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·Review·

In situ aneuploidy assessment in human sperm: the use of primed *in situ* and peptide nucleic acid–fluorescence *in situ* hybridization techniques

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Abstract

Both the primed *in situ* (PRINS) and the peptide nucleic acid–fluorescence *in situ* hybridization (PNA–FISH) techniques constitute alternatives to the conventional (fluorescence *in situ* hybridization, FISH) procedure for chromosomal investigations. The PRINS reaction is based on the use of a DNA polymerase and labeled nucleotide in an *in situ* primer extension reaction. Peptide nucleic acid probes are synthetic DNA analogs with uncharged polyamide backbones. The two procedures present several advantages (specificity, rapidity and discriminating ability) that make them very attractive for cytogenetic purposes. Their adaptation to human spermatozoa has allowed the development of new and fast procedures for the chromosomal screening of male gametes and has provided efficient complements to FISH for *in situ* assessment of aneuploidy in male gametes. *(Asian J Androl 2006 Jul; 8: 387–392)*

Keywords: aneuploidy; chromosomal screening; peptide nucleic acid–fluorescence *in situ* hybridization; primed *in situ*; spermatozoa

1 Introduction

The assessment of aneuploidy rate in human gametes is of critical importance because non-disjunction makes a major contribution to the chromosomal abnormalities found in humans. In particular, the chromosomal analysis of spermatozoa constitutes an essential approach for the investigation of the occurrence and etiology of chromosomal abnormalities under a wide variety of clinical conditions. To date, a lot of data were gathered thanks to the human sperm–hamster egg fusion technique [1, 2], but the procedure was so labor-intensive and time-consuming that its use was limited to a few laboratories. The advent of molecular genetic techniques has brought forth new procedures for *in situ* chromosomal analysis, and then has opened the way for comprehensive studies on aneuploidy occurrence.

Because of its relative simplicity and the availability of numerous probes, the fluorescence *in situ* hybridization (FISH) technique has become the method of choice in molecular cytogenetic investigations. Numerous chromosomal analyses on human sperm have been performed using FISH [3]. These reports have demonstrated the efficiency of the *in situ* labeling procedure on male gametes, but also pointed out the limitations of FISH on this biological material, which are essentially linked to the size of the probes and the reliability of the associated sperm decondensation treatments [4, 5]. During the last decade, alternative methods to FISH have been introduced and have shown to be valuable in detecting chromosomes and quantifying aneuploidies. These alternative procedures are the primed *in situ* (PRINS) labeling

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and the peptide nucleic acid–FISH (PNA–FISH) probes. The two procedures present several advantages for the *in situ* detection of nucleic acid sequences that make them very attractive for a number of cytogenetic purposes. Several recent studies have demonstrated that PRINS and PNA–FISH were as efficient as FISH on human gametes and exhibited higher specificity [6–8]. The present paper provides a brief overview of both the PRINS and PNA–FISH methods, and highlights recent applications of these techniques on human spermatozoa.

2 Primed *in situ* technique

2.1 Principles and methodology

Based on the use of chromosome-specific primers, the PRINS reaction combines the high sensitivity of polymerase chain reaction (PCR) with the cytological localization of DNA sequences [9]. The chromosomal identification is performed by *in situ* annealing of specific and unlabeled oligonucleotide primers to complementary sites on denatured chromosome spreads, nuclei or tissue sections. Cells or tissue samples are fixed and denatured before PRINS reaction, both to preserve morphology and to permit access of the reagents to the sequence target. The annealed primers provide initiation sites for chain elongation catalyzed by a Taq DNA polymerase in the presence of free nucleotides, of which at least one is labeled. The *in situ* visualization of generated fragments results from the incorporation of the labeled nucleotide (Figure 1) [10].

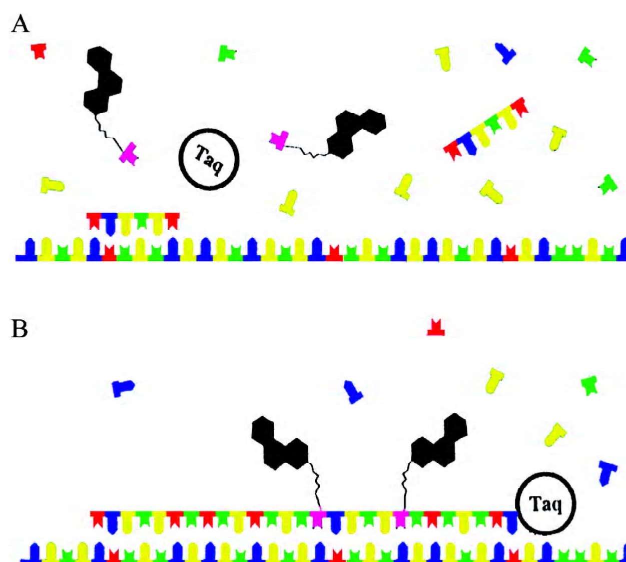

Figure 1. Principles of the primed *in situ* reaction. (A): Deposit of the reaction mixture on denatured preparations and specific annealing of primer to the DNA target sequence. (B): Primer extension by a Taq DNA polymerase with incorporation of the labeled nucleotide. Reprinted with permission from Pellestor [10].

PRINS labeling of human chromosomes is obtained using primers for repeated DNA sequences. An advantage of primers is their ability to differentiate between closely related sequences. This feature has been utilized for generating chromosome-specific primers from the alpha-satellite DNA motif. The lengths of the PRINS primers range from 18 to 30 nucleotides. Compared to the size of DNA repetitive probes (250–600 bp), this small size greatly facilitates their *in situ* accessibility to their genomic target sequences. This is particularly significant in cells with highly condensed nuclei, such as spermatozoa. Because they are unlabeled, high amounts of primers can also be used in PRINS reaction without inducing background signals. The complementation process between the primer and its centromeric target will be so specific that a simple mismatch between the 3'-end of the primer and the genomic sequence will prevent initiation of the *in situ* elongation by the Taq DNA polymerase. Thus, it has been possible to define specific alpha-satellite primers for some chromosomes undistinguishable by FISH with centromeric probes, such as chromosomes 13 and 21, which share 99.7% homology in their alpha-satellite DNA sequences [11].

Initially, PRINS reactions were performed either on a hotplate or a waterbath, but these procedures did not allow precise and durable temperature control. The PRINS protocol has been considerably improved and simplified by the introduction of programmable temperature cyclers equipped with a flat plate block. The use of automatic thermocyclers allows an optimization of both annealing and extension conditions. Thus, semi automatic PRINS protocols have been developed offering a high reproducibility in labeling reaction. An additional improvement was the direct use of fluorochromes in sequential PRINS reactions. Recently, a new multicolor PRINS protocol has been reported, allowing performance of ultra-rapid detection on several chromosomes, by mixing the different fluorochromes during the chain elongation reaction [12]. Each PRINS reaction consists of a unique 4-min step for annealing and elongation of each chromosome-specific primer. This new sequential procedure simplifies the PRINS technique and provides an easy way to carry out multicolor labeling. The PRINS reaction can also be combined with conventional FISH labeling

protocols. No further denaturation is required before the FISH reaction. This combined use may be an efficient approach to improve chromosomal detection.

2.2 Application to human sperm

In humans the PRINS method has been successfully tested for the assessment of aneuploidy in lymphocytes, amniocytes and preimplantation embryos [13–15]. The use of PRINS has also been reported for analysis of structural aberrations such as translocations and marker chromosomes as well as for the detection of fetal cells in peripheral venous blood of pregnant women [16, 17]. Further applications of PRINS have also been found in tumoral cytogenetics. The adaptation of PRINS to human spermatozoa has constituted a new step in the development of PRINS methodology and an interesting challenge because of the particularities of the human sperm nucleus in terms of genomic compaction and accessibility of DNA sequences.

The PRINS technique was combined with an efficient NaOH treatment of spermatozoa allowing the simultaneous decondensation and denaturation of sperm nuclei. In the PRINS reaction, the decondensation of the sperm head is a less limiting factor than in FISH because of the small size of the oligonucleotide primers. This greatly facilitates their penetration into sperm nuclei, resulting in a more homogeneous and more rapid labeling of sperm nuclei [18]. This might explain the extreme rapidity of PRINS labeling of human sperm. Indeed, a single-target PRINS reaction can be performed in 10 min. The time of optimal NaOH treatment depends on the age of the sperm preparation slides and the concentration of NaOH solution. Initially, we used 3 mol/L NaOH, but numerous experiments and a comparison of the results in terms of quality of the preparation obtained led us to adopt a 0.5 mol/L NaOH solution. Best results in labeling efficiency were obtained with 2-day-old slides and 5-min 0.5 mol/L NaOH pretreatment [18,19]. The NaOH treatment induces uniform swelling of the sperm nucleus to 1.5–2-fold its normal size, and maintains the characteristics and shape of the sperm nucleus, including the tail. This allows the differentiation between spermatozoa and other cells such as leukocytes or immature germ cells present in the ejaculate.

Double and triple PRINS reactions have been performed using various combinations of chromosome-specific primers (Figure 2) [10]. Both the efficiency and the reliability of the PRINS technique on sperm were tested by using different primers to label individual chromosomes. Using this PRINS protocol, we have directly estimated diploidy and disomy frequencies for 15 autosomes and sex chromosomes in sperm samples from several normal fertile donors [20]. The labeling efficiency usually ranges from 97% to 100% according to the primer tested. A minimum of 10 000 nuclei per chromosome was analyzed. For the autosomes, the mean disomy rate ranged from 0.18% to 0.36%. Among all donors, chromosome 21 exhibited the highest disomy rate (0.30–0.39%), suggesting that in male meiosis, chromosome 21 may be more susceptible to nondisjunction than the other autosomes. The frequency of disomy for gonosomes ranged from 0.08% to 0.13%.

Interindividual variations in disomy rates were observed but these were statistically non-significant. Interindividual heterogeneity was more evident in diploidy, where frequencies ranged from 0.08% to 0.45% among the normal subjects tested.

The efficiency of the new multicolor PRINS protocol, based on the direct mixing of the color of fluorochromes, was also tested and validated on human sperm [21]. Comparative estimates of disomy were performed for several chromosomes on sperm samples from two donors, either by the new three-color protocol or by the conventional dual-color PRINS procedure previously described. There was no statistical difference between the disomy rates obtained with the conventional dual-color PRINS technique and the fast three-color procedure. This new protocol provides significant simplifications in the multicolor PRINS protocol without modifying the efficiency and the specificity of the labeling reaction. The use of this multicolor protocol has advantages of rapidity and low cost as compared to FISH.

The PRINS procedure has also been used to directly

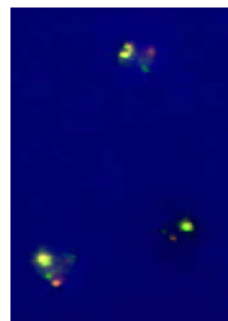

Figure 2. Example of three-color primed *in situ* (PRINS) labeling on human spermatozoa by using centromeric chromosome-specific PRINS primers. Chromosomes 1, 7 and 9 are labeled in yellow, green and red respectively. Reprinted with permission from Pellestor [10].

investigate in sperm the meiotic segregation patterns of reciprocal translocations [22] and to investigate the occurrence of interchromosomal effect in sperm of chromosomal rearrangement carriers [23].

3 Peptide nucleic acid–fluorescence *in situ* hybridization technique

3.1 Definition and properties

Peptide nucleic acids (PNAs) constitute a new class of DNA probes, which provide an interesting complement to FISH and PRINS. PNAs are synthetic mimics of DNA in which the deoxyribose phosphate backbone supporting the nucleic acid bases is replaced by a noncharged peptide backbone (Figure 3) [24]. The unique chemical makeup of these probes confers a number of beneficial properties, including enhanced hybridization rates, resistance to nucleases and proteases and the ability to penetrate condensed biological structures [25]. The neutral backbone of PNA provides strong binding between PNA–DNA or PNA–RNA strands and greater specificity of interaction than their DNA counterparts. While they hybridize according to normal Watson–Crick base pairing rules, PNA have been shown to bind to DNA or RNA targets with higher affinity than the corresponding oligonucleotides. Unlike DNA probes, which require high salt concentration to bind, PNA probes can bind to

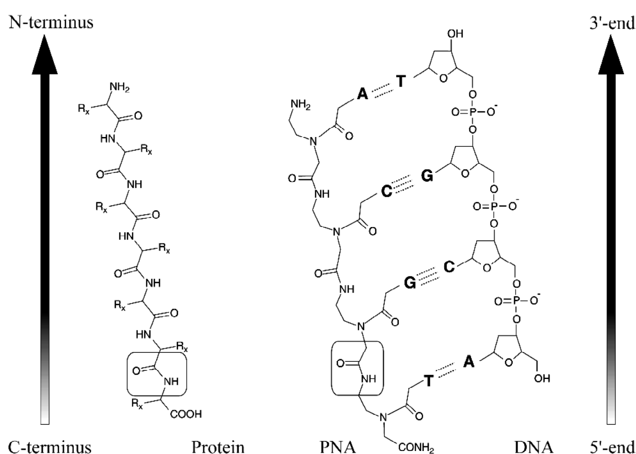

Figure 3. Chemical structures of peptide nucleic acid (PNA) as compared to DNA and protein (modified from Pellestor and Paulasova, Chromosoma 2004). Whereas the DNA involves a phosphate backbone supporting the nucleic acid bases, the PNA backbone is made from repeating *N*-(2-aminoethyl)-glycine units linked by peptide bonds. The acid nucleic bases are linked to the backbone by methylene carbonyl linkages. The amide bond characteritic for both PNA and protein is boxed in. Reprinted with permission from Pellestor *et al.* [24].

DNA or RNA under low ionic strength conditions that do not favor reannealing of complementary strands [26]. Experiments with homopyrimidine strands have shown that the melting temperature (Tm) of a 6-mer PNA deoxythymidine (PNAdT)–DNA deoxyadenosine (DNAdA) was 31ºC in comparison to a DNAdT–DNAdA 6-mer duplex that denatures at a temperature less than 10ºC. Experiments done with PNA probes containing all four bases have demonstrated that there is an increase of the Tm of approximately 1ºC per base pair in PNA hybrids compared to DNA–DNA or RNA–RNA duplex. In addition, a PNA–DNA mismatch is more destabilizing than a mismatch in a DNA–DNA duplex. A single mismatch in mixed PNA–DNA 15-mer decreases the Tm by 15ºC. In the corresponding DNA–DNA duplex, a single mismatch decreases the Tm by only 1ºC. Moreover, the use of even shorter PNA probes can further increase PNA specificity [27]. This advantage is particularly important for hybridization with short probes targeting repetitive sequences, because both the length and the repetitive nature of these genomic targets will favor renaturation over hybridization with probes.

This high level of discrimination at single-base level has indicated that short PNA probes could offer high specificity and has thus allowed the further development of several PNA-based strategies for molecular investigations and diagnosis. Short PNA oligomers, from17 to 22 base units, constitute efficient tools for detecting specific DNA sequences with fast hybridization kinetics over a wide pH range. In addition, PNA probes are not limited to any detection procedure. PNAs can be labeled with a large variety of reporter molecules (enzymes, haptens and fluorophores) [28].

3.2 Application to human sperm

The unique properties of PNA probes as a DNA mimic have led to the development of numerous applications. Most notably, PNAs find current uses in molecular biological techniques as specific and sensitive probes for complementary nucleic acids [27]. The introduction of PNA technology in cytogenetics is recent. First, it has been demonstrated that PNA probes were useful for detecting telomere repeat sequences [29]. Then, the availability of chromosome-specific centomeric PNA probes, directly fluorochrome-labeled, has led to the development of rapid and easy multicolor PNA-FISH protocols for the *in situ* detection and enumeration of human chromosomes in metaphases and interphase nuclei [30]. The procedure has been recently adapted to human blastomeres, and gametes [7, 8].

On human spermatozoa, decondensation pretreatment is also indispensable with PNA probes. No PNA labeling of sperm nuclei can be obtained without decondensation pretreatment. We have tested the two decondensation procedures that give efficient and reproducible results with FISH and PRINS reactions, namely, the dithiothreitol (DTT) and the NaOH treatments. The two procedures yielded satisfactory results with PNA probes and gave similar kinetics for the labeling reaction [31]. To estimate and validate the efficiency of PNA–FISH labeling on human sperm, comparative estimates of disomy X, Y and 1 were performed on sperm preparations from healthy subjects using multicolor FISH, PRINS and PNA–FISH procedures in parallel. An equivalent quality of *in situ* nuclear labeling (Figure 4) and similar disomy rates were obtained with the three methods. However, the hybridization timing of PNA probes (i.e. 40 min) was considerably shortened in comparison with the FISH reaction, which requires an overnight hybridization in order to be efficiently completed for sperm preparations. The fast hybridization kinetics of PNA–FISH labeling on sperm was comparable to the kinetics of the PRINS reaction. The similarity between PNA–FISH and PRINS might essentially be due to the small size of both PNA oligoprobes (18–20 bp) and PRINS primers, which do not exceed 30 bases in length.

Similar performance of PNA–FISH and PRINS methods has already been reported for the *in situ* detection and sizing of telomeric repeat sequences [32]. Both techniques presented comparable features in terms of specificity, staining intensity and efficiency, but PRINS always displayed a faster turnaround reaction time. This could reflect the fact that PRINS is an “active” reaction involving an ultrafast biochemical reaction of primer extension catalyzed by a Taq polymerase. In the case of PNA–FISH reaction, the rapidity of the labeling is due to the neutral backbone of the PNA molecules, which allows PNA–DNA binding to occur more rapidly and more tightly than DNA–DNA binding. The high affinity of PNA probes to DNA constitutes an important feature for chromosomal analysis. Previous studies have demonstrated that PNA probes could discriminate between two centromeric DNA repeats that differ by only a single base pair [33]. Similar results were obtained with PRINS primers and oligonucleotide probes whereas standard FISH probes are unable to discriminate between sequences with a single base resolution. The study of chromosomal polymorphism could benefit from the discriminating power of PNA probes. For sperm, this could be useful for the *in situ* distinction of autosomal non-disjunction occurring at meiosis I and meiosis II, when satellite polymorphisms exist between two homologous chromosomes.

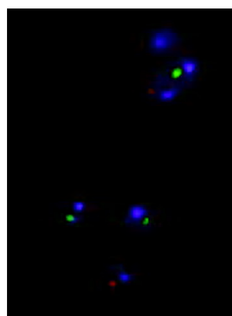

Figure 4. Example of peptide nucleic acid–fluorescence *in situ* hybridization (PNA–FISH) labeling on human sperm nuclei using centromeric chromosome-specific PNA probes. Chromosome 1 is labeled in blue, chromosome X in red and chromosome Y in green.

4 Conclusion

Since their introduction, both PRINS and PNA–FISH techniques have quickly evolved from basic research to diagnosis procedures. The fast hybridization, penetration and discrimination of both PRINS primers and PNA probes make them valuable tools for *in situ* chromosomal screening. Their successful use on human sperm has proven that these reagents could be used on difficult biological material, and consequently they have a great potential for clinical application. With the development of rapid and simplified protocols producing reliable and reproducible results, it has been proven that both PRINS and PNA–FISH techniques could become powerful tools for cytogenetic investigations and diagnosis. They can be used advantageously to complement FISH and PCR for the physical mapping of the human genome, and the possibility of combining PNA–FISH or PRINS with conventional FISH on the same preparation opens up interesting possibilities for multiplex assays.

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