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Physiology

Cytogenetic Analysis of Spontaneously Activated Noninseminated Oocytes and Parthenogenetically Activated Failed Fertilized Human Oocytes—Implications for the Use of Primate Parthenotes for Stem Cell Production

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***Purpose*:** Spontaneous parthenogenetically activated noninseminated oocytes and failed fertilized oocytes after ART activated by puromycin were studied to assess cleavage ability and the cytogenetic constitution of the resulting embryos.
***Methods*:** Failed fertilized oocytes were exposed to puromycin, and whenever activation occurred, they were further cultured until arrest of development. FISH was used to assess the ploidy of spontaneous (group A) and induced parthenotes (group B).
***Results*:** The mean number of oocytes exposed to puromycin and the percentage and type of activation were identical in IVF and ICSI patients. The more frequent types of activation were one or two *pronuclei* and one polar body suggesting that retention of the second polar body is a common event after parthenogenetic activation.
***Conclusions*:** Retention of the second polar body and chromosome malsegregation were observed after parthenogenetic activation, either spontaneous or induced by puromycin. This means that using parthenogenetic embryos for stem cell research will require great care and attention.

KEY WORDS: FISH; human oocytes; parthenogenetic activation; puromycin; stem cell production.

INTRODUCTION

Spontaneous parthenogenesis of freshly collected noninseminated human oocytes is a rare event not yet completely understood. Oocyte activation has been described after mechanical, chemical, or electrical *stimuli* (1,2). Spontaneous parthenogenetic activation of human oocytes has been observed after ageing in vitro (3) but there are few data concerning cleavage of such activated oocytes.

Experimental parthenogenetic activation of human oocytes has been reported using different activating agents (1,4–6). Puromycin is a protein synthesis inhibitor that interferes with spindle proteins and proved (6) to be an effective activator of failed fertilized human oocytes; calcium ionophore A23187 is able to induce parthenogenetic activation by causing a rise in intracellular calcium (5,7).

Recent reports (8) have suggested the possibility of generating cloned stem cells from the unfertilized eggs of primates, thus solving some of the ethical dilemmas of producing embryonic stem cells. This achievement suggests it may be possible to grow cells that can give rise to any tissue in the human body without cloning and destroying human embryos.

Although the genetic constitution of the analyzed cell lines from parthenogenetic embryos seemed

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to be normal (8), whether parthenotes will provide an alternative to therapeutic cloning is far from being determined as parthenote cells lack the influence of male chromosomes, which might have subtle effects on cell growth and development and those embryos usually die before developing normally.

We have been evaluating parthenogenethical activation after exposure of failed fertilized metaphase II oocytes to puromycin. The purpose of the study was to evaluate the behavior of aged oocytes that were not activated after IVF or ICSI in the presence of such a protein synthesis inhibitor thus assessing their potential to be activated and to cleave. We have also analyzed the cytogenetic constitution of such embryos to check for possible defects.

In another experimental group spontaneous parthenogenetic activation of noninseminated spare oocytes was observed in 12 patients in cycles that originated normal embryos and some of the patients achieved pregnancy suggesting that nuclear and cytoplasmic maturity are essential for activation either by sperm or after ageing in vitro.

We hypothesized that, excluding immunological cases, if oocytes were not fertilized by apparently normal spermatozoa, they would not be correctly activated in the presence of a protein synthesis inhibitor. If those aged oocytes were activated, they would cleave and develop chaotically as far as chromatin is concerned. The cytogenetic study of oocytes is a difficult task and even more difficult and inefficient in old cells as chromatin begins to degenerate and gets clumped. We used FISH analysis with X, Y, and an autosomic (8) probes to assess the ploidy of oocytes and parthenotes that resulted from induced activation. In one patient, 13 and 21 probes were also used to further analyze the abnormal pattern of chromosome disjunction. The systematic hybridization with a Y probe was chosen to detect abnormal fertilization with a Y carrying spermatozoa.

MATERIALS AND METHODS

Human oocytes were collected by ultrasound-guided puncture after pituitary desensitization with the GnRH analog buserelin in a long protocol and ovarian stimulation with hMG or pure FSH. Oocytes were either cultured in vitro in a commercially available medium used for Assisted Reproductive Technologies (ART) procedures (IVF Medicult, Denmark) for 48 h or inseminated with spermatozoa from the husband by using IVF or ICSI technique.

Group A

The grade IV non-inseminated spare oocytes (normal first polar body and normal perivitelline space) were cultured isolated at 37°C and 5% CO_2, each in a hole of a four-well plate and observed at 18–20, 24, 36, and 48 h after collection to evaluate the fate of the oocytes with ageing in vitro. When any sign of activation was observed (second polar body extrusion, pronuclear formation, or cleavage) oocytes were kept in culture in fresh medium until they arrested their development.

Group B

Oocytes that failed to fertilize and did not show any modification (displaying one polar body and absence of *pronuclei* at 48 h postinsemination) after insemination with 100,000 motile spermatozoa or microinjection with one morphologically normal motile sperm were selected for the study. Those failed fertilized oocytes were placed isolated in a four-well culture plate in IVF medium with puromycin 10 μg/mL in Ham F10 (prepared from a stock solution of puromycin 10 mg/mL in DMSO kept at −20°C) and allowed to develop in culture in the standard conditions. Two independent observers checked the morphology of oocytes at 6 h for signs of activation (presence of a second polar body or development of *pronuclei*) and when this was confirmed they were washed twice and kept in culture in fresh medium until arrest of development. If there were no signs of activation at 6 h, oocytes were kept in the protein synthesis inhibitor for 6 h more and rechecked. The two observers recorded independently all the data concerning the number of *pronuclei*, polar bodies, and blastomeres every 6 h until arrest of development (defined as absence of modification in a period of 24 h).

Technical Procedures

Spontaneous and induced parthenotes were exposed for 5 min to a solution of 1:3 trisodium citrate at 1.93% and potassium chloride at 0.56% and fixed individually under a Nikon inverted microscope with a 1:3 solution of cold absolute methanol/acetic acid for 5 min. Cells were then successively exposed to a solution of 1:1 methanol/acetic acid at 75%

Table I. Group A—Spontaneously Activated Noninseminated Oocytes

	Spontaneously cleaved oocytes/oocytes collected	Cleavage at 24 h	Arrest of development	FISH results	Interpretation
Patient 1	1/11	1PN2PB	1PN2PB	PB-XX, no 18	-
Patient 2	1/12	1 (2 cells)	6 cells	X, 18 in each nucleus (Fig. 1)	Normal haploid
Patient 3	1/4 (spermatozoa not available for insemination)	1 (2 cells)	Cytoplasmic fragmentation	Not successfull	
Patient 4	1/11	1 (2 cells)	3 cells	Not successfull	
Patient 5	1/17	1 (2 cells)	2 cells (a, b)	a – X, 18 b – X, 18 (Fig. 2(a)–(c))	Normal haploid
Patient 6	1/10	1 (imediate cleavage)	2 cells (c, d)	c – XX,no 18 spot d – XX, no 18 spot	Abnormal pattern
Patient 7	2/9	2 (imediate cleavage)	5 cells (e, f, g, h, i)	PB-X; PB-13/1818 5 Nuclei: Fig. 3	Abnormal pattern
				e – X/18/21;	e – Haploid pattern
				f – XX/1313/1818/2121	f – Diploid pattern
				g – X/1313/18/21	g – Abnormal pattern
				h – XX/1313/18	h – Abnormal pattern
				i – X/21 j-X/1313/18/21	i – Haploid pattern
			2 cells (j, k)	j – X/1313/18/21 k – XX/1313/18	j – Abnormal pattern k – Abnormal pattern
Patient 8	1/5	1 (2 cells)	2 cells (l, m)	l- 1818 m- No 18 spot	l – Normal pattern m – Abnormal pattern
Patient 9	1/19	1 (2 cells)	2 cells		
Patient 10	1/12	1 (2 cells)	2 cells		
Patient 11	1/16	1 (2 cells)	2 cells		
Patient 12	1/12	1 (2 cells)	6 cells		

Note. PB – polar body; PN – pronucleus/pronuclei; a – k – nuclei analyzed.

and 1:1 absolute methanol and acetic acid. Activated oocytes were stained with 2% Giemsa for 7 min and observed on the light microscope for chromatin analysis.

The slides were destained with a methanol/acetic acid 3:1 solution, air-dried, and then incubated in 0.01 M HCl containing 0.02% pepsin, for 5 min at 37°C. Slides were washed in phosphate-buffered saline (PBS; pH 7.4) and fixed in 1% formaldehyde for 2 min at 20°C and air-dried.

Centromeric fluorescent probes for the X, Y, and 18 chromosomes (Vysis) were used. In one patient we also analyzed the hybridization with locus-specific probes (LSI) for the 13 and the 21 chromosomes. The specimens were dehydrated in an ethanol series (70, 80, and 100%) and codenatured at 75°C for 5 min. The hybridization took place at 37°C for 18 h (overnight). Posthybridization washes were done at 72°C in 0.4 × SSC; 3% NP40 solution for 2 min, followed by a 2 × SSC, 1% NP40 solution, at room temperature, for 1 min. FISH efficiency was 95%, which is similar to that obtained on failed fertilized oocytes in our laboratory.

The slides were imaged using Ikaros software (Vysis) and analyzed with a Zeiss axiophot II microscope equipped with a triple band pass filter.

RESULTS

Group A

Thirteen parthenogenetic embryos derived from spare oocytes of 12 patients who had several good-quality oocytes for collection. Four women achieved pregnancy following embryo transfer in that cycle. The majority of our spontaneously activated cleaving oocytes arrested their development at the two-cell stage. Four parthenotes reached the six- to eight-cell stage with a cleavage rate similar to normal embryos with blastomeres of equivalent size and without any cytoplasmic fragmentation.

We were able to perform FISH in six spontaneous parthenogenetic embryos (Table I). Two of them were normal for the chromosomes analyzed (X and 18 for patients 2 and 5) and the other four displayed abnormal patterns of chromosome disjunction (Table I).

Group B

In aged oocytes exposed to puromycin we achieved hybridization results in 12 zygotes/embryos (Table II). One normal haploid pattern was seen in a 1PN2PB cell of patient 13 and a normal diploid nucleus and an

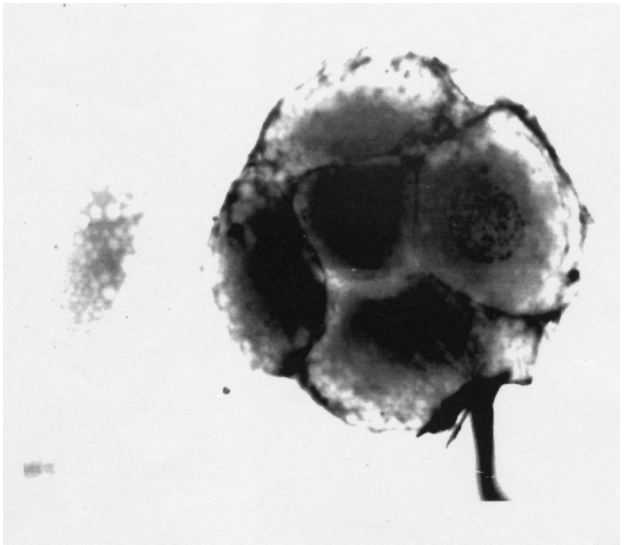

Fig. 1. Six-cell parthenogenetic embryo from patient 6. Each nucleus exhibited an X and an 18 spot after FISH analysis (haploid pattern).

haploid polar body in patient 15 (suggesting retention of the second polar body). One of the four-cell embryos of patient 16 had in fact been fertilized as we had hybridization with the Y probe, while the other embryo of the same patient exhibited an apparently normal pattern of X disjunction with one X spot for each of the four *nuclei* and the two polar bodies. All the other zygotes/embryos displayed abnormal patterns of chromosome segregation for the probes used (Table II).

Although IVF patients are older and have a higher number of unfertilized oocytes than do ICSI patients, the mean number of oocytes exposed to puromycin is identical in the two groups. The percentage and the type of activation are not different in the two treatment groups, the more frequent types of activation being 1PN1PB (36%) and 2PN1PB (27.5%) what means that retention of the second polar body is a common event. The age of the patients was not correlated with the type of activation (Table III).

DISCUSSION

The oocytes of group A were not exposed to spermatozoa, and so we are certain of their parthenogenetic activation. Van Blerkom et al. (8) reported the observation of 29 spontaneously activated oocytes on the basis of the presence of criteria such as the presence of a single enlarged pronucleus subjacent to the first polar body and no light microscopic evidence of sperm penetration or second polar body formation. Nevertheless, after DNA fluorescence analysis those authors found that those criteria were not sufficient as in 52% of the apparently spontaneously activated oocytes they detected the presence of a sperm nucleus. We have also detected the presence of a Y chromosome in a patient suggesting that, at least in this case, sperm penetration and chromatin decondensation had occurred. This does not necessarily mean that none of the other oocytes had been fertilized as this could have happened with an X carrying spermatozoon. However, there is a low probability that activation was a consequence of late sperm penetration or the microinjection procedure itself as the percentage of activation is not significantly different with the two ART techniques used (Table III). There is also the possibility that a spermatozoon reached the ooplasm after IVF or microinjection but remained intact without chromatin decondensation (as it seems to be the case in the 1PN1PB oocyte shown in Fig. 4(a) and (b)).

The majority of parthenogenetic embryos did not cleave after the two-cell stage which might be due to the phenomenon of genomic imprinting as has been suggested elsewhere (9). In fact, parthenogenetic mouse embryos never develop to the blastocyst stage in the same numbers as do fertilized ones, and up to 50% fail to implant, suggesting that genomic imprinting may be responsible for that lack of viability (9).

The fact that in our work parthenogenetic embryos were obtained from oocytes collected in cycles originating normal embryos, and achieving pregnancy in some cases, suggests that nuclear and cytoplasmic maturity are essential for activation either by sperm or after ageing in vitro. The observation of four good-looking six- to eight-cell stage parthenogenetic embryos which could have been taken for normal embryos if the oocytes had been inseminated leads us to call the attention to the possibility that when performing ART the repeated observation of the inseminated oocytes is an essential step to be aware of normal fertilization (2PN2PB) and to select the embryos with double pronuclear formation, timely syngamy, and normal cleavage rate. If the 2PN stage is not observed, parthenogenetic embryos can be taken for normal embryos reducing implantation and development rates of IVF embryos. Even after the observation of two *pronuclei*, parthenogenetical activation cannot be excluded, considering the possibility described by Winston (10) that polar body extrusion

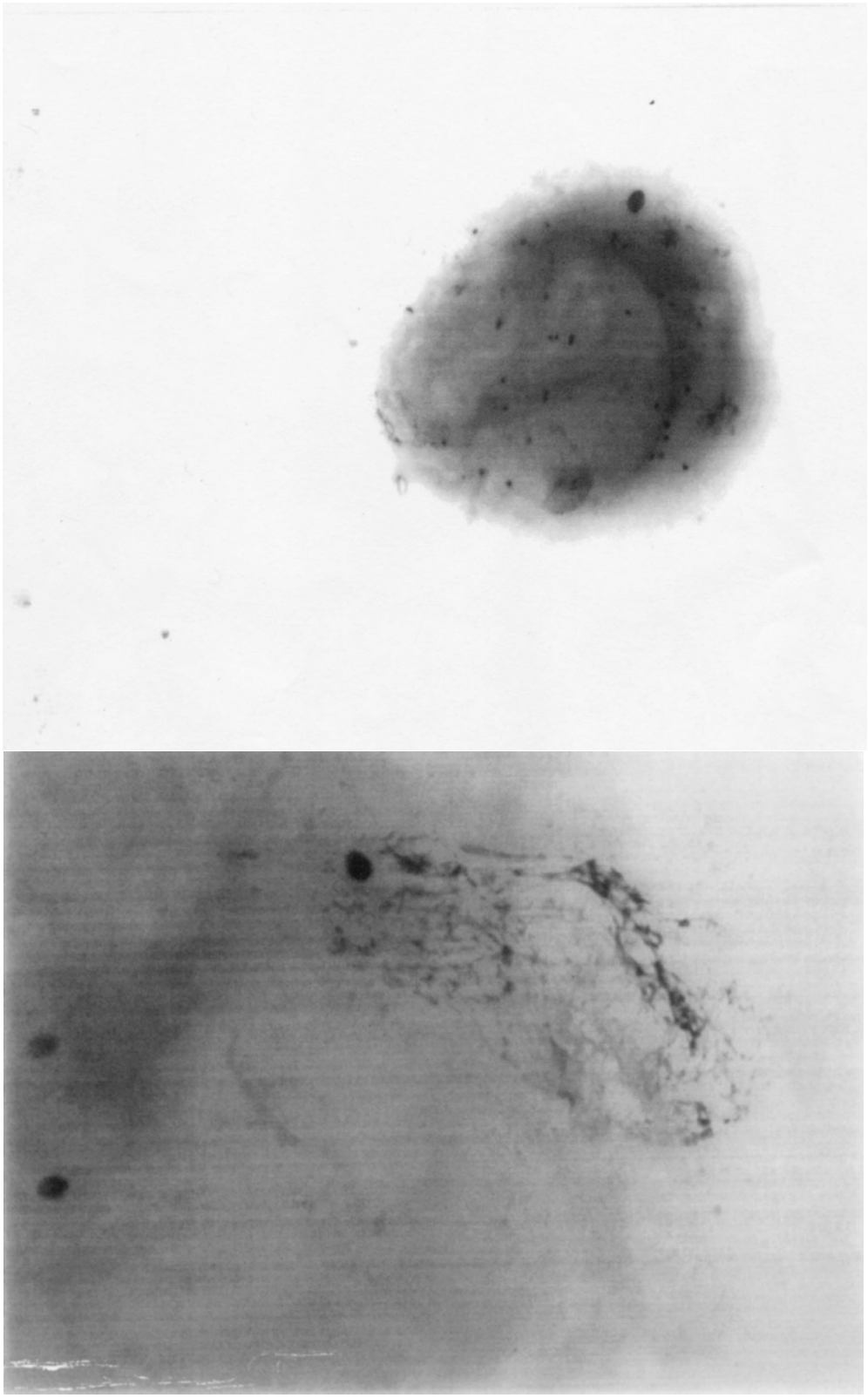

Fig. 2. Two-cell parthenogenetic embryo from patient 5. Each nucleus displayed an X and an 18 spot after FISH analysis (haploid pattern).

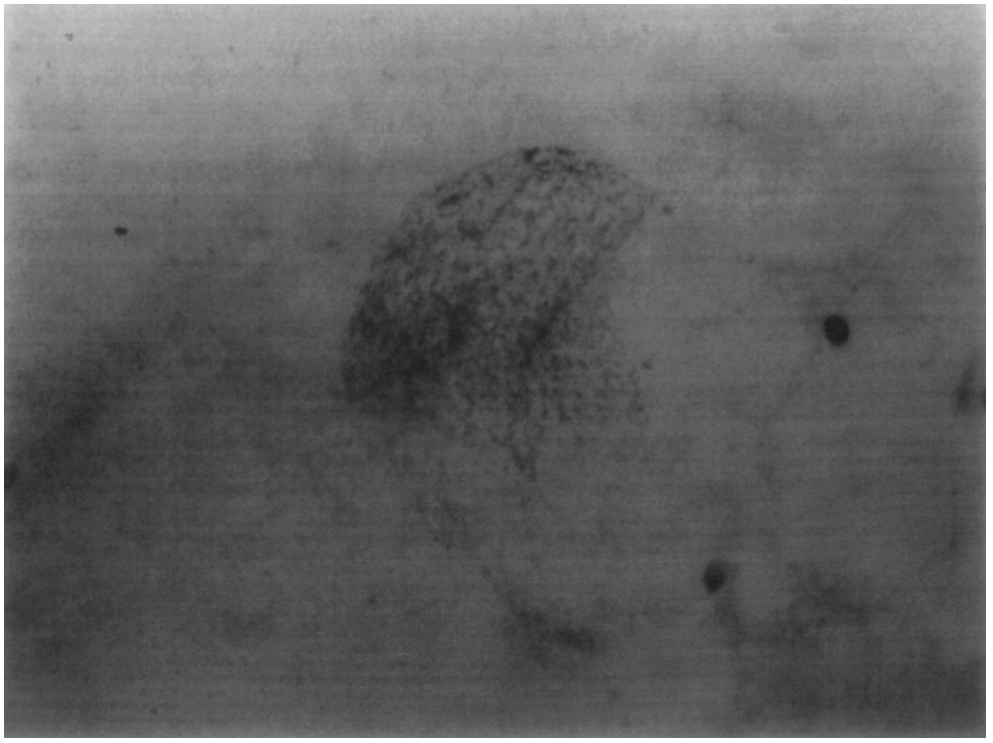

Fig. 2. (*Continued*)

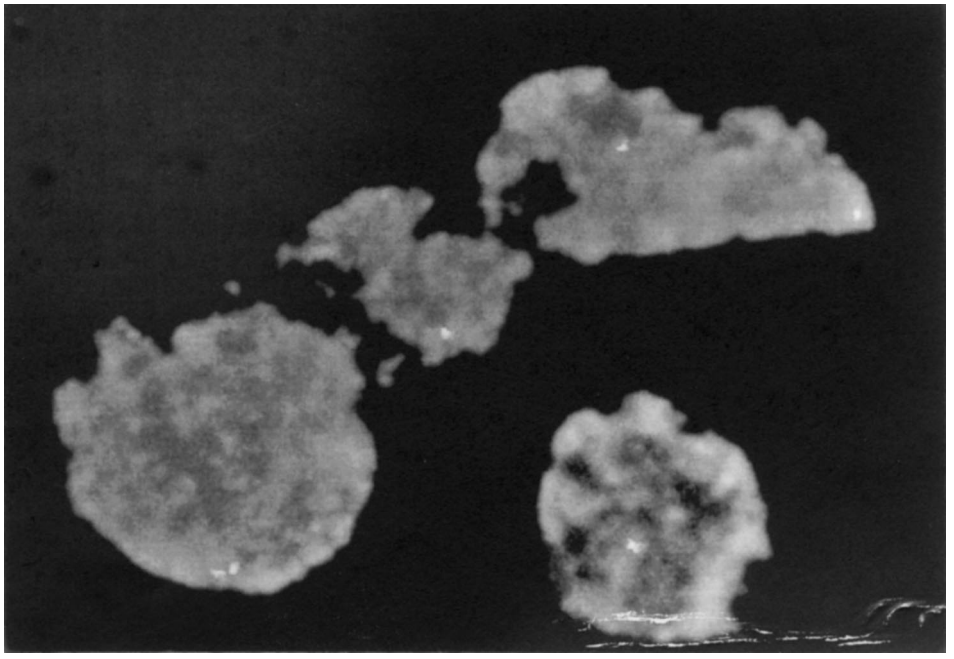

Fig. 3. Five-cell parthenogenetic embryo after immediate cleavage (patient 7). FISH analysis revealed a mosaic with haploid, diploid, and abnormal pattern of chromosome segregation. Hybridization with the X probe.

Table II. Group B-Failed Fertilized (NF) Oocytes Exposed to Puromycin at 48 h After Collection

	NF oocytes/ inseminated oocytes	Oocytes exposed to puromycin	Activated oocytes	Cleavage at 24 h	Fish results	Interpretation
Patient 13	3/3	3	*1PN2PB **2PN2PB	*1PN2PB **3 cells	*PN-X, 18 *PB-X,18 *PB-X,18	Normal haploid
Patient 14	8/20	8	+2PN1PB		+PB-X,18; PN-X; PN-no spot for the X or 18	+Abnormal pattern
			++2PN1PB		++PB-X,18; PN-XX; PN-XX,18	++Abnormal pattern
			+++2PN1PB		+++PN-X,18; PN-X,18 PB-no spot for the X and 18	+++Haploid PN. PB?
			++++2PN1PB		++++PB-18,18 No X spot; PN-XX; PN-XX	++++Abnormal pattern ?
Patient 15	11/12	4	1PN1PB	1PN1PB	PB-X,18 PN-XX,1818	Diploid PN (retention of the 2nd PB?)
Patient 16	3/8	3	*2PB	*4cells	*XY/XXXY/XX/XXY PB-no spots for the X	*Abnormal fertilization
			**2PB	**4cells	**X/X/X/X- PB-X; PB-X	**4 haploid nuclei and PBs
Patient 17	2/4	2	+1PN1PB		+PB-No X; PN-XX	+Abnormal pattern
			++1PN1PB		++PB-XX ; PN-noX or 18	++Abnormal pattern
Patient 18	4/7	4	+2PN1PB	+2cells	+No 18 spot/one cell lost	+Abnormal pattern
			++2PN1PB	++2 cells	++1818/181818	++Abnormal pattern
Patient 19	19/28	4	*1PN1PB	*4 cells	*Not successfull	
			**1PN1PB	**chaotic cleavage	**	
			***1PN1PB	***chaotic cleavage	***2 nuclei XX/1818	

was suppressed to yield an activated oocyte with two *pronuclei*. The timing of developmental events, such as extrusion of the second polar body, appearance and disappearance of *pronuclei*, and the first two divisions is similar to that seen in fertilized oocytes (11). Thus, although parthenogenesis is considered a rare event, the similarities previously described raise the question whether the transfer of parthenogenetically activated oocytes could explain early pregnancy losses after ART and emphasizes the need to adequately diagnose parthenotes.

The developmental arrest of oocytes at MII may prevent activation by sperm penetration but sometimes this can be overcome by exposition to an inhibitor of protein synthesis. This may lead us to think that the cytoplasmic conditions that are responsible for chromosome condensation are dependent on newly synthetized proteins. Nakagawa (11) exposed unfertilized human oocytes after ICSI to calcium ionophore A23187 and puromycin and reported a significant activation rate with 30% of the oocytes displaying two *pronuclei* and extrusion of the second polar body. Boosting of oocyte activation, after spermatid injection by a short incubation with calcium ionophore, yields high fertilization rates, although the ionophore treatment by itself was insufficient

Table III. Distribution of Unfertilized Oocytes Exposed to Puromycin According to the ART Technique Used

	54 IVF patients		16 ICSI patients		
	Median	SD	Median	SD	*P*
Pregnancy rate/cycle	20.4% (11/54)		37.5% (6/16)		
Age	32.4[a]	4	29.6[a]	4.3	0.01
Unfertilized oocytes	4.63[a]	2.7	3[a]	1.5	0.01
Oocytes in puromycin	4	2.3	2.9	1.5	ns
Absence of activation	1.1	1.4	1.1	1.2	ns
1PN2PB	0.3	0.5	0.1	0.3	ns
2PN1PB	0.5	0.8	0.6	1.1	ns
2PN2PB	0.3	0.5	0.1	0.4	ns
1PN1PB	0.7	1.1	0.5	1.2	ns
2PB	0.2	0.7	0.1	0.4	ns

Note. ns–not significant.
[a]Mann–Whitney test.

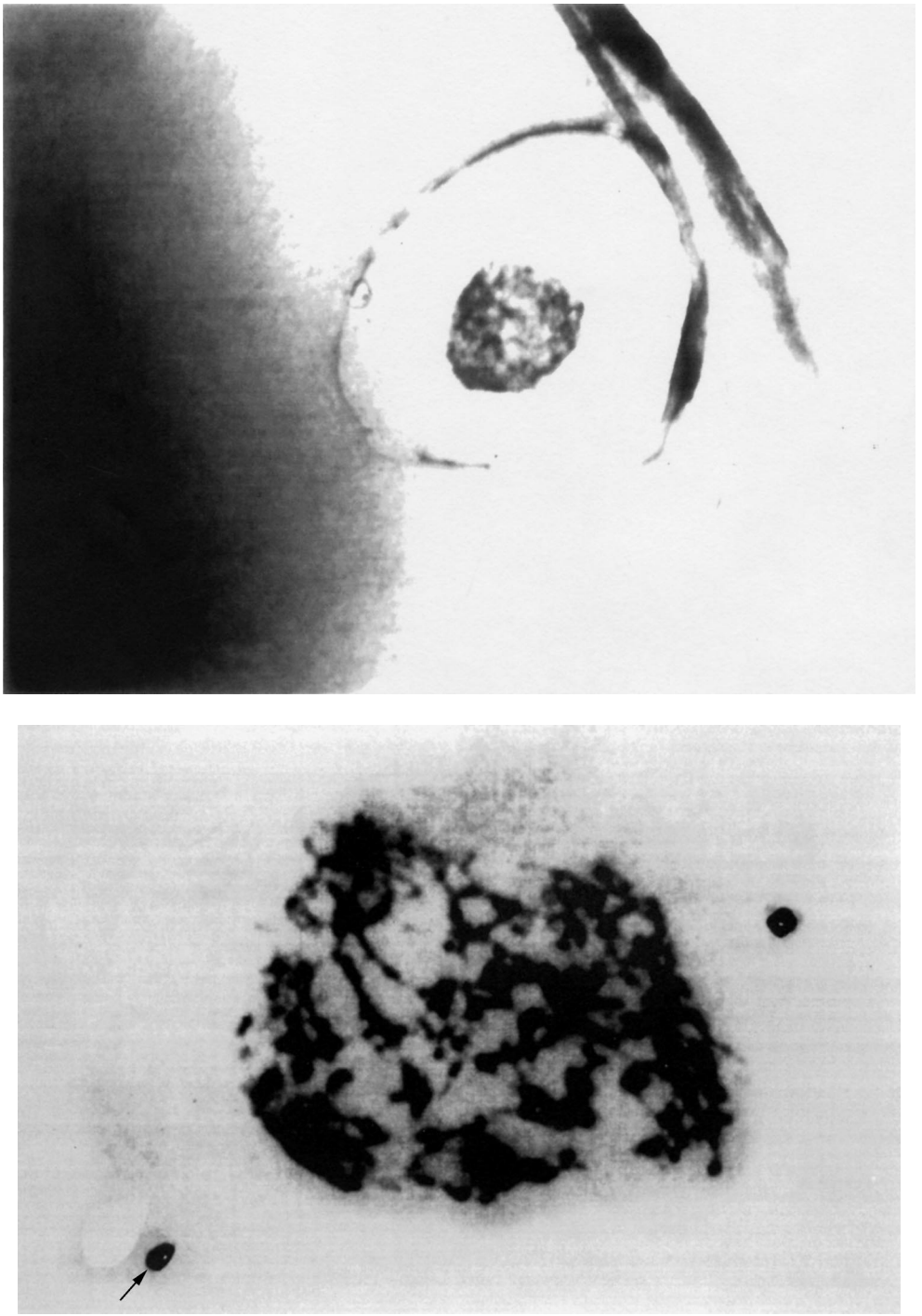

Fig. 4. (a, b). Activated oocyte (1PN1PB) after ICSI. The pronucleus exhibited an X and an 18 spot (normal haploid?). Notice the sperm head in the ooplasm with normal X and 18 signals, suggesting activation without sperm chromatin decondensation.

to activate human oocytes under the conditions described by Tesarik (12).

If a normal pattern of activation could be obtained with exposition to ionophores or other activators, these procedures could be used to generate embryos both for patients with low fertilization rates and for the production of parthenote blastocysts for stem cell research as reported recently (7).

Our preliminary results however suggest that, at least in failed fertilized aged oocytes, there is a trend towards retention of the second polar body and thus possible cytogenetic abnormalities including aneuploidy which might be explained by the fact that puromycin interferes with spindle proteins (11). In spontaneously activated oocytes we also found abnormal patterns of chromosome disjunction.

Further studies are needed to evaluate the activation pattern and cytogenetic complement of freshly collected oocytes exposed to parthenogenetic activators as our preliminary results suggest that a remarkable number of parthenogenetically activated oocytes have chromosome abnormalities, even if activation occurred spontaneously few hours after oocyte collection. This is in agreement with the findings in normally fertilized embryos after ART that show a high level of aneuploidy and mosaicism in human embryos produced in vitro and raises important questions on the use of unfertilized eggs of primates for generating cloned stem cells for research.

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REFERENCES

1. Johnson MH, Pickering SJ, Braude PR, Vincent C, Cant A, Currie J: Acid Tyrode's solution can stimulate parthenogenetic activation of human and mouse oocytes. Fertil Steril 1990;53:266–270
2. Gook DA, Osborn SM, Johnston, WIH: Parthenogenetic activation of human oocytes following cryopreservation using 1,2-propanediol. Hum Reprod 1995;10:654–658
3. Abramczuk JW, Lopata A: Resistance of the human follicular oocytes to parthenogenetic activation: DNA distribution and content in oocytes maintained in vitro. Hum Reprod 1990;5:578–581
4. Balakier H, Casper RF: Experimentally induced parthenogenetic activation of human oocytes. Hum Reprod 1993;8:740–743
5. Taylor AS, Braude PR: The early development and DNA content of activated human oocytes and parthenogenetic human embryos. Hum Reprod 1994;9:2389–2397
6. De Sutter P, Desmet R, Dhont M: Cytogenetic analysis of human oocytes parthenogenetically activated by puromycin. Hum Reprod 1993;8:141–142
7. Cibelli JB, Grant KA, Chapman KB, Cunniff K, Worst T, Green HL, Walker SJ, Gutin PH, Vilner L, Tabar V, Dominko T, Kane J, Wettstein PJ, Lanza RP, Studer L, Vranz KE, West MD: Parthenogenetic stem cells in nonhuman primates. Science 2002;295:819
8. Van Blerkom J, Davis PW, Merriam J: A retrospective analysis of unfertilized and presumed parthenogenetically activated human oocytes demonstrates a high frequency of sperm penetration. Hum Reprod 1994;9:2381–2388
9. Uranga JA, Arechaga J: Cell proliferation is reduced in parthenogenetic mouse embryos at the blastocyst stage: A quantitative study. Anat Rec 1997;247:243–247
10. Winston N, Johnson M, Pickering S, Braude P: Parthenogenetic activation and development of fresh and aged human oocytes. Fertil Steril 1991;56:904–912
11. Nakagawa K, Yamano S, Moride N, Yamashita M, Yoshizawa M, Aono T: Effect of activation with Ca ionophore A23187 and puromycin on the development of human oocytes that fail to fertilize after intracytoplasmic sperm injection. Fertil Steril 2001;76:148–152
12. Tesarik J, Mendoza C, Greco E: The activity (calcium oscillator?) responsible for human oocytes activation after injection with round spermatids is associated with spermatid nuclei. Fertil Steril 2000;74:1245–1247