Cardiac Malformation Rate: A Link to Embryo-Culture in Medium Containing Serum?

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Abstract: This retrospective observational study reports on the higher incidence of cardiac defects in newborns after *in vitro* fertilization (IVF) at a single center. The incidence of major congenital malformations of 379 children born between 1985 and 1989 (group A) following IVF after culture in medium containing human serum was compared to that of 441 children born between 1993 and 1997 (group B) after IVF using commercially produced media in which serum is substituted by synthetic serum replacement/ human serum albumin. In group A, major birth defects were noticed in 4.2% of cases with 2.9% clinically apparent congenital heart defects (CHD) at birth compared to an incidence of major birth defects of 0.7% in group B with no clinically apparent CHD ($P < 0.001$). Components in the *in vitro* culture system containing serum are likely related to the congenital malformations observed in this study.

Keywords: congenital malformations, embryo culture medium, epigenetic, *in vitro* fertilization, serum.

INTRODUCTION

Early follow-up studies on children conceived by the help of assisted reproduction technologies (ART) suggested that frequencies of birth anomalies resembled those arising with natural conception [1-3]. Recent epidemiological studies on ART outcomes now question such earlier findings reporting on the increased risk of birth defects in children conceived by *in vitro* fertilization (IVF) compared to those conceived naturally [4-6].

It is now generally agreed that there is a slightly increased risk of birth defects in children born after ART [7-10].

In vitro exposure of embryos to various culture systems is commonly associated with a higher incidence of unusually large offspring in animals, often with organ defects termed "large offspring syndrome (LOS)" [11-13]. Moreover, LOS has been described in animals produced by cloning, and researchers argue that perhaps some of the adverse consequences of cloning could be due to the presence of serum in most cloning experiments [14]. To further investigate this subject of the adverse effects of serum on embryos, Fernàndez-Gonzalez and collaborators created a mouse model and were able to show an effect of *in vitro* culture of embryos with serum on mRNA expression of imprinted genes, development, and behaviour [15].

Recently, it was demonstrated that serum also negatively affects cardiomyocyte differentiation of human embryonic stem (ES) cells, which are usually derivatives of the inner cell mass of day 5 embryos [16].

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Knowing the adverse effect of serum in diverse culture conditions on animals as mentioned above, and especially on the differentiation between ES cells and human cardiomyocytes, it is interesting to note that different studies in human ART refer to an increased risk for heart malformations [6, 17-19]. Unfortunately, all of these studies are based on birth registries, which lack the information on specific culture conditions and thus can only speculate on the underlying cause of the cardiac malformations.

We report on the observation of a higher rate of severe congenital heart defects (CHD) in children conceived by ART in two periods, which mainly differ in an exposition of oocytes and embryos to a serum-containing medium in the first period.

MATERIALS AND METHODS

Between 1985 and 1989 (period A), medium supplemented with serum of the mother (medium A) was used for *in vitro* culture. The stimulation protocol was based on clomiphene and human menopausal gonadotropin (hMG). From 1990 to 1992, both medium A as well as a commercially produced medium (Medicult, Copenhagen, Denmark), in which serum is substituted by synthetic serum replacement (SSR, USA: ART supplement) and human serum albumin (medium B), were used for *in vitro* culture. Follicle aspiration was performed transvesically with ultrasound guidance.

From the beginning of 1993 it seemed reasonable to us at our IVF unit to switch to only commercially available media B for culture. For our study we decided to choose as a control group for media B the time period after 1992 and used the same time interval of 5 years (1993-1997) (period B). In period B, only the IVF cycles with the same stimulation protocol as in period A was included in the analysis. The retrieval of oocytes in this period was performed transvagi-

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nally. While the sperm preparation protocols in period B were more sophisticated with the addition of Percoll gradients for a less damaging centrifugation of sperm, the media used were similar. Information on the results after embryo transfer and on the outcome of children born was gathered throughout both periods by the same person and in a systematic manner around 18 months after embryo transfer by intensively communicating with the couples as well as with the physicians or hospitals in charge. For all reported malformations, copies of relevant documents of paediatric examinations were made available for clinical validation.

Major congenital malformations (MCM) are defined here as those seen at birth or in the first few weeks thereafter that require surgery and that result in severe dysfunction or death. All other malformations referred to in this paper that are conspicuous at birth or shortly thereafter are considered minor congenital malformations.

Only those pregnancies were included in which a heartbeat was visible on ultrasound examination in the first trimester. We kept records on how many pregnancy terminations were carried out because of intrauterine ultrasound findings justifying such a procedure and on the number of miscarriages. Data on complications otherwise associated with pregnancy, such as preterm premature rupture of the membranes, preterm delivery, pre-eclampsia, or intrauterine growth retardation, were not considered for this evaluation.

STATISTICAL ANALYSIS

A retrospective explorative observational study was set up with a group of children born between 1985 and 1989 and a second group born between 1993 and 1997, both conceived by ART and differing as described above. To compare the distribution of congenital malformations in the two groups, a double-sided Fisher's exact test or a X2 test - where appropriate - were conducted, and odds ratios of respectively 95% CI were calculated using SPSS for Windows 11.0 (Chicago, IL, USA). Percentages were rounded up to the first decimal place. P-values below 0.05 were considered as statistically significant. Statistical analysis were performed only for MCM and CHD, because of the low rates and consequently overall lower statistical power for other malformations and the primary endpoint being to show higher rates of MCM.

STIMULATION PROTOCOLS AND CULTURE PROCEDURES IN DETAIL

Stimulation Protocol in Period A and B

For stimulation, clomiphene (Arcana, Vienna, Austria) in combination with hMG (Fertinorm, Serono, Austria) (150 IU per day) was administered for five days as described elsewhere [20]. When the leading follicle reached 18 mm, the endogenous LH surge was monitored in 4-hourly urine portions using HI-Gonavis (Mochida Pharmaceuticals, Tokyo, Japan). In case of an endogenous LH surge the cycle was cancelled. An ovulatory dose of 10,000 IU of hCG (Pregnyl, Organon, Holland) was given, and follicle aspiration was performed 34-36 hours thereafter. Follicular growth was monitored, and follicle aspiration was performed transvesically with ultrasound guidance in period A and with a transvaginal probe in period B. Luteal phase support consisted of administering 50 mg progesterone i.m. daily (Streule, Richterswil, Switzerland). In case of a positive pregnancy test 14 days after oocyte retrieval, progesterone supplementation was continued until gestational sac(s) with fetal heart activities was/were visible at 30 days after transfer.

Culture Procedures

In period A, Ham's F10 medium (Gibco, Aldrich, Austria), supplemented with penicilline/ streptomycine (INC, Biomedicals Inc., USA) and 8 to 15% (v/v) heat inactivated patients' serum (30 min at 56°C), was used for sperm preparation, oocytes and embryo culture. For oocyte retrieval Hepes buffered Ham's F10 supplemented with 40 IE/ml heparine (Sandoparin, Sandoz, Switzerland) was used. One day before follicle puncture (day 0), the culture medium was supplemented with heat inactivated patients' serum and incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

In period B, universal IVF medium (Medicult, Copenhagen, Denmark) was supplemented with 10% human serum albumin (5 mg/mL) instead of maternal serum for gametes preparation and embryo culture.

During both periods, a maximum of 10 cumulus oocyte complexes per well was inseminated with 100,000 motile spermatozoa 4 hours after oocyte retrieval.

The 'swim-up' technique and density gradients are the most commonly used techniques for sperm washing and preparation.

The 'swim-up' procedure was the standard procedure for the washing and preparation of the spermatozoa during period A. One mL of semen was washed with 3 mL of Ham's F10 medium (Gibco, Aldrich, Austria) containing 10% serum (v/v) in a 15 ml Falcon tube (Astromed, Austria). Following centrifugation at 250 g for 5 min, the supernatant was discarded and the washing procedure repeated. After a second washing, 1 mL of medium was gently dripped onto the pellet and incubated at 37°C for 30 min. The upper part (200 µL) of the supernatant was carefully removed for further use. Sperm were prepared with the same stock solution containing Ham's F10 medium (Gibco, Aldrich, Austria) supplemented with 10% (v/v) serum as described previously [21].

During period B, sperm preparation was performed using a discontinuous percoll gradient centrifugation technique.

The 100% percoll solution, which was medium designed, was prepared by diluting one part of Ham's F10 medium (10-fold concentrated) with nine parts of percoll (Pharmacia, Sweden). Combining the 100% percoll solution with Ham's F10 medium, different gradients of 40%, 60%, 70%, 80%, and 90% were prepared and the gradients kept iso-osmotic. Percoll density gradients were formed by layering 1 mL of each different concentration of percoll solution into a Falcon tube, starting with the highest density solution at the bottom. In case of normal semen parameters, all the gradients were used, and in case of semen with sub-optimal parameters, only layers of 100%, 70%, and 40% composed the column. Once the gradient had been prepared, 1 mL of semen was placed on the top of the gradient and the tube centrifuged for 15 min at 250 g. After centrifugation, only the 100% part was aspirated and washed by dilution with 10 mL of medium (Ham's F10 plus serum) before another 5 min of centrifuga-

tion. The supernatant was removed, and the pellet was re-suspended before insemination.

Fertilization was assessed 16–20 hours after insemination. Normal fertilization was confirmed by the presence of two pronuclei (2 PN) with two distinct polar bodies and placed in fresh medium. Embryo quality on day 3 was scored, and 2 to 3 embryos were selected for transfer.

For both periods A and B, oocyte and embryo cultures were performed in 4-well multidishes (Nunc, Roskilde, Denmark), each containing 500 µL media at 37°C in a humidified atmosphere of 5% CO2 in air.

Using 20-30 µL of medium, the embryos were placed into the Wallace catheter (HG Wallace Ltd, Colchester, Essex, UK) between two small areas of air, and a gentle transcervical embryo transfer without ultrasound guidance was performed, placing the embryos 0.5 cm apart from the fundus.

RESULTS

Period A

In period A, 1,852 embryo transfers (ET) were conducted resulting in 366 (19.8%) pregnancies with at least one embryo with positive heartbeat on ultrasound. Patient characteristics, data on pregnant women not included in the study, quality, and fertilization rates of retrieved oocytes are shown in Tables **1** and **2**.

Sixteen MCM were detected as described and subdivided below and shown in Table **3**. Of the 16 MCM, 7 boys and 9 girls were affected, showing no differences in sex distribution. Severe CHD occurred 6 times in singleton pregnancies with term delivery, twice in one of the babies of twin pregnancies with deliveries in pregnancy week 32 and 34 respectively, and 3 times in one of the babies of a triplet pregnancy, with deliveries of all three between pregnancy week 31 and 33. Three times, a transposition of the great arteries (TGA) was diagnosed, 3 times, a patent ductus arteriosus (PDA) with an atrial septal defect (ASD), and one case of a medium-sized pulmonic stenosis (PS) were observed. Two babies presented with TGA and additionally had PDA as well as an ASD. One child had a complex heart defect with a double-outlet-right-ventricle, a sub-aortal and a second ventricular muscular septum defect, a hypoplastic mitral annulus with a medium-sized mitral stenosis, a disrupted aortal arc behind the left arteria subclavia (Type A), a hypoplasia of the aortic arc with branching off from the truncus brachiocephalicus, and the right kidney located in the pelvis. Another child presented with a PS and had an atrial septal aneurysm.

In children with MCM and severe CHD, the medical history of the mothers/parents reveals no pre-existing causative factors (e.g. diabetes or adipositas of the mother, genetic predisposition of either couple).

Period B

In period B, 1,653 ET were conducted resulting in 457 (27.6%) pregnancies with at least one embryo with positive heartbeat on ultrasound. Patient characteristics, data on preg-

Table 1. Data on Patients and Children Included in the Study

Characteristic	Period A	Period B	Odds Ratio (95% CI)[B]	P-Value
Patients with ET (n)	1852	1653		
Age (yr)	33.0±3.85	33.0±3.81[A]		.85
Indication for treatment, n (%)				
Tubal factor	1259 (67.9)	1103 (66.7)	1.06 (0.92-1.22)	.43
Immunological and/or idiopathic	242 (13.0)	207 (12.5)	1.05 (0.86-1.28)	.63
Andrological factor	351 (18.9)	343 (20.8)	0.89 (0.76-1.05)	.18
Pregnant women, n (%)[C]	366 (19.8)	457 (27.6)	0.64 (0.55-0.75)	<0.001
Lost to follow-up, n (%)	7 (1.9)	11 (2.4)	0.79 (0.30-2.06)	.63
Miscarriage, n (%)	36 (9.8)	41 (8.9)	1.11 (0.69-1.77)	.67
Pregnancy termination, n (%)				
Pathologic amniocentesis finding	17 (4.6)	19 (4.2)	1.12 (0.58-2.19)	.73
Pathologic ultrasound finding	5 (1.4)	4 (0.9)	1.57 (0.42-5.89)	.52
Neural tube defect	4 (1.1)	3 (0.7)		
Potter syndrome	1 (0.3)	0		
Caudal regression syndrome	0	1 (0.2)		
Live birth, n	382	448		
Lost to follow-up, n	3	7[D]	0.49 (0.13-1.94)	.36
No. of Children Included in Study	**379**	**441**		
Singletons, n (%)	221 (58.3)	315 (71.4)	0.56 (0.42-0.75)	<0.001
Twins, n (%)	146 (38.5)	120 (27.2)	1.68 (1.25-2.25)	<0.001
Triplets, n (%)	12 (3.2)	6 (1.4)	2.37 (0.88-6.38)	.08

[A]Plus-minus values are means ±SD, [B]CI denotes confidence interval, [C]Pregnancy was defined by a positive heartbeat on ultrasound examination in the first trimester, [D]2 twins.

nant women not included in the study, quality, and fertilization rates of retrieved oocytes are shown in Tables **1** and **2**. In this time period, 441 children were born in the IVF group. Three major birth defects were detected as shown in Table **3**. No CHD was diagnosed.

Table 2. Analysis of Data on Oocyte Yield, Oocyte Morphology and Fertilization Rates

Variable	Period A	Period B
Patients with ET, n	1852	1653
Oocytes, n[A]	12651	13653
Immature, n (%)	1490 (11.8)	1489 (10.9)
Degenerative, n (%)	604 (4.8)	807 (5.9)
Preovulatory, n (%)	10557 (83.4)	11357 (83.2)
Fertilized oocytes, n	8098	9201
Oocytes per patient, n (± diff.%)[B]	6.8	8.2 (+ 20.91)
Fertilized oocytes per patient, n (± diff.%)[B]	4.4	5.7 (+ 27.29)
Fertilized oocytes per oocytes, n (± diff.%)[B]	0.64	0.67 (+ 5.28)

[A]Classified according to Veeck [22], [B]± differences in percent from period A to period B.

DISCUSSION AND CONCLUSIONS

To the best of our knowledge, this is the largest study of its kind on a link between culture conditions and the appearance of MCM observed in children born after IVF at a single center in Europe which parallels observations made on adverse effects of serum containing culture conditions on animals and on human ES cells differentiating to cardiomyocytes [14-16].

In our study, we observed a high rate of severe CHD of 2.9% in children conceived after IVF with culture in medium containing serum versus no CHD in group B ($P < 0.001$), in which serum was substituted by synthetic serum replacement and human serum albumin.

The incidence of severe CHD has been quite stable for many years in naturally conceived pregnancies, ranging from 2.5 to 3 live births per 1,000 [23]. If the incidence of moderate and severe CHD is looked at simultaneously, the number rises to approximately 6 to 10 per 1,000 live [23]. The differences in reported incidences of CHD are mainly due to the different abilities to detect trivial lesions. Still, these numbers are much lower than the 11 severe CHD (29 per 1,000 live births) seen in our study group A, and it is very noteworthy to find such a high number of primarily severe CHD (with apparent symptoms or complications and the need for CHD expertise) in this collective.

The presented data have both several potentially strong and weak points. The interpretation of collected data is first of all limited by the study itself, which is retrospective, observational, and originates from a single center. The observational bias was kept to a minimum, as the interviews were conducted in a structured way and always by the same person and included both the couples and the physicians, respectively, or the hospitals in charge where copies of medical records were available for the reported malformations.

Although single center studies harbor the danger of cluster formations, this can also be seen as a strength in our study, because data on the stimulation protocols and culture procedures were fully available, which is in contrast to published multi-center studies that refer to birth registries lacking detailed information on the whole procedure. Another indicator for low bias is the minimal loss of follow-up in both groups. Furthermore, the miscarriage rate, the rate of first or second trimester pregnancy termination due to pathologic findings with prenatal ultrasound screening as well as after invasive diagnosis did not differ significantly between the two groups.

Table 3. Distribution on Major Congenital Malformations in Periods A and B

Variable	Period A (N=379)	Period B (N=441)	Odds Ratio (95% CI)[A]	P- Value
MCM	16	3	6.4 (1.86-22.3)	<0.001
Severe CHD	11[B]	0		<0.001
Omphalocele	1	0		
Neural tube defect	1	0		
Cataract	1	1[C]		
Hydrocephalus	2	0		
Meningomyelocele	0	1		
Club feet	0	1		
Subgroup Analysis				
Singletons	221	315		
MCM	11	2	8.2 (1.79-37.35)	0.001
CHD	6	0		0.003
Twins	146	120		
MCM	2	1		1.00
CHD	2	0		0.50
Triplets	12	6		
MCM	3[D]	0		0.52
CHD	3	0		0.52

[A]CI denotes confidence interval, [B]Six singletons, two times one child of a twin pregnancy, [C]One child of a twin pregnancy, [D]Three times one child of a triplet pregnancy.

One could argue that the differences in pregnancy rates between group A (19.8%) and group B (27.6%) are the result of different unknown baseline characteristics of the parents seeking ART. However, this difference is not reflected in other parameters dependent on baseline characteristics, such as in miscarriage rates, pregnancy terminations due to pathologic ultrasound findings or due to pathologic amniocentesis.

In our opinion, the higher pregnancy rate is mainly caused by an overall increase in oocyte yield per woman by 20.9% and a consecutive increase of fertilized oocytes per woman by 27.3% from period A to B. This is mainly due to an improvement in the oocyte retrieval technique from transvesical to transvaginal puncture [24]. The increase of fertilized oocytes seems not to be an effect of oocyte quality, be-

cause period A and B show a similar distribution of mature, degenerative, and preovulatory oocytes as shown in Table **3**.

Templeton and Morris [25] already showed that the chances of live birth are related to the number of oocytes fertilized. Moreover, we could show an increase in the fertilization rate of oocytes by 5.3% from period A to B, which also contributes to the increased pregnancy rate.

On the other hand, one could speculate that serum in the culture medium has influenced the pregnancy rate and fertilization rate per oocyte, which would add an additional dimension to our study. In the mouse model, it was shown, that serum inhibits the growth of embryos to the blastocyst stage and reduces post implantation viability of embryos, even if they had a normal appearance in the blastocyst stage [15]. In the human model, it has been shown that, even though embryos cultured in serum produce more blastocysts, the implantation and pregnancy rates are low [26, 27]. Thus, despite embryos appeared morphologically similar in both periods on day 3 in our study, their intrinsic capability for further development could have been diminished due to serum. In this respect it would be interesting to know if the steady overall increase in the ART success rate observed worldwide was due to the new stimulation protocols available or the fact that fewer and fewer embryo culture protocols harbored serum as an ingredient.

We want to stress that no changes in culture conditions for oocytes and embryos other than the introduction of commercially produced medium for culture, in which serum is substituted by synthetic serum replacement and human serum albumin, occurred at our IVF unit between the period during which a high rate of birth defects was observed and the interval thereafter. The period in which embryos were kept in culture was not changed. To the best of our knowledge, no increase in the rate of such lesions was reported elsewhere for those years, not even in studies about the effect of ionizing radiation in the post-Chernobyl period [28]. Intracytoplasmatic sperm injection and new stimulation protocols due to new medications available on the market [highly purified follicle stimulating hormone (FSH-HP) and gonadotropin releasing hormone (GnRH)- agonist] were introduced in period B, but these patients were excluded from the study to guarantee comparability. It could be argued that this imposes a bias. When all ART procedures in period B were combined and compared to period A, a significant higher rate of MCM and CHD in period A still remained (data not shown).

Further substantiating our results, by overlooking the period after the study up to February 2008 with more than 6000 live births and where embryos were no longer cultured in serum-containing media, overall MCM rates as well as CHD resemble those found in period B (data not shown).

We therefore put forward the hypothesis that most probably "sub-optimal *in vitro* culture conditions" together with the induction of an abnormal embryo development under the use of serum could have resulted in the higher rate of CHD. We are aware of the fact that Ham`s F-10 is a complex medium. Furthermore we know of the theoretical possibility that ingredients other than serum exerts the negative effects observed.

But, on the other hand, there is a large body of evidence that the presence of serum in *in vitro* culture systems exerts adverse effects on embryonic development in various animals [29]. In case of adverse effects of serum during preimplantation embryo culture, one may expect to find major defects in many systems and not specifically in the cardiovascular system. But at least four human studies dealing with IVF predominantly show malformations in the cardiovascular system ([6, 17-19], however, the underlying cause could not be elucidated. In this context, it is noteworthy that certain alterations in the levels of gene expression after *in vitro* culture sometimes seem to be tissue specific (Blondin BR 2000) [30]. Unfortunately, no exact data on stimulation protocols, techniques, or procedures are available in most of the ART studies. It would be interesting to know whether serum was used as part of the culture medium in studies with a high rate of cardiac malformations. Of note, to our knowledge only the publications of Lancaster [17] and Licata [18], which were published before the introduction of serum free culture media, were able to show significant higher rates of CHD.

The formation of the heart involves a complex but sophisticated regulatory network of molecular events directly impacting on gene expression besides indirect mechanisms, such as protein-protein interactions modulating the activities of transcription factors bound to DNA and epigenetic mechanisms [31, 32]. A steadily growing number of genes relevant to normal heart morphogenesis have been discovered [33], and attempts have been made to link molecular events to normal and abnormal cardiac development [34]. In our study, *in vitro* cultured embryos were at a very early stage of development and exposed to serum for only a few days. Cardiogenesis, however, covers a longer time period in embryogenesis and follows a precisely orchestrated series of molecular and morphogenetic events. Thus, rather than molecular events acting directly on gene expression, a more plausible explanation for our findings is that unknown ingredients or an unnatural mixture of cytokines in the embryo culture medium containing serum indirectly exerted lasting effects *via* epigenetic perturbations on the transcriptional network controlling cardiac development.

The long-term developmental and behavioural consequences of mammalian embryo culture are still largely unknown [35]. Thus, the effects of different culture systems on embryos should be intensively investigated. In addition, ES-cell research can help to address some of the questions arising from observations made in preimplantation embryo culture experiments.

To substantiate our hypothesis on the effect of serum on CHD, prospective randomized studies would again be most suitable but no longer eligible in humans in light of our findings and only acceptable for investigating the effects of serum on CHD in animal models.

For general conclusions about serum-containing media and the rate of cardiac malformation further investigations are needed. What we can, however, learn from our study is that even the smallest changes in the "overall " procedure could have rather a dramatic influence in any field of embryo and child development [35]. Here we want to emphasize that many hypotheses which are proposed on the aetiology of various diseases and malformations for which little is known about the underlying mechanisms, could be elegantly tested

with the help of stem cell research and that this discipline could also assist scientists in finding possible explanations for their observations. Concerning ART, which is already in worldwide use, our responsibility is to initiate a population based prospective study with well-known and defined techniques to be able to give evidence based information to the increasing number of couples who are seeking help because of infertility problems.

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