Hum. Reprod. Advance Access published March 22, 2012
Human Reproduction, Vol.0, No.0 pp. 1–7, 2012
doi:10.1093/humrep/des088

human reproduction

ORIGINAL ARTICLE *Embryology*

Consistent and predictable delivery rates after oocyte vitrification: an observational longitudinal cohort multicentric study

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Submitted on September 11, 2011; resubmitted on January 16, 2012; accepted on January 31, 2012

BACKGROUND: An efficient method for cryopreservation of human oocytes may offer solutions to legal and ethical problems in routine infertility programs and may also be used for fertility preservation for medical and social reasons.

METHODS: We conducted an observational longitudinal cohort multicentric study to investigate the efficacy and reproducibility of oocyte cryopreservation outcomes in IVF/ICSI cycles. Moreover, the effects of patient and cycle characteristics on the delivery rate (DR) were analyzed.

RESULTS: In 486 cycles performed in 450 couples, 2721 oocytes were warmed and 2304 of them survived cryopreservation (84.7%). Of the 2182 oocytes subjected to ICSI, the rates of fertilization and development to top-quality embryos were 75.2 and 48.1%, respectively. A total of 128 deliveries were obtained (26.3% per cycle and 29.4% per transfer) for 450 patients (28.4%) and 147 babies were live born from 929 embryos transferred (15.8%). The forward logistic regression analysis on a per patient basis showed that female age [odds ratio (OR): 0.93, 95% confidence interval (CI): 0.88–0.98], number of vitrified oocytes (OR: 1.08, 95% CI: 1.01–1.17) and the day of transfer (OR: 1.97, 95% CI: 1.14–3.42) influenced DR. By recursive partitioning analysis, it can be estimated that more than eight oocytes vitrified are required to improve the outcome (22.6 versus 46.4% DR, respectively). When fewer oocytes are available in women aged >38 years, results are dramatically reduced (12.6 versus 27.5% DR, respectively). Conversely, when >8 oocytes are available, blastocyst culture represents the most efficient policy (62.1% DR; data from one center only).

CONCLUSIONS: Oocyte vitrification is an efficient and reliable approach, with consistent results between centers and predictable DRs. It should be applied routinely for various indications. A predictive model is proposed to help patient counselling and selection.

Key words: oocyte vitrification / female age / delivery rate

Introduction

One of the most important achievements in human assisted reproduction in the past decade has been the dramatically increased efficiency of cryopreservation of human oocytes in the second phase of meiotic division (MII oocytes). The procedure may offer solutions to a number of clinical, logistic and social problems including fertility preservation before systemic anticancer treatment, ovary removal or premature menopause; storage in cases of difficulty with sperm collection or inadequate seminal samples; cryobanking oocytes for oocyte donation or to delay motherhood; and ethical concerns and legal restrictions associated with embryo cryopreservation (Nagy *et al.*, 2009b).

Although the first report of birth after oocyte cryopreservation was published 25 years ago (Chen, 1986), for ~15 years the overall efficiency remained low hampering widespread application. The large size and spherical shape of oocytes interfere with even distribution

of cryoprotective additives. Moreover, some subcellular structures are specifically sensitive to cryoinjuries, and the fact that oocytes consist of one cell decreases the chance of recovery from a serious injury (Vajta and Nagy, 2006). Eventually, improvements in traditional slow-rate freezing, and especially application of an ice-free cryopreservation, vitrification, resulted in a breakthrough in oocyte cryopreservation (Fabbri *et al.*, 2001; Borini *et al.*, 2004; Kuwayama *et al.*, 2005). Recent reports demonstrate that oocyte cryopreservation does not compromise *in vitro* development and pregnancy rates (Cobo *et al.*, 2009; Rienzi *et al.*, 2010; Ubaldi *et al.*, 2010) and does not increase the frequency of complications and developmental abnormalities (Chian *et al.*, 2008; Noyes *et al.*, 2009); in oocyte donation programs, the clinical outcome with vitrified oocytes is not different from those with fresh counterparts (Nagy *et al.*, 2009a; Cobo *et al.*, 2010). Studies have also been published about the potential application of oocyte vitrification in standard infertility programs to replace embryo cryopreservation (Schoolcraft *et al.*, 2009; Rienzi *et al.*, 2010; Ubaldi *et al.*, 2010).

The primary aim of this study was to determine the efficiency, reproducibility and consistency of the overall outcome, i.e. live birth rate after oocyte vitrification in unselected infertility cycles performed in three different infertility clinics of two European countries. Additionally, the predictive value of various factors potentially influencing the outcome (legal environment, female age, causes of infertility, number of retrieved and cryopreserved oocytes, sperm parameters, incubation period of oocytes before cryopreservation, endometrial preparation and the day of transfer) were investigated. The different legal situation in the two countries has resulted in two differences: in two clinics, a maximum of four oocytes were warmed in one cycle, as a maximum of three embryos were allowed to be produced in Italy and Day 5 transfers were only performed in one of the three centers, because in Italy, all produced embryos had to be transferred regardless of their quality. The overall outcome was measured as the rates of deliveries.

Materials and Methods

Study design and participants

An observational longitudinal cohort multicentric study was conducted. Between October 2006 and April 2010, all consecutive treatment cycles of patients enrolled to routine infertility programs in three European clinics (G.E.N.E.R.A., Rome, Italy; Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy and IVI, Valencia, Spain) were involved in the study. Inclusion criteria were female age <43 years, oocyte vitrification, oocyte warming and available data about the pregnancy and delivery including developmental abnormalities. Preimplantation genetic diagnosis was regarded as an exclusion criterion. Infertility treatment protocols including hormonal stimulation, oocyte retrieval, *in vitro* fertilization, embryo culture and transfer methods applied in these clinics were based on the principles described by Rienzi *et al.* (2010).

Reasons of oocyte cryopreservation were the following: supernumerary oocytes (203; 45.1%), risk of hyperstimulation (22; 4.8%), no available sperm (29; 6.4%), repeated previous implantation failures with good quality embryos (166; 36.8%) and patient request (30; 6.7%). Most frequent causes of infertility were female factors including endometriosis, tubal and ovulatory factors (165; 36.7%) and male factors (150; 33.4%), while the rest of the cases were related to either combined (21; 4.7%), idiopathic infertility (80; 17.8%) or other factors (34; 7.6%).

The study was approved by the Institutional Review Board of the Clinics and informed consent was obtained from the patients.

Cryopreservation of oocytes

After transvaginal ultrasound-guided aspiration and recovery, oocytes were incubated for 2–8 h in commercial culture medium [Quinn's Advantage® Fertilization (HTF) Universal Medium, Sage, USA] in 6% CO_2 and 5% O_2 at 37°C. The timing for oocyte cryopreservation was not fixed and related to laboratory organization. This parameter was in fact found not to affect the final outcomes in previous studies (Ubaldi *et al.*, 2010). MII phase oocytes were selected under a stereomicroscope according to the presence of the first polar body and intact extra- and intracytoplasmic morphology (Rienzi *et al.*, 2010).

The vitrification and warming procedures have been described in detail (Kuwayama *et al.*, 2005; Cobo *et al.*, 2008; Rienzi *et al.*, 2010). No important changes were made in the vitrification protocol during the investigation period. All supernumerary MII phase oocytes were vitrified. Vitrification was performed by using the Cryotop device (Kitazato Co., Fujinomiya, Japan) consisting of a thin film strip attached to a plastic holder and a separate protective cap to avoid mechanical damage of the strip during manipulation and storage. Cryotop devices and ready-to-use vitrification and warming solutions were obtained from Kitazato Co. The first equilibration was performed in 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) at 24–26°C for 12–15 min. Subsequently, oocytes were transferred to a 24–26°C 15% EG, 15% DMSO and 0.5 M sucrose solution for 1 min, then placed on the film strip of the Cryotop as a single small drop. The excess solution was removed and the Cryotop was submerged into liquid nitrogen. The strip was covered with the protective cap and the sample was stored submerged in liquid or in vapor phase nitrogen.

At warming, the cap was removed under liquid nitrogen and the film strip of Cryotop was quickly submerged in 1 ml of 37°C warming solution containing 1.0 M sucrose for 1 min. During this period, oocytes were gently forced to float away from the filmstrip. Subsequently, oocytes were transferred to a 24–26°C solution containing 0.5 M sucrose and incubated for 3 min. After two subsequent washes in basic medium at 26°C for 5 min each, oocytes were transferred into culture medium. Degenerated oocytes were removed from the cohort. Surviving oocytes were cultured for 2–3 h before insemination with ICSI.

Embryo culture and transfer

Fertilized oocytes were incubated in culture medium for 2–5 days after insemination under conditions mentioned above, with a medium change on Day 3 (Quinn's Advantage® Cleavage Medium, Quinn's Advantage® Blastocyst Medium, Sage, CooperSurgical, Pasadena, CA, USA). Embryo quality was evaluated as described previously (Rienzi *et al.*, 2002, 2010). Briefly, cleaving embryos were evaluated taking into account cleavage speed, blastomere symmetry, extent of fragmentation and the presence or absence of multinucleated blastomeres. The embryo was defined as 'top quality' when the number of cells was four to five on Day 2 or eight on Day 3, with symmetrical blastomers, no multinucleation and when the fragmentation was not more of 10% of the volume (according to the Asebir criteria summarized in the Istanbul consensus workshop on embryo assessment, 2011).

Blastocyst transfer was proposed only when three or more top-quality embryos were available on Day 3. Embryo transfer was performed in the course of a natural cycle or after endometrial preparation (Glujovsky *et al.*, 2010). The luteal phase was supported by vaginal micronized progesterone (Progeffik 200 mg, Effik, Cinisello Balsamo, Milan, Italy; Progeffik, Effik Laboratories, Madrid, Spain).

Statistical analysis

Continuous data (female age, number of cumulus corona cell oocyte complexes retrieved, number of MII phase oocytes obtained, number of vitrified, warmed, survived, inseminated and fertilized oocytes, number of top-quality embryos, number of embryo transferred and cryopreserved) are presented as absolute, mean with 95% confidence interval (CI). Categorical variables (infertility factor, stimulation protocol, sperm origin and clinical outcomes) are presented as absolute, percentage frequency with 95% CI.

Clinical pregnancy was determined by ultrasound demonstration of a gestional sac at 7 weeks. The delivery rate (DR) was calculated as the number of deliveries per cycle, per transfer or per patient. Implantation rate (IR) was calculated as the number of newborns per transferred embryo. These rates were expressed as percentage probabilities with 95% CI.

The effect of covariates (female age, infertility factor, stimulation protocol, number of retrieved and vitrified oocytes, sperm quality, oocyte incubation time between retrieval and vitrification procedure, age of embryos at transfer and endometrial preparation at warming) on delivery were assessed using forward logistic regression analysis on a per patient basis.

Recursive partitioning analysis was used to stratify the sample according to predictive variables on DR (Sox, 1988). Accordingly, a decision-making model was computed.

Results

Study subjects

During the study period ~14 000 ovum pick-ups were performed in the centers. A total of 486 oocyte warming cycles performed in 450 couples were enrolled in the study. In case of supernumerary oocytes, all couples were involved in the oocyte warming cycles because no deliveries from the fresh transfer cycles were obtained.

The average age of females was 36.0 (95% CI: 35.64–36.37) years at the time of oocyte collection.

Oocyte collection cycles

For female hormonal stimulation, FSH was administered in an average total dose of 2060 IU (95% CI: 1973–2147) for a mean period of 11.7 (95% CI: 10.01–13.52) days. Hormonal stimulation was performed with gonadotrophin agonists (250) and antagonists (200) in almost equal proportion (55.6 versus 44.4%, respectively).

The average total number of collected cumulus–oocyte complexes (COCs) was 11.0 (95% CI: 10.37–11.55). Of these, 78% contained an MII phase oocyte. The mean number of oocytes vitrified was 6.55 (95% CI: 6.17–6.92). The average time of incubation between oocyte collection and vitrification was 2.47 h (95% CI: 2.39–2.55). Male gametes originated from ejaculated sperm (432 patients), or epididymal (4 patients) and testicular (14 patients) spermatozoa were used (96.0, 0.8 and 3.1% of total cases, respectively). For the ejaculated sperm, the number of spermatozoa was $>1 \times 10^6$/ml in 76.4% of cycles. Fresh ICSI was performed in 245 cycles. The average number of oocytes fertilized was 2.43 (95% CI: 2.29–2.56). Transfer of fresh embryos was performed in 220 cycles (89.8% of the cycles where fresh insemination was performed), with a mean number of 2.1 embryos (95% CI: 2.03–2.26).

Oocyte warming cycles

Characteristics of oocyte warming cycles are presented in Table I. A total of 486 warming cycles were performed, 6.4 and 1.0% of them

Table I Characteristics of the oocyte warming cycles.

Characteristics	Absolute number	Mean (95% CI)	% (95% CI)
Cycles	486		
Warmed oocytes	2721	5.62 (5.32–5.93)	
Survived oocytes	2304	4.78 (4.54–5.03)	84.7 (83.3–86.0)[a]
Inseminated oocytes	2182	4.53 (4.28–4.77)	94.7 (93.7–95.5)[b]
Fertilized oocytes	1642	3.41 (3.19–3.62)	75.2 (73.4–77.0)[c]
Days 2–3 top-quality embryos	796	1.89 (1.57–2.26)	48.1 (45.7–50.5)[d]
Embryo transfers	436		89.7 (86.7–92.1)
Embryos transferred	929	1.91 (1.83–1.99)	
Embryos cryopreserved	184	0.38 (0.29–0.48)	
Delivery	128		26.3 (22.6–30.4)[e]
			29.4 (25.2-33.8)[f]
Newborn	147		15.8 (13.6–18.3)[g]

CI, confidence interval.
[a]Of warmed oocytes.
[b]Of survived oocytes.
[c]Of inseminated oocytes.
[d]Of fertilized oocytes.
[e]Of cycles.
[f]Of embryo transfers.
[g]Of transferred embryos.

Table II Differences between centers.

	Center 1	Center 2	Center 3
No. warming cycles	231	174	81
No. patients	230	146	74
Mean female age (95% CI)	36.2 (35.7–36.7)	36.2 (35.6–36.9)	34.9 (33.9–35.9)
Mean no. CCOCS retrieved (95% CI)	9.1 (8.21–10.1)[a]	13.2 (12.4–14.0)	12.2 (11.4–13.0)
Mean no. MII phase oocytes (95% CI)	7.18 (6.49–7.87)[a]	10.4 (9.79–11.0)	9.61 (8.97–10.2)
Mean no. vitrified MII (95% CI)	6.80 (6.19–7.41)	6.45 (5.88–7.02)	5.95 (5.37–6.52)
Mean no. warmed MII (95% CI)	6.70 (6.14–7.26)[a]	4.40 (4.19–4.61)	5.11 (4.75–5.47)
No. embryo transfers (% and 95% CI)	200/231 (85.8; 80.8–89.7)	146/174 (83.9; 77.7–88.6)	74/81 (91.3; 83.2–95.7)
Mean no. embryos transferred (95% CI)	1.57 (1.47–1.67)[a]	2.26 (2.12–2.41)	2.14 (1.95–2.33)
No. cleavage stage (Days 2–3) transfers (%)	127/200 (63.5)	160/160 (100)	76/76 (100)
No. blastocyst stage transfers (%)	73/200 (36.5)		
Deliveries per cleavage stage (Days 2–3) transfers (% and 95% CI)	41/127 (32.3; 25.0–41.1)	40/160 (25.0; 17.5–29.9)	14/76 (18.4; 10.6–27.0)
Deliveries per blastocyst stage transfers (% and 95% CI)	33/73 (45.2; 34.2–56.6)		
Total deliveries per cycle (% and 95% CI)	74/231 (32.0; 26.1–38.0)	40/174 (23.0; 17.4–29.8)	14/81 (17.3; 10.6–27.0)
Total deliveries per transfer (% and 95% CI)	74/200 (37.0; 30.6–43.9)[b]	40/160 (25.0; 18.9–32.2)	14/76 (18.7; 11.5–29.0)

CI, confidence interval; CCOCs, cumulus corona cell oocyte complexes.
[a]Significant differences between center 1 versus 2 and 3.
[b]Significant difference between centers 1 and 3.

were second and third repeats, respectively. An average of 5.62 (95% CI: 5.32–5.93) oocytes/cycles were warmed. Percentages of survived, injected and fertilized oocytes were 84.7, 94.7 and 75.2%, respectively. The difference between the numbers survived and injected oocytes is the result of the legal restrictions in two laboratories described in the section Introduction. There were 929 embryos transferred in 436 cycles (mean number: 1.91, 95% CI: 1.83–1.99). Embryo transfers were performed at cleavage (363) and blastocyst (73) stages (83.2 and 16.7% of total transfers, respectively). Endometrial preparation was performed in 48.2% of the warming transfers (210/436). A total of 166 clinical pregnancies (34.2%, 95% CI: 30.1–38.5 per cycle; 38.1%, 95% CI: 33.6–42.7 per transfer) were obtained. The DR was 26.3% per cycle (95% CI: 22.6–30.4) and 29.4% per transfer (95% CI: 25.3–33.8). A total of 147 babies were born. Two common congenital anomalies were recorded (1.4%): one cleft lip and palate and one craniosynostosis.

Differences between centers

Main characteristics of vitrification and warming cycles were compared in the three centers in Table II. No significant differences were observed in laboratory outcomes. MII phase oocytes survival, fertilization and embryo development (cleavage and top-quality embryo rate on Days 2 or 3) were consistent between the three centers (84.0, 85.9 and 84.8; 74.1, 73.5 and 77.6; 45.3, 48.3 and 47.7, respectively, NS). Clinical outcomes were also consistent when the embryos were transferred at the same stage of development. A significantly higher number of oocytes were warmed in Center 1, which allowed the application of a blastocyst transfer policy. This approach was found to be highly efficient.

Evaluation of prognostic factors

According to the forward logistic regression analysis on patient basis, infertility factors, stimulation protocols, number of retrieved COCs and MII oocytes, sperm quality and oocyte incubation time between retrieval and vitrification and endometrial preparation at warming did not influence the DR. An inverse correlation was found between maternal age and DR [odds ratio (OR): 0.93, 95% CI: 0.88–0.98]. The number of vitrified oocytes (OR: 1.08, 95% CI: 1.01–1.17) and the day of transfer (OR: 1.97, 95% CI: 1.14–3.42) were also found to be associated with the primary outcome measure (Table III).

By recursive partitioning analysis, a general intuitive model, able to predict the probability of obtaining a delivery per patient according to her characteristics (number of MII oocytes vitrified, age and day of transfer) has been calculated (Fig. 1). It can be estimated that more than eight oocytes vitrified are required to improve the outcome (22.6 versus 46.4% DR, 95% CI: 18.5–27.4 and 37.3–55.6, respectively). When less, i.e. eight or less than eight oocytes are available, the results are dramatically reduced for women aged >38 years compared with younger women (12.6 versus 27.5% DR, 95% CI: 7.7– 14.1 and 22.1–33.6, respectively). Conversely, when more than eight oocytes are available, the culture to blastocyst stage represents the most efficient policy (62.1% DR, 95% CI: 43.8–77.3).

Discussion

The application of vitrification, i.e. ice-free cryopreservation in human assisted reproduction (Kuwayama *et al.*, 2005; Vajta and Nagy, 2006; Cobo *et al.*, 2010; Rienzi *et al.*, 2010), has opened new perspectives

Table III Forward logistic regression analysis to evaluate the effect of patients and cycle characteristics on delivery obtained with vitrified oocytes (per patient basis).

Covariate	*P*-value	OR	95% CI
Female age	0.01	0.93	0.88–0.98
Infertility factor			
Male (reference)			
Tubal	0.67	1.2	0.51–2.85
Endometriosis	0.77	0.77	0.22–3.08
Ovulatory	0.72	0.72	0.40–3.80
Idiopathic	0.95	0.97	0.38–2.45
Combined	0.3	0.56	0.34–1.23
Other	0.21	0.38	0.08–1.73
Stimulation protocol			
Agonist (reference)			
Antagonist	0.14	0.83	0.49–1.41
Sperm quality			
$>1 \times 10^6$/ml (reference)			
$<1 \times 10^6$/ml	0.07	1.75	0.99–3.10
MII phase oocytes obtained	0.81	0.98	0.85–1.14
Incubation time (h) before vitrification	0.87	1.08	0.92–1.25
MII phase oocytes vitrified	0	1.08	1.01–1.17
Day of transfer			
Cleavage stage (reference)			
Blastocyst stage	0.02	1.97	1.14–3.42
Endometrial preparation			
Natural cycles (reference)			
Prepared cycles	0.07	0.68	0.035–1.01

OR, odds ratio; CI, confidence interval.

in cryopreservation of blastocysts and MII phase oocytes. These improvements, especially for the latter, may have a profound effect on applicable strategies not only for infertility treatments, but also fertility preservation, for both medical and social indications, and could involve a wide range of patients. However, in spite of the excellent results achieved by several centers and the confirmed harmlessness of oocyte cryopreservation (Chian *et al.*, 2008; Noyes *et al.*, 2009), the overall acceptance of vitrification is still controversial (Vajta *et al.*, 2009) and the application is restricted to a relatively low number of countries while whole regions seem to ignore the new possibility.

To achieve the primary aim, i.e. to investigate the overall efficiency and consistency of oocyte cryopreservation, our present study was performed in an unselected population of patients in various geographical regions under different legislative environments, and treated by three different clinical and laboratory teams. Our results show high survival, fertilization and development rates, with clinical pregnancy, implantation and birth rates comparable with those achieved with embryos cryopreserved at various stages of development (Debrock *et al.*, 2011) or embryos from non-vitrified oocytes in routine infertility programs (Levi Setti *et al.*, 2008; La Sala *et al.*, 2009). Two of the 147 babies suffered common major malformations. This incidence is comparable with the occurrence in naturally conceived infants, confirming earlier observations that oocyte vitrification is not related to an increased risk of adverse perinatal outcomes (Noyes *et al.*, 2009).

New and reassuring information derived from our study was that no significant differences were found after oocyte vitrification between the three centers in the laboratory results including survival, fertilization and *in vitro* embryo development rates. Moreover, the pregnancy rate and DRs achieved with the transfer of cleavage stage embryos were also similar. The only difference was the direct consequence of the different legal situation. While in Centers 2 and 3, production of a maximum three embryos was allowed, and subsequently all of them had to be transferred to the uterus, while no such law restricted the freedom to select the fertilization, embryo culture and transfer policy in Center 1. Accordingly, at the latter center, a considerable number of embryos were *in vitro* developed to, and transferred at, the blastocyst stage.

The third, and probably most important piece of information derived from our study was the evaluation of the predictive value of different, commonly considered infertility factors and the day of transfer after fertilization. The DR was not influenced by the stimulation protocol, the number of retrieved COCs and oocytes, the incubation time between oocyte retrieval and the start of the vitrification procedure or quality of the sperm. However, a strong inverse correlation was found between the maternal age and DR. In practical terms, it means that in the investigated population, each year of maternal age decreased the DR by 7%.

Another factor that has been found correlated with the outcome was the number of vitrified MII stage oocytes. The statistical calculation revealed that with each available MII stage oocyte, an 8% increase in DRs could be obtained, provided that all other conditions were fixed. DRs double (from 22.6 to 46.4%) when the number of vitrified oocytes is higher than eight.

The third factor that has been found to influence the DRs after oocyte vitrification was the day of transfer after fertilization. With more than eight available vitrified oocytes and good-quality embryos on Day 3, Day 5 embryo transfer allowed a delivery in ~60% of patients.

Among the factors associated with the outcome, female age and the number of available MII stage oocytes are factors independent from clinical decision, but data can be used to select the right population for fertility preservation and for counselling to advise patients about their possible chances to obtain pregnancies. On the other hand, the day of transfer depends on clinical decision, and our data may help to make the right decision to obtain the highest possible pregnancy rates.

In conclusion, our study has confirmed that vitrification is a safe and efficient method for cryopreservation of human MII phase oocytes. The overall efficiency was found to be consistent in different centers and different geographical regions. Women younger than 39 years old, with more than eight available mature oocytes have the highest chance for clinical delivery. Transfer of blastocyst stage embryos in these women further increases the chances of successful

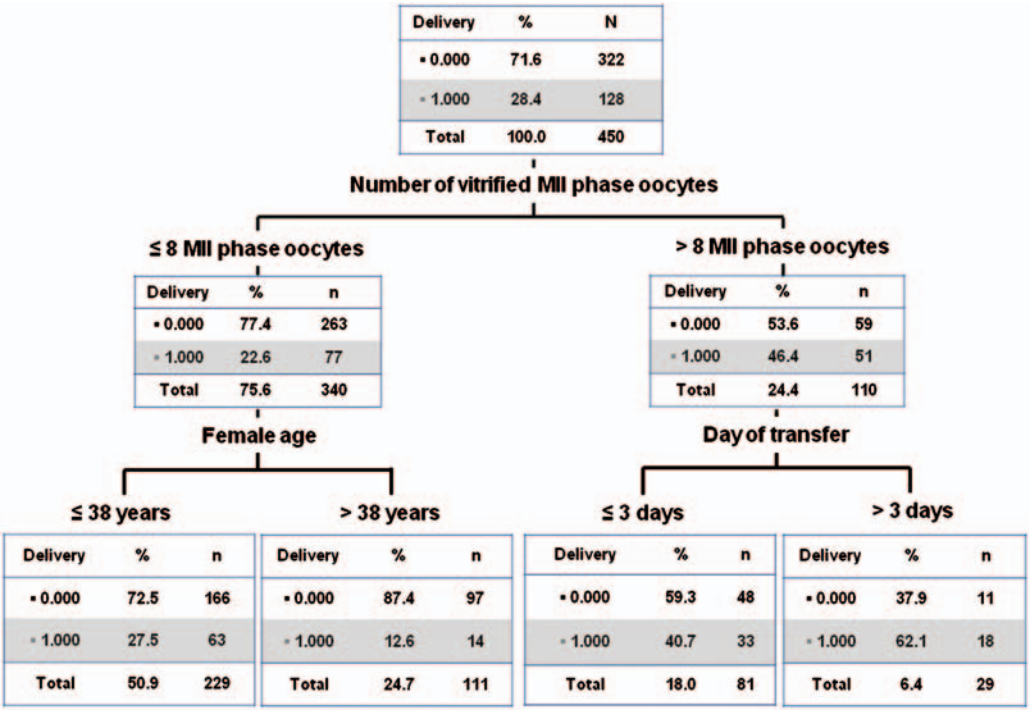

Figure 1 Decision-making model based on recursive partitioning analysis (per patient basis; black squares, 0 = delivery not obtained; grey squares, 1 = delivery obtained).

outcome. These data may provide help for patient counseling and selection.

Authors' roles

L.R. and A.C. developed and finalized the data set and performed the data analysis. L.R. and G.V. participated in study design and took the lead in writing the manuscript. A.C., A.P. and C.S. participated in study design, oversaw the data analysis and participated in editing the manuscript. J.R., G.R. and F.M.U. were responsible for overseeing the completion of the study, and editing and finalizing the manuscript.

Funding

No external funding was either sought or obtained for this study.

Conflict of interest

None declared.

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