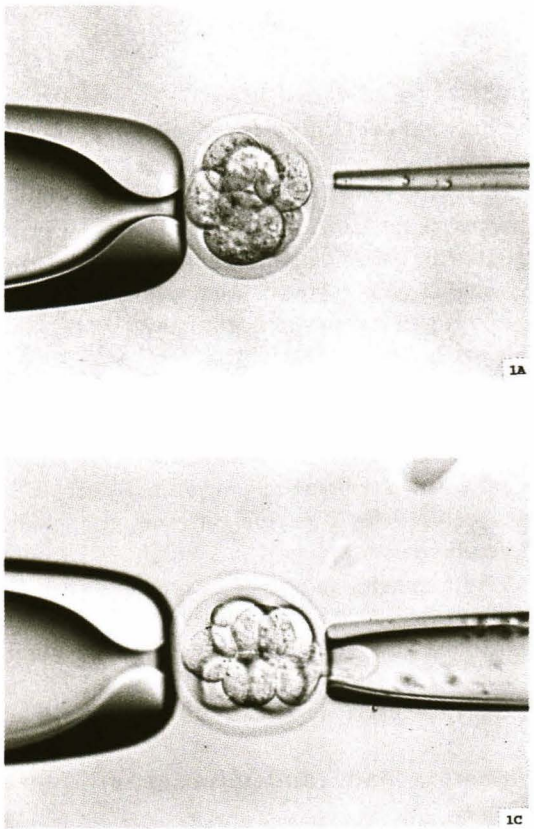

Fig. 1. Embryo biopsy, using acid Tyrode's solution for zona pellucida drilling.

A) Acid Tyrode's solution was expelled to dissolve the zona pellucida by drilling micropipette.
B) The drilled embryo with an openning in zona pellucida (arrow).
C) A blastomere was removed by biopsy micropipette.

acid Tyrode's, laser assisted and control groups. The embryos in the acid Tyrode's group were biopsied with acid Tyrode's solution. The embryos in the laser assisted group were biopsied with 1.48 μm diode laser. The embryos in the control group were transferred to a tissue culture dish containing 20 μl drops of preequilibrated IVF medium covered by mineral oil and incubated at culture condition. The embryos of the three groups were scored on day 4 and 5 of embryonic development, using the criteria in Table 1.

Embryo biopsy with acid Tyrode's solution

Three micromanipulators (Research Instruments Limited, UK), with an Olympus microscope were used. A stage warmer (Research Instruments Limited, UK) was used to maintain the temperature at 37°C during micromanipulation. The embryos were transferred to a biopsy dish (Falcon, USA) containing 20 μl drops of Ca^{2+} and Mg^{2+} free medium (IVF Science Scandinavia, Sweden) and a 20 μl drop of acid Tyrode's solution (IVF Science Scandinavia, Sweden) covered by mineral oil (Sigma, UK). The first micromanipulator was used to control a holding micropipette (Cook, Australia). The second and third controlled zona drilling (Cook, Australia) and biopsy micropipettes (Cook, Australia), respectively. The holding and biopsy micropipettes were filled with the embryo biopsy medium, and the zona drilling micropipette was filled with acid Tyrode's solution.

The embryo was immobilised by gentle suction on a holding micropipette controlled by a micromanipulator. The drilling micropipette was moved close to the zona pellucida and acid Tyrode's solution (pH 2.3) was gently expelled to gradually create an opening in the zona pellucida at 3 o'clock position, shown in Fig. 1A, B. The drilling micropipette was immediately withdrawn when the opening occurred. The biopsy micropipette was moved to the opening and contacted with the blastomere to be biopsied. Usually only one blastomere was aspirated, shown in Fig. 1C. The embryo post biopsy was transferred to a 20 μl drop of preequilibrated IVF medium covered by mineral oil and incubated at culture condition.

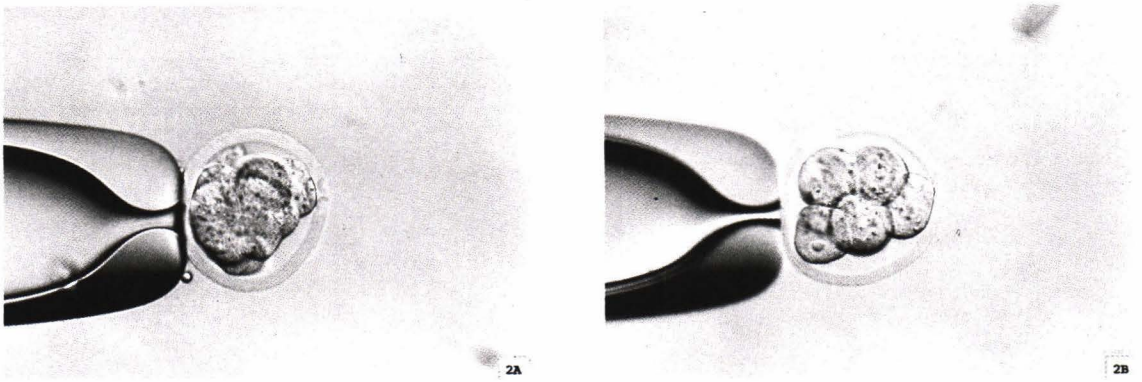

Fig. 2. Embryo biopsy, using laser assisted technique for zona pellucida drilling.

A) An opening in the zona pellucida was created by the laser beam (arrow).
B) The drilled embryo with an opening in the zona pellucida (arrow).

Embryo biopsy with laser assisted micromanipulator

The laser system (Saturn laser system) was provided by Research Instruments Limited (UK). The set-up for zona pellucida drilling was used as described by Germond et al (1995, 1996). The 670 nm diode laser aiming beam and the collimated 1.48 μm laser beam were fed into an inverted microscope through several mirrors and focused by the microscope objective (x100) within the microscope field in a spot of 4 μm in diameter. The power routinely available at the image plane of the objective was 1,480 nm/110 mW, pulse mode was up to 750 ms, corresponding to a maximal power density of 380 kW/cm^2.

The embryo was immobilised and positioned at 3 o'clock of the zona pellucida on the aiming laser spot by gentle suction on a holding micropipette. The zona pellucida was exposed to the laser beam with the lowest power that was enough to gradually dissolve the zona pellucida from the outer surface until the perforation of the zona pellucida occurred, shown in Fig. 2A, B. It was important to perforate the zona pellucida completely but not to harm the nearby blastomeres. The diameter of the opening was approximately two-thirds of the diameter of the blastomere to be aspirated. The biopsy micropipette was moved into contact with the opening and usually one blastomere was removed. The biopsied embryo was finally transferred to a 20 μl drop of preequilibrated IVF medium covered by mineral oil and incubated at culture condition.

The outcome measurements and statistical analysis

Statistical analysis was performed with the use of epi-info programme (version 6.0). Embryonic development, blastocyst formation, grade of the blastocysts and hatching rate were statistically analysed by chi-square and student's *t*-test. Statistical significance was defined as P value ≤ 0.05.

RESULTS

One hundred and thirty thawed embryos were included in the study. The number of embryos in the acid Tyrode's, laser assisted and control group were 50, 47 and 33, respectively. The embryonic development of all three groups is shown in Table 2. There was a significant delay of growth development on day 4 of the acid Tyrode's and laser assisted groups. Only 13 of 50 (26.0%) in the acid Tyrode's group and 16 of 47 (34.0%) in the laser assisted group became cavitating morulae, meanwhile 23 of 33 (69.7%) in the control group reached this stage.

The number of blastocysts obtained from the acid Tyrode's, laser assisted and control groups were 47, 46 and 33, respectively. The blastocyst formation of the acid Tyrode's, laser assisted and control groups were 94.0, 97.8 and 100.0 per cent, respectively. No significant difference in blastocyst

Table 2. Embryonic development, blastocyst formation, grade of the blastocysts and hatching rates of the studied groups.

Variables	Acid Tyrode's	Laser assisted	Control
No. of embryos	50	47	33
Day 4 embryonic development stage[a]			
- Degenerative	-	-	-
- Arrested	1	-	-
- Morulae	36	31	10
- Cavitating morulae	13	15	23
- Blastocyst	-	1	-
Day 5 embryonic development stage			
- Degenerative	1	-	-
- Arrested	-	-	-
- Morulae	1	-	-
- Cavitating morulae	1	1	-
- Blastocyst	47	46	33
Blastocyst formation (%)	47/50 (94.0%)	46/47 (97.8%)	33/33 (100.0%)
Blastocyst grading[b]			
- Grade 1	32	35	32
- Grade 2	12	9	1
- Grade 3	3	2	-
Hatching rate (%)	37/47 (78.7%)	39/46 (84.7%)	21/33 (63.6%)

a, b Significant difference between acid Tyrode's and control group, and laser assisted and control group

formation was found in all groups. The percentage of grade 1 blastocysts in the control group (32/33, 96.9%) was significantly higher than those of the acid Tyrode's (32/47, 68.0%) and laser assisted group (35/46, 76.0%). There was no significant difference in the percentage of grade 1 blastocysts between the acid Tyrode's and laser assisted group. The hatching rates of the three groups were not significantly different. The hatching rates of the acid Tyrode's, laser assisted and control groups were 78.7, 84.7 and 63.6 per cent, respectively.

DISCUSSION

Isolation of the genetic materials from preconception oocytes or preimplantation embryos is one of the most important and sensitive processes in PGD. Several methods have been introduced to improve the success rate of the procedure and survival of the embryos. Among the developed methods, drilling the zona pellucida of the oocytes and embryos with acid Tyrode's solution has been commonly used by most centres[5]. However, the main drawback of the use of acid Tyrode's solution was its toxicity and cytoplasmic acidification leading to cytoplasmic degeneration[15]. Exposure of 4-cell stage embryos to acid Tyrode's solution has been proposed to reduce their viability[16,17] and preimplantation development[16,18]. Although Cui et al[19,20] and Viville et al[21] reported that no significant adverse effect was found in the embryos biopsied by acid Tyrode's solution and more biopsy methods should be developed for substitution of this conventional method.

Several wavelengths of laser have been studied for their effectiveness and safety[22-26]. The infrared 1.48 μm wavelength laser has been reported to be an ideal laser with its effectiveness, safety and non-contact mode[7,27]. Several studies of the infrared 1.48 μm wavelength laser in mouse and human gametes and embryos have been performed to demonstrate its performance[6,8,9,12]. However, the comparison of this laser and the conventional acid Tyrode's solution, in terms of effectiveness and safety for embryo biopsy, has not been reported. The infrared 1.48 μm wavelength laser, therefore, was used in this study.

The biopsy procedures, both acid Tyrode's solution and laser assisted techniques, may produce growth delay on day 4 and 5 of embryonic development (Table 2). The growth delay might be the effect of either the biopsy procedures or Ca^{2+} and Mg^{2+} free medium. The Ca^{2+}-dependent cell adhesion molecule E-cadherin has been thought to be an essential factor in the formation of intercellular contact between blastomeres resulting in initiation and maintenance of compaction[28,29].

Prolonged exposure to Ca^{2+} and Mg^{2+} free medium may result in decreased survival rate and impaired development in mouse embryos[30-32]. Some studies, however, have shown no adverse developmental potential in the exposure of this substance to mouse and human embryos[33,34]. The exposure time of Ca^{2+} and Mg^{2+} free medium to the embryos in this study was less than that in the study of Dumoulin et al[34]. Therefore, the length of exposure time might not be the cause of growth delay. If the embryos in the control group were also incubated in Ca^{2+} and Mg^{2+} free medium, the effect of the biopsy procedures on embryonic development would be clearly determined. Embryo grading has been reported to influence the decision of which embryo should be transferred in IVF[35,36]. Since the embryos following biopsy are usually evaluated and transferred on day 0 or 1 post biopsy, the growth delay on day 1 post biopsy might affect the decision making and also prognosis interpretation. More research to determine the effect on day 1 post biopsy of the Ca^{2+} and Mg^{2+} free medium and biopsy procedures should be carried out.

The blastocyst formation of all studied groups was similar (Table 2). The growth delay on day 1 post biopsy of the acid Tyrode's and laser assisted groups did not influence the blastocyst formation. The biopsied embryos could overcome the growth delay and became blastocysts on day 5 of embryonic development. Grading of human blastocysts has been suggested to be useful for selecting the blastocysts for transferring or cryopreservation[14,37]. Transferring of one high-grade blastocyst was reported to give an implantation rate of 50 per cent and pregnancy rate of 70 per cent[37]. Although the percentages of grade 1 blastocysts in both laser assisted and acid Tyrode's groups were significantly lower than that in the control group and grade of the blastocysts in laser assisted group was similar to that in acid Tyrode's group. The effectiveness and safety of the laser assisted technique may be equal to that of the acid Tyrode's. Furthermore, the laser assisted technique was found to be an easier and quicker procedure without the requirement of a drilling micropipette, micropipette changing and the third micromanipulator. The size of the opening in the zona pellucida created by the laser was found to be under control, whereas the use of acid Tyrode's solution could not offer this capability.

The hatching rates of acid Tyrode's and laser assisted groups were higher than that of the control group but the difference was not statistically significant (Table 2). The zona pellucida of embryos *in vitro* has been thought to be hard due to a suboptimal culture condition and only 38.5 per cent of blastocysts hatched[38]. From the observation in this study, hatching embryos herniated through the openings created by biopsy procedures. The opening in the zona pellucida might offer an easier chance of hatching. However, the opening in zona pelucida created by the biopsy procedures may not increase implantation and pregnancy rates since the implantation and pregnancy rates following embryo biopsies for PGD were lower than those of normal infertile patients[5].

In conclusion, the infrared 1.48 μm wavelength laser may be an alternative to acid Tyrode's solution in embryo biopsy. The laser-assisted technique also has been found to be an easy and quick procedure. The effect of Ca^{2+} and Mg^{2+} free medium in the biopsied groups, however, might influence the embryonic development in this study. It is important to ensure that the new equipment is effective and safe for use in PGD. Other parameters, such as number of blastocyst nuclei including inner cell mass and trophectoderm cells, secreted HCG levels and pyruvate uptake of the embryos should be determined. *In vivo* animal studies and, eventually, *in vitro* studies in human embryos should be performed before accepting this biopsy technique for universal clinical practice.

(Received for publication on March 18, 2001)

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เปรียบเทียบการเจริญของตัวอ่อนหนูหลังการแยกบลาสโตเมียร์ระยะคลีเวจระหว่างวิธีใช้สารละลายกรดธัยโรดส์และเลเซอร์

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ได้ทำการศึกษาเปรียบเทียบถึงประสิทธิผลและความปลอดภัยระหว่างการใช้เลเซอร์ความยาวคลื่น 1.48 ไมโครเมตร และสารละลายกรดธัยโรดส์ในการแยกบลาสโตเมียร์ออกมาจากตัวอ่อนหนูแซ่แข็ง โดยศึกษาจากตัวอ่อนหนูแซ่แข็งจำนวน 130 ตัว ตัวอ่อนหนูจำนวน 50 ตัวถูกแยกบลาสโตเมียร์ออกมาโดยใช้สารละลายกรดธัยโรดส์สลายโซนา เพลลูซิดา จำนวน 47 ตัวถูกแยกเซลล์บลาสโตเมียร์โดยใช้เลเซอร์ความยาวคลื่น 1.48 ไมโครเมตรสลายโซนาเพลลูซิดา และจำนวน 33 ตัวไม่ได้ถูกแยกบลาสโตเมียร์ออกจัดเป็นกลุ่มควบคุม ตัวอ่อนหนูในกลุ่มสารละลายกรดธัยโรดส์จำนวน 13 ใน 50 และตัวอ่อนหนูในกลุ่มเลเซอร์จำนวน 16 ใน 47 เจริญเป็นตัวอ่อนระยะโมรูล่าที่เริ่มปรากฏช่องภายใน ในขณะที่ตัวอ่อนหนูในกลุ่มควบคุมจำนวน 23 ใน 33 สามารถเจริญเป็นตัวอ่อนในระยะนี้ในวันที่ 4 ของการเจริญเติบโต อัตราการเจริญเป็นตัวอ่อนระยะบลาสโตซิสต์ของกลุ่มสารละลายกรดธัยโรดส์ กลุ่มเลเซอร์ และกลุ่มควบคุมได้แก่ร้อยละ 94.0, 97.8 และ 100, ตามลำดับ อัตราการฟักตัวของตัวอ่อนระยะบลาสโตซิสต์ของกลุ่มสารละลายกรดธัยโรดส์ กลุ่มเลเซอร์ และกลุ่มควบคุมได้แก่ร้อยละ 78.7, 84.7 และ 63.6, ตามลำดับ ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติในอัตราการเจริญเป็นตัวอ่อนระยะบลาสโตซิสต์และการฟักตัวของตัวอ่อนระยะบลาสโตซิสต์ของทั้งสามกลุ่ม ร้อยละของตัวอ่อนระยะบลาสโตซิสต์ที่มีลักษณะดีชั้นหนึ่งในกลุ่มควบคุมสูงกว่ากลุ่มสารละลายกรดธัยโรดส์และเลเซอร์อย่างมีนัยสำคัญทางสถิติ แต่ไม่พบความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่มสารละลายกรดธัยโรดส์และเลเซอร์ สรุปได้ว่า เลเซอร์ความยาวคลื่น 1.48 ไมโครเมตรอาจสามารถถูกนำมาใช้แทนสารละลายกรดธัยโรดส์ในการสลายโซนา เพลลูซิดาสำหรับขั้นตอนแยกเซลล์บลาสโตเมียร์ในการวินิจฉัยทางพันธุกรรมในตัวอ่อนระยะก่อนฝังตัว

คำสำคัญ : เลเซอร์, สารละลายกรดธัยโรดส์, การแยกบลาสโตเมียร์จากตัวอ่อน, การวินิจฉัยทางพันธุกรรมในตัวอ่อนระยะก่อนฝังตัว, บลาสโตซิสต์

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จดหมายเหตุทางแพทย์ ฯ 2544; 84: 1190–1198

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