



HAL
open science

Does Embryo Culture Medium Influence the Health and Development of Children Born after In Vitro Fertilization?

Céline Bouillon, Roger Léandri, Laurent Desch, Alexandra Ernst, Céline Bruno, Charline Cerf, Alexandra Chiron, Céline Souchay, Antoine Burguet, Clément Jimenez, et al.

► **To cite this version:**

Céline Bouillon, Roger Léandri, Laurent Desch, Alexandra Ernst, Céline Bruno, et al.. Does Embryo Culture Medium Influence the Health and Development of Children Born after In Vitro Fertilization?. PLoS ONE, 2016, 11 (3), pp.e0150857. 10.1371/journal.pone.0150857 . hal-01569820

HAL Id: hal-01569820

<https://hal.science/hal-01569820>

Submitted on 27 Jul 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

RESEARCH ARTICLE

Does Embryo Culture Medium Influence the Health and Development of Children Born after *In Vitro* Fertilization?

Céline Bouillon¹, Roger Léandri², Laurent Desch³, Alexandra Ernst^{4^{‡‡}}, Céline Bruno³, Charline Cerf⁴, Alexandra Chiron⁵, Céline Souchay^{4^{‡‡}}, Antoine Burguet⁶, Clément Jimenez⁷, Paul Sagot⁸, Patricia Fauque^{3*}

1 Service de Médecine et Biologie de la Reproduction, Centre Hospitalier Régional Universitaire de Tours, Tours, France, **2** Centre d'Assistance Médicale à la Procréation, Hôpital Paule de Viguier, Groupe de Recherche en Fertilité Humaine, EA 3694, Toulouse, France, **3** Laboratoire de Biologie de la Reproduction, Hôpital de Dijon, Equipe GAD, Génétique des Anomalies du Développement, EA 4271, Université de Bourgogne, Dijon, France, **4** LEAD—CNRS UMR 5022, Université de Bourgogne, Pôle AAFE, Dijon, France, **5** Laboratoire de Biologie de la Reproduction, SELAFA BIOFFICE—Clinique Jean Villar, Bruges, France, **6** Service de Pédiatrie, Hôpital de Dijon, Université de Bourgogne, Dijon, France, **7** Service de Biologie de la Reproduction-CECOS, Institut des maladies neurodégénératives CNRS UMR 5293, Université de Bordeaux, Bordeaux, France, **8** Service de Gynécologie-Obstétrique, Médecine Fœtale et Stérilité Conjugale, Hôpital de Dijon, Université de Bourgogne, Dijon, France

^{‡‡} Current address: Department of Psychology—Cognition and Behavior, University of Liège, Liège, Belgium

^{‡‡} Current address: Laboratoire de Psychologie et Neurocognition, CNRS-UMR 5105, Université de Grenoble, Grenoble, France

* patricia.fauque@chu-dijon.fr



OPEN ACCESS

Citation: Bouillon C, Léandri R, Desch L, Ernst A, Bruno C, Cerf C, et al. (2016) Does Embryo Culture Medium Influence the Health and Development of Children Born after *In Vitro* Fertilization? PLoS ONE 11(3): e0150857. doi:10.1371/journal.pone.0150857

Editor: Wilfried A. Kues, Friedrich-Loeffler-Institute, GERMANY

Received: December 21, 2015

Accepted: February 19, 2016

Published: March 23, 2016

Copyright: © 2016 Bouillon et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

In animal studies, extensive data revealed the influence of culture medium on embryonic development, foetal growth and the behaviour of offspring. However, this impact has never been investigated in humans. For the first time, we investigated in depth the effects of embryo culture media on health, growth and development of infants conceived by *In Vitro* Fertilization until the age of 5 years old. This single-centre cohort study was based on an earlier randomized study. During six months, *in vitro* fertilization attempts (No. 371) were randomized according to two media (Single Step Medium—SSM group) or Global medium (Global group). This randomized study was stopped prematurely as significantly lower pregnancy and implantation rates were observed in the SSM group. Singletons (No. 73) conceived in the randomized study were included (42 for Global and 31 for SSM). The medical data for gestational, neonatal and early childhood periods were extracted from medical records and parental interviews (256 variables recorded). The developmental profiles of the children in eight domains (social, self-help, gross motor, fine motor, expressive language, language comprehension, letter knowledge and number knowledge – 270 items) were compared in relation to the culture medium. The delivery rate was significantly lower in the SSM group than in the Global group ($p < 0.05$). The culture medium had no significant effect on birthweight, risk of malformation (minor and major), growth and the frequency of medical concerns. However, the children of the Global group were less likely than those of the SSM group to show developmental problems ($p = 0.002$), irrespective of the different domains. In

conclusion, our findings showed that the embryo culture medium may have an impact on further development.

Introduction

Experimental animal studies on maternal diet during early embryonic development clearly showed that even subtle changes in the nutritional environment could alter phenotypes in offspring (like insulin resistance and vascular system dysfunction with high blood pressure) [1]. Similarly, it has been shown that individuals conceived during a period of famine have an increased risk of diseases like obesity, coronary artery disease and cognitive schizophrenia [2–4]. In addition, there is a lot of evidence from mouse studies that environmental culture conditions could affect the birthweight of offspring and have later consequences. Some authors recently reported that the type of culture medium used in human reproduction may also influence the birthweight of the children thus conceived [5–8]. Thus, the health of children born following Assisted Reproductive Technologies (ART) needs to be monitored with regard to the efficacy of the embryo culture medium used.

In our lab, an earlier randomized study was conducted to assess the effectiveness of two different culture media on *In Vitro* Fertilization (IVF) outcomes. However, six months later, this study was prematurely stopped as one medium was found to be significantly underperforming (in terms of pregnancy rate).

Thus, the purpose of the current study was to investigate in depth the effects of this underperforming embryo culture medium on children's health at birth and in the longer term, until the age of 5 years old. Our findings showed that embryo culture may have an impact on further development. These results confirm that the preimplantation period is a critical window of development. This is aligned with the consensus hypothesis that ART procedures increase epigenetic perturbations, potentially causing long-term health outcomes.

Materials and Methods

Study design and Population

This single-centre cohort study is based on an earlier randomized study conducted in 2008 at the University Hospital of Dijon, which compared the IVF outcomes following use of the two media: Global medium (LifeGlobal) and Single Step Medium (SSM, Irvine Scientific), the components are presented in [Table 1](#). Indeed, during this study, both embryo culture media were randomly used from the day of oocyte retrieval to day 2 or day 3; all other culture conditions in the laboratory were identical. Patients were randomized by the embryologist on the day the cycle was started according to a computer-generated randomization table. Clinicians performing the embryo transfer and patients were blinded for the allocation. However, as this randomized study showed that Global medium was significantly superior to SSM medium in terms of pregnancy and implantation rates, the study was stopped 6 months later.

Global medium was used for 179 attempts and SSM medium for 192 attempts of conventional IVF or ICSI (IntraCyttoplasmic Sperm Injection). The difference corresponded to cases without embryo transfer (ovarian hyperstimulation syndrome, high progesterone hormone levels and no oocyte at retrieval). For the present study, all singleton live births after embryo transfers of this randomized study were included. Altogether, 73 singleton births occurred including 42 for Global medium and 31 for SSM medium.

All participating couples had given their written informed consent to participate in the earlier randomized study and also in the children health study. This consent procedure was

Table 1. Components of both culture media according to published analyses*.

Component Type		Global medium	SSM medium
Salts and Ions	Sodium Chloride	+	+
	Potassium Chloride	+	+
	Magnesium Sulfate	+	+
	Calcium Chloride	+	+
	Potassium Phosphate	+	+
Buffer	Sodium Bicarbonate	+	+
Energy Substrates	Glucose	+	+
	Sodium Pyruvate	+	+
	Sodium Lactate	+	+
Amino Acids	<i>Essential amino acids</i>		
	Arginine	+	+
	Cysteine	+	+
	Histidine	+	+
	Isoleucine	+	+
	Leucine	+	+
	Lysine	+	+
	Methionine	+	+
	Phenylalanine	+	+
	Threonine	+	+
	Tryptophan	+	+
	Tyrosine	+	+
	Valine	+	+
	<i>Non-essential amino acids</i>		
	Alanine	+	+
	Asparagine	+	+
	Aspartic acid	+	+
	Glutamic acid	+	+
	Glycine	+	+
Proline	+	+	
Serine	+	+	
Taurine	-	+	
<i>Di-peptide</i>			
	Glycyl-L-Glutamine		Alanyl-L-Glutamine
Chelator	EDTA	+	+
Indicator	Phenol Red	+	+
Antibiotic	Gentamicin	+	+

* Zhao et al., 2013; Morbeck et al., 2014

doi:10.1371/journal.pone.0150857.t001

approved by the Institutional Review Board of Burgundy in France, and registered under number “2008-A01491-54”.

IVF procedure and outcome measurements

The choice between ICSI and IVF as the fertilization method depended upon semen sample characteristics and the history of the couples involved.

Oocytes and embryos were individually cultured in 30µl of either Global or SSM, both of which were supplemented identically with Human Serum Albumin 10%, Irvine Scientific) at 37°C under a humidified 3-gas atmosphere (5% CO₂, 5% O₂ and 90% N₂). The mineral oil

used to recover the culture droplets (Nidacon), culture dishes (BD Falcon) and incubators were also identical for both groups. The pH of the equilibrated media was around 7.3.

Depending on the age of the women, the number of previous cycles, and the number and quality of embryos available, one or two embryos were transferred at day 2 or day 3.

Data collection

The collected items were established in collaboration with the Officials of ELFE study (French longitudinal study of children) [9]. Deliveries occurred in 11 maternity hospitals. Gestational and neonatal medical data were extracted from the medical record on site (97 variables recorded). Of the 73 deliveries, there was only one delivery for which we were unable to obtain information.

Regarding the data on the long-term health, growth and development of children, couples were first contacted by mail. In the absence of a refusal to continue to participate, they were contacted for a telephone interview. Of the 73 couples, seven could not be reached. All of the couples contacted agreed to the interview, and their responses were based on the national medical health record book (*carnet de santé*) of the children (159 variables recorded). This child's *carnet de santé* includes compulsory medical examinations performed at 8 days, at 2, 4, 9, and 24 months, and then at 3 and 4 years. Weight, height, and head circumference were recorded at the age of 2 months, 4 months, 9 months, 24 months, 3 and 4 years. Head circumference was only measured until the age of 24 months. Post-natal weight and height measurements were expressed as standard deviation scores, normalized for age and gender (according to AUDI-POG database [10]). Any hospitalization, surgery or disease was noted. The various malformations detected at birth and after the neonatal period were classified as minor or major malformations according to the EUROCAT (European Surveillance of Congenital Anomalies) classification [11].

Around five years after delivery, parents completed the French version of the Child Development Inventory (CDI) based on 270 items [12], which aims to assess the developmental profile of children in eight domains, including social, self-help, gross motor, fine motor, expressive language, language comprehension, letter knowledge and number knowledge. By the CDI analysis, two measurements of the children's development were provided: the developmental age for each domain, which corresponds to the age at which behaviors first appear in children in general and the general developmental score, which takes into account the chronological age of children and includes 70 items extracted from all 270 items previously demonstrated to be age-discriminating (Instructions and examples of items are provided in [S1 File](#)). This questionnaire has been selected based on its psychometric properties showing a high sensitivity and specificity as well as a good predictive value [12–14]. The CDI has been identified as a useful and cost-effective screening measure for determining developmental outcomes among high-risk infants [14].

Statistical Analysis

Before comparing the effects of both culture media on the health of children, the homogeneity between the two groups (Global and SSM media) was tested for maternal age, distribution between IVF and ICSI, indication and attempt number. A Chi² test was used for binary variables. The Fisher test was used to compare small sample sizes. For continuous variables, analysis of variance was performed. If necessary, a Kruskal-Wallis test was applied. Since the CDI results were not normally distributed, data were first transformed to normality, for both the developmental age and the general developmental score. The absence of any between-group difference for the parental socio-economic level (according to the INSEE database, i.e. the French National Institute of Statistics and Economic Studies) was first verified using Chi² test.

Table 2. Pregnancy outcomes.

	Global group	SSM group	Rate Ratio	p
	(No. 179)	(No. 192)	(95% CI)	
Implantation (% per embryo transferred ^a)	82 (29.1%)	48 (16.3%)	1.78 [1.31–2.43]	0.001
Clinical pregnancies (% per cycle ^b)	66 (36.9%)	42 (21.9%)	1.68 [1.22–2.33]	0.001
Twin pregnancies (% per clinical pregnancy)	8 (12.1%)	4 (9.5%)	1.27 [0.41–3.96]	0.76
Early spontaneous abortion (% by positive pregnancy test)	18 (24.7%)	9 (18.0%)	1.37 [0.68–2.78]	0.38
Ectopic pregnancy (% by positive pregnancy test)	1 (1.4%)	0 (0%)	NC	-
Therapeutic abortions (% by positive pregnancy test)	1* (1.4%)	0 (0%)	NC	-
Delivery of live-born children (% per cycle ^c)	50 (27.9%)	35 (18.2%)	1.53 [1.05–2.23]	0.03
Delivery of singleton (% per total number of live birth deliveries)	42 (84.0%)	31 (88.6%)	0.95 [0.80–1.13]	0.75
Delivery of twin (% per total number of live birth deliveries)	8 (16.0%)	4 (11.4%)	1.40 [0.46–4.26]	0.75

Data are presented as numbers (%)

No.: number of cycles

NC: Not calculable

*: for exencephaly

^a: The implantation rate was the ratio between the number of gestational sacs and the number of transferred embryos.

^b: Clinical pregnancy was determined by the presence of an intrauterine gestational sac with a foetal heartbeat on ultrasound examination 4–5 weeks after the embryo transfer.

^c: The delivery rate was the ratio between the number of deliveries and the number of embryo transfers.

doi:10.1371/journal.pone.0150857.t002

Correlations between the CDI domains and gender, term of birth, mother and father’s socio-economic status were explored. In case of significant correlation, these variables were included as covariable. Regarding the developmental profile in the eight domains of the CDI, the effect of the culture medium was explored in a blinded manner using repeated measures ANOVA. Similar analyses were conducted to investigate the effect of the fertilization method. Concerning the general developmental score, a t-test for independent samples was used. The difference was considered significant if $p \leq 0.05$.

Results

Preimplantation and prenatal periods

In the randomized study conducted in 2008, the implantation and delivery rates were significantly lower in the SSM group than in the Global group ($p < 0.05$ —[Table 2](#)). For each group, the characteristics of the parents and cycles of the 73 singletons born after embryo transfer are shown in [S1 Table](#). For these singletons, no difference was found between the groups except for early embryo development (embryos in the SSM group exhibited significantly more fragmentation and, their development was significantly late in comparison with embryos cultured in Global medium, data not shown).

For the gestational complications and distribution of the delivery mode, no significant difference was found between the groups ([S2 Table](#)).

Birthweight and malformations

At birth, no difference was noted between the two groups for gestational age, sex distribution, proportion of children admitted to a neonatal intensive care unit and days of hospitalization ([S3 Table](#)). The birthweight of singletons was $3131\text{g} \pm 505\text{g}$ and $3145\text{g} \pm 448\text{g}$ for the Global and SSM groups, respectively. Taking into account the criteria considered relevant following

univariate analyses (S1 Table), namely male smoker, gestational diabetes, term birth and gender, the culture medium had no significant effect on birthweight ($F(1, 68) = 1.16, p = 0.28; \eta^2_p < 1\%$). None of the offspring had a very low birthweight (<1500g) or high birthweight (>4500g).

Defects detected in singletons were classified as minor and major malformations according to the EUROCAT classification (Table 3, S2 File). Statistical analysis showed no difference between the groups regarding defects, whether minor or major.

Growth and medical concerns

No significant differences between the groups emerged for weight, height, body mass index and head circumference measured at the age of 9 months, 24 months and 4 years (S1 Fig).

Up to 4 years old, various medical histories were studied regarding hospitalization, medical or surgical treatment and different chronic diseases (Table 4, S3 File). No differences between the groups were found.

Development

No significant difference was found between the groups regarding the socioeconomic status of mothers ($p = 0.45$) or fathers ($p = 0.68$).

A first set of analyses was conducted on the developmental age of 55 children (31 from the Global group and 24 from the SSM group, established on complete data) in each CDI domain. Statistical analysis revealed a main effect of the culture medium ($F(1, 53) = 5.32, p = 0.02, \eta^2_p = 0.09$), with a significantly higher developmental age in children from the Global group. In other words, children from the Global group overall obtained better developmental scores than those from the SSM group, in developmental domains including social skills, self-help, gross and fine motor skills, expressive language, language comprehension, as well as letter and number knowledge.

To control for the influence of potential confounding factors, correlations between gender, term birth, mother and father's socioeconomic status with the CDI domains were explored (S4 Table). Based on these results, mother's socioeconomic status was included as a covariable in

Table 3. Malformations.

		<i>Global group*</i>	<i>SSM group*</i>
		<i>(No. 40)</i>	<i>(No. 31)</i>
Major malformations (according to EUROCAT)	Detected during the neonatal period	2 (5.1%)	1 (3.2%)
	Detected after the neonatal period	1 (2.8%)	1 (3.3%)
Minor malformations	Detected during the neonatal period	7 (18.0%)	3 (9.7%)
	Detected after the neonatal period	9 (25.0%)	7 (23.3%)
Major malformations by organ type	Cardiac ^a	2 (5.6%)	1 (3.3%)
	Nervous system ^b	0 (0%)	1 (3.3%)
	Limbs ^c	1 (2.8%)	0 (0%)

Available data are presented as numbers (%).

No.: number of singletons

^aAll cardiac malformations were ventricular septal defect

^bThe nervous system abnormality was a macrocrania with hydrocephalus but without intracranial hypertension and with a normal neurological development of the child.

^c The limb abnormality was a bilateral syndactyly of the first knuckle of the 2nd and 3rd toes.

* Statistical analysis showed no difference between the groups, $p > 0.05$

doi:10.1371/journal.pone.0150857.t003

Table 4. Singletons' medical history at 4 years old.

	<i>Global group*</i>	<i>SSM group*</i>
	<i>(No. 36)</i>	<i>(No. 30)</i>
Age (years)	4.2 (0.2)	4.1 (0.2)
Weight (kg)	16.2 (2.3)	16.0 (1.9)
Height (cm)	103.4 (4.3)	104.2 (4.1)
BMI	15.1 (1.3)	14.8 (1.1)
Asthmatic medication	5 (13.9%)	5 (16.7%)
Treatment > 1 month	18 (50.0%)	14 (46.7%)
Eczema > 1 flare	7 (19.4%)	9 (30.0%)
Psychotherapy	6 (16.7%)	4 (13.3%)
Hospitalizations (night)	10 (27.8%)	13 (43.3%)
Hospitalizations (day)	9 (25.0%)	5 (16.7%)
Chronic diseases	8 (22.2%)	6 (20.0%)
Cardiac	2 (5.6%)	1 (3.3%)
Pulmonary (asthma)	4 (11.1%)	4 (13.3%)
Neurological	1 (2.8%)	1 (3.3%)
ENT with secondary deafness	1 (2.8%)	1 (3.3%)
Digestive	1 (2.8%)	0 (0%)
Haematological	1 (2.8%)	0 (0%)
Other diseases	0 (0%)	1 (3.3%)
Surgery	8 (22.2%)	5 (16.7%)
Circumcision	1 (2.8%)	3 (10.0%)
Ventilating tube treatment	4 (11.1%)	3 (10.0%)
Tonsillectomy	1 (2.8%)	1 (3.3%)
Adenoid removal	2 (5.6%)	1 (3.3%)
Other surgeries	3 (8.3%)	0 (0%)

Available data are presented as numbers (%) or mean (SD)

BMI: Body Mass Index, ENT: Ear Nose and Throat, No.: number of singletons

* Statistical analysis showed no difference between the groups, $p > 0.05$

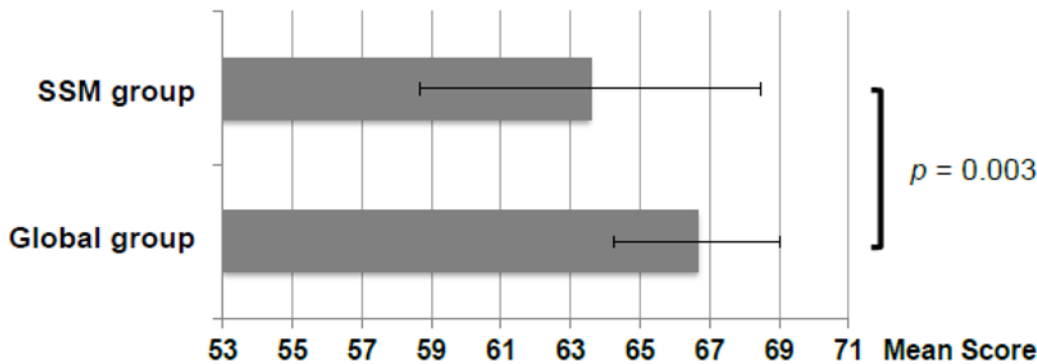
doi:10.1371/journal.pone.0150857.t004

ANOVA analysis. The effect of culture medium type on developmental age was still significant ($F(1, 52) = 4.06, p = 0.04, \eta^2_p = 0.07$), showing a higher developmental age in children from the Global Group than in those from the SSM group. No significant culture medium x CDI domains interaction was obtained ($F(7, 371) = 1.98, p = 0.06, \eta^2_p = 0.03$). The absence of any significant interaction means that the effect of culture medium on developmental problems is the same in every CDI domain.

The second set of statistical analyses focused on the general developmental score, which provides a general developmental indicator taking into account the chronological age, with no distinction of particular developmental domains (see [S1 File](#) for more details on the scoring). The comparison of this measure between the groups showed a significant difference ($t = 3.04, p = 0.003$), with a higher score for the Global group than for the SSM group ([Fig 1](#)).

A complementary analysis was run to explore the number of children with significant developmental problems (according to the CDI norms) in each group. The number of children presenting with developmental problems in at least one CDI domain was significantly lower in the Global group (8 out of 31 children) than in the SSM group (16 out of 24 children) ($\chi^2 = 9.18, p = 0.002$ —[Fig 1](#)).

A: General developmental score (mean)



B: Proportion of children with developmental problems in at least one domain

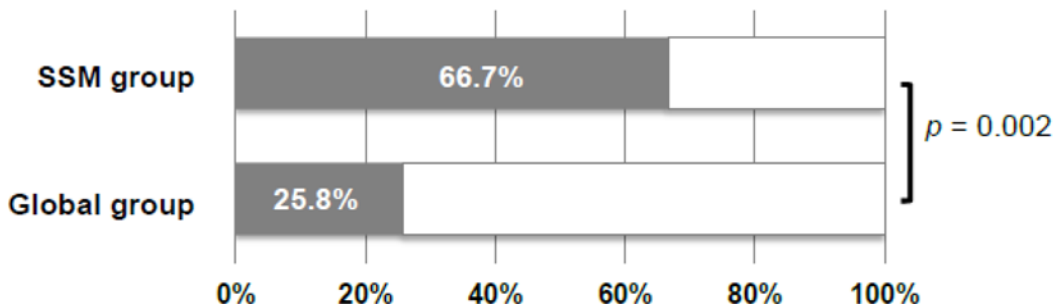


Fig 1. Development. General developmental score (A) and proportion of children with developmental problems in at least one out of eight domains according to the CDI norms (B) in Global and SSM groups.

doi:10.1371/journal.pone.0150857.g001

The comparison of the fertilization method yielded no significant results (for each CDI domain: $F(1, 53) = 0.71, p = 0.40, \eta^2_p = 0.01$ or regarding the general developmental score: $p = 0.55$) and no significant fertilization method x CDI domains interaction was found ($F(7, 371) = 1.23, p = 0.28, \eta^2_p = 0.02$).

Discussion

To our knowledge, no prior studies have explored the longer-term effects of culture media on health, growth and development of infants until the age of 5 years old. Our data highlight significant differences on the developmental age. These findings of the effect of embryo culture medium are crucial and raise important points.

Studies published so far have mainly focused on the health of children according to the IVF method used [15]. The influence of the culture medium was assessed with regard to gestational development, birthweight and health at birth [5]. Only one recent study on culture media has been published, and this concerned weight during the first two years of life [16]. However, in animal studies, extensive data are available and reveal the influence of the type of culture medium on embryonic development and foetal growth of offspring [5,17,18]. In our study, we observed poorer embryo scoring with SSM than with Global. It is important to know what

differences in culture media composition may explain this negative impact on embryo development. We can exclude the protein source, as the supplements were the same in both media. From manufacturers' data and previous published analyses, the composition of both media used in this study seems to be roughly identical except for two components [19,20]. First, SSM medium contains the amino acid taurine whereas Global medium does not. Taurine has been added to culture media for its antioxidant properties even though its ability to increase blastocyst formation in humans has not been proven [21]. A recent study performed in chick embryos even suggested that exogenous taurine increased brain oxidative stress and apoptosis during embryo development [22]. Second, glutamine, a deliberately omitted labile amino acid, is supplied in both media by different heat-stable di-peptides (Alanyl-L-Glutamine (AlaGln) in SSM and Glycyl-L-Glutamine (GlyGln) in Global) in order to prevent ammonium production, which has long been known to retard foetal development and cause neural tube defect exencephaly [23] even after short exposure especially during the cleavage stages [24]. However, in line with our results, the lower efficacy of AlaGln as compared with GlyGln has also been reported in mouse preimplantation embryos [25]. Potentially, ammonium release could be different depending on the di-peptide form used. Recently, Wale and Gardner showed that embryo culture performed under 5% oxygen induced glutamine production and could alleviate ammonium stress in the mouse embryo [26]. In the current study, 5% oxygen was employed. Another important method to reduce ammonium production is to titrate down the levels of amino acids in the medium [27]. Regrettably, as the manufacturers didn't disclose the specific concentration of components, a valid analysis of the levels of amino acids in the two media is currently impossible.

In humans, studies were essentially based on early embryo development or implantation rates [28], and those analysing birthweight and post-natal growth remain limited [5,16]. However, some studies, again revealed that the composition of the culture medium could affect early embryonic and foetal development and even birthweight [7,8]. Meta-analyses reported, after correction for confounding factors, that ART-conceived singletons also had a higher risk of prematurity and perinatal morbidity than children conceived naturally [29,30]. In our study, after identical adjustments, no difference was highlighted between the two culture media at this step. Finally, we found no increased risk of either minor and major malformations or physical health problems until the age of five years in singletons from the SSM group although this medium was underachieving. We have to keep in mind that this absence of difference is probably due to the fact that the size of our children cohort is relatively small to evidence non-major differences between groups. However, this study revealed that the children from the Global group were significantly less likely to display developmental problems than were those of the SSM group. Importantly, this effect of culture medium on child development was the same in every CDI domain, including social skills, self-help, gross and fine motor skills, language comprehension, expressive language, letter and number knowledge. The method used in this work to determine the developmental outcomes has been selected based on its psychometric properties showing a high sensitivity and specificity as well as a good predictive value (i.e. in comparison with standard measure of developmental status such as neuropsychological tests) [12–14]. To our knowledge, no study evaluated the developmental and cognitive status of ART-singletons according to the type of culture medium. However, some studies on mental and motor development of ART-children showed that children were more likely to have delayed mental and motor development and a lower intelligence quotient [31–33] whereas other studies reported no adverse cognitive and motor outcomes [32,34–36].

The current study provides a valid and detailed comparison. Indeed, the initial randomized study performed in a single centre over a short period of time ensured that the environment of the lab, especially the culture equipment (i.e. incubator, plastic dishes, oil, etc. . .) was similar.

In addition, the participation was very high (90.4%) as was the completeness of data (89.6%). The absence of significant differences for health issues should not reassure us. Differences in the health status may occur later, which is why this cohort study requires a longer follow-up. Indeed, Ceelen and colleagues reported an increase in the sum of skinfolds and a higher risk of cardiovascular disease at age 8–18 years in IVF-children than in children from the general population (2009) [37]. In addition, several authors reported that new-borns that are either too small or too big may be vulnerable to heart disease, hypertension, type II diabetes and obesity in adulthood [38,39]. As reported in animal models, the embryo culture step itself could perturb embryo metabolism and gene expression [40] and have consequences on behaviour [41,42]. Even though the developmental delay at five years observed in SSM group may disappear later, it could be the first sign of more long-term neuromotor developmental concerns.

These embryonic responses to early environmental stress could be kept in memory and provoke adverse effects in infancy and even in adulthood, potentially through epigenetic changes (such as DNA methylation). Indeed, in recent years, it has been shown that the risk of epigenetic disorders is greater in ART-children than in naturally conceived children [43] and that any stress during embryo culture can cause epigenetic changes in humans and in animals [44–48] which are potentially responsible for the occurrence of disease not only at birth but also in adulthood [49]. Furthermore, in a recent study some epigenetic errors can still be observed during childhood in ART infants [50].

Thus, it would be interesting to carry out studies comparing the epigenetic landscape of children born following the use of both media. Regions involved in neuronal development should be specifically studied since DNA methylation was recently found to be altered in the sperm of fathers of infants with autism signs [51].

Conclusions

For the first time, we have observed that embryo culture medium can have an impact on development in humans. These findings are crucial and further investigations are needed. Indeed, they could also enhance our knowledge about maternal nutrition during the periconception period. Media are optimized in animal models. However, in view of our results and the literature, the precise formulation of culture media should be disclosed and we recommend more evaluations dedicated to human embryo culture before their commercialization, for example by including epigenetic assays on mouse embryos. Our findings also plead for both neonatal (only demanded in France) and long-term follow-up of ART-children.

Supporting Information

S1 Fig. Comparison of singletons' weight (A), height (B), BMI (C) and head circumference (D) at the age of 9 months, 24 months and 4 years old.

(TIF)

S1 File. Instructions and examples of items for each domain of the CDI.

(DOCX)

S2 File. Details on malformations.

(DOCX)

S3 File. Details on growth and medical concerns.

(DOCX)

S1 Table. Parental and cycle characteristics of singletons.

(DOCX)

S2 Table. Maternal health during pregnancy.
(DOCX)

S3 Table. Neonatal data of singletons.
(DOCX)

S4 Table. Correlations between gender, term birth, mother and father socio-economic status with the CDI domains.
(DOCX)

Acknowledgments

The authors thank Officials of ELFE (Etude Longitudinale Française depuis l'Enfance) for their collaboration, the technical team of the IVF Center of Dijon for their technical help, Pierre Jouannet for his comments on the manuscript and Philip Bastable for proof-reading.

Author Contributions

Conceived and designed the experiments: CB CC CS CJ PS PF. Performed the experiments: CB LD AE CB CC AC CS PF. Analyzed the data: CB RL LD AE CB AB CS CJ PS PF. Contributed reagents/materials/analysis tools: CB AB CJ PS PF. Wrote the paper: CB RL AE PF.

References

1. Velazquez MA. Impact of maternal malnutrition during the periconceptional period on mammalian pre-implantation embryo development. *Domest Anim Endocrinol.* 2015; 51:27–45. doi: [10.1016/j.domaniend.2014.10.003](https://doi.org/10.1016/j.domaniend.2014.10.003) PMID: [25498236](https://pubmed.ncbi.nlm.nih.gov/25498236/).
2. de Rooij SR, Wouters H, Yonker JE, Painter RC, Roseboom TJ. Prenatal undernutrition and cognitive function in late adulthood. *Proc Natl Acad Sci U S A.* 2010; 107(39):16881–16886. doi: [10.1073/pnas.1009459107](https://doi.org/10.1073/pnas.1009459107) PMID: [20837515](https://pubmed.ncbi.nlm.nih.gov/20837515/); PubMed Central PMCID: PMC2947913.
3. Painter RC, de Rooij SR, Bossuyt PM, Simmers TA, Osmond C, Barker DJ, et al. Early onset of coronary artery disease after prenatal exposure to the Dutch famine. *Am J Clin Nutr.* 2006; 84(2):322–327; quiz 466–327. PMID: [16895878](https://pubmed.ncbi.nlm.nih.gov/16895878/).
4. Stein AD, Kahn HS, Rundle A, Zybert PA, van der Pal-de Bruin K, Lumey LH. Anthropometric measures in middle age after exposure to famine during gestation: evidence from the Dutch famine. *Am J Clin Nutr.* 2007; 85(3):869–876. PMID: [17344511](https://pubmed.ncbi.nlm.nih.gov/17344511/).
5. Zandstra H, Van Montfoort AP, Dumoulin JC. Does the type of culture medium used influence birth-weight of children born after IVF? *Hum Reprod.* 2015; 30(3):530–542. doi: [10.1093/humrep/deu346](https://doi.org/10.1093/humrep/deu346) PMID: [25574031](https://pubmed.ncbi.nlm.nih.gov/25574031/).
6. Hassani F, Eftekhari-Yazdi P, Karimian L, Rezazadeh Valojerdi M, Movaghgar B, Fazel M, et al. The Effects of ISM1 Medium on Embryo Quality and Outcomes of IVF/ICSI Cycles. *Int J Fertil Steril.* 2013; 7(2):108–115. PMID: [24520472](https://pubmed.ncbi.nlm.nih.gov/24520472/); PubMed Central PMCID: PMC3850342.
7. 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. doi: [10.1093/humrep/dep456](https://doi.org/10.1093/humrep/dep456) PMID: [20085915](https://pubmed.ncbi.nlm.nih.gov/20085915/).
8. Nelissen EC, Van Montfoort AP, Coonen E, Derhaag JG, Geraedts JP, Smits LJ, et al. Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos. *Hum Reprod.* 2012; 27(7):1966–1976. doi: [10.1093/humrep/des145](https://doi.org/10.1093/humrep/des145) PMID: [22552689](https://pubmed.ncbi.nlm.nih.gov/22552689/).
9. Vandentorren S, Bois C, Pirus C, Sarter H, Salines G, Leridon H, et al. Rationales, design and recruitment for the Elfe longitudinal study. *BMC Pediatr.* 2009; 9:58. doi: [10.1186/1471-2431-9-58](https://doi.org/10.1186/1471-2431-9-58) PMID: [19772571](https://pubmed.ncbi.nlm.nih.gov/19772571/); PubMed Central PMCID: PMC2754449.
10. Vendittelli F, Riviere O, Crenn-Hebert C, Claris O, Tessier V, Pinquier D, et al. [Audipog perinatal network. Part 1: principal perinatal health indicators, 2004–2005]. *Gynecol Obstet Fertil.* 2008; 36(11):1091–1100. doi: [10.1016/j.gyobfe.2008.08.010](https://doi.org/10.1016/j.gyobfe.2008.08.010) PMID: [18926760](https://pubmed.ncbi.nlm.nih.gov/18926760/).
11. EUROCAT. Guide 1.4 and Reference Documents: Instructions for the Registration and Surveillance of Congenital Anomalies. European Surveillance of Congenital Anomalies. 2013.

12. Duyme M, Zorman M, Tervo R, Capron C. French norms and validation of the Child Development Inventory (CDI): Inventaire du Developpement de l'Enfant (IDE). *Clin Pediatr (Phila)*. 2011; 50(7):636–647. doi: [10.1177/0009922811398390](https://doi.org/10.1177/0009922811398390) PMID: [21339250](https://pubmed.ncbi.nlm.nih.gov/21339250/).
13. Chaffee CA, Cunningham CE, Secord-Gilbert M, Elbard H, Richards J. The influence of parenting stress and child behavior problems on parental estimates of expressive and receptive language development. *J Abnorm Child Psychol*. 1991; 19(1):65–74. PMID: [2030248](https://pubmed.ncbi.nlm.nih.gov/2030248/).
14. Doig KB, Macias MM, Saylor CF, Craver JR, Ingram PE. The Child Development Inventory: A developmental outcome measure for follow-up of the high-risk infant. *J Pediatr*. 1999; 135(3):358–362. PMID: [10484803](https://pubmed.ncbi.nlm.nih.gov/10484803/).
15. Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment: Part I—General health outcomes. *Hum Reprod Update*. 2013; 19(3):232–243. doi: [10.1093/humupd/dms062](https://doi.org/10.1093/humupd/dms062) PMID: [23449642](https://pubmed.ncbi.nlm.nih.gov/23449642/).
16. Kleijkers SH, van Montfoort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC, et al. IVF culture medium affects post-natal weight in humans during the first 2 years of life. *Hum Reprod*. 2014; 29(4):661–669. doi: [10.1093/humrep/deu025](https://doi.org/10.1093/humrep/deu025) PMID: [24549211](https://pubmed.ncbi.nlm.nih.gov/24549211/).
17. Schwarzer C, Esteves TC, Arauzo-Bravo MJ, Le Gac S, Nordhoff V, Schlatt S, et al. ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses. *Hum Reprod*. 2012; 27(9):2627–2640. doi: [10.1093/humrep/des223](https://doi.org/10.1093/humrep/des223) PMID: [22736328](https://pubmed.ncbi.nlm.nih.gov/22736328/).
18. Morbeck DE, Paczkowski M, Fredrickson JR, Krisher RL, Hoff HS, Baumann NA, et al. Composition of protein supplements used for human embryo culture. *J Assist Reprod Genet*. 2014; 31(12):1703–1711. doi: [10.1007/s10815-014-0349-2](https://doi.org/10.1007/s10815-014-0349-2) PMID: [25261352](https://pubmed.ncbi.nlm.nih.gov/25261352/); PubMed Central PMCID: PMC4250459.
19. Morbeck DE, Krisher RL, Herrick JR, Baumann NA, Matern D, Moyer T. Composition of commercial media used for human embryo culture. *Fertil Steril*. 2014; 102(3):759–766 e759. doi: [10.1016/j.fertnstert.2014.05.043](https://doi.org/10.1016/j.fertnstert.2014.05.043) PMID: [24998366](https://pubmed.ncbi.nlm.nih.gov/24998366/).
20. Zhao Q, Yin T, Peng J, Zou Y, Yang J, Shen A, et al. Noninvasive metabolomic profiling of human embryo culture media using a simple spectroscopy adjunct to morphology for embryo assessment in in vitro fertilization (IVF). *Int J Mol Sci*. 2013; 14(4):6556–6570. doi: [10.3390/ijms14046556](https://doi.org/10.3390/ijms14046556) PMID: [23528887](https://pubmed.ncbi.nlm.nih.gov/23528887/); PubMed Central PMCID: PMC3645654.
21. Devreker F, Van den Bergh M, Biramane J, Winston RL, Englert Y, Hardy K. Effects of taurine on human embryo development in vitro. *Hum Reprod*. 1999; 14(9):2350–2356. PMID: [10469709](https://pubmed.ncbi.nlm.nih.gov/10469709/).
22. Berning EJ, Bernhardson N, Coleman K, Farhat DA, Gushrowski CM, Lanctot A, et al. Ethanol- and/or Taurine-Induced Oxidative Stress in Chick Embryos. *J Amino Acids*. 2013; 2013:240537. doi: [10.1155/2013/240537](https://doi.org/10.1155/2013/240537) PMID: [23606945](https://pubmed.ncbi.nlm.nih.gov/23606945/); PubMed Central PMCID: PMC3628655.
23. Lane M, Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil*. 1994; 102(2):305–312. PMID: [7861382](https://pubmed.ncbi.nlm.nih.gov/7861382/).
24. Zander DL, Thompson JG, Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod*. 2006; 74(2):288–294. doi: [10.1095/biolreprod.105.046235](https://doi.org/10.1095/biolreprod.105.046235) PMID: [16221986](https://pubmed.ncbi.nlm.nih.gov/16221986/).
25. Biggers JD, McGinnis LK, Lawitts JA. Enhanced effect of glycyl-L-glutamine on mouse preimplantation embryos in vitro. *Reprod Biomed Online*. 2004; 9(1):59–69. PMID: [15257821](https://pubmed.ncbi.nlm.nih.gov/15257821/).
26. Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod*. 2013; 89(3):75. doi: [10.1095/biolreprod.113.109256](https://doi.org/10.1095/biolreprod.113.109256) PMID: [23803557](https://pubmed.ncbi.nlm.nih.gov/23803557/).
27. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update*. 2015. doi: [10.1093/humupd/dmv034](https://doi.org/10.1093/humupd/dmv034) PMID: [26207016](https://pubmed.ncbi.nlm.nih.gov/26207016/).
28. Mantikou E, Youssef MA, van Wely M, van der Veen F, Al-Inany HG, Repping S, et al. Embryo culture media and IVF/ICSI success rates: a systematic review. *Hum Reprod Update*. 2013; 19(3):210–220. doi: [10.1093/humupd/dms061](https://doi.org/10.1093/humupd/dms061) PMID: [23385469](https://pubmed.ncbi.nlm.nih.gov/23385469/).
29. Helmerhorst FM, Perquin DA, Donker D, Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. *BMJ*. 2004; 328(7434):261. Epub 2004/01/27. doi: [10.1136/bmj.37957.560278.EE](https://doi.org/10.1136/bmj.37957.560278.EE) PMID: [14742347](https://pubmed.ncbi.nlm.nih.gov/14742347/); PubMed Central PMCID: PMC324454.
30. Wen J, Jiang J, Ding C, Dai J, Liu Y, Xia Y, et al. Birth defects in children conceived by in vitro fertilization and intracytoplasmic sperm injection: a meta-analysis. *Fertil Steril*. 2012; 97(6):1331–1337 e1331-1334. Epub 2012/04/07. doi: [10.1016/j.fertnstert.2012.02.053](https://doi.org/10.1016/j.fertnstert.2012.02.053) PMID: [22480819](https://pubmed.ncbi.nlm.nih.gov/22480819/).
31. Bowen JR, Gibson FL, Leslie GI, Saunders DM. Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. *Lancet*. 1998; 351(9115):1529–1534. doi: [10.1016/S0140-6736\(98\)10168-X](https://doi.org/10.1016/S0140-6736(98)10168-X) PMID: [10326534](https://pubmed.ncbi.nlm.nih.gov/10326534/).

32. Knoester M, Helmerhorst FM, Vandenbroucke JP, van der Westerlaken LA, Walther FJ, Veen S, et al. Cognitive development of singletons born after intracytoplasmic sperm injection compared with in vitro fertilization and natural conception. *Fertil Steril*. 2008; 90(2):289–296. doi: [10.1016/j.fertnstert.2007.06.090](https://doi.org/10.1016/j.fertnstert.2007.06.090) PMID: [17980875](https://pubmed.ncbi.nlm.nih.gov/17980875/).
33. Soderstrom-Anttila V, Salokorpi T, Pihlaja M, Serenius-Sirve S, Suikkari AM. Obstetric and perinatal outcome and preliminary results of development of children born after in vitro maturation of oocytes. *Hum Reprod*. 2006; 21(6):1508–1513. doi: [10.1093/humrep/dei503](https://doi.org/10.1093/humrep/dei503) PMID: [16449308](https://pubmed.ncbi.nlm.nih.gov/16449308/).
34. Leslie GI, Gibson FL, McMahon C, Cohen J, Saunders DM, Tennant C. Children conceived using ICSI do not have an increased risk of delayed mental development at 5 years of age. *Hum Reprod*. 2003; 18(10):2067–2072. PMID: [14507822](https://pubmed.ncbi.nlm.nih.gov/14507822/).
35. Nekkebroeck J, Bonduelle M, Desmyttere S, Van den Broeck W, Ponjaert-Kristoffersen I. Mental and psychomotor development of 2-year-old children born after preimplantation genetic diagnosis/screening. *Hum Reprod*. 2008; 23(7):1560–1566. doi: [10.1093/humrep/den033](https://doi.org/10.1093/humrep/den033) PMID: [18285321](https://pubmed.ncbi.nlm.nih.gov/18285321/).
36. Ponjaert-Kristoffersen I, Bonduelle M, Barnes J, Nekkebroeck J, Loft A, Wennerholm UB, et al. International collaborative study of intracytoplasmic sperm injection-conceived, in vitro fertilization-conceived, and naturally conceived 5-year-old child outcomes: cognitive and motor assessments. *Pediatrics*. 2005; 115(3):e283–289. doi: [10.1542/peds.2004-1445](https://doi.org/10.1542/peds.2004-1445) PMID: [15741353](https://pubmed.ncbi.nlm.nih.gov/15741353/).
37. Ceelen M, van Weissenbruch MM, Prein J, Smit JJ, Vermeiden JP, Spreeuwenberg M, et al. Growth during infancy and early childhood in relation to blood pressure and body fat measures at age 8–18 years of IVF children and spontaneously conceived controls born to subfertile parents. *Hum Reprod*. 2009; 24(11):2788–2795. doi: [10.1093/humrep/dep273](https://doi.org/10.1093/humrep/dep273) PMID: [19648588](https://pubmed.ncbi.nlm.nih.gov/19648588/).
38. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*. 2008; 359(1):61–73. Epub 2008/07/04. doi: [10.1056/NEJMra0708473](https://doi.org/10.1056/NEJMra0708473) PMID: [18596274](https://pubmed.ncbi.nlm.nih.gov/18596274/); PubMed Central PMCID: [PMC3923653](https://pubmed.ncbi.nlm.nih.gov/PMC3923653/).
39. Simmons RA. Developmental origins of adult disease. *Pediatr Clin North Am*. 2009; 56(3):449–466. Epub 2009/06/09. doi: [10.1016/j.pcl.2009.03.004](https://doi.org/10.1016/j.pcl.2009.03.004) PMID: [19501686](https://pubmed.ncbi.nlm.nih.gov/19501686/); PubMed Central PMCID: [PMC3357632](https://pubmed.ncbi.nlm.nih.gov/PMC3357632/).
40. Rinaudo P, Schultz RM. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction*. 2004; 128(3):301–311. Epub 2004/08/31. doi: [10.1530/rep.1.00297](https://doi.org/10.1530/rep.1.00297) PMID: [15333781](https://pubmed.ncbi.nlm.nih.gov/15333781/).
41. 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. doi: [10.1073/pnas.0306846101](https://doi.org/10.1073/pnas.0306846101) PMID: [14747652](https://pubmed.ncbi.nlm.nih.gov/14747652/); PubMed Central PMCID: [PMC341785](https://pubmed.ncbi.nlm.nih.gov/PMC341785/).
42. Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez 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. Epub 2004/04/14. doi: [10.1073/pnas.0308560101](https://doi.org/10.1073/pnas.0308560101) PMID: [15079084](https://pubmed.ncbi.nlm.nih.gov/15079084/); PubMed Central PMCID: [PMC395892](https://pubmed.ncbi.nlm.nih.gov/PMC395892/).
43. Vermeiden JP, Bernardus RE. Are imprinting disorders more prevalent after human in vitro fertilization or intracytoplasmic sperm injection? *Fertil Steril*. 2013; 99(3):642–651. doi: [10.1016/j.fertnstert.2013.01.125](https://doi.org/10.1016/j.fertnstert.2013.01.125) PMID: [23714438](https://pubmed.ncbi.nlm.nih.gov/23714438/).
44. Fauque P, Ripoché 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. Epub 2010/02/13. doi: [10.1093/hmg/ddq059](https://doi.org/10.1093/hmg/ddq059) PMID: [20150233](https://pubmed.ncbi.nlm.nih.gov/20150233/).
45. 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. Epub 2004/07/09. doi: [10.1242/dev.01241](https://doi.org/10.1242/dev.01241) PMID: [15240554](https://pubmed.ncbi.nlm.nih.gov/15240554/).
46. 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. Epub 2010/08/13. doi: [10.1095/biolreprod.110.085480](https://doi.org/10.1095/biolreprod.110.085480) PMID: [20702853](https://pubmed.ncbi.nlm.nih.gov/20702853/).
47. 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. doi: [10.1095/biolreprod.103.018093](https://doi.org/10.1095/biolreprod.103.018093) PMID: [12773416](https://pubmed.ncbi.nlm.nih.gov/12773416/).
48. de Waal E, Vrooman LA, Fischer E, Ord T, Mainigi MA, Coutifaris C, et al. The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model. *Hum Mol Genet*. 2015; 24(24):6975–6985. doi: [10.1093/hmg/ddv400](https://doi.org/10.1093/hmg/ddv400) PMID: [26401051](https://pubmed.ncbi.nlm.nih.gov/26401051/); PubMed Central PMCID: [PMC4654053](https://pubmed.ncbi.nlm.nih.gov/PMC4654053/).
49. El Hajj N, Haaf T. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. *Fertil Steril*. 2013; 99(3):632–641. doi: [10.1016/j.fertnstert.2012.12.044](https://doi.org/10.1016/j.fertnstert.2012.12.044) PMID: [23357453](https://pubmed.ncbi.nlm.nih.gov/23357453/).

50. Whitelaw N, Bhattacharya S, Hoad G, Horgan GW, Hamilton M, Haggarty P. Epigenetic status in the offspring of spontaneous and assisted conception. *Hum Reprod.* 2014; 29(7):1452–1458. doi: [10.1093/humrep/deu094](https://doi.org/10.1093/humrep/deu094) PMID: [24812310](https://pubmed.ncbi.nlm.nih.gov/24812310/).
51. Feinberg JI, Bakulski KM, Jaffe AE, Tryggvadottir R, Brown SC, Goldman LR, et al. Paternal sperm DNA methylation associated with early signs of autism risk in an autism-enriched cohort. *Int J Epidemiol.* 2015. doi: [10.1093/ije/dyv028](https://doi.org/10.1093/ije/dyv028) PMID: [25878217](https://pubmed.ncbi.nlm.nih.gov/25878217/).