Introduction

During in vitro fertilization (IVF), multipronuclear zygotes account for approximately 5–8% of all fertilized oocytes, the majority of which are trippronuclear (3PN) zygotes [1–3]. A study of the chromosomal constitution of paternal pronuclei in zygotes derived from intracytoplasmic sperm injection (ICSI) revealed that 33.3% of the abnormal male pronuclei are diploid [4]. An increased risk of polyspermy is associated with early or late insemination [5], rupture of the zona pellucida [6], and insemination with high numbers of motile sperm [7]. Triploidy does not only occur in vitro; triploid embryos resulting from conception in vivo lead to spontaneous abortion [8], the formation of hydatidiform moles or even triploid infants.

However, birth of tetraploid infants and prolonged survival of triploid children have been reported [9,10]. These live births are complicated by severe malformations and multiple anomalies, and so 3PN zygotes are not utilized during IVF. The high frequency of triploidy has generated numerous investigations of the parents, the mechanism underlying the additional haploid...
chromosome set, and the relationship between 3PN origin and phenotype. However, despite these efforts, the origin of triploidy remains controversial. The greatest difficulty with karyotype analyses is in distinguishing dispermia from digynia. As demonstrated in early cytogenetic studies of triploidy, over two-thirds of cases originate from the father (i.e. diandric triploidy), typically as a result of dispermia [11–14].

Normally, only one sperm fertilizes an oocyte because oocytes undergo the zona reaction, which prevents polyspermy. During conventional IVF, however, inhibition of polyspermy might not always occur. 3PN zygotes generated via conventional IVF largely arise from dispermic fertilization, including regular extrusion of the second polar body. During ICSI, only one spermatozoan is injected into a mature metaphase II oocyte, but occasionally, 3PN zygotes are formed when the first polar body is retained. It has been assumed that this results from non-extrusion of the second polar body [15]. After conception in vivo, dispermia is the most common cause of 3PN, although some digynic triploids have been reported. The fluorescence in situ hybridization (FISH) technique is simple and enables direct visualization of spermatozoa. We applied this technique in our evaluation of 3PN zygotes because, compared to classical staining methods, it is a more accurate method for the quantification of sperm tails. The purpose of our study, therefore, was to enumerate sperm tails via FISH to ascertain the origin of 3PN zygotes.

Materials and Methods

IVF protocol
Between November 2000 and December 2002, all IVF cases (149 cycles) were performed in the assisted reproductive technology program at Mackay Memorial Hospital. Twenty-one zygotes with 3PN were identified and included in our study. The long protocol of ovulation induction was followed, in which gonadotropin-releasing hormone agonist (GnRHa) combined with human menopausal gonadotropins (HMG) (Pergonal®, Serono, Rome, Italy) and recombinant follicle stimulating hormone (rFSH) (Gonal-F®, Serono, Aubonne, Switzerland) were used. Subcutaneous injection of leuprolide acetate (Lupron; Abbott Laboratories, Chicago, IL, USA) for pituitary desensitization, 0.5 mg per day, was started on the 21st day of the previous menstrual cycle. HMGs were given, beginning on day 3 of menstruation. When at least two leading follicles reached a mean diameter of 18 mm, 10,000 IU of human chorionic gonadotropin (Pregnyl; Organon, Oss, The Netherlands) was administered. Transvaginal ovum pick-up (OPU) was carried out under ultrasonographic guidance 34–36 hours later. Oocytes were examined under an inverted microscope to determine whether fertilization or pronuclei existed 16–18 hours after fertilization, and 3PN zygotes were collected 1 day after insemination.

Zygote fixation
After zona pellucida removal with acidified Tyrode’s solution, zygotes were placed in hypotonic solution for 2–5 minutes, then transferred to clean slides and fixed with several drops of a freshly prepared mixture of methanol and acetic acid (3:1).

FISH
Slides with fixed 3PN zygotes were dehydrated in ethanol (70%, 85% and 100%), and 10 μL of X and Y chromosome probes were added to each sample. After covering with a glass slide and sealing with rubber cement, the pronuclei were denatured with probes on a hotplate at 75°C for 2 minutes. Hybridization was completed within 1 hour in a humidified chamber at 37°C. The slides were washed in 4X saline sodium citrate (SSC) at 72°C for 1.5 minutes and 2X SSC/0.05% Tween-20 (Sigma Chemical Co., St Louis, MO, USA) at room temperature for 30 seconds, and 10 μL of 4’,6-diamidino-2-phenylindole (DAPI; Vysis, Downers Grove, IL, USA) were added before inspection. After FISH, the slides were washed in phosphate-buffered saline (PBS) three times for 5 minutes before immunofluorescent staining [16].

Immunofluorescent staining
Briefly, slides were denatured in 70% formamide (Sigma Chemical Co.), 2X SSC at 80°C for 1 minute, and then incubated with blocking solution (3% bovine serum albumin, 0.1% Tween-20, 4X SSC) in a Coplin jar for 30 minutes. Mouse monoclonal anti-acetylated-α-tubulin antibody (Sigma T 6793) was added to the samples. After incubation in a humidified chamber at 37°C for 2 hours, slides were washed in PBS three times for 10 minutes before incubation with fluorescein isothiocyanate (FITC) conjugated goat (Chemicon International Inc., Temecula, CA, USA) anti-mouse immunoglobulin G (Molecular Probes Inc., Eugene, OR, USA) for 30 minutes.

Image observation
The slides were examined under an Olympus BX 40 microscope (1000× magnification; Olympus Corp., Tokyo, Japan) fitted with a single band pass filter for FITC Texas Red and Aqua Blue, and a triple-band filter for DAPI/Texas Red/FITC. Hoechst dye 33342 was used for visualization of the number of pronuclei. All
the slides were observed and interpreted by the same observer. Fixed spermatozoa from semen samples were processed in parallel as controls.

Results

A total of 149 cycles were enrolled in this study. There were 21 zygotes with three pronuclei. The number of 3PN zygotes combined with one, two and four sperm tails were two (10%), 18 (85%) and one (5%), respectively (Table). Therefore, in excess of 90% of 3PN zygotes were multispermic (Figure). At the same time, in the control group, stain efficiency was > 97%.

Discussion

Triploidy is one of the most common chromosome abnormalities in humans. Four situations generally lead to triploid fertilization: (1) penetration of an oocyte by two spermatozoa; (2) retention of the first or second polar body; (3) penetration of an oocyte by a binucleate spermatozoan; (4) penetration of a binucleate oocyte by a single spermatozoan. Dispermy is the most common fertilization anomaly in humans [17]. Most of these embryos cleave but arrest prior to differentiation. Most dispermic zygotes divide into three or four cells after the first division, but only a minority of dispermic zygotes divide into two cells [17]. However, dispermy could not be distinguished from digyny in the published reports of karyotype analyses [18]. In some cases, observation of the polar body can be difficult and the possibility of polar body degeneration after several hours of culture in vitro cannot be disregarded. A peculiarity of ICSI is the occurrence of abnormally fertilized oocytes with three pronuclei and one polar body. It is generally accepted that these 3PN are digynic in their origin and the supernumerary pronucleus is supposed to be due to the non-extrusion of the second polar body [15,19]. Polar bodies are occasionally fragmented and it is difficult to discriminate between one fragmented polar body and two polar bodies. Moreover, the morphologic evaluation of the zygotes can lead to misinterpretation of the ploidy when supernumerary micropronuclei are observed [4].

In human zygotes, sperm tail remnants can almost never be identified by light microscopic examination [20]. The FISH technique is relatively simple and enables large numbers of spermatozoa to be examined at a

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Table. Number of sperm tails in tripronuclear zygotes

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<th>Zygote</th>
<th>Fertilization</th>
<th>Number of pronuclei</th>
<th>Number of sperm tails</th>
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IVF = in vitro fertilization.

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Figure. Fluorescent micrographs of triploid zygotes. Note the number of sperm tails (arrowheads) that appear in (A) digynic or (B) diandric zygotes.
time. Some studies using FISH for disomy rates in spermatozoa have yielded a fairly wide range of values for chromosomes [21–27]. The lack of fluorescent signals of the sperm tail in a few zygotes was, in our view, primarily due to some errors occurring in the preparative procedure. To our knowledge, this is the first report of the application of the FISH technique to detect the number of sperm tails and has provided direct evidence that the origin of 3PN zygotes after IVF is the result of penetration with two spermatozoa in 85% of 3PN oocytes. Of note, two conditions cannot be ruled out: (1) 3PN zygotes with one sperm tail; and (2) the presence of four sperm tails in three pronuclei. In our opinion, the former condition may be the failure of extrusion of the polar body and the latter condition may be due to inactivation of sperm after penetration. According to our observations, the extra haploid chromosome is of paternal origin in the majority of cases. However, further larger clinical studies are necessary to validate our results.

References


27. Pang MG, Hoegerman SF, Cuticchia AJ, Moon SY, Doncel GF, Acosta AA, Kearns WG. Detection of aneuploidy for chromosomes 4, 6, 7, 8, 9, 10, 11, 12, 13, 17, 18, 21, X and Y by fluorescence in situ hybridization in spermatozoa from nine patients with oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection. *Hum Reprod* 1999; 14:1266–73.