Embryo culture does not affect the longevity of offspring in mice

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Abstract

The oldest assisted reproductive technologies (ART)-conceived child is only 27 years old. Thus, the effects of ART on longevity are unknown, and it will be many years before this can be assessed in humans. We recently reported that culturing pre-implantation mouse embryos under suboptimal conditions results in differences in how the offspring perform in behavioral assays that reflect anxiety (elevated zero maze) and spatial memory (Morris hidden water maze; Ecker *et al.* 2004). Here we monitored the mice generated in our previous study and found no difference in their longevity.

Reproduction (2005) **130** 599–601

Longevity and embryo culture

Since the birth of Louise Brown in 1978, use of assisted reproductive technologies (ART) to treat human infertility has grown at a rapid rate, accelerating with the implementation of intracytoplasmic sperm injection (ICSI) in the early 90's such that in many developed countries ART accounts for 1–3% of births (Maher 2005). Culture of pre-implantation embryos prior to embryo transfer is typically an intrinsic part of ART. Culture, however, is known to affect both embryo metabolism (Gardner *et al.* 2000) and the global pattern of gene expression (Rinaudo & Schultz 2004), including imprinted genes (Doherty *et al.* 2000, Khosla *et al.* 2001, Mann *et al.* 2004). Regarding imprinting, culture can lead to bi-allelic *H19* expression that is linked with loss of DNA methylation of the paternal allele in the differentially methylated region (Mann *et al.* 2004); DNA methylation of this region is essential for repression of the paternal allele. Loss of imprinting can lead to specific syndromes in humans, including Angelman and Beckwith–Weidemann syndromes. Of particular note is that a significantly higher incidence of these syndromes is observed in ART-conceived children, and in most instances this is due to loss of DNA methylation in critical regulatory sequences (Maher 2005), i.e. the syndrome is due to an epigenetic change. In addition, there are reports of an increased incidence of cerebral palsy (Strömberg *et al.* 2002) and low birth-weight (Schieve *et al.* 2002) in ART-conceived children. Because the effects of ART on longevity in humans will not be known for many years, we monitored the mice generated in our previous study to ascertain

if there were any significant differences in their longevity. Our analysis, which did not find any significant difference in lifespan after culture (Fig. 1A–C), is described below.

We compared the lifetimes of mice that were from two difference sources, namely, those that developed *in vivo* and those that developed *in vitro* in either KSOM or Whitten's medium. Because there are sex differences in mouse lifetimes (Fig. 1D), an adjustment was made for sex. In addition, in the original experimental design three different genotypes were generated due to mating females with either wild-type males or males harboring either a Teto or Line30 transgene. See Ecker *et al.* (2004) for further discussion on the rationale for this experimental design. Adjustment for genotype was also considered. In addition, interaction effects on lifetime between sex, genotype, and *in vivo/in vitro* were considered. Although the complete natural lifetime was observed for most mice, there were some cases of incomplete lifetimes and some mice required euthanasia. The lifetimes were compared using the Cox proportional hazards regression model, which accommodates such incomplete or censored observations (Cox 1972).

There were a total of 290 mice. Complete natural lifetime in 237 mice (53 *in vivo*, 88 KSOM, 96 Whitten's). Death time was known within an interval but the death date was not precisely known for 40 mice (10 *in vivo*, 15 KSOM, 15 Whitten's) in which case the interval midpoint was used as time of death. 2 mice were euthanized after developing lethal conditions (0 *in vivo*, 1 KSOM, 1 Whitten's; euthanization date taken as time of death). 9 mice were euthanized with non-lethal ailments such as

Figure 1 Percentage survival in offspring following culture *in vitro* or development *in vivo*. A; survival of all animals, B; survival as a function of sex for males, C; survival as a function of sex for females, D; survival as a function of sex. See text for discussion of statistical analysis.

fighting wounds (1 *in vivo*, 5 KSOM, 3 Whitten's; euthanization date taken as time of death), and 2 mice were still alive at the time of this analysis (0 *in vivo*, 0 KSOM, 2 Whitten's; the last observed time was used as the censored time).

The Cox regression model included *in vivo/in vitro* and sex as predictors of lifetime. The Cox model results are in terms of hazard ratios or instantaneous relative risk. For example, the hazard ratio (HR) for the *in vivo* variable is interpreted as the instantaneous relative risk of death at any time for an *in vitro*-derived mouse compared with an *in vivo*-derived mouse, where a significant hazard ratio less than 1 would indicate that at any point in time the risk of death for an *in vivo*-derived mouse is less than for an *in vitro*-derived mouse, indicating a longer lifetime for an *in vivo*-derived mouse.

The result indicates that *in vivo* status does not impact the lifetimes (HR = 0.916, $P = 0.541$, 95% confidence interval 0.692–1.213), while sex does impact the lifetimes (HR = 0.677, $P = 0.001$, 95% confidence interval 0.535–0.857), with males having longer lifetimes. Thus, after adjusting for sex, there is no difference in lifetimes between *in vivo* and *in vitro*-derived mice. An additional model was fit to consider the three mouse genotypes included in the study. Including genotype in the above Cox model does not alter the conclusions. The result indicates that *in vivo* status does not impact on lifetime (HR = 0.815, $P = 0.176$, 95% confidence interval 0.606–1.096), while the male sex has longer lifetimes (HR = 0.652, $P < 0.001$, 95% confidence interval 0.513–0.827) compared to females. Genotype does

impact on lifetime ($P = 0.021$), with wild type and Teto lifetimes not significantly different to each other ($P = 0.801$), but line30 mice having shorter lifetimes compared to both wild type (HR = 1.442, $P = 0.020$, 95% confidence interval 1.059–1.966) and to Teto mice (HR = 1.495, $P = 0.010$, 95% confidence interval 1.103–2.027). The proportional hazards assumption of the Cox model was assessed both graphically and via a hypothesis test based on Schoenfeld residuals (Schoenfeld 1982), and both indicate that the required assumption is satisfied for these models. Each of the potential two-way interactions between *in vivo/in vitro*, genotype, and sex were all considered for inclusion in the model. None of these interactions were significant and there was no impact on the non-significant result obtained for the *in vivo/in vitro* status.

In summary, results using a mouse model in which embryo culture had long-term effects on behavior in the offspring had no effect on longevity. These results suggest that although there is a significant increase in risk associated with ART, a detrimental effect on longevity is unlikely to be one of them.

Acknowledgements

This research was supported by a grant from the NIH (U01 HD 44575 to RM Schultz and T Abel as part of the NICHD Cooperative Program on Female Health and Egg Quality and P50 MH 64045 to RE Gur). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 14 June 2005

First decision 6 July 2005

Revised manuscript received 6 July 2005

Accepted 22 August 2005