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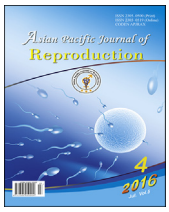

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Minute changes to the culture environment of mouse pre-implantation embryos affect the health of the conceptus

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ABSTRACT

Objective: To investigate the consequences of removing embryos from the incubator daily, and exposing them to ambient air at 37.0 °C for short periods of time, on embryo development, gene expression and the health of the conceptus.

Methods: An *in vitro* cross-sectional study was undertaken using CBA F_1 mouse zygotes exposed to ambient air at 37.0 °C for 3, 5, and 8 min daily during their entire *in vitro* development. Blastocysts were analyzed for total cell numbers, apoptosis, and H_{19} and Igf_2 expression and transferred to pseudopregnant recipient females to examine pregnancy, fetal and placental weight, and placental H_{19} and Igf_2 expression.

Results: Short exposure of pre-implantation embryos to ambient air at 37.0 °C resulted in a statistically significant reduction in blastocyst development and total cell numbers, increased apoptotic cell index, and impaired H_{19} and Igf_2 expression in the blastocyst. Implantation rates were significantly reduced and fetuses and placentae displayed a significant reduced weight on day 18 of pregnancy. Placentae displayed significantly reduced levels of H_{19} and Igf_2 expression.

Conclusions: Exposing mouse pre-implantation embryos to ambient air at 37.0 °C, even for brief periods for routine micromanipulations is detrimental to normal embryonic development. Our results highlight the importance of how small alterations in the culture environment can have major consequences for the health of the embryo.

1. Introduction

As the success of assisted reproductive technology (ART) has improved over the last decade, concern has grown about the safety and long-term consequences for children born *via* these complex procedures. Methods including *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and *in vitro* culture have been associated with impaired pre-implantation embryo development, leading to restricted fetal growth and significant phenotypic and behavioral changes in adult offspring [1–4]. Studies have demonstrated a strong association between ART and altered gene expression affecting methylation and expression of imprinted genes in the blastocyst, placenta, and fetus [5–11].

In vitro manipulation of gametes and pre-implantation embryos as part of ART may lead to an increased risk of congenital malformations and diseases caused by abnormal genomic imprinting [12–18]. This is corroborated by reports correlating abnormal genomic imprinting to epigenetic disorders, including Beckwith–Wiedman, Angleman, and Silver–Russell syndromes [17–20]. While infertility *per se* may signal a genetic predisposition to epigenetic instability, it does not account for all anomalies, and it is evident that reproductive technology applied to gametes and pre-implantation embryos contributes to these adverse outcomes [21].

Although the effects of a range of ART techniques on embryo growth and subsequent fetal development have been extensively investigated, the effects of routine daily manipulations have not been studied. Removing the embryo culture system from the incubator and exposing it to ambient air causes minute changes to the culture environment. However, such removal and exposure may be necessary for routine procedures, such as assessing fertilization, grading embryos, changing culture media, and transferring embryos. The time needed for these

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procedures (3, 5 and 8 min) is directly related to the proficiency of the individual embryologist.

Using a mouse model, we tested the hypothesis that *in vitro* development of the pre-implantation embryo and growth of the resulting conceptus are affected by even minute daily changes to the culture environment. We show that exposing the embryo culture to ambient air at 37.0 °C daily for very short periods throughout *in vitro* pre-implantation impaired embryo development. In addition, the expression patterns of the two important imprinted growth-related genes, insulin-like growth factor (Igf_2) and H_{19}, were altered in embryos and placentas.

2. Materials and methods

2.1. Animals and embryo collection

Animal procedures followed the Australian Code of Practice for the Use of Animals for Scientific Purposes (7^{th} edition) under ethics approval from The University of Sydney, Sydney West Area Health Service Animal Ethics Committee (Approval Number 4155).

All handling and culture media used in this study were from Vitrolife AB, Vastra Frolunda, Sweden, which are commonly in IVF. Pre-pubertal (3–4 week) C57 × CBA, F_1 (Monash Animal Services, Monash University, Victoria, Australia) female mice were injected intraperitoneally with 5 IU pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet, Australia Pty. Ltd., Bendigo, Australia) followed 48 h later by intraperitoneal injection of 5 IU human chorionic gonadotropin (hCG; Chorulon, Intervet, Victoria, Australia) and placed one per cage with 8- to 10-week-old C57 × CBA, F1 males. Female mice were sacrificed by cervical dislocation at 21 h post hCG (day 1 of pregnancy). Pronucleate oocytes were recovered in G-MOPS™ PLUS and denuded using 80 IU HYASE™ 10× in 100 μL drops under OVOIL™ and washed in 50 μL drops of G-MOPS™ PLUS under OVOIL™.

2.2. Experimental design and embryo culture

Mouse zygotes were randomly allocated into experimental and control groups. These groups included (i) embryos not removed from the incubator other than for change of culture medium [day 3, (26.0 ± 2.1) s; control], (ii) embryos removed from the incubator for 3 min daily, (iii) 5 min daily, and (iv) 8 min daily. Whenever embryos were removed from the incubator, they were held on a controlled heated stage assuring (37.0 ± 0.2) °C in the culture medium drop. Embryos were cultured in groups of 10 in 20 μL drops of G-1™ PLUS culture medium under OVOIL™ for 48 h. On day 3 of development, the embryos were transferred in 20 μL drops of G-2™ PLUS blastocyst medium until day 4 of development at the blastocyst stage. All cultures were performed at 37.0 °C and 6% CO_2.

2.3. Cell number, allocation, and apoptosis

Blastocyst total cell, trophectoderm (TE), and inner cell mass (ICM) numbers and apoptosis were determined as described previously [22]. Blastocysts were washed three times in phosphate-buffered saline containing polyvinylpyrrolidone (PBS)/PVP (1 μg/mL), fixed in 3.7% paraformaldehyde in PBS, pH 7.3, overnight at 4.0 °C, then washed again in PBS/PVP, and stored at 4.0 °C until analysis. Blastocysts were permeabilized in 0.5% Triton X-100 (Sigma–Aldrich Chemical Co., St. Louis, MO) for 1 h at room temperature, washed three times in PBS/PVP, and incubated in TUNEL reagent (Roche Molecular Biochemicals, Indianapolis, IN) for 1 h at 37.0 °C in the dark. A TUNEL-negative control was obtained by omitting Terminal deoxynucleotidyl transferase (TdT) from the labeling mix. Embryos were then washed in PBS/PVP and counterstained with the fluorochrome propidium iodide (PI; 50 mg/mL in PBS; Sigma–Aldrich Chemical Co.) for 60 min at room temperature in the dark. To avoid background staining from cytoplasmic RNA, RNase A (60 mg/mL; Sigma–Aldrich Chemical Co.) was added to the PI labeling mix. After extensive washing, the embryos were mounted under cover-slips in mounting medium (Invitrogen Molecular Probes, Eugene, OR). Slides were sealed with nail varnish and stored at 4 °C until confocal analysis. Fluorescence was detected using a Leica scanning confocal microscope (Leica TC_2 SP_2 Laser Scanning Spectral Confocal Microscope, Leica Microsystems, Wetzlar, Germany). Blastocysts were scanned in two channels to detect PI and TUNEL. At least 18–20 optical sections were taken through each blastocyst at 3- to 4-μm intervals. TE and ICM were distinguished according to morphology and position described previously [23].

2.4. RNA preparation, cDNA synthesis and real time qRT-PCR

To achieve a homogenous population for RNA purification, total RNA isolation from pools of 30 blastocysts or placental tissue was obtained using the RNeasy Micro Kit Plus (Qiagen Pty. Ltd., Valencia, CA) following the manufacturer's instructions. First strand cDNA libraries were generated using the SuperScript™ III First-Strand Synthesis SuperMix for real-time qRT-PCR (Invitrogen Molecular Probes), according to the manufacturer's protocol. Six microliters of total RNA were combined with 10 μL of 2× RT Enzyme mix (including oligo-dT_{20}, random hexamers, $MgCl_2$, and deoxynucleotide triphosphates [dNTPs]) and 2 μL of RT Enzyme mix and incubated at 50.0 °C for 30 min, followed by incubation at 25.0 °C for 10 min. Reactions were terminated by denaturing the reverse transcriptase enzyme at 85.0 °C for 5 min, following incubation on ice for 2 min. Finally, 1 μL (2 U) of *Escherichia coli* RNase H was added, and reactions were incubated at 37.0 °C for 20 min.

Quantitative gene expression analyses of genes H_{19} and Igf_2 were performed, using the housekeeping gene Sdha as an endogenous control. Each primer was first tested for amplification on cDNA standards from day 17 mouse placenta. Real-time qRT-PCR was performed on the Corbett Rotorgene 6000 (Corbett Research Pty. Ltd, Sydney, Australia) by TaqMan® technology using TaqMan® assays (Gene IDs; Sdha: Mm_01352366_m1; H_{19}: Mm00469706_g1; and Igf_2: Mm00439565_g1). All samples were amplified in triplicate. Each 10-μL reaction contained 5.0 μL of 2× TaqMan® Gene Expression Master Mix (ABI-Life Technologies, Carlsbad, CA), 0.5 μL of 20× TaqMan® Gene Expression Assay (ABI-Life Technologies), 2.5 μL of nuclease-free water, and 2 μL of cDNA template. The template was replaced by nuclease-free water in the negative controls. The thermal program included

40 cycles of 95.0 °C for 10 min, followed by 40 cycles of 95.0 °C for 15 s and 40 cycles of 60.0 °C for 60 s. The relative mRNA expression was calculated by comparison, with standard curves generated from serial dilutions (1:1; 1:5; 1:25, and 1:125) of pooled day-17 mouse placental cDNA, and normalized to the average abundance of mRNA for the reference gene Sdha.

2.5. Embryo transfers

Embryo transfers were carried out as described previously [2]. Naturally ovulating 9- to 10-week-old recipient C57 × CBA, F1 female mice were mated to vasectomized 10-week-old C57 × CBA, F_1 males to establish pseudopregnancy. On day 4 of development, the *in vitro* cultured blastocysts were transferred to pseudopregnant females on day 3 of pseudopregnancy. Five blastocysts were transferred to the left uterine horn and five control blastocysts to the right uterine horn of the mouse (i.e., test and control groups were transferred to opposite sides). On day 18 of pregnancy, implantation sites and live fetuses were determined, and fetal and placental measurements were recorded.

2.6. Statistical analysis

All statistical analyses were carried out using SPSS (SPSS, Chicago, IL). Data of blastocyst total cell numbers, allocation measurements (ICM and TE), levels of apoptosis, blastocyst, and placental gene expression, and fetal and placental weights are presented as mean ± SEM. Differences in the percentages of embryos developed to two cells, blastocysts, and hatching blastocysts in different treatment groups were compared by χ^2 test. Total blastocyst cell numbers, levels of apoptosis in blastocyst, allocation measurements (ICM and TE), fetal and placental weights, and blastocyst gene expression levels were analyzed using one-way ANOVA test followed by post hoc Fisher's least significance difference (LSD) test. Gene expression levels in placentae were not normally distributed and were analyzed using a nonparametric Mann–Whitney *U* test. *P* values < 0.05 were considered significant.

3. Results

3.1. In vitro culture and embryo development

A total of 2192 zygotes were cultured from the one-cell to blastocyst stage at 37.0 °C, 6% CO_2 in air. The number of one-cell embryos that developed to two cells was not affected by daily exposure to 3 min of ambient air at 37.0 °C; however, in the 5- and 8-min groups, the first cleavage was significantly reduced compared to the control (91.8% and 91.0% vs. 95.4%; $P < 0.05$; Table 1). Blastocyst development was affected when embryos were exposed for 8 min on a daily basis (67.5% vs. 80.0%; $P < 0.05$; Table 1). Daily exposure of embryos to ambient air at 37.0 °C for even 3 min was associated with a significant reduction in the blastocyst hatching rate ($P < 0.05$; Table 1).

3.2. Blastocyst cell number, allocation and apoptosis

Assessment of blastocyst quality by determining blastocyst cell number and allocation revealed a significant decrease in total cell numbers in all groups, compared to the control. The 8-min group displayed a significant reduction of inner cell mass (ICM; 19.91 ± 1.03 cells vs. 15.09 ± 1.26 cells) and trophectoderm (TE; 40.17 ± 1.32 cells vs. 33.64 ± 2.07 cells) compared to control blastocysts ($P < 0.05$; Figure 1). However, the ratio of ICM to TE in the blastocyst was not affected ($P > 0.05$). The level of apoptosis was assessed in blastocysts. There was no significant increase in the levels of apoptosis in blastocysts in the 3- and 5-min groups ($P > 0.05$; Figure 1). However, the apoptotic cell index indicated that (5.60 ± 1.10)% of all cells in blastocysts in the 8-min group were apoptotic ($P < 0.05$; Figure 1).

Table 1
The *in vitro* development of mouse zygotes exposed to ambient air at 37.0 °C.*

Time exposed	% developed to two cells (*n*)#	% blastocysts (*n*)	% hatching blastocysts (*n*)
Control	95.4 (497/521)[a]	80.0 (417/521)[a]	56.1 (234/417)[a]
3 min	93.7 (502/536)[a]	77.4 (415/536)[a]	45.8 (190/415)[b]
5 min	91.8 (503/548)[b]	76.1 (417/548)[a]	38.4 (160/417)[c]
8 min	91.0 (534/587)[b]	67.5 (397/587)[b]	26.7 (106/397)[d]

* Data represent the number of embryos developed to two-cell, blastocyst, and hatching blastocyst stages of development, expressed as a percentage of the initial number of zygotes; #*n*, number of embryos in each group (10 replicate experiments). There were no significant differences among the 10 replicate experiments in the first cleavage, blastocyst, and hatching rates, analyzed using a one-way ANOVA test; [a–d] Different letters within each column denote significant differences, χ^2 test, $P < 0.05$.

3.3. Blastocyst expression of H_{19} and Igf_2

Exposing pre-implantation embryos for 3 and 5 min did not affect the levels of gene expression of H_{19} ($P > 0.05$; Figure 2A). Embryos exposed for 8 min had significantly increased expression levels of H_{19}, compared to the control blastocysts ($P < 0.05$; Figure 2A). Igf_2 was significantly down-regulated in all groups, compared to control blastocysts ($P < 0.05$; Figure 2B).

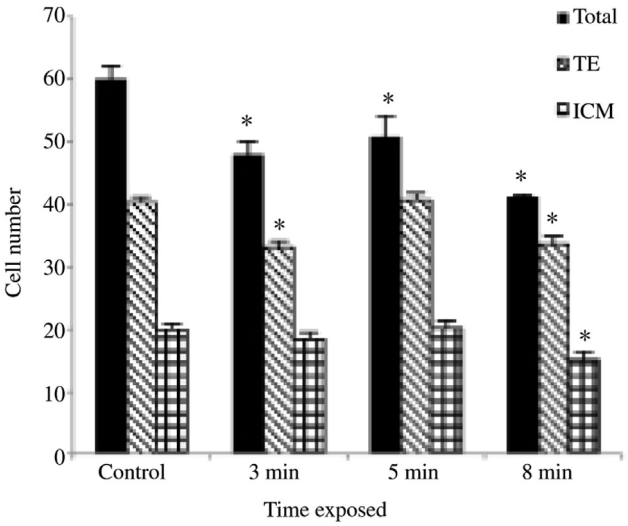

Figure 1. Ambient air at 37.0 °C reduced total cells and induced apoptosis in blastocysts.

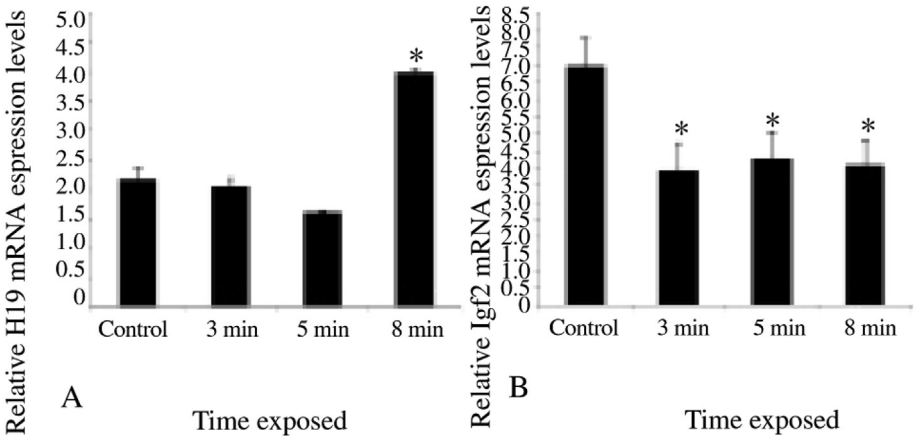

Figure 2. Real time qRT-PCR quantification of H_{19} and Igf_2 mRNA levels in blastocysts exposed to ambient air at 37.0 °C.
Relative abundance of the imprinted genes (A) H_{19} and (B) Igf_2 in blastocysts obtained from zygotes cultured *in vitro* and exposed to ambient air at 37.0 °C. The level of mRNA was quantified by real-time qRT-PCR and normalized to the expression of the reference gene Sdha. Data are mean ± SEM and were analyzed using a one-way ANOVA test, followed by post hoc Fisher's *LSD* test (three replicate experiments; three pools of 30 blastocysts). *Significant difference from the control group, $P < 0.05$.

3.4. Full-term development of blastocysts

The ability of blastocysts to implant and establish a pregnancy was assessed following transfer to pseudopregnant female mice. A significant reduction in implantation rate was observed in blastocysts exposed to ambient air at 37.0 °C for 8 min (52% vs 36%; $P < 0.05$, Table 2). There was a significant reduction in fetal ($P < 0.05$; Figure 3A) and placental weight ($P < 0.05$; Figure 3B) of conceptus resulting from implanted blastocysts in the 8-min group.

3.5. Placental expression of H_{19} and Igf_2

Blastocysts exposed to ambient air at 37.0 °C for 3, 5, and 8 min during the pre-implantation embryo development period were transferred to pseudopregnant recipient mice to assess if

Table 2
The effect of ambient air at 37.0 °C on implantation, fetal growth and placental development on day 18 of pregnancy following day 4 blastocyst transfers to day 3 pseudopregnant female mice.

Day 18 fetal and placental characteristics	Control	3 min	5 min	8 min
Total no. of BΦs[#] transferred (total recipients)	75	65	45	40
Total BΦs transferred (pregnant recipients)	60	50	45	25
BΦs implanted in pregnant recipients (%)[*]	31 (52)[a]	34 (68)[a]	27 (60)[a]	9 (36)[a]
Viable implants/BΦs transferred (%)	21/60 (35)[a]	23/50 (46)[a]	23/45 (51)[a]	7/25 (28)[a]
Viable implants/total implants (%)	21/29 (72)[a]	23/34 (67)[a]	23/27 (85)[a]	7/9 (78)[a]
Resorptions/total implants (%)	8/29 (28)[a]	8/34 (24)[a]	8/27 (29)[a]	2/9 (22)[a]

[#] BΦs = blastocysts; [*] Data were analyzed using χ^2 test (6 replicate experiments).
[a] Significant differences, $P < 0.05$.

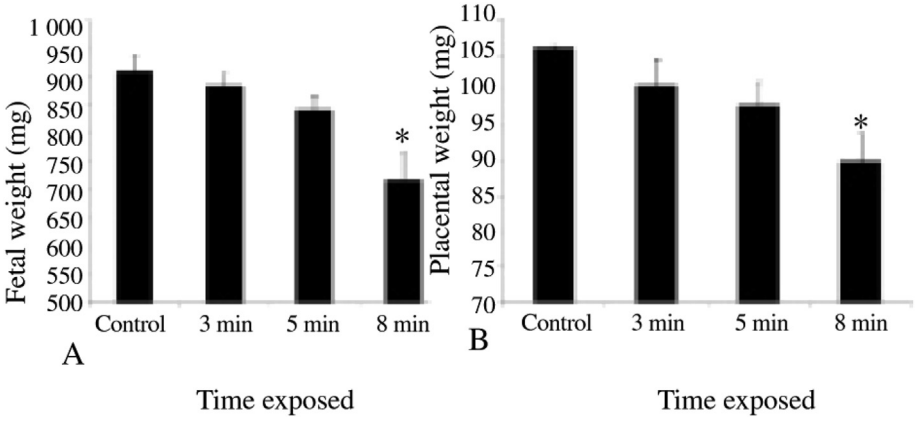

Figure 3. Fetal growth rates in utero on day 18 of pregnancy after transfer of blastocysts obtained from zygotes cultured *in vitro* and exposure to ambient air at 37.0 °C.
Fetal outcomes following embryo transfer for (A) fetal weight and (B) placental weight. Data are presented as mean ± SEM. Control, $n = 21$; 3 min, $n = 23$; 5 min, $n = 23$; and 8 min, $n = 7$ placentae and fetuses analyzed. Data were analyzed using a one-way ANOVA test, followed by post hoc Fisher's *LSD* test. *Significant difference from the control group, $P < 0.05$.

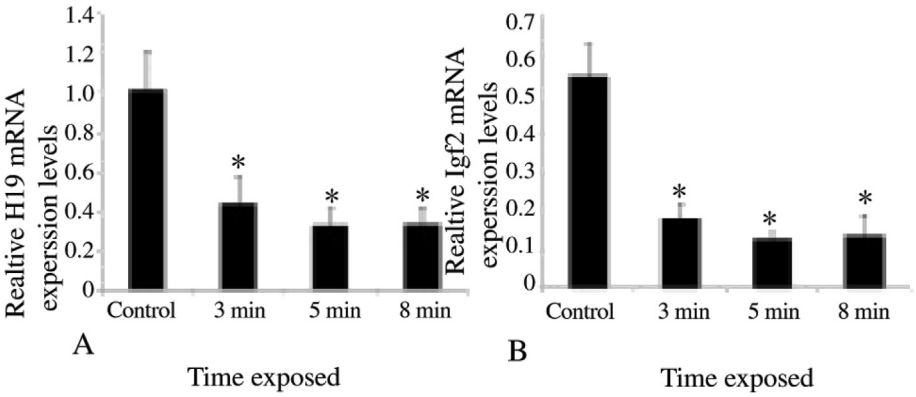

Figure 4. Real time qRT-PCR quantification of H_{19} and Igf_2 mRNA levels in placental tissue.
Relative abundance of the imprinted genes (A) H_{19} and (B) Igf_2 of day-18 placentae after transfer of blastocyst obtained from zygotes cultured *in vitro* and exposed to ambient air at 37.0 °C. The level of mRNA was quantified by real-time qRT-PCR and normalized to the expression of the reference gene Sdha. Data are mean ± SEM, and data were analyzed using Mann–Whitney *U* test. Control, $n = 21$; 3 min, $n = 23$; 5 min, $n = 23$; and 8 min, $n = 7$ placentae used for gene expression analysis. *Significant difference from the control group, $P < 0.05$.

the impaired H_{19} and Igf_2 persisted post-implantation. Placental H_{19} expression was found to be significantly down-regulated in all groups (3, 5, and 8 min), compared to the control ($P < 0.05$; Figure 4A). Similar to H_{19}, Igf_2 expression was significantly down-regulated in all groups ($P < 0.05$; Figure 4B).

4. Discussion

The risks of ART need to be considered in light of emerging evidence of increased incidence of congenital malformations and diseases associated with abnormal genomic imprinting in children conceived through IVF and ICSI. These adverse outcomes are at least partially a consequence of compromised pre-implantation embryo development that may result from suboptimal manipulation and culture. While several studies have linked genetic alterations in pre-implantation embryo and conceptus with laboratory-induced stressors, such as the culture environment and highly invasive techniques such as ICSI, the result of minute manipulations and embryo handling have not been investigated [24].

In our study, we used short exposures (0–8 min) to ambient air to introduce minute changes to the embryo culture system by removing embryos in culture from a controlled incubator environment for short periods daily. The effects of these minute changes were then assessed through analyses of embryo developmental capacity and expression profiles of two growth-related imprinted genes in embryos and placental tissue.

We found that the first cleavage of zygotes and their potential to form blastocysts were decreased when embryos were removed from the incubator for short times daily (5 and 8 min). Blastocysts in the culture systems that were exposed to daily 8-min periods of ambient air had a lower number of cells and an increased incidence of apoptosis. The viability of blastocysts, as determined by their ability to expand and fully hatch, was decreased with exposure to ambient air at 37.0 °C, for as short a time as 3 min daily. The ability of blastocysts to hatch is of great significance, because it affects subsequent implantation and establishment of pregnancy [25]. A blastocyst's failure to hatch, decreased cell numbers, and increased apoptosis are associated with suboptimal embryo culture conditions [25–29].

Removing a carbonate-buffered embryo culture system from a CO_2-containing environment inevitably could change the culture pH. The pH regulatory mechanisms in the embryo are activated immediately after fertilization and pronuclear formation, and the cleavage-stage embryo efficiently regulates the diverse alkaline and acidic loads it encounters during its passage through the fallopian tube to the uterus [30–32]. However, the pH of *in vivo* transition changes gradually and does not exhibit the specific fluctuating pattern found in an *in vitro* culture system, which is a rapid alkaline change induced by a short exposure to ambient air, followed by an equally rapid acidic change after being returned to the incubator. Similarly, small pH fluctuations in the culture environments brought on by increased incubator door openings and daily observations of human pre-implantation embryos have been associated with reduced quality and developmental rates of blastocysts [33]. Our data could suggest that even the smallest perturbations in the culture system may adversely contribute to developmental competence and viability of embryos.

Gene expression analysis of expanded blastocysts resulting from daily exposure of the culture system to ambient air for even short times (3, 5, and 8 min) resulted in significant decreases in Igf_2 expression, while exposure for 8 min was associated with a significant increase in H_{19} expression. The imprinted genes H_{19} and Igf_2 play a critical role in regulating fetal and placental development and in the pathogenesis of imprinting disorders observed in human, which a main reason why they were selected for analysis in this study. *In vitro* culture and IVF are associated with altered expression of Igf_2 and H_{19} in blastocysts, indicating that these genes are extremely sensitive to *in vitro* manipulation, compared with other imprinted genes [7,34]. Suboptimal culture conditions have also been linked with perturbed and -even complete loss of H_{19} expression in blastocysts [35–37].

Embryos were transferred to pseudopregnant recipient mice, and our results showed that the conceptus resulting from the exposed embryos were significantly affected. Eight-minute exposure was associated with significantly decreased implantation rate as well as fetal and placental weight. This finding is of particular importance, because correct development and function of the placenta are crucial for subsequent fetal development and fetal programming [38]. In addition, the placenta, which facilitates physiologic intrauterine development and fetal growth, may be a good indicator of fetal health [39]. It is therefore not surprising that minute changes in the environment during *in vitro* culture, which likely undermined

the adequate development of the placenta, led to fetuses with body weights that averaged 22% less than the control.

Further gene expression analysis of conceptus from embryos with daily exposure to ambient air (3, 5, and 8 min) showed aberrant expression of H_{19} and Igf_2 in the placentae. Other studies have found that IVF and *in vitro* culture of mouse embryos affect not only the expression of H_{19} and Igf_2 in placentae but can lead to impaired expression of a large number of placental genes. H_{19} expression is limited to the TE cells of the blastocyst, so these cells might be more sensitive to epigenetic disturbances caused by *in vitro* culture and manipulation [6,40]. Pre-implantation effects on H_{19} persist post-implantation in a tissue-specific manner, because of a loss of specific methylation of the H_{19} differentially methylated domain in blastocysts and placentae. Since H_{19} and Igf_2 are regulated by the same ICR in opposite ways methylation analysis of ICR would clarify contradictory upregulation of H_{19} at the blastocyst stage and down-regulation in placentae [41].

This effect indicates a possible inability of TE cells to restore genomic imprints in the resulting placenta, whereas it has been suggested that ICM cells exhibit robust mechanisms that protect imprinting in the embryo. The sensitivity of TE cells could result from their closer contact with culture medium and the fact that they are the first cells to differentiate in the developing embryo. In addition, studies associate the inability of TE cells to restore methylation errors in the placenta to a lack of DNA methyltransferases, which maintain methylation in imprinted genes [42–44]. The function of H_{19} in placental development is currently unknown, although H_{19} is associated with the oppositely imprinted gene Igf_2, which is a major regulator of fetal growth [39]. Abnormal expression of H_{19} and Igf_2 is closely linked with aberrant fetal and placental growth, and abnormal regulation of the H_{19} promoter or deletion of H_{19} results in abnormal expression of Igf_2, creating disturbed regulation of fetal and placental growth [45–47]. Loss or deregulation of Igf_2 has been linked with up-regulation or loss of expression of H_{19}, resulting in offspring with affected weight in both human and mouse [39,48–55].

Together the results of our study show that there is a domino effect, in which even minute changes to the mouse embryo cultures significantly affect the developmental competence and viability of the embryos, leading to blastocysts with lower cell numbers and altered gene expression. While these changes do not prevent embryos from implanting and forming a conceptus, the fetal and placental weights were significantly decreased and the genetic perturbations were memorized in the conceptus. Numerous studies in animal models have demonstrated that many components of ART have a negative impact on the offspring, including impaired growth and perturbed gene expression in the conceptus and offspring as a result of IVF and *in vitro* culture; oxygen tension during culture; exposure to fluorescent light and sunlight; disturbances in embryo metabolism; and ICSI [2,24,56–61]. More concerning, several reports in humans demonstrate that placentae from growth-restricted pregnancies exhibit reduced expression of Igf_2 [62–64]. Also, both singleton and twin babies conceived through ART exhibit preterm birth and low birth weight [65–68].

Taken together, these reports and the results of this study suggest that IVF laboratory methods may be correlated with aberrant H_{19} and Igf_2 expression and reduced birth weight of children conceived through ART and that individual embryologists' proficiency may play a role as well.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References

[1] Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, et al. Effect of *in vitro* culture of human embryos on birthweight of newborns. *Hum Reprod* 2010; **25**(3): 605-612.

[2] Sjöblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology* 2005; **146**(5): 2142-2153.

[3] Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, Bilker WB, et al. Long-term effects of culture of preimplantation mouse embryos on behavior. *Proc Natl Acad Sci U S A* 2004; **101**(6): 1595-1600.

[4] Fernández-Gonzalez R, Moreira P, Bilbao A, Jiménez A, Pérez-Crespo M, Ramírez MA, et al. Long-term effect of *in vitro* culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proc Natl Acad Sci U S A* 2004; **101**(16): 5880-5885.

[5] Giritharan G, Talbi S, Donjacour A, Di Sebastiano F, Dobson AT, Rinaudo PF. Effect of *in vitro* fertilization on gene expression and development of mouse preimplantation embryos. *Reproduction* 2007; **134**(1): 63-72.

[6] Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod* 2000; **62**(6): 1526-1535.

[7] Li T, Vu TH, Ulaner GA, Littman E, Ling JQ, Chen HL, et al. IVF results in *de novo* DNA methylation and histone methylation at an *Igf2-H19* imprinting epigenetic switch. *Mol Hum Reprod* 2005; **11**(9): 631-640.

[8] Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod* 2001; **64**(3): 918-926.

[9] Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008; **17**(1): 1-14.

[10] Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, et al. *In vitro* fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010; **5**(2): e9218.

[11] Fauque P, Ripoche MA, Tost J, Journot L, Gabory A, Busato F, et al. Modulation of imprinted gene network in placenta results in normal development of *in vitro* manipulated mouse embryos. *Hum Mol Genet* 2010; **19**(9): 1779-1790.

[12] Hansen M, Bower C, Milne E, de Klerk N, Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects – a systematic review. *Hum Reprod* 2005; **20**(2): 328-338.

[13] Yu L, Chen M, Zhao D, Yi P, Lu L, Han J, et al. The H19 gene imprinting in normal pregnancy and pre-eclampsia. *Placenta* 2009; **30**(5): 443-447.

[14] Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R, Horsthemke B. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. *J Med Genet* 2005; **42**(4): 289-291.

[15] Stinshoff H, Wilkening S, Hanstedt A, Brüning K, Wrenzycki C. Cryopreservation affects the quality of *in vitro* produced bovine

embryos at the molecular level. *Theriogenology* 2011; **76**(8): 1433-1441.
[16] Sutcliffe AG, Derom C. Follow-up of twins: health, behaviour, speech, language outcomes and implications for parents. *Early Hum Dev* 2006; **82**(6): 379-386.
[17] Cox GF, Bürger J, Lip V, Mau UA, Sperling K, Wu BL, et al. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet* 2002; **71**(1): 162-164.
[18] Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y. *In vitro* fertilization may increase the risk of Beckwith–Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *Am J Hum Genet* 2003; **72**(5): 1338-1341.
[19] Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, et al. Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet* 2003; **40**(1): 62-64.
[20] Olson CK, Keppler-Noreuil KM, Romitti PA, Budelier WT, Ryan G, Sparks AE, et al. *In vitro* fertilization is associated with an increase in major birth defects. *Fertil Steril* 2005; **84**(5): 1308-1315.
[21] Kovalevsky G, Rinaudo P, Coutifaris C. Do assisted reproductive technologies cause adverse fetal outcomes? *Fertil Steril* 2003; **79**(6): 1270-1272.
[22] Sjöblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. *Biol Reprod* 2002; **67**(6): 1817-1823.
[23] Brison DR, Schultz RM. Increased incidence of apoptosis in transforming growth factor alpha-deficient mouse blastocysts. *Biol Reprod* 1998; **59**(1): 136-144.
[24] de Waal E, Yamazaki Y, Ingale P, Bartolomei M, Yanagimachi R, McCarrey JR. Primary epimutations introduced during intracytoplasmic sperm injection (ICSI) are corrected by germline-specific epigenetic reprogramming. *Proc Natl Acad Sci U S A* 2012; **109**(11): 4163-4168.
[25] Seshagiri PB, Sen Roy S, Sireesha G, Rao RP. Cellular and molecular regulation of mammalian blastocyst hatching. *J Reprod Immunol* 2009; **83**(1–2): 79-84.
[26] Sjöblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development in vitro. *Hum Reprod* 1999; **14**(12): 3069-3076.
[27] Kamjoo M, Brison DR, Kimber SJ. Apoptosis in the preimplantation mouse embryo: effect of strain difference and *in vitro* culture. *Mol Reprod Dev* 2002; **61**(1): 67-77.
[28] Bain NT, Madan P, Betts DH. The early embryo response to intracellular reactive oxygen species is developmentally regulated. *Reprod Fertil Dev* 2011; **23**(4): 561-575.
[29] Felmer RN, Arias ME, Muñoz GA, Rio JH. Effect of different sequential and two-step culture systems on the development, quality, and RNA expression profile of bovine blastocysts produced in vitro. *Mol Reprod Dev* 2011; **78**(6): 403-414.
[30] Phillips KP, Baltz JM. Intracellular pH regulation by HCO_3^-/Cl^- exchange is activated during early mouse zygote development. *Dev Biol* 1999; **208**(2): 392-405.
[31] Zhao Y, Baltz JM. Bicarbonate/chloride exchange and intracellular pH throughout preimplantation mouse embryo development. *Am J Physiol* 1996; **271**(5 Pt 1): C1512-C1520.
[32] Dale B, Menezo Y, Cohen J, DiMatteo L, Wilding M. Intracellular pH regulation in the human oocyte. *Hum Reprod* 1998; **13**(4): 964-970.
[33] Zhang JQ, Li XL, Peng Y, Guo X, Heng BC, Tong GQ. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. *Reprod Biomed Online* 2010; **20**(4): 510-515.
[34] Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D, et al. Assisted reproductive technology affects developmental kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos. *BMC Dev Biol* 2007; **7**: 116.
[35] Lane M, Gardner DK. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol Reprod* 2003; **69**(4): 1109-1117.
[36] Wu Q, Ohsako S, Ishimura R, Suzuki JS, Tohyama C. Exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the methylation status of imprinted genes H19 and Igf2. *Biol Reprod* 2004; **70**(6): 1790-1797.
[37] Market-Velker BA, Fernandes AD, Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal *in vitro* culture interferes with imprint maintenance. *Biol Reprod* 2010; **83**(6): 938-950.
[38] Godfrey KM. The role of the placenta in fetal programming – a review. *Placenta* 2002; **23**(Suppl. A): S20-S27.
[39] Sibley CP, Coan PM, Ferguson-Smith AC, Dean W, Hughes J, Smith P, et al. Placental-specific insulin-like growth factor 2 (*Igf2*) regulates the diffusional exchange characteristics of the mouse placenta. *Proc Natl Acad Sci U S A* 2004; **101**(21): 8204-8208.
[40] Poirier F, Chan CT, Timmons PM, Robertson EJ, Evans MJ, Rigby PW. The murine H19 gene is activated during embryonic stem cell differentiation *in vitro* and at the time of implantation in the developing embryo. *Development* 1991; **113**(4): 1105-1114.
[41] Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, et al. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004; **131**(15): 3727-3735.
[42] Lei H, Oh SP, Okano M, Jüttermann R, Goss KA, Jaenisch R, et al. *De novo* DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* 1996; **122**(10): 3195-3205.
[43] Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 1998; **19**(3): 219-220.
[44] Chen T, Ueda Y, Dodge JE, Wang Z, Li E. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol Cell Biol* 2003; **23**(16): 5594-5605.
[45] Leighton PA, Saam JR, Ingram RS, Stewart CL, Tilghman SM. An enhancer deletion affects both H19 and Igf2 expression. *Genes Dev* 1995; **9**(17): 2079-2089.
[46] Tabano S, Colapietro P, Cetin I, Grati FR, Zanutto S, Mandò C, et al. Epigenetic modulation of the IGF2/H19 imprinted domain in human embryonic and extra-embryonic compartments and its possible role in fetal growth restriction. *Epigenetics* 2010; **5**(4): 313-324.
[47] Angiolini E, Coan PM, Sandovici I, Iwajomo OH, Peck G, Burton GJ, et al. Developmental adaptations to increased fetal nutrient demand in mouse genetic models of Igf2-mediated overgrowth. *FASEB J* 2011; **25**(5): 1737-1745.
[48] DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 1990; **345**(6270): 78-80.
[49] Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 1993; **75**(1): 73-82.
[50] Kang M. Inherited microdeletions that give rise to Beckwith–Wiedmann Syndrome. *Clin Genet* 2005; **67**(4): 299-300.
[51] Kim M, Geum D, Khang I, Park YM, Kang BM, Lee KA, et al. Expression pattern of HSP25 in mouse preimplantation embryo: heat shock responses during oocyte maturation. *Mol Reprod Dev* 2002; **61**(1): 3-13.
[52] Bressan FF, De Bem TH, Perecin F, Lopes FL, Ambrosio CE, Meirelles FV, et al. Unearthing the roles of imprinted genes in the placenta. *Placenta* 2009; **30**(10): 823-834.
[53] Apostolidou S, Abu-Amero S, O'Donoghue K, Frost J, Olafsdottir O, Chavele KM, et al. Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J Mol Med (Berl)* 2007; **85**(4): 379-387.
[54] Lim D, Bowdin SC, Tee L, Kirby GA, Blair E, Fryer A, et al. Clinical and molecular genetic features of Beckwith–Wiedemann syndrome associated with assisted reproductive technologies. *Hum Reprod* 2009; **24**(3): 741-747.
[55] Demars J, Shmela ME, Rossignol S, Okabe J, Netchine I, Azzi S, et al. Analysis of the IGF2/H19 imprinting control region uncovers

new genetic defects, including mutations of OCT-binding sequences, in patients with 11p15 fetal growth disorders. *Hum Mol Genet* 2010; **19**(5): 803-814.

[56] Thompson JG, Kind KL, Roberts CT, Robertson SA, Robinson JS. Epigenetic risks related to assisted reproductive technologies: short- and long-term consequences for the health of children conceived through assisted reproduction technology: more reason for caution? *Hum Reprod* 2002; **17**(11): 2783-2786.

[57] Delle Piane L, Lin W, Liu X, Donjacour A, Minasi P, Revelli A, et al. Effect of the method of conception and embryo transfer procedure on mid-gestation placenta and fetal development in an IVF mouse model. *Hum Reprod* 2010; **25**(8): 2039-2046.

[58] Feil D, Lane M, Roberts CT, Kelley RL, Edwards LJ, Thompson JG, et al. Effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. *J Physiol* 2006; **572**(Pt 1): 87-96.

[59] Takenaka M, Horiuchi T, Yanagimachi R. Effects of light on development of mammalian zygotes. *Proc Natl Acad Sci U S A* 2007; **104**(36): 14289-14293.

[60] Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 2009; **80**(2): 295-301.

[61] Wakefield SL, Lane M, Mitchell M. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. *Biol Reprod* 2011; **84**(3): 572-580.

[62] Frost JM, Moore GE. The importance of imprinting in the human placenta. *PLoS Genet* 2010; **6**(7): e1001015.

[63] McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, et al. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta* 2006; **27**(6–7): 540-549.

[64] Bourque DK, Avila L, Peñaherrera M, von Dadelszen P, Robinson WP. Decreased placental methylation at the H19/IGF2 imprinting control region is associated with normotensive intrauterine growth restriction but not preeclampsia. *Placenta* 2010; **31**(3): 197-202.

[65] Collier AC, Miyagi SJ, Yamauchi Y, Ward MA. Assisted reproduction technologies impair placental steroid metabolism. *J Steroid Biochem Mol Biol* 2009; **116**(1–2): 21-28.

[66] McDonald SD, Han Z, Mulla S, Murphy KE, Beyene J, Ohlsson A, et al. Preterm birth and low birth weight among *in vitro* fertilization singletons: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2009; **146**(2): 138-148.

[67] McDonald SD, Han Z, Mulla S, Ohlsson A, Beyene J, Murphy KE, et al. Preterm birth and low birth weight among *in vitro* fertilization twins: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2010; **148**(2): 105-113.

[68] Wisborg K, Ingerslev HJ, Henriksen TB. *In vitro* fertilization and preterm delivery, low birth weight, and admission to the neonatal intensive care unit: a prospective follow-up study. *Fertil Steril* 2010; **94**(6): 2102-2106.