นิพนธ์ต้นฉบับ • Original Article

การลดลงของศักยภาพในการฝังตัวของตัวอ่อน เนื่องจากผลของแอมโมเนียมคลอไรด์

สุพัชญ์ สีนะวัฒน์, กนก สีจร
หน่วยชีววิทยาการเจริญพันธุ์ ภาควิชาสูติศาสตร์และนรีเวชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

Impaired Implantation Potential, A Dose-Dependent Effect of Preimplantation Exposure to Ammonium Chloride

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วัตถุประสงค์การวิจัย : เพื่อศึกษาผลของแอมโมเนียมคลอไรด์ (NH4Cl) ขนาดความเข้มข้น 0.3 และ 0.6 มิลลิโมลาร์ ในน้ำยาเลี้ยงตัวอ่อนของหนูสายพันธุ์ (F_1 x F_1) ที่มีต่อการเจริญของหนูทั้งในระยะก่อนและระยะหลังการฝังตัวของตัวอ่อน

กระบวนการวิจัย : ตัวอ่อนของหนูสายพันธุ์ (F_1 x F_1) ระยะ 1 เซลล์ จำนวน 267 ตัว ได้ถูกแบ่งเป็นสามกลุ่มโดยวิธีการสุ่มเพื่อเลี้ยงต่อในน้ำยาเลี้ยงตัวอ่อน 3 ชนิด คือ น้ำยา M16, น้ำยา M16+ แอมโมเนียมคลอไรด์ ในขนาด 0.3 มิลลิโมลาร์ และน้ำยา M16+ แอมโมเนียมคลอไรด์ในขนาด 0.6 มิลลิโมลาร์ ตัวอ่อนทั้งสามกลุ่มจะถูกเลี้ยงในน้ำยาเลี้ยงตัวอ่อนเป็นเวลา 3 วัน ก่อนที่จะถูกย้ายฝากเข้าไปในโพรงมดลูกของหนูตัวเมียที่เป็นแม่อุ้มบุญ รูปร่างของตัวอ่อนจะถูกประเมินทุกวันในระยะ 3 วันที่ตัวอ่อนถูกเลี้ยงในน้ำยาเลี้ยงตัวอ่อน หนูที่เป็นแม่อุ้มบุญจะถูกฆ่าเมื่อตั้งครรภ์ได้ 15.5 วัน เพื่อตรวจประเมินโพรงมดลูกถึงจำนวนของการฝังตัว จำนวนของทารก และจำนวนของการแท้ง ตลอดจนประเมินลักษณะทั่ว ๆ ไปของทารกด้วย

ผลการวิจัย : เมื่อเปรียบเทียบระหว่างกลุ่มทดลองทั้งสองกลุ่ม (คือ กลุ่มที่ได้รับแอมโมเนียมคลอไรด์ขนาด 0.3 และ 0.6 มิลลิโมลาร์) ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างจำนวนของตัวอ่อนที่เจริญถึงระยะมอร์รูลา (morula) หลังจากเลี้ยงตัวอ่อนในน้ำยาเลี้ยงตัวอ่อนเป็นเวลา 3 วัน แต่เมื่อพิจารณาผลการทดลองหลังจากย้ายฝากตัวอ่อนเข้าสู่โพรงมดลูกจะพบว่าอัตราการฝังตัวของตัวอ่อนในกลุ่มที่ได้รับแอมโมเนียมคลอไรด์ 0.3 มิลลิโมลาร์ สูงกว่ากลุ่มที่ได้รับแอมโมเนียมคลอไรด์ 0.6 มิลลิโมลาร์ อย่างมีนัยสำคัญ (Fisher Exact test, $p < 0.05$) ยิ่งไปกว่านั้น preimplantation pregnancy loss ในกลุ่มที่ได้รับแอมโมเนียมคลอไรด์ขนาดความเข้มข้นสูง (0.6 มิลลิโมลาร์) ยังสูงกว่ากลุ่มที่ได้รับแอมโมเนียม- คลอไรด์ขนาดความเข้มข้นต่ำ (0.3 มิลลิโมลาร์) อย่างมีนัยสำคัญ (Fisher exact test, $p < 0.05$) นอกจากนั้นยังพบอุบัติการของภาวะ

Objectives : To investigate the effects of preimplantation exposure of (F1 x F1) strain mouse embryos to different dosages (0.3 VS 0.6mM) of ammonium chloride.

Study Methods : Total of 267, one-cell stage mouse embryos were randomly allocated to culture in either M16 medium (control), or the other two experimental groups which are M16+0.3 mM ammonium chloride or M16+0.6 mM ammonium chloride. Embryos were left in culture for 3 days before being transferred to 2.5 day pseudopregnant recipients. Embryo morphology was assessed after 1, 2 and 3 day of culture. The number of implantation site, fetuses, moles and any gross abnormality found were noted.

Results : There was no significant difference in the number of embryos achieved morula or more advanced stage after three days of culture between the two treatment groups. Implantation rate was significantly higher in the group of embryos exposed to 0.3 mM ammonium chloride compared to those exposed to 0.6 mM ammonium chloride (Fisher Exact test, $P<0.05$). Moreover, preimplantation pregnancy loss was significantly higher in the group of embryos exposed to 0.6 mM ammonium chloride compared to the other treatment group (Fisher exact test, $P<0.05$). There was one grossly abnormal fetus detected in the group of embryos exposed to 0.3mM ammonium chloride. The abnormality observed was polydactyly of both hind limbs with the incidence of 20% per fetus obtained.

Conclusions : The study showed that preimplantation exposure of mouse embryos to higher dosage of ammonium chloride (0.6 mM) resulted in a significant decrease in implantation rate and significant increase in preimplantation pregnancy loss when compared to a lower

polydactyly ที่ขาหลังทั้งสองข้างในตัวอ่อนที่ได้รับแอมโมเนียมคลอไรด์ในขนาด 0.3 มิลลิโมลาร์ ในอัตราร้อยละ 20 ต่อจำนวนตัวอ่อน (fetus) ที่เกิดขึ้นทั้งหมด

สรุป : การศึกษานี้บ่งชี้ว่าการสัมผัสของตัวอ่อนหนูสายพันธุ์ (F1 x F1) ต่อแอมโมเนียมคลอไรด์ในระยะก่อนการฝังตัว (preimplantation period) อาจทำให้ความสามารถในการฝังตัวของตัวอ่อนลดลงในแบบ dose - dependent

dosage of ammonium chloride (0.3 mM). This implies that preimplantation exposure to ammonium chloride impairs implantation potentials of mouse embryos, possibly in a dose-depending fashion.

ศรีนครินทร์เวชสาร 2543; 15(3), 166-173 • Srinagarind Med J 2000; 15(3), 166-173

Introduction

The developments of preimplantation stage mammalian embryos normally occur within the protected environment of the female reproductive tract. At present, preimplantation embryos of numerous experimentally useful species, plus human embryos used in the treatment of infertility, can be routinely cultured to the blastocyst stage, usually with the production of normal offspring following embryo transfer. In spite of this, embryo development *in vitro* is far from satisfactory, and much remains to be discovered. In general, development of embryos in culture is slower than *in vivo,* as manifested by reduced cell number, consistent with a progressive loss of viability and reduced metabolism[1-4]. This indicates that media currently used for embryo culture are sub-optimal. In addition, the culture environment used for preimplantation embryos can profoundly affect post-implantation events. These findings, together with the more recent report by Lane and Gardner of which demonstrated that the presence of ammonium ions in the medium of preimplantation mouse embryos was associated with fetal retardation and exencephaly in a time-and concentration-dependent manner[5], indicate that sub-optimal culture conditions may have long term effects on the offspring. If such effects occurred in the course of human *in vitro* fertilization, it could be devastating. This study is thus designed to test whether the presence of either 0.3 or 0.6 mM ammonium chloride in M16 medium is associated with abnormality in both preimplantation and postimplantation development of (F1x F1) strain mouse embryos when the embryos are cultured from 1 cell stage for the duration of 3 days.

Materials and Methods

Animals and superovulation procedure

Before the procedure of superovulation proceeded, all the mice were raised in the light-cycle controlled rooms in which they should be in for at least a fortnight. Embryos were obtained from (C5BL/Ola x CBA/Ca) F1 hybrid females. Virgin females 4-6 weeks old were superovulated with an intraperitoneal injection of 5 iu pregnant mare's serum gonadotropin (PMSG) at 12.00 hr, and followed 48 hours later by 5 iu of human chorionic gonadotropin (HCG). The superovulated females were placed with F1 males immediately following the second injection. The presence of a vaginal plug the following morning indicated that mating has taken place.

Pseudopregnant state

Pseudopregnant mice were prepared by mating 6-8 weeks old CF1 females in natural estrus with vasectomized CF1 males and the females with vaginal plugs the following morning were designated 0.5 day of pseudopregnancy. This procedure was performed on the same day that the donors of 1-cell stage embryos were checked for the vaginal plugs.

Embryo collection

Collection of 1-cell stage embryos was performed on the morning after HCG injection (day 1.5 of pregnancy). The 1 cell stage embryos, surrounded by cumulus cells, were normally found in the upper part of the oviduct which at this time was much larger and could easily be located under 20x magnification. The oviduct was placed in a drop containing M2 medium on a 35mm petri dish and was viewed through the stereomicroscope at 20 x or 40 x magnifications. One watchmaker's forceps was used to grasp the oviduct at the position next to the swollen infundibulum and it was held firmly on the bottom of the dish. The other forceps were used to tear the oviduct close to where the embryos were located, releasing the groups of embryos from the oviduct.

The 1 cell stage embryos were transferred to the glassdish containing 100 unit/ml of hyaluronidase at room temperature and were allowed to incubate in the solution

for a few minutes until the cumulus cells fell off. The embryos would not be left in the hyaluronidase for more than 1 minute after the cumulus cells were shed. The transfer pipette was used to pick up the 1-cell embryos, without cumulus cells, and transfer them to a fresh dish of M2 to rinse off the hyaluronidase. The embryos were then washed in M2 medium three times before being transferred to specific medium of interest (M16, M16 + 0.3 mM ammonium chloride, or M16 + 0.6 mM ammonium chloride) for culture at 37 °C.

Embryo culture

All culture dishes were prepared 1 day before embryo collection by dispensing 20 ul drops of each medium (M16 medium, M16 + 0.3mM ammonium chloride, or M16 +0.6mM ammonium chloride) in an array on the bottom of the 35 mm petri dish. The dish was then flooded with paraffin oil. The dishes were equilibrated in a gas of 5% CO_2 in air at 37 ° C overnight.

The embryos were transferred into the culture dishes on the following morning after the culture dishes were prepared. All embryos were cultured in group of ten in 20 ul drops of the medium under a layer of light weight paraffin oil.

Assessment of embryo morphology

Embryo morphology was determined after 22, 46, or 70 hours of culture using phase contrast microscope. Before the eight-cell stage, development was judged by counting the number of blastomeres. Embryos showing compaction and blastocoel cavity formation were classified as morulae and blastocysts, respectively. Blastocysts in the process of emerging and having emerged from the zonae pellucidae were classified as hatching and hatched blastocysts, respectively.

Classification of the embryo morphology was as follows:

2 cell stage embryo
3-4 cell stage embryo
5-8 cell stage embryo
Compacted morula
Blastocyst

After the assessment of embryo morphology was completed, the culture dishes were returned to the gas-equilibrated incubator and maintained at 37 ° C. The time periods in which the embryos assessed were minimized as much as possible to ensure that the embryos were maintained at 37 ° C most of the time.

Embryo transfer

5-7 embryos at either morula or blastocyst stage from each treatment group were separately transferred to the uterine horn of 2.5 day pseudopregnant females. After the recipient females recovered from the embryo transfer process, they were kept in the light-cycle controlled room and left for another 13 days until day 15.5 of gestation at which they would finally be sacrificed.

Assessment of the fetuses

On day 15.5 of pregnancy, the recipient females were killed. The uterine cavities were carefully examined to determine the number of implantation sites, fetuses and resorptions (or moles). Fetal growth was subsequently assessed by the scoring system developed by Wahlsten and Wainwright in 1977 [6] which was based on the development of the external features including skin, limbs, eyes, and ears. Crow-rump length, weight of the fetus and other morphological presentations of the fetus were also evaluated and recorded. Any morphological abnormalities being found were noted. Photography and bone staining of the fetus with limb abnormalities were also performed.

Media

There were four media being used in this study. The M2 medium was used for collecting and transferring embryos. The standard M16 medium was utilized for culture embryos in the control group while the M16 medium added with 0.3 or 0.6 mM ammonium chloride were used to culture embryos in the experimental groups.

The M2, M16, M16 + 0.3mM ammonium chloride, and M16 + 0.6 mM ammonium chloride were made up from individual stock solutions (as shown in table 1 and 2). All stocks were pushed through the Millipore filter and stored in refrigerator at 4°C in Falcon plastic tubes. All stocks can be kept for 3 months except stock B and C that are needed to be prepared every other week. Bovine serum albumin (BSA) was prepared every time media were made up. All salts and glucose were of Analar grade (BDH, Poole, Dordet, UK) Sodium pyruvate, sodium lactate, glutamine, ammonium chloride, and phenol red were of cell culture grade (Sigma Chemical Co, Poole, UK) Bovine serum albumin, lot 90 (Miles Pentex Crystalline) was purchased from Bayer Diagnostics.

Data collection

The embryo morphology was scored daily, in the morning (from 10.30am to 11.00 am) for the whole duration of culture. Postimplantation evaluation was performed in the morning (from 10.30 am to 11.30 am) of day 15.5 of pregnancy.

Table 1: Stock solutions for making medium M2, M16, M16+0.3mM ammonium chloride and M16 + 0.6mM ammonium chloride

Stock	Component	g/100ml
A (10 x conc)	NaCl	5.534
	KCl	0.356
	KH_2PO_4	0.162
	$MgSO_4.7H_2O$	0.294
	NaLactate	2.608
	Glucose	1.000
	Penicillin	0.060
	Streptomycin	0.050
B (10 x conc)	$NaHCO_3$	2.106
	Phenol red	0.010
C (100 x conc)	NaPyruvate	0.036/10 ml
D (100 x conc)	$CaCL_2.2H_2O$	0.252/10 ml
E (10 x conc)	Hepes*	5.957
	Phenol Red	0.010
NH_4Cl	NH_4Cl	0.016

* To make up Hepes, dissolve the solid in 40 ml H_2O + 30 ml M/5 NaOH (0.8g/ml). Adjust with more NaOH to 7.4 before making up to 100 ml.

Experimental design

There were three experiments carried out in this study. The total number of embryos being studied were 87, 80 and 70 in the first, second and third experiment, respectively. In this study, embryos were collected at 1 cell stage (day 0.5 of pregnancy). Cumulus cells were removed from the 1-cell stage embryos by incubating these embryos in 100 unit/ml of hyaluronidase and gently pipeting embryos up and down. After removing cumulus cells, the embryos were washed in M2 medium three times before being randomly allocated into one of the three study media (which was M16 medium, M16 + 0.3 mM ammonium chloride, and M16 + 0.6mM ammonium chloride). Embryos were left in culture for 3 days before being transferred to the surrogated females. Preimplantation evaluation was carried out after 1, 2 and 3 days of culture. The embryos were left in the recipients until day 15.5 of gestation at which the recipients were sacrificed and examined.

Statistical analysis

All statistical analysis was done with the software package, Multistat (Biosoft, Cambridge), on the MacIntosh computer. The data was keyed in and the type of statistical analysis chose was performed on the data. Chi-squared test was used to compare the number of embryos that achieved morula or more developmentally advanced stage, preimplantation pregnancy, postim plantation pregnancy loss, the number of fetuses obtained, and the number of fetus per implantation, between the treated group and the control group. The test gave us a value for Chi-square (x^2) and also a probability (P). Yates correction was applied when appropriate. The Fisher exact test was used where the use of the Chi-square test was inappropriate. This test gave us the probability (P) directly. Student t-test (unpaired) was also used to determine whether or not there were significant differences between weight, crown-rump length, and estimated age of the fetuses in the treated group and those from the control group. This test provided us the t-value (t), and the probability (P).

Results

When preimplantation development in culture media was started from 1 cell stage, the results turned out surprisingly (Table 3, 4, and 5). There was high incidence of developmental arrest at 2 cell stage or less in groups of embryos cultured in M16 medium, medium M16+ 0.3 mM ammonium chloride, and medium M16+ 0.6mM ammonium chloride (50.47 %, 25 %, and 16.66 %, respectively). The incidence of developmental arrest or so-called the "two cell block" of embryos cultured in M16 was significantly higher than those cultured in medium M16+0.3mM ammonium chloride or medium M16 + 0.6mM ammonium chloride ($X^2 = 14.20$, $P<0.05$; and $X^2 = 18.58$, $P<0.05$, respectively). There was no significant difference in the incidence of the "two cell block" in the groups of embryos cultured in medium M16 + 0.3mM and medium M16 + 0.6mM ammonium chloride ($X^2 = 1.52$, $P>0.05$). Although the 2 cell block is common in M16 medium for embryos from most inbred and random bred strains, it was not expected for the (F1 x F1) embryos from F1 hybrid females.

When the number of embryos that achieved morula or more developmentally advanced stages after three days of culture was considered, embryos cultured in M16 significantly achieved such stage at lower proportions when compared to those cultured in medium M16 + 0.3mM ammonium chloride or medium M16 + 0.6mM ammonium chloride ($X^2 = 14.40$, $P<0.05$; and $X^2 = 11.52$, $P<0.05$, respectively). There was no significant difference in the

Table 2 Preparations of medium from stock solutions

Stock	M2 (ml)	M16 (ml)	M16+0.3 mM NH_4Cl (ml)	M16+0.6mM NH_4Cl (ml)
A	1.0	1.0	1.0	1.0
B	0.16	1.0	1.0	1.0
C	0.1	0.1	0.1	0.1
D	0.1	0.1	0.1	0.1
E	0.84	-	-	-
NH_4Cl	-	-	1.0	2.0
H_2O	7.8	7.8	6.8	5.8
BSA	40 mg	40 mg	40 mg	40 mg

Table 3 Preimplantation development of embryos in M16 medium (summarized)

Stage	Day 0.5	Day 1.5	Day 2.5	Day 3.5
1 cell (%)	100.00	17.76	10.28	8.41
2 cell (%)	-	82.24	46.73	42.06
3-4 cell (%)	-	-	42.99	18.69
5-8 cell (%)	-	-	-	0.93
morula (%)	-	-	-	29.91
blastocyst	-	-	-	-
Total number Of embryo	107	107	107	107

Table 4 Preimplantation development of embryos in medium M16+0.3mM NH_4CL

Stage	Day 0.5	Day 1.5	Day 2.5	Day 3.5
1cell (%)	100.00	18.00	15.00	15.00
2 cell (%)	-	82.00	19.00	10.00
3-4 cell (%)	-	-	66.00	15.00
5-8 cell (%)	-	-	-	4.00
morula (%)	-	-	-	56.00
blastocyst	-	-	-	-
Total number Of embryo	100	100	100	100

number of embryos achieved morula or more advanced stage after the same period of culture between those cultured in medium M16 + 0.3mM ammonium chloride and medium M16 + 0.6mM ammonium chloride (X^2 = 0.006, P>0.05). There was no blastocyst obtained from

Table 5 Preimplantation development of embryos in medium M16+0.6 mM NH_4Cl

Stage	Day 0.5	Day 1.5	Day 2.5	Day 3.5
1 cell (%)	100.00	20.00	8.33	8.33
2 cell (%)	-	80.00	16.67	8.33
3-4 cell (%)	-	-	75.00	15.00
5-8 cell (%)	-	-	-	11.67
morula (%)	-	-	-	56.67
blastocyst (%)	-	-	-	-
Total number Of embryo	60	60	60	60

embryos cultured in any of the three culture media being investigated in this study.

Evaluation of postimplantation development in this study (Table 6) revealed that implantation rate was significantly higher in group of embryos cultured in medium M16 + 0.3mM ammonium chloride when compared to those cultured in medium M16 + 0.6mM ammonium chloride (Fisher Exact test, P<0.05). Moreover, preimplantation pregnancy loss was significantly higher in the group of embryos cultured in medium M16 + 0.6mM ammonium chloride than those cultured in medium M16 + 0.3mM ammonium chloride (Fisher Exact test, P<0.05). However, there were no significant differences in postimplantation pregnancy loss and the number of fetus obtained between the two groups (Fisher Exact test, P<0.05; and Fisher Exact test, P>0.05, respectively).

In this study, there was one grossly abnormal fetus detected in the group of embryos cultured in medium M16+ 0.3mM ammonium chloride. The abnormality found was polydactyly of both hind limbs. The percentage of abnormal fetus was 20 % per fetus obtained, or 4.35 % per embryo transferred.

Discussion

The cleavage rate and viability of mammalian preimplantation embryos is greatly reduced by culture *in vitro* [1, 2, 7], indicating that the present culture systems are far from optimal. More recently, Gardner and Lane (1993) demonstrated that, while started in culture from 1cell stage, the presence of ammonium in mMTF medium significantly decreased the number of (F1 x F1) strain mouse embryos reaching the morula stage after 72 hours and the blastocyst stage after 96 hours of culture, at the concentration 0.62 and 0.15mM, respectively [8].

Table 6 Postimplantation development of fetuses previously cultured in M16 medium, medium M16+0.3 mM NH_4Cl, or medium M16+0.6 mM NH_4Cl (summarized results)

	Parameters	M16	M16+0.3	M16+0.6
1.	Number of embryos transferred	32	56	34
2.	Number of recipients	6	9	5
3.	Number of pregnant recipients	0	4	1
4.	Number of implantation sites	0	14	1
5.	Number of fetuses	0	5	0
6.	Number of moles	0	9	1
7.	Number of abnormal fetuses	0	1	0
8.	Number of embryos transferred to pregnant recipients	0	23	7
9.	Preimplantation pregnancy loss	0	9	6
10.	Percentage of implantation	0	60.87*	14.28*
11.	Percentage of fetuses obtained	0	21.74	0
12.	Percentage of fetuses per implantation	-	35.71	0
13.	Percentage of preimplantation pregnancy loss	-	39.13*	85.71*
14.	Percentage of postimplantation pregnancy loss	-	64.29	100
15.	Weight of fetus(mean+SEM), (g)	-	0.427±0.020	-
16.	CRL(mean+SEM), (mm)	-	13.682+0.295	-
17.	Average age(mean+SEM), (days)	-	14.900+0.061	-

*Significantly different between the two treatment groups ($P<0.05$).

The presence of ammonium in culture medium may affect the developing embryo in several ways: Ammonium could decrease the concentration of alpha-ketoglutarate by its conversion to glutamate. This would impair the flux through the TCA cycle leading to serious depletion of ATP in the cell. Furthermore, ammonium can activate the enzyme phosphofructokinase resulting in increased glycolytic activity, a pathway that appears to be detrimental to early cleavage stages[9, 10]. Alternatively, ammonium as a weak base could elevate intracellular pH, against which the mouse embryo appears to have no regulatory mechanism [11, 12].

In this study another medium, M16, was investigated since it is a commonly utilized medium for mouse embryo culture in most laboratories. We surprisingly found that the presence of ammonium chloride in this medium did not show adverse effect on preimplantation development of embryos. Moreover, supplementation of M16 medium with ammonium chloride significantly increased number of embryos reaching morula or more developmentally advanced stage when these embryos were cultured from 1 cell stage. This unexpected result could, partly, be explained by the fact that there was high incidence of the "two cell block" in (F1 x F1) strain embryos when they were cultured in M16 medium in comparison to when mMTF was used [5]. This high incidence of the "two cell block" precludes most of the embryos in both the control and experimental groups to develop beyond the 2-cell stage and, hence, leads to difficulty in interpreting, or even misleads, the results of preimplantation development.

The unexpected discovery of high incidence of the "two cell block" in embryos from F1 strain donors found in this study leaded us to speculate that the type of medium used in culturing mouse embryos may differently affect the preimplantation development of such embryos since such event had not been found in the previous reports when mMTF medium was utilized for embryo culture [5, 8].

Components of M16 medium being used in this study are very similar to those present in mMTF medium except that there are lower concentration of NaCl, and higher concentration of both NaLactate and glucose in M16 medium than in mMTF medium (94.70 VS 103.40 mMol/L, 23.30 VS 4.79 mMol/L, and 5.55 VS 3.40 mMol/L, respectively). It was reported that increasing the concentration of NaCl and glucose independently decreased the proportions of CF1 embryos reaching 4 cell

stage while varying NaLactate had no effect [13]. Results from the same study also demonstrated that percentage of embryos reaching 4 cell stage in M16 medium was very low (3%) due to the high incidence of the "two cell block" but lowering concentration of glucose in the medium could partially overcome the block. The inhibitory effect of glucose on embryonic cleavage was found to be maximal at concentration 5.5 mMol/L that is the same concentration presented in M16 medium. Cross and Brinster showed that zygotes of a random-bred strain can develop through the " two cell block" in a mdeium containing lactate and pyruvate, but no glucose [14]. More recently, Menezo and Khatchadourian reported that they have overcome the "two cell block" in a random-bred Swiss mouse cultured in medium M16 by replacing glucose with fructose, and proposed that the "two cell block" is caused by a dysfunction in glucose-6-phosphate isomerase[15]. It is reasonable, therefore, to postulate that the higher incidence of the "two cell block", in M16 medium found in this study could possibly be attributed to the higher concentration of glucose presented in M16 medium than that in mMTF medium.

Another possible factor that might be attributed to the high incidence of the "two cell block" found in this study is the relatively high osmotic pressure of M16 medium being utilized in this study (270-306 mOsmol, MEAN $\pm$ SEM = 296.00 $\pm$ 3.56 mOsmol ; data not shown). Although early studies[16, 17] indicated that the embryos seem to have considerable ability to adjust to variations in osmotic pressure, these studies also demonstrated that there were limited ranges of osmolarity optimal for development of mouse one cell embryos (250-280 mOsmol). In one recent study, reducing the osmotic pressure to as low as 229 mOsmol has substantially improved in vitro development[18]. This result was argued to be due to reduced NaCl concentration (85 mM). In more recent study, however, it was shown that the osmolarity, and not the NaCl concentration, is responsible for maintaining the development of rat one-cell embryo[19]. This study concludes that the optimal osmolarity of the medium for early embryonic development is species specific and depends on developmental stages of embryos. We, thus, speculate that the high osmolarity pressure of M16 medium used in this study may possibly be another potential factor that resulted in the high incidence of the "two cell block". Further study comparing M16 medium and mMTF medium in culturing one cell stage embryos of (F1 x F1) hybrid strain is suggested to prove whether such speculations are true or not.

In this study, we interestingly found that, being cultured in M16 medium, the exposure of 1 cell stage (F1 x F1) strain mouse embryos to higher dose of ammonium chloride (0.6mM) significantly impaired implantation potentials in comparison to the lower dosage (0.3mM) as shown by reducing implantation rate and increasing preimplantation pregnancy loss. These results lead us to speculate that exposure of 1-cell stage embryos to ammonium chloride reduces implantation and increases preimplantation pregnancy loss in a dose-dependent manner. Unfortunately, in this study there were no pregnant recipients obtained from the control group, thus, the precise conclusion on the adverse effect of preimplantation exposure to ammonium chloride on implantation potentials of the embryos cannot be firmly addressed. Again, further studies should be helpful in elucidating this proposed speculation.

Besides the significant decrease in implantation potentials, preimplantation exposure to 0.3 mM ammonium chloride also resulted in gross abnormality of the fetus, preaxial polydactyly of both hind limbs. The incidence of fetal dysmorphogenesis found in this study was 20% per fetus, or 4.35 % per embryo transferred. The mechanism(s) underlined the association between preimplantation exposure to ammonium chloride and polydactyly found in this study need to be elucidated. Possible explanation is that ammonium chloride might induce mutation in genes related to limb formation and subsequently results in polydactyly. There are several genes involved in the determination of the outline of limb which could possibly be candidates for disorders like polydactyly and syndactyly. These include Indian hedgehog (Ihh), and triphalangeal thumb (TPT) genes [20, 21]. More detailed study will, hopefully, be able to uncover the molecular mechanism(s) behind the correlation between polydactyly and preimplantation exposure to ammonium chloride.

One major limitation of this study is that there was no pregnancy occurred in the control group. The comparison between the results obtained from treatment groups and the control group, thus, cannot be thoroughly achieved. Hence, relying solely on the results found in this study cannot make the reliable conclusion regarding the adverse effects of preimplantation exposure of mouse embryos to ammonium chloride.

The results found in this study, together with those prevoiusly reported by Lane and Gardner in 1994[5], are very surprising since they obviously contradict to the generally held convictions which indicate that preimplantation embryos are not susceptible to teratogens, a large insult may kill the embryos but the surviving

embryos usually manifest no organ-specific anomalies. We ultimately propose that exposing preimplantation embryos to insults, such as ammonium chloride, is capable to result in long-term adverse effects such as impaired implantation and fetal dysmorphogenesis. If such effects occurred in the course of human in vitro fertilization, it could be devastating. Further study in this aspect, therefore, is clinically very important in preventing the unwanted abnormalities that could arise from human IVF practices.

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