Fetal abnormalities produced after preimplantation exposure of mouse embryos to ammonium chloride

Supat Sinawat1,4, Wei-Chih Hsaio1,5, Jean H.Flockhart1, Matthew H.Kaufman2, John Keith3 and John D.West1,6

1Division of Reproductive and Developmental Sciences, Genes and Development Group, and 2Division of Biomedical Sciences, Genes and Development Group, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD and 3Assisted Conception Unit, Simpson Centre for Reproductive Health, The Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA, UK

4Present address: Department of Obstetrics and Gynecology, Faculty of Medicine, Khon Kaen University, 40002, Thailand

5Present address: North Town Women and Children’s Hospital, 7 Sec1 Chung Yang Road, Tu-Cheng City, Taipei 236, Taiwan

6To whom correspondence should be addressed. E-mail: John.West@ed.ac.uk

BACKGROUND: The aims of this study were to determine whether preimplantation exposure of mouse embryos to ammonium resulted in abnormal fetal development and to evaluate similar risks to the outcome of human assisted conception. METHODS: Mouse embryos cultured from the 1-cell stage were exposed to 0.3 mmol/l ammonium chloride for 3 days. Embryos cultured from the 2-cell stage were exposed to 0.3 or 0.6 mmol/l ammonium for 2 days. After transfer to the uteri of pseudopregnant recipient females, post-implantation development was evaluated on embryonic day 15.5 (E15.5) or E18.5. RESULTS: There was no consistent effect of preimplantation exposure to ammonium chloride on fetal or placental weights. All 101 E18.5 fetuses were normal but 5/217 E15.5 fetuses were abnormal (three exencephalic and two polydactylous), which was significantly higher than the 0/363 for the pooled groups of E15.5 control fetuses (P = 0.007). The combined E15.5 and E18.5 frequency was also significantly higher than the controls (5/318 versus 0/433; P = 0.013). CONCLUSIONS: These results support the conclusion that abnormal preimplantation culture conditions can cause fetal abnormalities in mice, but the risks may be lower than previously suggested. Further work is needed to evaluate the risk more fully but this risk should be considered when designing new strategies for human assisted conception.

Key words: ammonium/culture/fetal abnormality/preimplantation embryo/preimplantation teratology

Introduction

In recent years attention has focused on the potential dangers of some assisted conception techniques. Human IVF is accompanied by a higher frequency of twinning and other multiple births, premature birth and low birth weight, but there is little evidence that IVF causes developmental anomalies. Two recent studies showed that IVF was also associated with developmental delay and congenital abnormalities, but this was attributed mostly to maternal characteristics and the greatly increased incidence of multiple births rather than the IVF procedure itself (Anthony et al., 2002; Stromberg et al., 2002). Nevertheless, the outcome of assisted conception continues to be closely monitored and there is anxiety about both ovulation induction (Ertzeid and Storeng, 2001) and embryo culture conditions. Recent epidemiological reports have raised the possibility of an association between assisted conception and syndromes caused by imprinting anomalies (Cox et al., 2002; DeBaun et al., 2003; Maher et al., 2003).

Human IVF was developed in the 1970s (Steptoe and Edwards, 1978; Edwards, 1981) and is based partly on previous work with laboratory animals, particularly the mouse (Whittingham, 1968). Experimental work with preimplantation mouse embryos had shown that, up until the 8-cell stage, blastomeres could contribute to any developmental lineage (Tarkowski and Wroblewska, 1967; Hillman et al., 1972) and the embryo was also capable of size regulation after cells were either removed or added (Tarkowski, 1959; Buehr and McLaren, 1974). This suggested that accidental loss of cells would not result in loss of specific organs or even small or dysmorphic embryos. Thus the prevailing view at the time was that insults to the embryo at the preimplantation stage might kill it or prevent formation of sufficient inner cell mass cells to...
form a fetus, but they would not cause organ-specific developmental abnormalities. It is still widely believed that the embryo only becomes sensitive to teratological effects at later stages when organogenesis occurs, although this attitude is now beginning to change (see Discussion). More recently, developmental abnormalities of fetal sheep and cattle, often collectively referred to as the ‘large offspring syndrome’ or ‘large calf syndrome’, have resulted from manipulations of preimplantation sheep and cow embryos or their culture in non-physiological conditions, including media containing serum (Walker et al., 1996; Young et al., 1998; Sinclair et al., 1999). As the name implies, the large offspring syndrome phenotype is characterized by large size at birth. Other features include prolonged gestation, prenatal losses, respiratory problems and various anatomical abnormalities, including cerebellar dysplasia, skeletal malformations, facial malformations and increased muscle mass (reviewed by Young et al., 1998).

Fortunately, human and mouse preimplantation embryos appear to be less susceptible than embryos of ungulates to abnormal culture conditions. Nevertheless, some aspects of large offspring syndrome may also be relevant to mouse and human conceptuses (Leese et al., 1998; Sinclair et al., 2000; Khosla et al., 2001b). There is evidence that abnormal preimplantation culture conditions may affect fetal size and cause fetal abnormalities. One of the most striking examples stemmed from attempts to improve culture media for mouse and human embryos by including amino acids (Gardner and Lane, 1993; Lane and Gardner, 1994).

Non-essential amino acids improved blastocyst formation, blastocyst cell numbers and hatching, but detrimental effects were seen after 72 h that were attributed to the accumulation of ammonium from deamination of amino acids. Ammonium levels reached 0.3 mmol/l after 96 h at 37°C and the adverse effects on preimplantation development were avoided by transferring embryos to fresh medium after 48 h in culture. When 1-cell stage mouse embryos were cultured continuously in medium containing amino acids or in medium supplemented with up to 0.3 mmol/l ammonium chloride and then transferred to pseudopregnant recipient females, the resultant fetuses tended to be smaller and more retarded than in control groups, and many were exencephalic (Lane and Gardner, 1994). No other abnormalities were reported, but the frequency of exencephaly exceeded 20% in several treatment groups. It was higher among fetuses derived from embryos exposed for 93 h than for those exposed for 69 h, and showed a dose–response relationship between 0.037 and 0.3 mmol/l ammonium chloride.

In a more recent study by the same group, 1-cell stage mouse embryos were cultured from embryonic day 0.5 (E0.5) to E2.5 and then transferred to media with different concentrations of amino acids until E4.5, after which time they were transferred to recipient females (Lane et al., 2001). Unlike the earlier study of 1-cell stage embryos exposed to ammonium (Lane and Gardner, 1994), embryos exposed to higher amino acid concentrations (more ammonium) from E2.5 (~8-cell stage) produced heavier rather than smaller fetuses. No mention was made of exencephaly or other fetal abnormalities in the later study.

The extraordinarily high frequency of exencephaly found by Lane and Gardner (1994) could have significant implications for human IVF, but remains to be verified. The purpose of the present study was to evaluate this important claim for a teratological effect induced by exposure of preimplantation mouse embryos to ammonium chloride. Our experiments also extended the previous study by comparing the effects of two concentrations of ammonium in three different culture media on mouse embryos cultured from both the 1- and 2-cell stages. The significance of this work is discussed in the context of other studies to help evaluation of the risks involved in human-assisted conception.

Materials and methods

Embryo collection, culture and transfer

(C57BL/Ola × CBA/Ca)F1 hybrid mice were bred and maintained under conventional conditions at the University of Edinburgh. Females were superovulated by intraperitoneal injections of 5 IU pregnant mare’s serum gonadotrophin (Folligon; Intervet) at approximately 12 noon followed 48 h later by 5 IU HCG (Chorulon; Intervet). After HCG injections, the females were caged individually with (C57BL/Ola × CBA/Ca)F1 males overnight and mating was verified by the presence of a vaginal plug the following morning, which was designated E0.5. One- and 2-cell stage embryos were flushed from the oviducts at E0.5 and E1.5, respectively, at approximately 10:00 h with either M2 (Quinn et al., 1982) or KSOM-H (Summers et al., 1995) HEPES-buffered handling medium.

Groups of 10 embryos were cultured in pre-equilibrated 20-μl drops of culture media under mineral oil (M-8410; Sigma) or silicon oil (Dow Corning, 200/50 cS fluid; BDH, Poole UK) at 37°C in 5% CO2 in air. Oil was stored in the dark to avoid the production of embryotoxic compounds (Provo and Herr, 1998). The three control culture media used were M16 (Whittingham, 1971), mMTF (Gardner and Lane, 1993) and KSOM (Lawitts and Biggers, 1993; Erbach et al., 1994). The experimental media were M16, mMTF or KSOM supplemented with either 0.3 or 0.6 mmol/l ammonium chloride (abbreviated to M16 + 0.3 mmol/l, etc.). After culture, E3.5 embryos from the same treatment group were surgically transferred to one or both uterine horns (five to seven embryos per uterine horn) of pseudopregnant F1 hybrid female mice (abbreviated stock name ‘CF1’; West and Flockhart, 1994) at approximately 10:00 h on day 2.5 of pseudopregnancy, which was then designated E2.5. A control series of (C57BL/Ola × CBA/Ca)F1 hybrid conceptuses was produced by natural matings without any in vitro embryo culture.

Analysis of post-implantation development

Most conceptuses were analysed at E15.5. The recipient females were killed by cervical dislocation and the conceptuses removed from the uterus. The numbers of implantation sites, moles (resorbing conceptuses) and live fetuses were noted, and each live conceptus was weighed intact. The extraembryonic membranes were removed and the placentas and fetuses were weighed separately. Fetal crown–rump lengths were measured and fetuses were examined to determine developmental stage and identify any gross external abnormalities. Fetal development was staged according to the criteria of Wahlsten and Wainright (1977), and scores for skin, limbs, eyes and ears were averaged to give an overall stage score. Abnormal fetuses and normal littermates were photographed and further analysis of abnormalities...
was performed where appropriate. This included staining limb abnormalities with alcian blue, to check for the presence of skeletal elements, and routine histology of other abnormal specimens, which were fixed in Bouin’s fixative, embedded in paraffin wax, sectioned at 7 μm and stained with haematoxylin and eosin.

Some fetuses were analysed at E18.5 (~24 h before birth) for both external and internal abnormalities. Fetuses were fixed in Bouin’s fixative for one week and then transferred to 70% ethanol. After external examination, razor blade cuts were made in the positions shown in Figure 1. Bilateral cuts at positions 1 and 2 removed respectively both forelimbs and hindlimbs and cut 3 removed the tail. These were examined and the toes were counted. The top of the head was removed by cut 4 (through the jaws) and the tongue removed to allow inspection for cleft palate. The anterior head was then cut in three places (cuts 5, 6 and 7). Cut 5 revealed the bilateral nasal cavities, which should be open, vertical and uniform in shape; cut 6 passed through both eyes and was to allow identification of abnormalities such as microphthalmia; cut 7 was between the frontal and parietal bones and allowed inspection of the brain. Cuts 8–11 allowed examination of the body organs at different levels.

One-tailed Fisher’s exact tests were performed using the statistical package GenStat. Other statistical tests were performed on an Apple Macintosh computer using the statistical package StatView 4.1 (Abacus Concepts, Inc., Berkeley, CA, USA).

## Results

### Experimental strategy

(C57BL/Ola × CBA/Ca)F₂ hybrid conceptuses in control series 1 were produced by natural matings. These were analysed to control for effects of embryo culture and to establish the sporadic incidence of exencephaly and other external fetal anomalies in our mouse colony. Conceptuses in experimental series 2–7 were (C57BL/Ola × CBA/Ca)F₂ hybrids that were cultured in different media either for 3 days from the 1-cell stage, or for 2 days from the 2-cell stage, before being transferred surgically to pseudopregnant recipient females, as explained in Materials and methods. A preliminary account of the results from group 6 has been published elsewhere (Sinawat, 2001).

Lane and Gardner (1994) cultured 1-cell stage preimplantation embryos in up to 0.3 mmol/l ammonium chloride and we used a similar experimental design for our first experimental group (group 2 in Tables I and II). E0.5, 1-cell stage embryos were cultured in either M16 medium (our standard culture

---

### Table I. Survival after preimplantation exposure to ammonium chloride

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium a</th>
<th>Preimplantation embryos (M + B)/total</th>
<th>Embryos transferred b</th>
<th>Post-implantation development (E15.5)</th>
<th>Implanted (%)</th>
<th>Moles</th>
<th>Fetuses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-vivo controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultured from E0.5 for 3 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>M16</td>
<td>32/107</td>
<td>30</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>M16 + 0.3 mmol/l</td>
<td>56/100***</td>
<td>56</td>
<td>23</td>
<td>14 (61)</td>
<td>9</td>
<td>5 (36)</td>
</tr>
<tr>
<td>2c</td>
<td>M16 + 0.6 mmol/l</td>
<td>34/60**</td>
<td>57</td>
<td>7</td>
<td>1 (14)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3a</td>
<td>mMTF</td>
<td>154/246</td>
<td>62</td>
<td>63</td>
<td>39 (62)</td>
<td>17</td>
<td>22 (56)</td>
</tr>
<tr>
<td>3b</td>
<td>mMTF + 0.3 mmol/l</td>
<td>118/238**</td>
<td>50</td>
<td>58</td>
<td>30 (52)</td>
<td>14</td>
<td>16 (53)</td>
</tr>
<tr>
<td>4a</td>
<td>KSOM</td>
<td>171/218</td>
<td>78</td>
<td>94</td>
<td>53 (56)</td>
<td>35</td>
<td>18 (34)</td>
</tr>
<tr>
<td>4b</td>
<td>KSOM + 0.3 mmol/l</td>
<td>220/288</td>
<td>76</td>
<td>126</td>
<td>57 (45)</td>
<td>34</td>
<td>23 (40)</td>
</tr>
<tr>
<td>Cultured from E1.5 for 2 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>M16</td>
<td>118/140</td>
<td>84</td>
<td>67</td>
<td>43 (64)</td>
<td>6</td>
<td>37 (86)</td>
</tr>
<tr>
<td>5b</td>
<td>M16 + 0.3 mmol/l</td>
<td>134/140**</td>
<td>96</td>
<td>60</td>
<td>38 (63)</td>
<td>4</td>
<td>34 (89)</td>
</tr>
<tr>
<td>6a</td>
<td>M16</td>
<td>133/137</td>
<td>97</td>
<td>56</td>
<td>32 (57)</td>
<td>4</td>
<td>28 (88)</td>
</tr>
<tr>
<td>6b</td>
<td>M16 + 0.6 mmol/l</td>
<td>139/140</td>
<td>99</td>
<td>70</td>
<td>32 (46)</td>
<td>9</td>
<td>23 (72)</td>
</tr>
<tr>
<td>7a</td>
<td>KSOM</td>
<td>325/334</td>
<td>98</td>
<td>158</td>
<td>88 (56)</td>
<td>25</td>
<td>63 (72)</td>
</tr>
<tr>
<td>7b</td>
<td>KSOM + 0.3 mmol/l</td>
<td>230/230</td>
<td>100</td>
<td>120</td>
<td>83* (69)</td>
<td>24</td>
<td>59 (71)</td>
</tr>
<tr>
<td>7c</td>
<td>KSOM + 0.6 mmol/l</td>
<td>295/295</td>
<td>100</td>
<td>160</td>
<td>106 (66)</td>
<td>49</td>
<td>57* (54)</td>
</tr>
</tbody>
</table>

a0.3 mmol/l = medium supplemented with 0.3 mmol/l ammonium chloride; 0.6 mmol/l = medium supplemented with 0.6 mmol/l ammonium chloride.

b(M + B) = (morulae + blastocysts).

Embryos transferred excludes those transferred to females that failed to become pregnant.

*P < 0.05; **P < 0.01; ***P < 0.001; significantly different from appropriate control group (χ² test).

NA = not applicable.
medium at that time) or M16 medium supplemented with 0.3 mmol/l ammonium chloride (M16 + 0.3 mmol/l). Although we used (C57BL/Ola × CBA/Ca)F₂ embryos from (C57BL/ Ola × CBA/Ca)F₂ hybrid females, which are considered to be resistant to the ‘2-cell block’ (Whittingham, 1975; Goddard and Pratt, 1983), many of these embryos arrested at the 2-cell stage and so were unsuitable for transfer. To circumvent the difficulty incurred by the 2-cell block further experiments were performed, either starting the cultures at the 2-cell stage or using media designed to overcome the 2-cell block. In case 2-cell embryos were less sensitive than 1-cell embryos we compared the effects of 0.3 and 0.6 mmol/l ammonium chloride in M16 medium on 2-cell stage embryos (groups 5 and 6 in Tables I and II). We also cultured 1-cell embryos in mMTF and KSOM media, supplemented with 0.3 mmol/l ammonium, and we cultured 2-cell embryos in KSOM medium supplemented with 0.3 and 0.6 mmol/l ammonium.

### Preimplantation development

Table I shows that almost all of the embryos cultured for 2 days from the 2-cell stage (E1.5 to E3.5) reached the morula or blastocyst stage and were not visibly affected by the presence of either 0.3 or 0.6 mmol/l ammonium chloride in the culture medium. More embryos failed to reach the morula and blastocyst stages when cultured from the 1-cell stage (E0.5 to E3.5). The success rates varied among the three media, being higher for KSOM than either mMTF or M16, but were not affected in a consistent way by supplementation with ammonium chloride.

### Post-implantation survival

The results of the analysis of E15.5 conceptuses are shown in Tables I and II. As mentioned above, the 1-cell embryos cultured in M16 medium developed poorly and in control group 2a few were transferred and none implanted. In groups 3–7, neither 0.3 nor 0.6 mmol/l ammonium chloride had a consistent effect on the frequency of implantation. The frequency of surviving E15.5 fetuses among implanted embryos was lower among embryos cultured for 3 days from E0.5 than for those cultured for 2 days from E1.5, but was unaffected by 0.3 mmol/l ammonium chloride (Table I). A lower proportion of implanted conceptuses survived to form fetuses, after exposure to 0.6 mmol/l ammonium chloride from the 2-cell stage, in both M16 and KSOM media, but this difference was only significant for the KSOM group.

### Fetal growth

Some significant differences in developmental growth parameters are noted in Table II for conceptuses cultured in media with and without ammonium chloride. Culture in M16 or mMTF typically resulted in heavier placentas than culture in KSOM or the in-vivo controls, but exposure to ammonium had no consistent effect on placental weight. In some cases mean fetal size was reduced after exposure to ammonium, as found by Lane and Gardner (1994), and this was also noted for the E18.5 fetuses (see below). In some other groups fetuses were heavier after preimplantation exposure to ammonium, as found by Lane et al. (2001). Overall, however, embryonic exposure to ammonium chloride had no consistent effects on...
mean fetal weight, crown–rump length, developmental stage, placental weight or conceptus weight.

Fetal abnormalities

None of the 363 E15.5 control fetuses (195 group 1 in-vivo controls plus 168 fetuses derived from the cultured control groups) were morphologically abnormal, but five of the 217 E15.5 fetuses derived from preimplantation embryos exposed to ammonium chloride were abnormal (Table II). Two of these had polydactyly: one left hindlimb preaxial polydactyly was produced in group 5b (2-cell embryos cultured in M16 + 0.3 mmol/l) and one bilateral hindlimb preaxial polydactyly was produced in group 2b (1-cell embryos cultured in M16 + 0.3 mmol/l) (Figure 2). Staining confirmed that the extra digit included skeletal elements. In both of the hindlimbs of the group 2b fetus with polydactyly the extra digit had an additional small appendage, but it was not clear whether these appendages contained skeletal elements (Figure 2). Exencephalic fetuses were produced in groups 4b, 6b and 7b (Figure 3). Those in groups 4b and 6b were small and the one in group 4b was developmentally retarded, whereas the fetus in group 7b was more normal in size (Table II).

Overall 2.3% (5/217) of E15.5 fetuses derived from embryos exposed to ammonium chloride had visible external morphological abnormalities. Although the abnormalities occurred in five of the seven treated groups, they were more frequently produced by E0.5 embryos that had been exposed to ammonium for 3 days (two of 44; 4.5%) than by those exposed for 2 days from E1.5 (3/173; 1.7%). The incidence of abnormal fetuses derived from 1- or 2-cell embryos, exposed to ammonium for 2 or 3 days, respectively, did not differ

Figure 2. Polydactyly in E15.5 fetuses. (A, B) Preaxial polydactyly of (A) left hindlimb and (B) right hindlimb of fetus from group 2b. An additional small appendage is visible on the extra digits in (A) and (B). (C) Right hindlimb of normal fetus. (A small piece of dark card, positioned behind the digits for photography, is visible in A–C.)

Figure 3. Exencephaly in E15.5 fetuses. (A) Exencephalic fetus from group 4b (crown–rump length 11.8 mm). (B) Exencephalic fetus from group 6b (crown–rump length 11.0 mm). (C) Exencephalic fetus from group 7b after fixation (crown–rump length 15.1 mm). (D) Head of normal E15.5 fetus. (E, F) Coronal section of head, through eyes of (E) normal E15.5 fetus (oblique section) and (F) exencephalic E15.5 fetus (near symmetrical section) from group 6b, shown in (B).
significantly from one another or from their respective cultured control groups by one-tailed Fisher’s exact tests.

The overall frequency of abnormal fetuses from embryos exposed to ammonium chloride reached borderline statistical significance. One-tailed Fisher’s exact tests showed that although the frequency of abnormal fetuses in the treated group was not significantly higher than in the cultured control groups (5/217 versus 0/168; \( P = 0.056 \)), it was significantly higher than in the larger group of in-vivo controls (5/217 versus 0/195; \( P = 0.040 \)). The overall incidence of abnormal fetuses (exencephaly plus polydactyly) in the treated groups was very significantly higher than in the pooled in-vivo and cultured control groups (5/217 versus 0/363; \( P = 0.007 \)), but the frequency of exencephaly alone was not significantly higher than in the pooled controls (3/217 versus 0/363; \( P = 0.052 \)).

**Analysis of E18.5 conceptuses**

In addition to the results shown in Tables I and II, some fetuses derived from 2-cell embryos cultured for 2 days were allowed to develop until E18.5 to allow a more thorough search for morphological abnormalities. These comprised 70 live fetuses from control M16 cultures (equivalent to E15.5 groups 5a and 6a) and 101 live fetuses from ammonium-treated 2-cell embryos: 57 live fetuses plus one dead fetus derived from M16 + 0.3 mmol/l cultures (equivalent to E15.5 group 5b) and 44 live plus two dead fetuses derived from M16 + 0.6 mmol/l cultures (equivalent to E15.5 group 6b). These fetuses were weighed and fixed, and many were dissected as described in Materials and methods to allow a systematic examination of internal structures (64 from the M16 control group, 29 from the M16 + 0.3 mmol/l group and 27 from the M16 + 0.6 mmol/l group). The E18.5 fetuses from 2-cell embryos exposed to 0.3 and 0.6 mmol/l ammonium in M16 medium were significantly lighter (1249 ± 18 and 1251 ± 19 mg, respectively) than the M16 medium control group (1305 ± 14 mg; \( P < 0.05 \)). No external abnormalities were found among any of these fetuses (0/101) and no internal abnormalities were found among those that were dissected and examined in greater detail (0/56). When combined with the E15.5 fetuses the overall incidence of abnormal fetuses in the E15.5 and E18.5 treated groups was only 1.6% (5/318), but this was still significantly higher than in the pooled in-vivo and cultured E15.5 plus E18.5 control groups (5/318 versus 0/433; \( P = 0.013 \) by one-tailed Fisher’s exact test).

**Discussion**

**Effect of preimplantation exposure to ammonium on fetal development**

Lane and Gardner (1994) analysed day 15 (E14.5) conceptuses derived from (C57BL/6 × CBA/Ca)F2 embryos that were cultured from E0.5 for 48, 69 or 93 h in mMTF medium, with or without ammonium chloride. Exposure to ammonium reduced the frequency of implantation, caused retarded fetal development and produced a high frequency of exencephaly. In contrast, no consistent significant effect on implantation rate or fetal growth was found in the present study.

The experiment by Lane and Gardner (1994), comparing the duration of exposure to 0.3 mmol/l ammonium, produced 27% (3/11) exencephalic fetuses from embryos cultured for 69 h (E0.5 to E3.5) and 38% (6/16) from those cultured for 93 h (until E4.5). A parallel experiment comparing the effects of ammonium concentration in media used to culture embryos from E0.5 to E3.5 showed a dosage effect. Numbers deduced from their Table 5 and Figure 2 imply that the frequency of exencephaly increased from 7% (1/15) for 0.037 mmol/l, 10% (1/10) for 0.075 mmol/l, 21% (3/14) for 0.15 mmol/l to 29% (4/14) for 0.3 mmol/l ammonium chloride (Lane and Gardner, 1994). If these numbers are correct, overall 22.5% (18/80) of fetuses, produced by E0.5 embryos exposed to up to 0.3 mmol/l ammonium chloride, were exencephalic.

In the present study, all the abnormal fetuses were derived from embryos exposed to ammonium chloride, but the frequency was much lower. There were too few fetal abnormalities to determine whether 1-cell embryos were more susceptible than the 2-cell stage or whether 0.6 mmol/l ammonium chloride produced more abnormalities than 0.3 mmol/l. The highest frequency of exencephaly occurred in the group exposed to 0.3 mmol/l ammonium from E0.5 to E3.5, but this was only 2.3% (1/44). Even the incidence of exencephaly plus polydactyly (4.5%; 2/44) was significantly lower than the exencephaly frequency reported by Lane and Gardner (1994) for a similar exposure period and concentration (28%; 3/11 plus 4/14 in two experiments; \( P = 0.009 \) by two-tailed Fisher’s exact test).

Although Lane and Gardner (1994) found a 10-fold higher frequency of exencephaly, they did not report polydactyly or any other morphological abnormalities. Fetuses were examined 1 day later in the present study, but exencephaly and polydactyly are readily identifiable at both E14.5 and E15.5. The high proportion of exencephalic fetuses reported by Lane and Gardner (1994) may have also contributed to the more consistent reduction in fetal size found in their study. Both the spontaneous and induced frequencies of exencephaly depend on genetic background and they vary among different strains of mice (Sah et al., 1995; Hall et al., 1997; Vacha et al., 1997; Holvand et al., 1999; Juriloff et al., 2001). It seems unlikely that the mice used by Lane and Gardner (1994) had a lower susceptibility threshold for the induction of exencephaly, because (C57BL × CBA/ Ca)F2 embryos were used in both studies, so the genetic backgrounds would be almost identical. Different strains were used as pseudopregnant recipient females, and this could be a contributing factor.

Embryonic exposure to ammonium may also cause large offspring syndrome in sheep. McEvoy et al. (1997) reported that ewes fed a diet containing urea had elevated plasma levels of urea and ammonia. This was associated with preimplantation embryo mortality and abnormal embryonic metabolism, and the only fetus in this group was larger than controls.

**Evidence for preimplantation teratology in the mouse**

Studies in mutation research, teratology, developmental biology and reproductive biology provide other examples where exposure of preimplantation mouse embryos to a variety of
agents causes fetal abnormalities, implying that the preimplantation stage is sensitive to teratological effects. Treatment of pregnant mice with different mutagens during the preimplantation period or direct treatment of isolated preimplantation embryos has produced a spectrum of fetal abnormalities including exencephaly and polydactyly (Bossert and Iannaccone, 1985; Rutledge, 1997). Lithium is a post-implantation teratogen that disrupts the inositol pathway and can cause exencephaly (Jurand, 1988; Berridge et al., 1989). Transient exposure of preimplantation mouse embryos to lithium chloride produced a high proportion of post-implantation abnormalities, but showed no effects before gastrulation (Rogers and Varma, 1996; 2000). Fetal malformations have also been reported after treatment of pregnant rats with either aspirin or acetaminophen on day 3 of pregnancy (Ying and Lou, 1993) and after treatment of pregnant mice with retinoic acid (a known teratogen) at E5.5, shortly after implantation (Pauken et al., 1999), but these effects could be mediated via indirect effects on the maternal environment.

Possible mechanisms of preimplantation teratology

The half-life of many putative preimplantation teratogens makes it unlikely that they persist for long enough to act during organogenesis (Rutledge, 1997), which implies that a somatically inheritable change must occur in the preimplantation embryo. While some chemicals may induce somatic mutations (Bossert and Iannaccone, 1985), most fetal abnormalities probably result from epigenetic changes (Rutledge, 1997). These could include modifications of the chromatin, which do not alter the DNA sequence (e.g. changes in methylation), and other cellular changes, which could alter gene expression and thereby affect the phenotype at a later stage.

Although the two types of malformation found in the present study appear to be unrelated, they could be caused by disruption of a shared component of their separate developmental pathways. For example, polydactyly can result from disturbance of the sonic hedgehog (Shh) signalling pathway (Tanaka et al., 2000; Lettice et al., 2002), and mutants affecting the Shh pathway have faulty neural tube closure (Goodrich et al., 1999; Eggenschwiler et al., 2001). If ammonium ions interfered with Shh signalling this might cause both polydactyly and exencephaly.

Other observations raise the possibility that ammonium could cause exencephaly by disturbing expression of the p53 tumour suppressor gene or the Gas5 (growth arrest specific 5) gene. Mice lacking p53 expression are predisposed to exencephaly as well as tumours, and other developmental abnormalities (including polydactyly) have also been reported (Armstrong et al., 1995; Sah et al., 1995). Although we know of no direct effect of ammonia on p53 expression, there is some evidence for a link between ammonia and gastric carcinogenesis (Tsujii et al., 1997). Preimplantation exposure of mouse embryos to 0.3 mmol/l ammonium chloride results in elevated expression of Gas5 in blastocysts (Fontanier-Razzaq et al., 2000) and differential expression of Gas5 may affect susceptibility to exencephaly in mice (Vacha et al., 1997).

Possible mechanisms underlying large offspring syndrome

A large number of molecular and cellular changes induced by culture of preimplantation embryos has been documented in order to try to understand the underlying causes of large offspring syndrome (reviewed by McEvoy et al., 2001). Mouse embryos cultured in medium supplemented with serum produced small fetuses (Caro and Trounson, 1984; Arny et al., 1987; Khosla et al., 2001a) and abnormal expression of several imprinted and non-imprinted genes, including Grb10, H19, Igf2, Grb7 and Gas6 (Fontanier-Razzaq et al., 2000; Khosla et al., 2001a). Particular attention has been focused on the effects of embryo manipulations and culture on expression of imprinted genes. This is both because many imprinted genes are associated with growth disorders and because the altered methylation states associated with imprinted genes are acquired during gametogenesis or fertilization, and so could be modified during preimplantation development, whereas methylation patterns of non-imprinted genes are only established after implantation (Khosla et al., 2001b). Abnormal expression of several imprinted genes has been found in fetuses produced from cultured bovine embryos (Doherty et al., 2000) and mice cloned from embryonic stem cells (Humpherys et al., 2001). Moreover, expression of IGF2R was reduced in fetal sheep with large offspring syndrome, derived from cultured preimplantation embryos (Young et al., 2001).

Evaluating the risks of non-physiological embryo culture to human fetal development

The possibility that ICSI may be associated with an increased risk of Angelman syndrome (Cox et al., 2002) has rekindled the debate on the safety of human-assisted conception. Two further epidemiological reports suggested that the frequency of Beckwith±Wiedemann syndrome may be increased after IVF as well as the more invasive ICSI procedure (DeBaun et al., 2003; Maher et al., 2003). Both these syndromes can be caused by imprinting anomalies, prompting concern that cultured human preimplantation embryos may be susceptible to epigenetic alterations leading to fetal abnormalities. The present study is also relevant to the broader issue of culturing human embryos in non-physiological conditions.

The frequency of abnormal fetuses produced in the present study was much less than that reported by Lane and Gardner (1994) and only of borderline significance, unless all the control groups were pooled. Although our results suggest that there is a risk of fetal abnormalities arising from treatment of preimplantation mouse embryos with ammonium, this risk may be lower than suggested by Lane and Gardner (1994). Nevertheless, the present results provide support for the important conclusion that exposure of preimplantation mouse embryos to ammonium can cause abnormal fetal development. Further studies are needed to characterize the full spectrum of abnormalities that can be produced, define the critical exposure time, determine whether genetic background affects susceptibility and investigate the underlying mechanisms.

Supplementation of culture medium with ammonium is an artificial situation but it accumulates in normal culture medium containing amino acids (Lane and Gardner, 1994). Fortunately,
exposure to ammonium can be reduced by replacing the medium after 2 days (Lane and Gardner, 1994; Lane et al., 2001), so this specific risk can be minimized for human IVF. Improved culture systems may also help; for example, J.Keith (unpublished data) has suggested modifying the medium circulation chambers, used for post-implantation rodent embryos (New, 1978; 1990), for use with preimplantation embryos.

The important general point to emerge from this and other studies, discussed above, is that the preimplantation period is not immune from teratological effects. Although teratology is more usually associated with the period of organogenesis, there is a growing body of evidence that preimplantation teratology is a real phenomenon that affects mice as well as ungulates. This additional risk factor should be considered when evaluating the merits of prolonging the culture period before transferring human IVF embryos to the reproductive tract or designing new strategies for assisted conception.

Acknowledgements

We thank Professors Andrew Copp, Philip Iamacone and Michael Legge for much helpful discussion and their comments on an early draft of the manuscript. We are also grateful to Drs David Gardner and Kate Hardy for technical advice, Margaret Keighren for technical help, Anthea Springbett for performing the one-tailed Fisher’s exact tests, and Denis Doogan, Maureen Ross and Jim Macdonald for expert mouse husbandry.

References


*Submitted on April 7, 2003; accepted on July 9, 2003*