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ARTICLE

Neonatal and neurodevelopmental outcome of children aged 3–10 years born following assisted oocyte activation

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Frauke Vanden Meerschaut was born in Belgium in 1984 and concluded her medical studies at the University of Ghent, Faculty of Health Sciences and Medicine in 2009. Since then, she has been part of the research team of the Centre for Reproductive Medicine at the Ghent University Hospital. She is interested in different aspects of reproductive medicine. She recently started working as an obstetrician gynaecology trainee, while finalizing her PhD on assisted oocyte activation.

Abstract Assisted oocyte activation (AOA) using a calcium ionophore has been used for more than a decade following intracytoplasmic sperm injection (ICSI) fertilization failure. However, since AOA does not mimic precisely the physiological fertilization process, concerns exist about its use in human assisted reproduction. This study assessed the neonatal and neurodevelopmental outcome of children aged ≥3 years who had been born following AOA in our centre. Twenty-one children participated in the study (81% response rate; mean age 63.6 ± 21.07 months). Neonatal data were collected via questionnaires. Neurodevelopmental outcome was tested using the Reynell Developmental Language Scales or Clinical Evaluation of Language Fundamentals, Wechsler Preschool and Primary Scale of Intelligence or Wechsler Intelligence Scale for Children, and the Movement Assessment Battery for Children III. Behaviour was scored by the Social Communication Questionnaire, the Child Behaviour Checklist and the Teachers Report Form. For all tests and questionnaires, the mean outcomes lay within the expected ranges. These are first data on the developmental outcome of AOA children. The high response rate and the robustness of the tests support the data, which are reassuring although still considered preliminary. Therefore, AOA should still be performed only in selected couples.

KEYWORDS: assisted oocyte activation, child follow up, failed fertilization, ICSI, ionomycine

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Introduction

Intracytoplasmic sperm injection (ICSI) is typically used in cases of severe semen dysfunction or following failure of conventional IVF. In general, fertilization rates of 70—75% are obtained (Palermo et al., 2009). In contrast, failed fertilization still occurs in 1—3% of ICSI cycles, even when a sufficient number of mature oocytes and motile spermatozoa are available (Esfandiari et al., 2005; Flaherty et al., 1995). The principal reason for fertilization failure following ICSI is defective oocyte activation (Flaherty et al., 1998; Liu et al., 1995; Rawe et al., 2000). The primary cellular event leading to mammalian oocyte activation is the initiation of intracellular calcium oscillations in the oocyte's cytosol. Although doubts have risen recently, substantial evidence suggests that the spermatozoon supplies an activating protein, phospholipase C zeta, into the oocyte's cytosol which triggers calcium oscillations via an inositol-3-phosphate-mediated pathway (Aarabi et al., 2012; Saunders et al., 2002). The oocyte responds to this by the calcium-dependent activation of several down-stream pathways, which are necessary for successful oocyte activation, pre- and post-implantation embryo development (Ducibella et al., 2002; Kashir et al., 2010; Parrington et al., 2007). Therefore, both the spermatozoon and the oocyte are considered to be responsible for successful oocyte activation and embryo development. In this study centre, the mouse oocyte activation test (MOAT), a heterologous ICSI model, is performed to diagnose the activation capacity of a patient's spermatozoa (Rybouchkin et al., 1996).

Several reports show that most, but not all, of the couples suffering from ICSI failure benefit from the application of ICSI combined with assisted oocyte activation (AOA) (Heindryckx et al., 2005, 2008; Montag et al., 2012; Vanden Meerschaut et al., 2012). Different activating agents have been used successfully in human assisted reproduction, e.g. calcium ionophore, electrical pulses and strontium chloride (Baltaci et al., 2010; Kyono et al., 2008; Rybouchkin et al., 1997; Yanagida et al., 2006). However, until adequate scientific evidence is provided regarding its safety and efficacy, ICSI combined with AOA cannot yet be considered an established treatment. The current study centre has been performing ICSI combined with AOA during more than a decade, using the injection of calcium chloride ($CaCl_2$) along with the spermatozoon into the oocyte, followed by a 2-fold calcium ionophore exposure of the injected oocyte (Rybouchkin et al., 1997) and has built up a large experience in the treatment of this rare but challenging group of patients. Rewarding results have been obtained regarding fertilization and pregnancy rates (Heindryckx et al., 2008). Nevertheless, the artificially induced calcium rises do not mimic precisely the physiologically sperm-induced calcium oscillations and little is known yet about the possible adverse effects of ionophores on post-implantation embryo development. Therefore, concerns exist about the use of a calcium ionophore as oocyte-activating agent in human assisted reproduction. However, the influence of ionomycin exposure during the zygote stage on pre- and post-implantation development has been evaluated in the mouse (Heytens et al., 2008). Zygotes treated with ionomycin showed normal pre- and post-implantation development, and normally developing and fertile pups were born originating from ionomycin-treated zygotes. Nevertheless, since these results were obtained from highly fertile animals, results should not be extrapolated without caution to a subfertile human population (Harper et al., 2012). Even though the exposure to the calcium ionophore for the purpose of AOA is short and limited to the zygote stage, it is appropriate to examine possible adverse effects of ionomycin in children born following AOA.

Since the main aim of a IVF-clinic is to ensure the birth of healthy babies, the goal of this study was to report on the neonatal outcome of children born following AOA and on their cognitive, language and motor development and behaviour from the age of 3 years onwards and to compare their outcomes with age-matched norms.

Materials and methods

Participants

All children born following AOA at University Hospital Ghent from October 2001 until September 2008 were eligible for this study. Inclusion criteria for the neurodevelopmental and behavioural assessment were: (i) age $\geq$3 years; (ii) the ability to visit the outpatient clinic; and (iii) Dutch as the mother tongue. Written informed consent was given by the parents and the study was approved by the Ethical Committee of the Ghent University Hospital (EC number: 2011/188; Belgian registry number: B670201110978, approved 19 May 2011).

Assisted oocyte activation

ICSI combined with AOA was performed as previously described (Heindryckx et al., 2008). Briefly, a spermatozoon was injected into the oocyte together with a small amount of 0.1 mol/l $CaCl_2$ (corresponding to the diameter of the oocyte). Thereafter, the oocytes were incubated for 30 min at 37°C in a 6% CO_2 air atmosphere. Next, the oocytes were exposed to a calcium ionophore (10 μmol/l ionomycin, I9657; Sigma-Aldrich, Bornem, Belgium) for 10 min. Following ionophore exposure, the oocytes were washed with Cook Cleavage medium (Cook Ireland, Limerick, Ireland) and were incubated again. After another 30 min, the calcium ionophore treatment was repeated for 10 min. Finally, the oocytes were washed and incubated overnight under 5% O_2.

Neonatal data

A questionnaire concerning obstetric and neonatal parameters was filled in by the parents shortly after the birth of their child. This questionnaire was retrieved from the patients' file at the moment of this study and informed consent was given by the parents to use this questionnaire for data analysis. Additionally, some parents were consulted on the day of the neurodevelopmental assessment if data were missing or unclear.

Follow up by assessment

All children aged $\geq$3 years were invited to the study centre for neurodevelopmental follow up using a set of three

standardized tests. For each child, all tests took place on one single day and were performed with a period of rest in between. The order of testing could be different due to planning. The cognitive, language and motor development tests were performed by two clinical psychologists, one speech therapist and one physiotherapist, respectively.

Cognitive development

To assess cognitive development, the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III-NL, $\leq$7 years) or the Wechsler Intelligence Scale for Children (WISC-III-NL) was used (Hendriksen and Hurks, 2009; Kort et al., 2005). Both tests measure verbal IQ (VIQ), performance IQ (PIQ) and full-scale IQ (FSIQ) and can be completed without reading or writing. Dutch/Flemish norms were used for both tests.

Language development

Language skills were assessed by the Reynell Developmental Language Scales (*Reynell Taalontwikkelingsschalen*, RTOS) or by the Clinical Evaluation of Language Fundamentals (CELF-IV-NL) (Kort et al., 2008; Schaerlaekens et al., 2003). The RTOS is used for children <5 years and is a Dutch/Flemish norm-based practical tool for everyday clinical use. The CELF-IV-NL is used for children aged $\geq$5 years. For both tests, the outcomes are percentile scores and include two scales next to a total percentile score: the comprehension and the expressive scale.

Motor development

The Movement Assessment Battery for Children (Movement ABC-II-NL) was used to identify and evaluate movement problems (Henderson et al., 2010). It provides a comprehensive assessment of movement competence on age-appropriate tasks within three subtests: manual dexterity, ball skills and static and dynamic balance. Outcome score is a percentile score based on Flemish/Dutch norms. Additionally, a questionnaire regarding motor skills in everyday life was given to the parents (*Vragenlijst voor Motorische Vaardigheden van Kleuters* (VMVK, <5 years) or *Coördinatievragenlijst Voor Ouders* (CVO, $\geq$5 years)). The CVO was officially translated from Developmental Coordination Disorder Questionnaire (Wilson et al., 2000).

Follow up by questionnaires

The behaviour of the AOA children aged $\geq$3 years was assessed by three standardized questionnaires. The Social Communication Questionnaire (SCQ) is a worldwide-used screening tool for autism spectrum disorders (ASD) (Rutter et al., 2003a; Warreyn et al., 2004). Furthermore, the Child Behaviour Checklist (CBCL) and the Teacher's Report Form (TRF) were used. These questionnaires are identical, filled in by the parent and teacher, respectively, on which the child is rated on various behavioural and emotional aspects. The CBCL is one of the most widely used standardized measures in child psychology for evaluating maladaptive behavioural and emotional problems in children (Achenbach and Rescorla, 2001). For the CBCL, the usefulness of existing US norms within a Flemish community sample were explored before and the reliability was excellent (Braet et al., 2011). In addition to the total problem score, the CBCL and TRF give two broad syndrome scores: internalizing and externalizing behaviour. All the above mentioned questionnaires were in Dutch and filled in by one or both parents, except for the TRF, which was filled in by the teacher of the child. The TRF was a non-mandatory questionnaire.

Statistical analysis

To compare birthweight and gestational age between the singletons and twins, the Mann–Whitney *U*-test was used. The Fisher's Exact test was used to compare the amount of birth defects in the study population to the amount of birth defects present in a large ICSI population (Davies et al., 2012). The Mann–Whitney *U*-test was used to compare mean results on the developmental tests between study groups. The Wilson's confidence interval for a single proportion method was used to calculate the 95% confidence intervals around the proportion of children with test scores within pathological thresholds (Altman et al., 2008). The Statistical Package for the Social Sciences version 20 (SPSS Statistics, IBM Corp, NY, USA) and R version 2.15.1 (The R Foundation for Statistical Computing) were used for statistical analysis. A P-value < 0.05 was considered statistically significant.

Results

Study population

From 2001 onwards, 53 pregnancies were achieved following AOA in this study centre, of which 41 ended in childbirth (77.4% ongoing pregnancy rate). Six out of those 41 pregnancies were twin pregnancies (14.6%). Eight spontaneous miscarriages (15.1%) and three biochemical pregnancies (5.7%) occurred. One pregnancy was interrupted because of the fetus carrying trisomy 21. From those 47 children born following AOA, 26 were aged 3 years or older at the time of this study and therefore eligible for this study. Twenty-one out of those 26 children aged 3–11 years could be included in the study (81% response rate). The mean ± standard deviation and median (range) age of the included children was 63.6 ± 21.07 and 62.0 (38–124) months, respectively. The mean maternal and paternal age at childbirth was 32 ± 3.0 years and 34 ± 3.6 years, respectively. Two singleton sisters had a mother with Asian descent. All other parents were Caucasians. Two-thirds of the parents had a higher level of education (beyond 18 years of age). All children lived together with both their biological parents.

The fertility history and indications for AOA are shown in Table 1. In 11 couples, the indication for AOA was low or zero fertilization in previous conventional ICSI cycles, whereas for the remaining three couples, AOA had been advised because of total globozoospermia, a condition in which the sperm acrosome is lacking and typically leading to very low or zero fertilization rates using conventional ICSI. Without AOA the mean fertilization rate in this group of 14 couples was 19.9%, whereas following AOA treatment the fertilization rate was restored to 74.1%. A mean of

Table 1 Fertility history and indications for AOA.

Characteristic	*Study population (n = 14 patients)*
Fertility history[a]	
Conventional ICSI without pregnancy	
Cycles	2.1 ± 1.75
Fertilization rate (%)	19.9 ± 19.30
ICSI + AOA	
Cycles	2.5 ± 1.16
Fertilization rate (%)	74.1 ± 20.81
Indication to perform AOA[a]	
Zero fertilization following conventional ICSI	3 (21.4)
Low fertilization (<33.3%) following conventional ICSI	8 (57.1)
Globozoospermia	3 (21.4)
MOAT results[b]	
0—20% activation: certain sperm deficiency	5 (41.7)
21—84% activation: probable sperm deficiency	4 (33.3)
85—100% activation: no sperm deficiency	3 (25.0)

Values are mean ± standard deviation or *n* (%).
AOA = assisted oocyte activation; ICSI = intracytoplasmic sperm injection; MOAT = mouse oocyte activation test.
[a]Data for all 14 parent couples.
[b]Data for 12 couples.

2.1 ± 1.75 conventional ICSI cycles were performed in these couples before they were referred to this study centre. Pregnancies following AOA treatment were achieved after a mean of 2.5 ± 1.16 fresh AOA cycles. For 12 out of 14 couples, a diagnostic MOAT was performed before proceeding to AOA treatment. Nine of the couples were classified into MOAT group 1 or 2, which strongly suggests a sperm deficiency (Vanden Meerschaut et al., 2013).

Neonatal outcome and birth defects

Eleven singletons and 10 twins born following ICSI combined with AOA were included in this study. Neonatal data were retrieved for all of the included children (Table 2). The birthweight of the singletons was significantly higher compared with the twins ($P < 0.01$). Seven out of 21 children (33.3%) were born following a Caesarean section, four of whom were twins. Mean gestational age at birth was 39.4 ± 0.92 weeks and 36.8 ± 2.53 weeks for the singletons and twins, respectively ($P < 0.001$). Five children (solely twins) were admitted to the neonatal intensive care unit (NICU) (mean duration of stay 35 ± 18.8 days, range 1—44 days). Reasons for admission to the NICU were: prematurity ($n = 2$), poor condition of the mother ($n = 2$) and low temperature ($n = 1$). Birth defects were diagnosed in three children (14.3%, 95% CI 5.0—34.6%) at toddler age. In one child, a small arachnoid cyst was found by chance at the age of three. The condition is considered to be benign and did not require any intervention. In another child, a pectus excavatum and genu varum were found. In a third child, vesico-ureteral reflux was diagnosed at the age of two.

Non-respondent analysis

The five children who did not participate were excluded because of: (i) a mother tongue different from Dutch ($n = 3$); and (ii) parents were not able to visit the outpatient clinic ($n = 2$).

All five excluded children were singletons (three girls and two boys), and two of them were sisters. Indications for AOA were globozoospermia ($n = 4$) and failed fertilization following conventional ICSI ($n = 1$). Their mean ± standard deviation and median (range) age at the moment of recruitment for the study was 73.8 ± 33.66 and 77.0 (40—125) months, respectively. The maternal age at childbirth was 28 ± 4.0 years. Mean gestational age of these children was 39.0 ± 1.32 weeks, with none of them born before week 37 of pregnancy. Mean birthweight was 3107 ± 217.8 g.

Neurodevelopmental outcome

The majority of the children were younger than 7 years at the moment of testing. Seven of the 11 singletons were single child. Two singletons were sisters; another pair of singletons comprised a brother and a sister. The results are presented in Table 3.

Cognitive development

Cognitive development was assessed for all included children. Mean FSIQ, PIQ and VIQ scores on the WPPSI-III-NL were 104.2, 105.0 and 102.8, respectively (20 children: 10 singletons, 10 twins). One girl and one boy (not genetically related to each other) scored 77 and 74 for VIQ and PIQ, respectively. However, both their FSIQ scores were considered normal: 93 and 85, respectively. The oldest child was tested using the WISC-III-NL: FSIQ, PIQ and VIQ scores were 131, 130 and 123, respectively. Considering the pathological threshold for FSIQ scoring being <70 (cognitive disability), the proportion of children in this cohort falling below this threshold is 0% (95% CI 0—15.5%).

Language development

One child was excluded because of an incomplete test result due to a lack of co-operation. Eight children aged <5 years were tested using the RTOS. Their total percentile score for language development was 78.2, while their percentile scores on receptive and expressive language skills were 82.0 and 73.3. The remaining 12 children aged ≥5 years were tested using the CELF-IV-NL. Their total, receptive and expressive language percentile scores were 40.1, 32.5 and 40.9, respectively. If both groups were compared, the younger group scored significantly better than the older group of children ($P < 0.05$). Importantly, the older group consisted of eight twins (8/12, 67%), whereas in the younger group only two twins were present (2/8, 25%). Considering the pathological thresholds for language percentile scoring being <5th percentile (language disability), the proportion

Table 2 Neonatal data.

Outcome	*Singletons (n = 11)*	*Twins (n = 10)*	*Total (n = 21)*
Obstetric			
Delivery <37 weeks	0 (0)	2 (20.0)	2 (9.5)
Vaginal delivery	8 (72.7)	6 (60.0)	14 (66.7)
Caesarean section	3 (27.3)	4 (40.0)	7 (33.3)
Neonatal			
Birthweight (g)	3623 ± 324.5[a] (3085–4140)	2696 ± 676.7[a] (1640–3450)	3181 ± 695.5 (1640–4140)
Birthweight <2500 g	0 (0)	3 (30)	3 (14.3)
APGAR <7 at 5 min	0 (0)	0 (0)	0 (0)
Neonatal intensive care	0 (0)	5 (50.0)	5 (23.8)
Duration (days)			
≤7	0 (0)	1 (10.0)	1 (4.8)
>7	0 (0)	4 (40.0)[b]	4 (19.0)
Perinatal mortality	0 (0)	0 (0)	0 (0)
Gender			
Female	8 (72.7)	6 (60.0)	14 (66.7)
Male	3 (27.3)	4 (40.0)	7 (33.3)

Values are *n* (%) or mean ± standard deviation (range).
[a]$P < 0.01$ (Student's t-test). [b]Two of these children were admitted to NICU because of severe illness of their mother (aorta dissection).

of children in this cohort falling below this threshold for the expressive and total language score was 0% (95% CI 0–16.1%), whereas this was 5% (95% CI 0.01–23.6%) for the receptive language score.

Motor development

In the two youngest children, the test was cancelled because of fatigue. Seventeen children were tested using the Movement ABC-II-NL for children aged 3–6 years. The mean total percentile score was 37.1. The remaining two older children (both singletons) were tested using the Movement ABC-II-NL test for children aged 7–10 years. Their mean percentile score was 50.0. Subtest percentile scores are shown in Table 3. Six children (31.6%) scored below the 15th percentile for motor skill development. Among them, 5 (83.3%) were twins, of which two were born before week 37 of gestation. No correlation was found between the birthweight of the included children and the total or subtest scores on motor skill development. Considering the pathological thresholds for motor skill percentile scoring being <5th percentile (Developmental Coordination Disorder), the proportion of children in this cohort falling below this threshold is 10.5% (95% CI 2.9–31.4%) for manual dexterity, 5.3% (95% CI 0.01–24.6%) for aiming and catching and the total motor score, and 0% (95% CI 0–16.8%) for balance. Additionally, the parents were asked to fill out a questionnaire regarding motor skills in everyday life (VMVK or CVO questionnaire). All children ($n = 21$) tested within the normal ranges on these questionnaires (data not shown), meaning that according to the questionnaires no notable co-ordination disorders with influence on everyday life activities are present in these children.

Behaviour

The behaviour of all 21 AOA children was assessed using three validated questionnaires. The SCQ questionnaire was filled out and returned valid for 16 children. The mean score was 6.4 (range 0–13). No correlation was found between paternal and maternal age at birth, APGAR score at 5 min, birthweight and the SCQ score. The CBCL and TRF (non-mandatory) were returned for 19 and eight children, respectively. The mean scores for the CBCL and TRF were 43.8 and 44.0, respectively. Again, these scores fell within the normal range for children of similar age, being <70. Also, for both questionnaires, the mean scores for internalizing and externalizing behaviour lied within the normal range. One girl scored above the norm for internalizing behaviour (CBCL score: 74). Nevertheless, this was not confirmed by her teacher (TRF score: 53).

Discussion

This is the first report on the neurodevelopmental and behavioural outcome of children born following AOA, which is a rarely required, nonetheless highly specialized fertilization technique. The high response rate and the robustness of the performed tests and questionnaires support the validity of the results found in this study.

Others described a group of 22 babies born following AOA (Takisawa et al., 2011a,b). Of these babies, 10 were born following AOA by the calcium ionophore A23187 and 12 following $SrCl_2$ treatment. Growth and health of these babies at birth were not different between both AOA protocols. In a similar communication, weight and height of 21 AOA children was assessed up to the age of six (Sato et al., 2011).

Table 3 Scores of the follow up by assessment.

Test and age group	*No. of children*	*Norm*	*Score*			
Cognition	21		Verbal IQ	Performal IQ	Full scale IQ	
WPPSI-III-NL (≤7 years)	20	100	102.8 (96.4–109.2) (77–129)	105.0 (97.8–112.2) (74–142)	104.2 (97.5–110.8) (85–144)	
WISC-III-NL (>7 years)	1	100	123	130	131	
Language	20		Receptive scale	Expressive scale	Total percentile score	
RTOS (<5 years)	8	50	82.0 (63.4–100.7)[a] (35.5–99)	73.3 (49.4–97.2)[b] (30–99)	78.2 (57.2–99.2)[c] (33.3–99.0)	
CELF-IV-NL (≥5 years)	12	50	32.5 (7.3–57.7)[a] (3.6–94.5)	40.9 (16.6–65.2)[b] (7.1–88.5)	40.1 (16.8–63.4)[c] (9.1–89.7)	
Motor skills	19		Manual dexterity	Aiming and catching	Balance	Total percentile score
Movement ABC-II-NL (3–6 years)	17	50	42.0 (24.4–58.7) (1–95)	43.8 (29.5–58.0) (2–95)	41.5 (25.1–57.9) (5–91)	37.1 (22.3–52.0) (2–84)
Movement ABC-II-NL (7–10 years)	2	50	56.5 (NA) (50–63)	21.0 (NA) (5–37)	77.0 (NA) (63–91)	50.0 (NA) (37–63)
Autism screening	16		Score			
SCQ (≥2 years)	16	<15	6.4 (4.5–8.3) (0–13)			
Maladaptive behaviour screening	19		Internalizing broadband	Externalizing broadband	Total score	
CBCL (1.5–5 years)	11	<70	50.5 (42.7–58.2) (33–74)	47.6 (42.1–53.0) (35–60)	48.6 (42.5–54.8) (30–63)	
CBCL (6–18 years)	8	<70	38.4 (33.6–43.2) (33–50)	39.1 (35.0–43.2) (34–48)	37.3 (32.0–42.5) (29–45)	
TRF (1.5–5 years)	4	<70	47.5 (41.3–53.7) (44–53)	44.8 (36.8–52.7) (38–49)	45.5 (41.7–49.3) (44–49)	
TRF (6–18 years)	4	<70	43.5 (31.7–55.7) (37–52)	42.7 (40.9–44.1) (41–43)	42.5 (32.1–52.9) (33–48)	

Values are mean (95% CI) (min–max) or *n*. Norm: (i) the 50th percentile of the general population (for cognition, language and motor skill assessment); or (ii) the cut-off below which the behaviour is considered normal (for autism and maladaptive behaviour screening).
CBCL = Child Behaviour Checklist; CELF = Clinical Evaluation of Language Fundamentals; Movement ABC-II-NL = Movement Assessment Battery for Children III; NA = not applicable (*n* = 2); RTOS = Reynell Developmental Language Scales; SCQ = Social Communication Questionnaire; TRF = Teachers Report Form; WISC = Wechsler Intelligence Scale for Children; WPPSI = Wechsler Preschool and Primary Scale of Intelligence.
[a]$P < 0.01$. [b,c]$P < 0.05$ comparing RTOS and CELF scores (Mann–Whitney *U*-test), respectively.

For the singletons, the physical growth was within the 10–90% percentile. In the latter report, it was not mentioned whether the children were born following calcium ionophore or strontium treatment.

The neonatal data of this group of children were reassuring. No birth defects were reported at birth, whereas at toddler age birth defects were reported in 14.3% of the children followed up by assessment. However, the occurrence of birth defects in this cohort was not different from a recent reported birth defect rate of 9.7% in 939 ICSI children (Davies et al., 2012). Nonetheless, the sample size of the current study group was not adequate to allow for a meaningful conclusion, as it led to a wide confidence interval for the observed birth defect rate, which indicated that the observed birth defect rate is statistically compatible not only with the rate of the reference cohort (9.7%), but also with a rate that is 3-times higher, which should be interpreted with caution. One should also bear in mind that children born following assisted reproduction technology stay more often in neonatal care units compared with naturally conceived children and are checked very carefully, which might lead to overestimation of anomalies that create an observational bias.

For the assessment of neurodevelopmental health, only children aged 3 years or older were included. It is known that tests of cognitive ability performed on very young children (≤2 years) are not necessarily reliable predictors of long-term intelligence (Bowen et al., 1998; Leslie et al.,

2003). In several studies, no delay of cognitive development was found in ICSI children compared with IVF or naturally conceived controls (Cederblad et al., 1996; Leunens et al., 2008; Place and Englert, 2003; Ponjaert-Kristoffersen et al., 2005). However, one study suggested that IQ scores were significantly lower in ICSI children (Knoester et al., 2008). The latter could not be confirmed by the present study with normal IQ scores. One girl, who scored rather low for VIQ (<85), was known with distinct shyness, especially in new situations, which could have led to a false low score on VIQ. No gross problems were known for this girl at school or in relationship with classroom peers. One boy also scored relatively low for PIQ (<85). This boy was at the lower age limit to perform this test and since this test was performed at the end of the day, tiredness could have been the cause for the lower PIQ score (Murphy and Davidshofer, 1998). Both children had normal FSIQ scores.

At the group level, the mean scores for language development fell within the normal ranges. The younger children tested by the RTOS scored significantly higher on language development compared with the older group tested by the CELF-IV-NL. This difference might be attributed to the fact that the majority of the children in the older group were twins and several reports claim that twins on average score lower on language development tests compared with singletons (Rutter et al., 2003b; Thorpe, 2006).

It has been shown that motor skill development in ICSI children can lag behind (Knoester et al., 2007a). Although the overall results of the present cohort was reassuring, the twins tended to lag behind compared with the included singletons, although the difference was not significant. This might be explained by the significantly lower gestational age at birth for the twins compared with the singletons.

Extensive research regarding the influence of IVF and ICSI on children behaviour and the incidence of attention-deficit hyperactivity disorder and disorders in the autism spectrum (ASD) has been performed. It was shown that the prevalence of ASD was higher among ICSI children (3.4%) compared with the general population (0.6–0.7%) (Knoester et al., 2007b). Older paternal as well as maternal age are considered to be risk factors for ASD (Hultman et al., 2011; Sandin et al., 2012). On the contrary, another group could not confirm a relationship between IVF/ICSI and ASD when results were adjusted according to maternal age and educational level, parity, smoking, birthweight and multiplicity (Hvidtjørn et al., 2011). In this study, the SCQ questionnaire was used to assess the risk for ASD. The mean score for the AOA children fell within the normal range. Nevertheless, taking into account the research of Snow and Lecavalier (2008), one younger child (aged 37 months) scored just above the cut-off value. The latter researchers decreased the cut-off score from 15 to 13 for younger children. Importantly, one should keep in mind that a single questionnaire is not sufficient to diagnose ASD. No significant correlations were found in this study between the SCQ score and paternal and maternal age at birth, APGAR score at 5 min or birthweight. According to the CBCL questionnaire, this group of AOA children did not show any maladaptive behaviour. However, one girl scored above the norm for internalizing behaviour. This could be explained by the fact that ICSI girls tend to score higher on the externalizing and internalizing broadband as well as on the total CBCL score in comparison with IVF girls (Knoester et al., 2007b). Nevertheless, this girl's CBCL score was not confirmed by her teacher, since her TRF score was normal.

This cohort consists of almost 50% twins, which is essentially higher compared with the overall twinning rate following assisted reproduction technology, which was 11.2% in Belgium in 2010 (Belrap, 2012). Since 1 July 2003, assisted reproduction centres in Belgium are legally obliged to follow a strict regulation regarding embryo transfer. In women aged <36 years, only one embryo can be transferred in a first treatment cycle. From the second cycle on, two embryos might be transferred if only moderate embryos are available for transfer. From the third cycle onwards, a maximum of two embryos may be transferred. Since most of the couples undergoing AOA carry a history of several treatment cycles, most of these couples legally applied for the transfer of two embryos, which led to the high twinning rate in the current cohort of children. Since the high success rates of both the study centre's AOA procedure and the current cryopreservation techniques, the centre now encourages the transfer of only one embryo in AOA couples. Consequently, the twinning rate in this AOA population has gone down significantly in the last 5 years.

Four singletons of this cohort are siblings, which is one-third of the singleton cohort. Kramer and Conger (2009) pointed out the important role of brothers and sisters in a child's development. Sibling relations provide an important context for the development of children's understanding of their social, emotional, moral and cognitive worlds. This should be kept in mind when interpreting the results of the current study.

The strengths of this case series are both the high response rate and the use of a range of different validated age-adjusted tests and questionnaires. Although AOA is rarely necessary in modern assisted reproduction, its possible impact on offspring development should not be neglected. However, this follow-up study is also characterized by certain limitations that must be taken into consideration. First of all, since AOA is a rarely used technique, the absolute number of included children is very low which severely limits conclusion making. Also, the fact that all tests were pooled on one day could have influenced the results, since some children showed fatigue and a lack of interest during the course of the day. Unfortunately, due to practical considerations (mostly related to the parents' professional activities), all tests had to be performed on one day. Another concern which needs to be addressed is the fact that two-thirds of the parents of the children that were followed up by assessment had a higher level of education (beyond 18 years of age). Parental education, together with family income, occupational status and place of residence describes the socioeconomic status (SES) of an individual. The influence of SES on the development of children born at term is well established (Ardila et al., 2005). Research shows that SES is associated with a wide range of health, cognitive and socioemotional outcomes in children, with effects beginning prior to birth and continuing into adulthood (Bradley and Corwyn, 2002; Turkheimer et al., 2003). As a matter of fact, parental education is a major component of the SES variables. It is generally accepted that IVF/ICSI children comprises a selected group of children, born to parents with on average higher educa-

tional and socioeconomic levels. Therefore, appropriate reference values for IQ and percentile scores may be higher, so that the apparent lack of departure from the norm, or advantage over it, may be less reassuring than it looks. Finally, this analysis did not include a group of control children, since for all used tests and questionnaires, validated norm values were available. Individual performance was compared with established norms for Dutch-speaking children. Also, since the current cohort of AOA children is very small due to the fact that AOA is a rarely required technique, the prior aim was to describe the neurodevelopmental performance of those children specifically. In future, the optimal design would be a multicentre-based child age-matched control study.

The overall results of this case series of children born following ICSI combined with AOA showed that neonatal, developmental as well as behavioural outcome is reassuring, although the results are still to be considered preliminary. No serious adverse effects were observed; however, the size of this study is too small to reach a definite conclusion. Therefore, AOA should still not be considered routine practice yet. The sample size of this cohort was too small to detect chromosomal aberrations and malformations following AOA.

Couples undergoing AOA (or any other assisted reproduction procedure) should be counselled in advance regarding increased risks of pregnancy complications and higher rates of obstetric interventions. Although conflicting data exist, studies of children born following IVF and ICSI suggest increased rates of congenital malformations, imprinting disorders and a marginally increased risk of cancer (Basatemur and Sutcliffe, 2008). However, the current study and evidence is inadequate to draw definite conclusions about long-term outcomes after assisted reproduction technology and AOA specifically. Prospective multicentre long-term studies are needed to eliminate the effect of confounders. The long-term outcomes after AOA are difficult to evaluate because of the variability in AOA methods and data reporting. Therefore, clinicians should aim for standardization of the AOA procedure as well as the methodology for follow up of children born following AOA. ICSI combined with AOA should still be performed only in cases where a clear indication is present.

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