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Morphological scoring of human pronuclear zygotes for prediction of pregnancy outcome

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BACKGROUND: As embryo selection is not allowed by law in Switzerland, we need a single early scoring system to identify zygotes with high implantation potential and to select zygotes for fresh transfer or cryopreservation. The underlying aim is to maximize the cumulated pregnancy rate while limiting the number of multiple pregnancies. METHODS: In all, 613 fresh and 617 frozen–thawed zygotes were scored for proximity, orientation and centring of the pronuclei, cytoplasmic halo, and number and polarization of the nucleolar precursor bodies. From these individual scores, a cumulated pronuclear score (CPNS) was calculated. Correlation between CPNS and implantation was examined and compared between fresh and frozen–thawed zygotes. The effect of freezing on CPNS was also investigated. RESULTS: CPNS was positively associated with embryo implantation in both fresh and frozen zygotes. With similar CPNS, frozen zygotes presented implantation rates as high as those of fresh zygotes. Nucleolar precursor bodies pattern and cytoplasmic halo appeared as the most important factors predictive of implantation for both types of zygotes, while pronuclei position was specifically relevant for frozen–thawed zygotes. Freezing induced an alteration of most zygote parameters, resulting in a significantly lower CPNS and a lower pregnancy rate. CONCLUSIONS: CPNS may be used as a single prognostic tool for implantation of both fresh and frozen–thawed zygotes. Lower CPNS values of frozen–thawed zygotes may also be indicative of freezing damage to zygotes. Successful implantation of frozen zygotes despite lower CPNS suggests that they may recover after thawing and *in vitro* culture.

Key words: cryopreservation/IVF/morphology scoring/pregnancy outcome/zygote score

Introduction

While one major goal of IVF has long been the enhancement of the pregnancy rates per treatment cycle, the necessity to decrease IVF-induced iatrogenic multiple pregnancies has become a health, economic and legal issue in several countries (Adashi *et al.*, 2003). However, the transfer of a single embryo is still a novel and challenging approach, even for patients with favourable indications for whom a transfer of two embryos is still often proposed. Patients do not always accept this medical viewpoint unless strong reassurance may be given that the overall treatment outcome is not impaired. With this objective in mind, various strategies can be used to select out for the transfer, either at the pronuclear (Scott and Smith, 1998; Tesarik *et al.*, 2000; Salumets *et al.*, 2001) or at later stages of embryo development (Fisch *et al.*, 2001; De Placido *et al.*, 2002), the embryos which possess the highest implantation ability.

Early developmental characteristics of embryos based on their cleavage rate and morphological appearance have been used since the early days of IVF as tools for the evaluation of their biological quality and implantation ability (Puissant *et al.*, 1987; Shoukir *et al.*, 1997; Ziebe *et al.*, 1997). More recently, the *in vitro* culture of human embryos up to the blastocyst stage has been presented as a method of choice to decrease the number of transferred embryos and the ensuing multiple pregnancies (Gardner *et al.*, 1998). Unfortunately, approaches involving embryo selection cannot be implemented in countries with restrictive IVF legislation such as Switzerland and Germany (Germond and Senn, 1999; van der Ven *et al.*, 2002). Therefore, the use of an early scoring system permitting the identification of viable oocytes (Michelmann *et al.*, 1995) or pronuclear zygotes (Montag and van der Ven, 2001) remains the only realistic option to optimize the chances of both the fresh and frozen–thawed cycles. The initial work on pronuclear scoring (Scott and Smith, 1998) has given rise to several studies on the prognostic value of the nucleolar precursor bodies (NPB) pattern (Tesarik and Greco, 1999), cytoplasmic halo (Ebner *et al.*, 2003a; Stalf *et al.*, 2002) and pronuclei (PN) orientation (Garello *et al.*, 1999). So far, no study has extended the use of such a scoring system to frozen–thawed pronuclear zygotes.

The present study was aimed at investigating the usefulness of zygote morphometric measurements and of a cumulated pronuclear score (CPNS) to predict implantation competence

in both fresh and frozen–thawed cycles. The proposed CPNS is a combination of features described by various authors and consists of the sum of individual scores assigned to the position and orientation of the PN, the degree of cytoplasmic retraction (halo effect), the number and polarization of the NPB. A secondary objective was to use the scoring system as a tool to evaluate the effects of the freeze–thaw process on the 2PN zygote morphology.

Materials and methods

Patients, ovarian stimulation and management of the frozen–thawed cycles

Couples undergoing transfer of fresh (n = 304) or cryopreserved embryos (n = 345), frozen at the 2PN zygote stage, in the Reproductive Medicine Unit, Department of Gynaecology and Obstetrics, Centre Hospitalier Universitaire Vaudois (CHUV) from May 2001 to December 2002 were included in this retrospective study. For the controlled ovarian stimulation (Germond *et al*., 2002), patients were desensitized with GnRH agonist (Decapeptyl; Ferring, Zürich, Switzerland) and follicular growth stimulated with recombinant FSH (Gonal-F; Serono, Switzerland; Puregon, Organon, Switzerland). When at least two follicles reached ≥17 mm in diameter, hCG (Profasi; Serono, Switzerland) was administered. In frozen–thawed cycles, patients monitored their own urine using commercially available qualitative LH tests (OVU-LH; Intex, Muttenz, Switzerland). Transfers were planned 3 days after the LH peak. Thawing of the zygotes was planned 24–26 h earlier, in order to allow for at least one mitotic division to occur. In cases of anovulation, embryos were replaced in artifical cycles using pituitary desensitization in association with estradiol (Progynova; Schering, Baar) and progesterone (Utrogestan; Vifor, Fribourg, Switzerland) administration (de Ziegler and Frydman, 1990).

Oocyte retrieval, IVF and embryo culture

Oocyte retrieval was performed via vaginal ultrasound-guided follicular puncture 36 h after HCG injection. In conventional IVF, fertilization of the collected cumulus–oocyte complexes was performed in 4-well dishes using 50 000 motile sperm. The use of ICSI was restricted to severe male factor cases or when, due to the couple's history, a failure of conventional IVF was anticipated. Oocytes, zygotes and embryos were cultured in large volumes (1 ml) of a commercially available medium (IVF-50; Vitrolife, JCD, Lyon, France) in 4-well Nunc dishes (Nagel Nunc International, Life Technologies, Zürich, Switzerland) under 5% CO_2, 5% O_2 and 90% N_2. At 16–18 h after insemination, fertilization was assessed by observing the presence of 2PN and two polar bodies under an inverted microscope (IX-70; Olympus, Zürich, Switzerland) equipped with Hoffman modulation contrast. Fertilized oocytes were randomly allocated to fresh transfer or cryopreservation without any criteria of selection, but a maximum of two zygotes, exceptionally three, were further cultured in IVF-50 until fresh embryo transfer on day 2. All supernumerary zygotes were frozen before syngamy (16–18 h after insemination) using a slow freezing protocol as previously described (Senn *et al*., 2000). Clinical pregnancies were assessed by the presence of a fetal sac, with cardiac activity, 5 weeks after embryo transfer.

Zygote scoring

Fresh zygotes observed under Hoffman contrast (×200 magnification) were photographed at the pronuclear stage 16–18 h after insemination. Thawed zygotes were photographed after complete removal of propanediol and sucrose. An Octax Eyeware camera (Octax, Herborn, Germany) attached to the microscope was used and digital images were then stored in the Eyeware database prior to morphological analysis. Using the Octax software, the following parameters were measured on each 2PN zygote kept in culture: external diameter of the oocyte (D1), mean zona pellucida (ZP) thickness, mean cytoplasm diameter (D2). The cumulated pronuclear score (CPNS) was the sum of scores assigned to the six following parameters: proximity of the PN, orientation of the PN with respect to the polar bodies, centring of the two PN, cytoplasmic halo, number of NPB, polarization of the NPB. Grading from worst to best were 1, 2 or 3 for each parameter. Various scoring examples are shown in Figure 1. The CPNS for each zygote may thus vary from 6 to 18. The same observer scored all zygotes.

Statistics

All data were analysed using STATA (version 8) for Windows. Following the assessment of normality with the Shapiro–Wilk test and equality of the variances, mean values were compared using unpaired Student's *t*-test. In the cases where features were not normally distributed, the non-parametric Mann–Whitney test was used in addition to Student's *t*-test. Differences were considered as significant when both tests gave $P < 0.05$. Frequencies were compared by using χ^2.

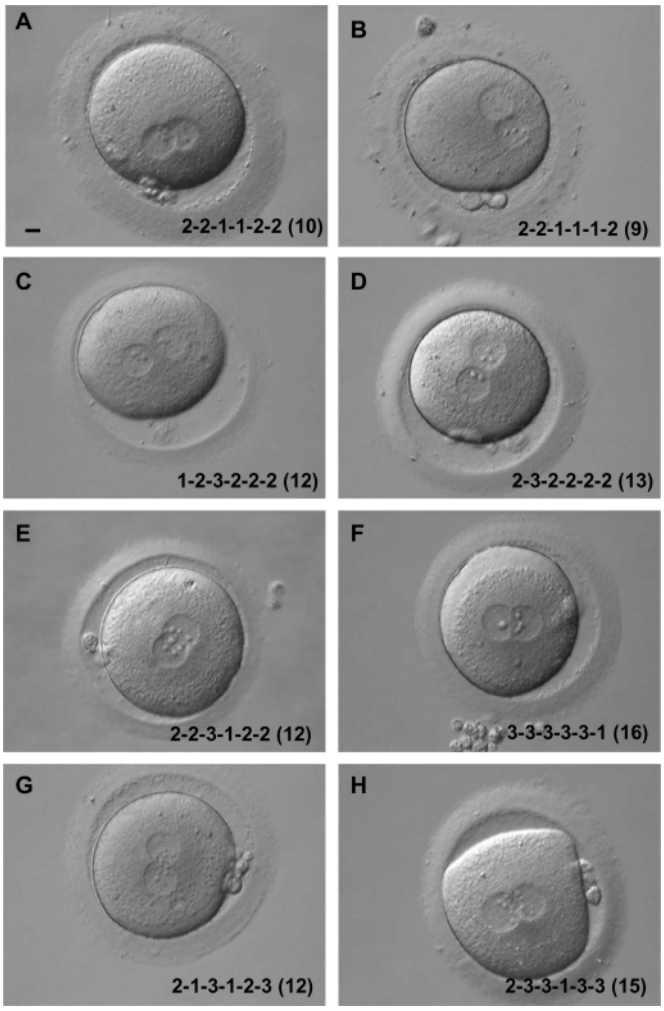

Figure 1. Examples of zygote scoring. Scores (1, 2 or 3) assigned to each individual parameter are indicated for each zygote. Cumulated pronuclear score is indicated in parentheses. Bar = 10 μm.

Results

Relationship between zygote scores and pregnancy

Description and outcome of the cycles included in this study are summarized in Table I. To determine which morphometric or morphological parameters were positively associated with clinical pregnancy, the various measured diameters of the zygotes transferred were compared between cycles with and without conception. The mean diameters D1 (external diameter of the oocyte) and D2 (cytoplasm) and the ZP thickness were not different between the pregnant and non-pregnant group (data not shown). When compared to fresh zygotes, frozen–thawed zygotes presented significantly smaller cytoplasm (D2) diameters in both non-pregnant (109.9 ± 4.2 versus 105.6 ± 5.3, $P < 0.0001$) and pregnant (109.9 ± 6.5 versus 105.9 ± 7.2, $P < 0.005$) patients.

When the mean scores of the six individual parameters (PN proximity, PN orientation, PN centring, cytoplasmic halo, NPB polarization, NPB number) used to calculate CPNS were compared between pregnant and non-pregnant groups, different patterns were observed for fresh and frozen–thawed zygotes (Table II). For fresh zygotes, the mean scores for cytoplasmic halo, NPB polarization and NPB number were significantly higher in the pregnant group, while those for PN centring, proximity and orientation were not. For frozen–thawed zygotes all mean scores, except that of NPB number, were significantly higher in the pregnant group. For both fresh and frozen–thawed zygotes, CPNS was significantly higher in the pregnant group.

To determine whether the frozen–thawed zygotes belonging to the pregnant group presented scores similar to those of their fresh counterparts, the mean scores of the different parameters were compared between fresh and frozen–thawed zygotes of this group. We found that all mean scores, as well as the calculated CPNS, were significantly lower in frozen–thawed than in fresh zygotes for all the parameters, except orientation and centring of the PN. Because not all the transferred zygotes had implanted into the patients who achieved pregnancy, the same analysis was performed on a subgroup of patients in whom all the transferred embryos had implanted, i.e. single pregnancies after the transfer of one embryo and twin pregnancies after the transfer of two embryos. In this group, the mean scores of cytoplasmic halo, NPB polarization, NPB number were still significantly different between fresh and frozen–thawed zygotes but the parameters describing PN position (proximity, orientation and centring), although slightly increased, were not significantly different.

Relationship between zygote scores and embryo implantation

The respective influence on implantation of the six individual parameters used for CPNS calculation was then investigated for fresh zygotes. After removal of the transfers in which the two embryos transferred presented different scores for a given parameter, the remaining transfers were analysed according to the zygote scores and the corresponding implantation rates were calculated. This analysis was repeated for each individual parameter. Figure 2 shows that implantation rates increased significantly when the scores for cytoplasmic halo and NPB polarization increased, but zygotes presenting NPB undergoing

Table I. Outcomes of the cycles included in this study

	Fresh cycles	Frozen cycles
Age (years) (mean ± SD)	34.5 ± 4.4	33.3 ± 4.3
No. of transfer cycles	304	345
No. of embryos	613	617
No. of embryos transferred (mean ± SD)	2.02 ± 0.44	1.79 ± 0.53
Clinical pregnancy rate	79/304 (26.0)	54/345 (15.7)
Implantation rate	103/613 (16.8)	61/617 (9.9)

Values in parentheses are percentages.

Table II. Scores (mean ± SD) of individual parameters of the cumulated pronuclear score (CPNS) according to clinical pregnancy

	Non-pregnant group	Pregnant group	Pregnancies with all embryos implanted
Fresh zygotes			
No. of transferred zygotes (*n*)	449	163	45
PN proximity	2.57 ± 0.56^{A}	2.63 ± 0.52^{G}	2.51 ± 0.50
PN orientation	1.93 ± 0.79^{B}	2.03 ± 0.76	1.93 ± 0.75
PN centring	2.52 ± 0.57	2.60 ± 0.51	2.60 ± 0.57
Cytoplasmic halo	$1.98 \pm 0.75^{a,C}$	$2.35 \pm 0.73^{a,H}$	2.31 ± 0.76^{L}
NPB polarization	$1.76 \pm 0.78^{b,D}$	$1.91 \pm 0.76^{b,I}$	2.00 ± 0.73^{M}
NPB number	$2.24 \pm 0.68^{c,E}$	$2.46 \pm 0.58^{c,J}$	2.44 ± 0.62^{N}
CPNS	$13.02 \pm 2.15^{d,F}$	$14.00 \pm 1.74^{d,K}$	13.80 ± 1.64
Frozen–thawed zygotes			
No. of transferred zygotes (*n*)	514	103	20
PN proximity	$2.17 \pm 0.74^{e,A}$	$2.33 \pm 0.73^{e,G}$	2.55 ± 0.68
PN orientation	$1.79 \pm 0.75^{f,B}$	1.97 ± 0.78^{f}	2.10 ± 0.85
PN centring	2.48 ± 0.59^{g}	2.63 ± 0.55^{g}	2.70 ± 0.65
Cytoplasmic halo	$1.49 \pm 0.66^{h,C}$	$1.69 \pm 0.63^{h,H}$	1.65 ± 0.74^{L}
NPB polarization	$1.26 \pm 0.49^{i,D}$	$1.43 \pm 0.60^{i,I}$	1.60 ± 0.68^{M}
NPB number	1.84 ± 0.66^{E}	1.95 ± 0.70^{J}	2.05 ± 0.60^{N}
CPNS	$11.06 \pm 1.86^{j,F}$	$12.02 \pm 2.13^{j,K}$	12.65 ± 2.30

The no. of transferred zygotes (*n*) was used to calculate the score means in each group.
Data with the same lower-case superscripts are significantly different ($^{a,c,d,j}P < 0.0001$, $^{h,i}P < 0.005$, $^{b,e,f,g}P < 0.05$). Within the same columns, data with the same upper-case superscripts are significantly different ($^{A,C–F,H–K}P < 0.0001$, $^{B,G,L}P < 0.005$, $^{M–N}P < 0.05$).
PN = pronuclei; NPB = nucleolar precursor bodies.

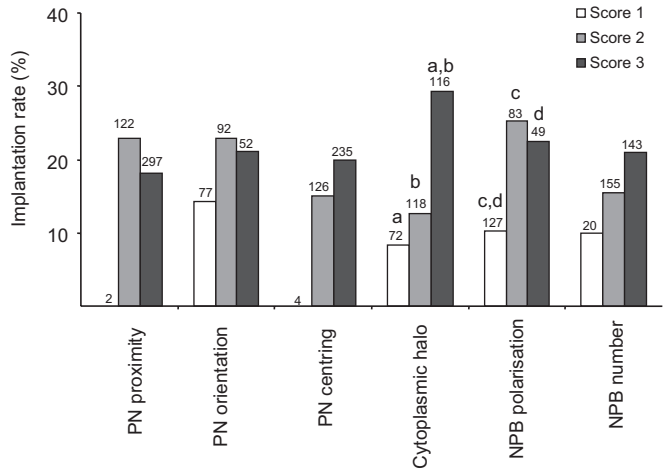

Figure 2. Influence of the individual parameters of the cumulated pronuclear score (CPNS) on implantation rates after fresh transfers. All the transfers of embryos presenting the same score at the zygote stage (1, 2 or 3) were pooled and the corresponding implantation rates were calculated. The numbers of transferred embryos are indicated on the top of each bar. Bars with the same superscripts are significantly different (χ^2, $^{a,b,c}P < 0.005$, $^{d}P < 0.05$).

polarization (score 2) implanted as well as those with maximum polarization (score 3). For NPB number, a trend towards an increased implantation rate was observed but without reaching significance. Parameters describing PN position did not result in higher implantation rates when highly scored. However, the numbers of zygotes exhibiting a score of 1 for PN proximity and centring were too low to be included in the statistical analysis.

The CPNS values, which include the scores of all the above parameters, were then correlated to the implantation rates of both fresh and frozen–thawed zygotes. Implantation rates reach a plateau when at least one of the transferred embryos had a $CPNS_{max}$ ≥15 (Figure 3A) or when the mean CPNS of the embryos transferred was ≥14 (Figure 3B). Similar implantation rates were found when fresh and frozen–thawed zygotes displayed the same CPNS.

Influence of freezing on zygote scores

Although an increase in CPNS was positively associated with pregnancy, the mean CPNS was significantly lower for frozen–thawed than for fresh zygotes (Table II), suggesting that freezing may affect some parameters predictive of zygote quality. To determine which parameters were altered by freezing, the frequencies of the different scores (1–2–3) were calculated for each parameter and compared between fresh and frozen–thawed zygotes. Figure 4 shows that freezing was associated with a significant decrease in the proportion of high scores for PN proximity and orientation, cytoplasmic halo, NPB polarization, and NPB number. By contrast, PN centring was not significantly affected by freezing.

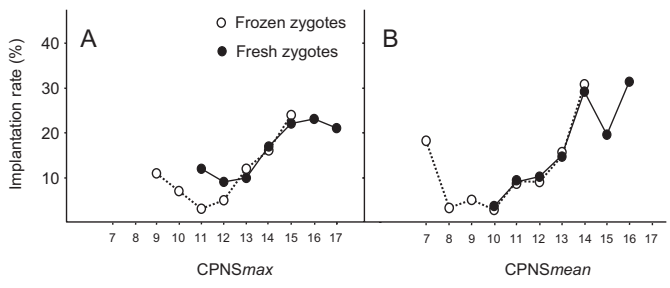

Figure 3. Implantation rates of fresh and frozen–thawed zygotes presenting similar cumulated pronuclear score (CPNS). Implantation rate was calculated according to the maximum CPNS of the transferred zygotes (**A**) or to the mean CPNS of the transferred zygotes (**B**). Implantation rates are not shown for the scores corresponding to <10 transferred embryos.

Discussion

Assessment of developmental and implantation potential of embryos is of great importance for selection of embryos for transfer, so as to achieve high pregnancy rates associated with a low number of multiple pregnancies. For that purpose, scoring of zygotes, cleavage stage embryos or blastocysts has been proposed to identify and to select embryos for fresh transfer (Fisch *et al.*, 2001; Ebner *et al.*, 2003b). In the present study, we focused on scoring of fresh and frozen–thawed zygotes and we proposed a cumulated pronuclear score (CPNS) predictive of implantation competence of embryos.

Dynamic sequences of morphological changes occurring in the pronuclear zygote play a critical role for subsequent embryo development and may be used as markers of implantation potential of the embryo. Following their formation, the

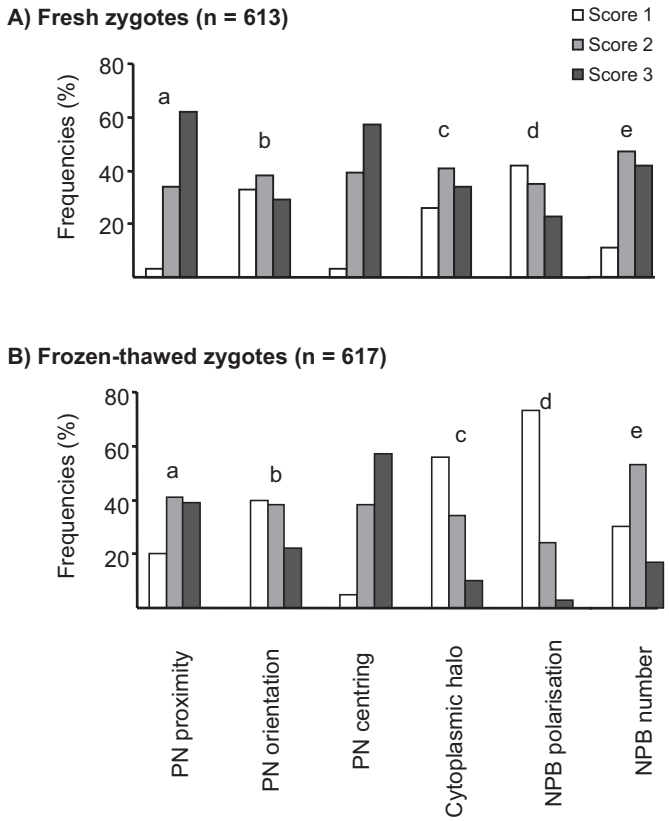

Figure 4. Frequencies of the individual parameter scores of the cumulated pronuclear score (CPNS) in fresh and frozen–thawed zygotes. Frequencies with the same superscripts are significantly different (χ^2, $^{a,c,d,e}P < 0.0001$, $^{b}P < 0.01$).

female pronucleus migrates towards the male pronucleus until they become in close apposition; then they move together toward the centre of the oocyte and change their orientation in order to align with the second polar body (Payne *et al.*, 1997). Orientation of the PN determines the first cleavage plane and may be important for embryo polarity (Edwards and Beard, 1997). Inside the pronuclei, PNB follow a dynamic pattern during PN movement; they first appear scattered, followed by their polarization and alignment along the line of PN contact (Tesarik and Kopecny, 1989). In the cytoplasm, the appearance of a clear halo during fertilization results from the movement of organelles such as mitochondria from the cortex towards the centre of the oocyte around the PN (Bavister and Squirrell, 2000).

In our study, all these morphological features were examined for pronuclear zygote scoring. Defined as their proximity, centring and orientation in the ooplasm, PN position did appear to influence implantation rates of frozen–thawed but not of fresh zygotes. Distant and eccentric PN were reported as bad prognostic for implantation (Gianaroli *et al.*, 2003), but only a minority of zygotes presented this pattern in our study. As a consequence, PN centring and proximity may not be useful to predict the implantation potential of fresh zygotes. However, a more precise evaluation of PN centring and apposition would be needed as it may reveal subtle differences between zygotes, which may account for different implantation potentials. Several authors have reported discrepant results regarding the influence of PN orientation on embryo development and implantation (Garello *et al.*, 1999; Gianaroli *et al.*, 2003; Kattera and Chen, 2004). According to our results, PN alignment with polar bodies was not specifically required for successful implantation of fresh embryos.In contrast to PN position, we found that NPB patterns and cytoplasmic halo were relevant indicators of implantation for both fresh and frozen–thawed zygotes. A minimum number of three NPB in each pronucleus, and the same distribution of the NPB in the two PN have been positively correlated with pregnancy (Tesarik *et al.*, 2000; Wittemer *et al.*, 2000; Balaban *et al.*, 2001). Among this type of zygote, those presenting polarized NPB appear to have a better implantation potential (Scott *et al.*, 2000; Montag and van der Ven, 2001). In the present study, we confirmed that zygotes with a large number of NPB, undergoing polarization or fully polarized, had a higher probability of implanting. Our results showing an increase in embryo implantation when cytoplasmic halo was present in zygotes confirm previous observations (Scott and Smith, 1998; Ludwig *et al.*, 2000; Salumets *et al.*, 2001; Stalf *et al.*, 2002; Zollner *et al.*, 2002; Ebner *et al.*, 2003a), and indicate that changes in organelle localization is of critical importance for subsequent implantation.

Microscopic examination of zygotes after freezing and thawing revealed that certain structures used for scoring were affected by freezing, resulting in lower CPNS. This was not the consequence of a selection of zygotes with low CPNS for freezing, as they were randomly allocated to fresh transfer or to cryopreservation. Cooling is known to affect microtubule organization by inducing depolymerization of microtubules (Mandelbaum *et al.*, 2004). During the first cell cycle, microtubules play a key role in PN migration and apposition (Simerly *et al.*, 1995), and organelle movement to the oocyte centre (Bavister and Squirrell, 2000; Ebner *et al.*, 2003a), while pronuclear centring appears to depend on microfilaments (Terada *et al.*, 2000). Freezing at the zygote stage may alter the microtubule network, resulting in displacement of PN and organelles that explains a lower CPNS. On the other hand, alteration in NPB number and polarization in our study is consistent with the reported deleterious effect of freezing on the organization of the nucleus and nucleoli in human germinal vesicle oocytes (Van Blerkom and Davis, 1994). Interestingly, a number of frozen–thawed zygotes implanted despite significantly lower scores for cytoplasmic halo, NPB number and distribution after thawing, emphasizing that a proportion of zygotes may recover from freeze–thawing-induced damage.

Zygote scoring may be combined with embryo scoring for optimum embryo evaluation and selection (Nagy *et al.*, 2003). However, in Switzerland and Germany, we have to rely on zygote scoring to determine which embryos present high implantation potential. Using embryo selection based exclusively on zygote scoring, pregnancy rates were improved significantly when high score zygotes were transferred (Ludwig *et al.*, 2000; Montag and van der Ven, 2001). The CPNS we proposed in this study correlated positively to implantation rates and may be used to identify and to select zygotes for fresh embryo transfer, without additional embryo criteria. CPNS of frozen–thawed zygotes may be useful for selection when the number of zygotes that can be thawed in one cycle is not limited. Although it is beyond the scope of this study, CPNS determination before freezing would allow better characterization of the zygotes' resistance to cryo-damage and might be used to select zygotes for freezing. Finally, CPNS of frozen–thawed zygotes may be used as a tool to assess the freezing-dependent damage to the cytoskeleton.

In conclusion, CPNS may be used as a single prognostic tool for implantation of both fresh and frozen–thawed zygotes. Among the parameters used for CPNS calculation, NPB patterns and cytoplasmic halo are the most useful indicators for both fresh and frozen–thawed zygotes, whereas PN position appeared more specifically relevant for frozen–thawed zygotes.

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