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Large Offspring Arising as A Result of Preimplantation Exposure of Mouse Embryos to Ammonium Chloride

Supat Sinawat MD, MSc,
Konok Seejorn MD.

Reproductive Biology Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Khon Kaen University, 40002, Thailand

ABSTRACT

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

Study Methods Total of 484, one-cell stage mouse embryos were randomly allocated to culture in either mMTF medium or mMTF added with 0.3mM ammonium chloride 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 Exposure of one-cell stage embryos to 0.3mM ammonium chloride resulted in significant decrease in number of embryos reaching morula stage after three days of culture (X^2=5.36, P<0.05). The presence of ammonium chloride in culture medium also gave rise to significant increase in preimplantation pregnancy loss, weight of the fetuses and crown-rump lengths (X^2=7.83, P<0.01; t=3.22, P<0.01; t=3.19, P<0.01, respectively).

Conclusions Preimplantation exposure of 1-cell stage mouse embryos to ammonium chloride interestingly resulted in abnormally large offspring as well as impaired implantation potential. Further study is required since these findings are clinically very important to human IVF practices.

Key words : Large offspring, preimplantation exposure, mouse embryos, ammonium chloride

It is fortunate that the developments of preimplantation stage mammalian embryos in vivo are safely occurred 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 M ammonium chloride in mMTF medium is associated with abnormality in both preimplantation and postimplantation development of (F1xF1) 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 (mMTF medium or mMTF added with 0.3mM 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 (mMTF medium or mMTF added with 0.3mM 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 the group of ten in 20 ul drops of the medium under a layer of lightweight 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. Crown-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 are three media being used in this study. The M2 medium was used for collecting and transferring embryos. The mMTF medium was utilized for culture embryos in the control group while the mMTF medium added with 0.3mM ammonium chloride was used to culture embryos in the experimental groups.

The M2, mMTF, and mMTF+0.3mM ammonium were made up from individual stock solutions (as shown in table 1 and 3). 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.

Experimental design

There were five experiments carried out in this study. The total number of embryos being studied in the control (mMTF) group and the experimental group (mMTF+0.3mM ammonium chloride) were 246 and 238, 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 either mMTF medium or mMTF added with 0.3mM 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, postimplantation 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

The results from this study (Table 5 and 6) showed that, being cultured from 1-cell stage onwards, the number of embryos that achieved morula or more developmentally advanced stages was significantly higher in the group of embryos cultures in mMTF than those cultured in mMTF supplemented by 0.3 mM of ammonium chloride (X2=8.33, P<0.55). In addition, the incidencs of the "two cell block" was significantly lower in embryos cultured in mMTF than those cultured in ammonium-supplemented mMTF (X^2=5.36, P<0.05).

Statistical analysis of postimplantation development (Table 7) revealed that, being in culture from 1-cell stage onwards, the presence of 0.3 mM ammonium chloride in mMTF medium resulted in significantly increased preimplantation pregnancy loss (X^2=7.83, P<0.01). In addition, weight of fetuses cultured in ammonium chloride-supplemented mMTF medium and their crown-rump lengths are significantly higher than those of the control group (t=3.22, P<0.01; and t=3.19, P<0.01, respectively). There were, however, no significant differences in implantation rate, postimplantation pregnancy loss, number of fetus obtained, number of fetus per implantation, and the averaged age of the fetuses between the treated and the control groups.

Discussion

Previous studies by several investigators have clearly shown that 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 1-cell stage, the presence of 0.62 mM of ammonium chloride in mMTF medium significantly decreased the number of (F1 x F1) strain mouse embryos reaching the morula stage after 72 hours of culture.[8]

In the present study we found that supplementation of mMTF medium with 0.3 mM of ammonium chloride was able to significantly reduced the number of (F1 xF1) strain mouse embryos reaching morula or more developmentally advanced stages when these 1-cell stage embryos were cultured for duration of 3 days. This result agreed with and emphasized the previously claimed adverse effects of ammonium chloride on preimplantation development of (F1xF1) strain mouse embryos.

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 pHi, against which the mouse embryo appears to have no regulatory mechanism.[11, 12]

The effects of preimplantation exposure to ammonium chloride on postimplantation development were more striking. This study demonstrated that exposure of 1 cell stage (F1xF1) strain embryos to 0.3mM ammonium chloride resulted in significant increase in preimplantation pregnancy loss. We also found that implantation rate and percentage of fetuses obtained were decreased in the treated group although statistical analysis revealed no significance. Moreover, we interestingly found that preimplantation exposure to 0.3mM ammonium chloride from 1-cell stage onwards resulted in significantly increase in both crown-rump length (CRL) and weight of the fetuses compared to those of the control group. In addition, there was no developmentally retardation found in the fetuses obtained from ammonium chloride-supplemented medium in comparison to that obtained from the control group. These results are contradictory to those previously reported by Lane and Gardner (1994) which demonstrated that the presence of 0.15 or 0.3mM ammonium chloride in mMTF medium significantly reduced crown-rump length and retarded fetal development.[5]

There has been no previous report on the association between the presence of ammonium chloride in preimplantation culture medium and the increase in size and weight of fetuses or offspring. However, there is accumulating evidence that simple experimental manipulations of early embryonic stages of wide range of mammals result in a variety of startling growth phenotypes. In vitro fertilization, nuclear transfer, and embryo transfer of bovine embryos result in dramatic overgrowth, lengthened gestation, and increased perinatal mortality, the so-called "large calf syndrome".[13-15] Manipulation of mouse embryos increases variability in body mass,[16] or causes reduced body mass.[1, 17] In addition, alterations in behavior and a range of morphophysiological parameters (including increased body mass) have been demonstrated in mice derived from frozen embryo.[18] Human babies resulting from in vitro fertilization and embryo transfer exhibit low birth mass,[19] and hypertension occurs in IVF-ET pregnancies more commonly than in pregnancies conceived spontaneously.[20] The primacy of growth defects observed across a range of species in these studies suggests that expression at a variety of growth related loci is adversely affected by manipulation of early embryos. However, it is not clear whether there is a common factor associated with all or some of these abnormalities. Neither the environmental factor(s), nor the underlying mechanism(s) resulting in the oversized phenotype have been elucidated.

Parental imprinting may explain why the manipulation of early mammalian embryos in vitro frequently results in a range of abnormalities, with aberrant growth predominating.[21] Such growth abnormality may reflect genetic conflict between maternal and paternal alleles over growth program (conflict theory), and that manipulation during preimplantation development in vitro, such as exposure of embryos to ammonium chloride in this study, may coincide with a particularly sensitive period of programming of growth-related genes.

One of the likely molecular components of the imprinting mechanism is the methylation of DNA at CpG dinucleotide, a view that is supported by description of putative DNA methylation germline imprints in Igf2r,[22] H19[23] and Xist[24] genes.

It was reported that mice that are null mutants for Igf2, Igf2r and H19 exhibit marked growth phenotypes.[25] This suggests that imprinted gene can be strongly implicated in fetal and placental growth. It is also worth noting that, while allelic methylation of imprinted genes may be extensively (and variously) remodeled between fertilization and the blastocyst stage, non-imprinted genes undergo an invariant

global demethylation by the morula to blastocyst stage, and remethylation is not observed until around gastrulation. This provides a basis for the differential susceptibility on imprinted and non-imprinted genes to environment in the preimplantation period.

The variability in growth responses following insults during preimplantation period in different species suggests that several genetic loci may be involved, or that aberrant expression of a smaller number of genes may vary depending on the precise nature or duration of the insults. Species differences in the identity of susceptible loci may also be expected. We, thus, propose that identification of the altered gene expression between the normal and the large fetuses may provide clues as to the nature of the underlying mechanism(s) of the large fetuses obtained after preimplantation exposure to ammonium chloride found in this study.

The results found in this study, together with those previously 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 potential and abnormally large fetuses. 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.

Table 1. Stock solutions for making M2 medium

Stock	Component	g/100ml
A (10 x conc)	Nacl	5.534
	KCl	0.356
	KH2PO4	0.162
	MgSO4.7H2O	0.294
	NaLactate	2.608
	Glucose	1.000
	Penicillin	0.060
	Streptomycin	0.050
B (10 x conc)	NaHCO3	2.106
	Phenol red	0.010
C (100 x conc)	NaPyruvate	0.036/10 ml
D (100 x conc)	CaCL2.2H2O	0.252/10 ml
E (10 x conc)	Hepes*	5.957
	Phenol Red	0.010
NH4Cl	NH4Cl	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.

Table 2. Preparations of M2 medium from stock solutions

Stock	M2 (ml)
A	1.0
B	0.16
C	0.1
D	0.1
E	0.84
H_2O	7.8

Table 3. Stock solutions for making mMTF medium and mMTF+0.3 mM NH4Cl

Stock	Components	g/100ml
A (10 x conc)	Nacl	5.746
	KCl	0.356
	KH_2PO_4	0.162
	$MgSO_4.7H_2O$	0.293
	NaLactate	0.897
	Glucose	0.612
	Penicillin	0.060
	Streptomycin	0.050
B (10 x conc)	$NaHCO_3$	2.101
	Phenol red	0.010
C (100 x conc)	Pyruvate	0.041/10 ml
D (100 x conc)	$CaCL_2.2H_2O$	0.252/10 ml
E	NaCl	0.292
NH4Cl	NH_4Cl	0.016

Table 4. Preparations of mMTF medium and mMTF+0.3mM ammonium chloride

Stock	mMTF (ml)	mMTF+0.3mM NH_4Cl
A	1.0	1.0
B	1.0	1.0
C	0.1	0.1
D	0.1	0.1
E	0.1	0.1
NH_4Cl	-	1.0
H_2O	7.7	6.7
BSA	40 mg	40 mg

Table 5. Preimplantation development of embryos in mMTF medium

Stage	Day0.5	Day1.5	Day2.5	Day3.5
1cell (%)	100.00	10.16	8.13	8.12
2 cell (%)	-	89.84	17.48	12.20
3-4 cell (%)	-	-	73.17	12.20
5-8 cell (%)	-	-	1.22	4.88
morula (%)	-	-	-	61.38
blastocyst	-	-	-	1.22
Total number of embryo	**246**	**246**	**246**	**246**

Table 6. Preimplantation development of embryos in medium mMTF+0.3mM NH_4Cl

Stage	Day 0.5	Day1.5	Day 2.5	Day3.5
1cell (%)	100.00	6.30	5.04	3.36
2 cell (%)	-	92.44	28.15	26.05
3-4 cell (%)	-	1.26	66.39	17.65
5-8 cell (%)	-	-	0.42	3.36
morula (%)	-	-	-	49.16
blastocyst	-	-	-	0.42
Total number of embryo	**238**	**238**	**238**	**238**

Table 7. Postimplantation development of fetuses previously cultured in mMTF medium and medium mMTF+0.3mM NH_4Cl

Parameters	mMTF	mMTF+0.3 NH4Cl
1. Number of embryos transferred	137	120
2. Number of recipients	18	15
3. Number of pregnant recipients	8	7
4. Number of implantation sites	39	30
5. Number of fetuses	22	16
6. Number of moles	17	14
7. Number of abnormal fetuses	0	0
8. Number of embryos transferred to pregnant recipients	63	58
9. Preimplantation pregnancy loss	24	28
10. Percentage of implantation	61.90	51.72

Parameters	mMTF	mMTF+0.3 NH4Cl
11. Percentage of fetuses obtained	34.92	27.59
12. Percentage of fetuses per implantation	56.41	53.33
13. Percentage of preimplantation pregnancy loss	38.09	48.28
14. Percentage of postimplantation pregnancy loss	43.59	46.67
15. Weight of fetus(mean±SEM), (g)	0.335±0.017	0.405±0.012*
16. CRL(mean±SEM), (mm)	12.755±0.308	14.001±0.82*
17. Average age(mean±SEM), (days)	14.920±0.067	15.047±0.041

* Significantly different from the control value ($P<0.01$).

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