Zygote **12** (November), pp. 339–344. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002989 Printed in the United Kingdom

Chromosomal analysis of mouse spermatozoa following physical and chemical treatments that are effective in inactivating HIV

Kazuto Morozumi[1], *Hiroyuki Tateno*[2], *Kaoru Yanagida*[3], *Haruo Katayose*[1], *Yujiroh Kamiguchi*[2] *and Akira Sato*[1]

Fukushima Medical University, Fukushima, Asahikawa Medical College, Asahikawa, and International University of Health and Welfare, Ootawara, Japan

Date submitted: 23.9.04. Date accepted: 21.9.04

Summary

Human immunodeficiency virus (HIV) can be inactivated by heating at 56 °C for 30 min, treating with 50% ethanol at room temperature for 10 min, or treating with 2% sodium hypochlorite solution (NaClO) at room temperature for 60 min. Using a mouse model, we evaluated the risk of generating chromosome damage in spermatozoa following these treatments. The spermatozoa were all dead after the treatments. Although 41.3% of oocytes injected with ethanol-treated spermatozoa successfully activated, none of the oocytes injected with heated or NaClO-treated spermatozoa activated. When artificial stimulation with strontium was used, the fertilization of oocytes with heated or ethanol-treated spermatozoa was completely rescued. Sperm nuclei treated with NaClO neither decondensed nor developed to a male pronucleus. The incidences of structural chromosome aberrations in 1-cell zygotes derived from the heated spermatozoa (45.6%) and ethanol-treated spermatozoa (91.2%) were significantly higher than those in the matched controls (5.5% and 10.5%, respectively). Further study is needed to develop a methodology for the protection of spermatozoa against chromosome damage or the separation of damaged spermatozoa before intracytoplasmic sperm injection.

Keywords: Chromosome, Ethanol treatment, Heating, Mouse spermatozoa

Introduction

Given the prevalence of the human immunodeficiency virus (HIV), we are facing the issue of how to remove HIV from infected semen samples to prevent vertical transmission of the disease in assisted reproductive medicine. Semprini *et al*. (1997) reported the lack of secondary transmission in 1954 insemination attempts with washed spermatozoa. However, Baccetti *et al*. (1994) reported that semen washing cannot entirely eliminate HIV-1 RNA from HIV-1 infected semen. Hanabusa *et al*. (2000) showed that swim-up processing after Percoll gradient centrifugation of the infected semen reduced the HIV-1 RNA and HIV-1 proviral DNA to undetectable levels. Politch *et al*. (2004) also reported that the double tube gradient method was effective in removing HIV. Since it is known that HIV is inactivated by heating at 56 °C for 30 min, treating with 50% ethanol at room temperature (RT) for 10 min, or treating with 2% sodium hypochlorite solution (NaClO) at RT for 60 min (Ball, 1987), if these treatments are shown to have less of an effect on the chromosomes in the spermatozoa, they would offer another option for removing HIV from infected semen.

As spermatozoa pass through the epididymides, nuclear protamines are extensively crosslinked by disulfide bonds (Bedford & Calvin, 1974). Previous studies have suggested that mammalian spermatozoal chromatin is resistant to various physical and chemical disruptions. Even though mature hamster, mouse and human spermatozoa were heated at 90 °C for 30 min,

All correspondence to: K. Morozumi, Department of Obstetrics and Gynecology, Fukushima Medical University, 1, Hikarigaoka, Fukushima 960-1295, Japan. Tel: +81 24 5471290. Fax: +81 24 5483878. e-mail: morozumi@fmu.ac.jp
[1]Department of Obstetrics and Gynecology, Fukushima Medical University, Fukushima, Japan.
[2]Department of Biological Sciences, Asahikawa Medical College, Asahikawa, Japan.
[3]Clinical Research Center, International University of Health and Welfare, Ootawara, Japan.

they were capable of undergoing pronuclear formation and DNA synthesis when they were artificially injected into oocytes (Yanagida *et al*., 1991). Rabbit oocytes injected with spermatozoa heated at 60 °C for 30 min could develop to 8-cell embryos (Hoshi *et al*., 1992). Furthermore, mouse spermatozoa heated at 56 °C for 30 min supported full embryonic development (Cozzi *et al*., 2001). Ethanol has no harmful effect on the primary structure of sperm protamines (Lee *et al*., 1991) and does not damage DNA molecules (Sambrook *et al*., 1989). Sperm nuclei could be transformed into pronuclei after being stored in 100% ethanol (Katayose *et al*., 1992). However, male pronuclear formation does not necessarily reflect the chromosomal integrity of the spermatozoa, since more than 80% of mouse zygotes derived from spermatozoa stored in 70% ethanol for 24 h at −20 °C displayed structural chromosome aberrations (Tateno *et al*., 1998).

To explore the feasibility of clinically using HIV-infected spermatozoa in assisted reproduction, we evaluated the cytogenetic effects of heating at 56 °C for 30 min, treating with 50% ethanol at RT for 10 min or treating with 2% NaClO at RT for 60 min on spermatozoa using a mouse model.

Materials and methods

Reagents and media

All inorganic and organic reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. The medium used for culturing the eggs after intracytoplasmic sperm injection (ICSI) was a bicarbonate-buffered CZB supplemented with 5.56 mM D-glucose and 4 mg/ml bovine serum albumin (BSA) (Chatot *et al*., 1989). The medium used for oocyte collection and the ICSI procedure was a modified CZB with 20 mM HEPES-Na, 5 mM $NaHCO_3$ and 0.1 mg/ml polyvinyl alcohol (PVA; cold-water-soluble) instead of BSA. Calcium-free CZB medium containing 10 mM $SrCl_2$ (Sr^{2+}-CZB) was used for the artificial activation of oocytes, because strontium itself does not affect oocytes and sperm chromatin. Both CZB and Sr^{2+}-CZB media were used under 5% CO_2 in air, and HEPES-CZB was used under 100% air. The pH of these media was approximately 7.4.

Preparation of mouse oocytes

Female B6D2F1 mice, 8–13 weeks of age, were superovulated by the intraperitoneal injection of 5 IU PMSG followed 48 h later by the intraperitoneal injection of 5 IU hCG. Mature oocytes were collected from the oviducts approximately 15 h after hCG injection. They were freed from the cumulus cells by treatment for 5 min with 0.1% (w/v) bovine testicular hyaluronidase in HEPES-CZB. The cumulus-free oocytes were thoroughly rinsed and temporarily kept in CZB medium.

Preparation of spermatozoa

A dense sperm mass was collected from the cauda epididymides and placed in the bottom of a 1.5 ml centrifuge tube containing 200 µl of CZB medium. The spermatozoa were allowed to swim up in the medium for 10–20 min at 37 °C. The supernatant including motile spermatozoa was then transferred to 500 µl microtubes. The microtubes were placed in a water bath, and the spermatozoa were heated at 56 °C for 30 min. Immediately, the microtubes were cooled with tap water. To treat the spermatozoa with 50% ethanol at RT for 10 min, the same volume of 100% ethanol was added to the microtubes. To treat the spermatozoa with 2% NaClO at RT for 60 min, 2 µl of 100% NaClO was added to 98 µl of sperm suspension. After the treatments with ethanol or NaClO, the spermatozoa were washed twice with HEPES-CZB by centrifugation.

The vitality of the spermatozoa following each treatment was assessed using a commercially available cell viability test kit (Live/dead FertiLight; Molecular Probes, Eugene, OR). The assay was performed according to the manufacturer's instructions.

ICSI protocol

ICSI was carried out according to the method described by Kimura & Yanagimachi (1995) with some modifications. Within 30 min after the respective treatments, a small amount (25 µl) of sperm suspension was mixed thoroughly with 50 µl of HEPES-CZB medium containing 10% (w/v) polyvinylpyrrolidone. A small portion of this suspension was transferred to an ICSI chamber which had been prepared on the stage of an inverted microscope equipped with a micromanipulation system. A single heated spermatozoon was drawn head first, and the head was separated from the tail by applying a few high-intensity piezo pulses to the neck region. Immediately, the sperm head was injected into an oocyte. However, the entire ethanol-treated spermatozoon was injected into an oocyte, because it was difficult to separate the head from the tail due to hardening of the spermatozoon. In treatment with 2% NaClO, the sperm head was separated from the tail, and the head was then neatly injected into the oocyte. Ten to thirty oocytes were employed for every operation cycle, and sperm injection into them was completed within 30 min. The entire ICSI procedure was completed within 2 h to minimize chromatin degradation due to a long storage of dead spermatozoa. ICSI was also carried out with fresh sperm heads

https://doi.org/10.1017/S0967199404002989 Published online by Cambridge University Press

Table 1 Nuclear development in mouse eggs 6 h after injection of spermatozoa that had undergone various physical and chemical treatments

Treatments	$SrCl_2$ (10 mM)	No. of eggs examined (no. of expts)	Oocyte nuclei			Sperm nuclei				Fertilization (%)
			Met II	Met III	PN	Intact	Decondensed	PCC	PN	
Heat, 56 °C,	–	32 (2)	32	0	0	0	0	32	0	0.0
30 min	+	27 (2)	0	0	27	0	0	0	27	100.0
50% ethanol, RT,	–	46 (2)	22	5	19	0	7	20	19	41.3
10 min	+	16 (2)	0	0	16	0	0	0	16	100.0
2% NaClO, RT,	–	19 (2)	19	0	0	19	0	0	0	0.0
60 min	+	14 (1)	0	0	14	14	0	0	0	0.0

Met II, metaphase II; Met III, metaphase III; PN, pronucleus; PCC, premature chromosome condensation.

following application of a high-intensity piezo pulses and with whole spermatozoa as matched controls for heat treatment and ethanol treatment, respectively. The ICSI procedure was carried out in 100% air at RT under paraffin oil (Merck Japan, Tokyo).

Artificial oocyte activation and culture of injected oocytes

Surviving oocytes after sperm injection were transferred into a droplet (approximately 100 µl) of CZB medium and Sr^{2+}-CZB medium under paraffin oil. The latter group was incubated for 2 h to induce artificial activation, washed with normal CZB medium and then transferred into the same medium for further culture. Six to eight hours after ICSI, the eggs were examined under an inverted microscope to determine whether successful fertilization had occurred. Some unfertilized eggs without pronuclei and/or the second polar body were fixed for cytological preparation.

Chromosome preparation and analysis

Approximately 8 h after the ICSI, fertilized eggs with two distinct pronuclei and the second polar body were transferred into a drop (0.2 ml) of CZB medium containing 0.01 µg/ml vinblastine sulfate and cultured until they reached the first cleavage metaphase. Between 19 and 21 h after ICSI, the eggs at the first cleavage metaphase were treated with 0.5% (w/v) protease (Kaken Pharmaceuticals, Tokyo) for 7–8 min to loosen the zona pellucida, followed by incubation in a hypotonic solution (1:1 mixture of 1% sodium citrate and 30% fetal bovine serum) for 8 min at RT. Fixation of the eggs and the spreading of the chromosomes were performed according to Mikamo & Kamiguchi (1983). The chromosome slides were stained with 2% Giemsa (Merck) in buffered saline solution (pH 6.8) for 8 min for conventional chromosome analysis.

Statistical analysis

The difference in the percentage of chromosome aberrations between the experimental group and matched control group was compared using Fisher's exact probability test or the chi-square test. Differences were considered significant at the $p < 0.05$ level.

Results

Vitality of spermatozoa after treatments

Predictably, more than 90% of the fresh spermatozoa collected by the swim-up method were alive and motile, while 100% of the spermatozoa became immotile and died after the heat, ethanol and NaClO treatments.

Fertilization after ICSI

The nuclear development and fertilization rates in oocytes injected with treated spermatozoa were examined 6–8 h after ICSI (Table 1). When the oocytes injected with heated sperm heads were cultured in CZB medium [$SrCl_2$ (–)], the oocyte nuclei remained in metaphase II and the sperm chromatin displayed partial premature chromosome condensation (PCC) (Fig. 1A). Following 2 h of incubation in Sr^{2+}-CZB medium [$SrCl_2$ (+)], however, 100% of the eggs had formed both female and male pronuclei. Among the 46 oocytes injected with ethanol-treated spermatozoa, 19 (41.3%) underwent successful fertilization without incubation in Sr^{2+}-CZB medium. Twenty-two (47.8%) were at metaphase II and the rest were arrested at metaphase III following extrusion of the second polar body (Fig. 1B). In these non-activated oocytes, the sperm chromatin underwent decondensation or partial PCC. Predictably the 2 h incubation in Sr^{2+}-CZB medium was effective in activating the oocytes injected with ethanol-treated spermatozoa. Oocytes injected with NaClO-treated sperm heads never activated in

https://doi.org/10.1017/S0967199404002989 Published online by Cambridge University Press

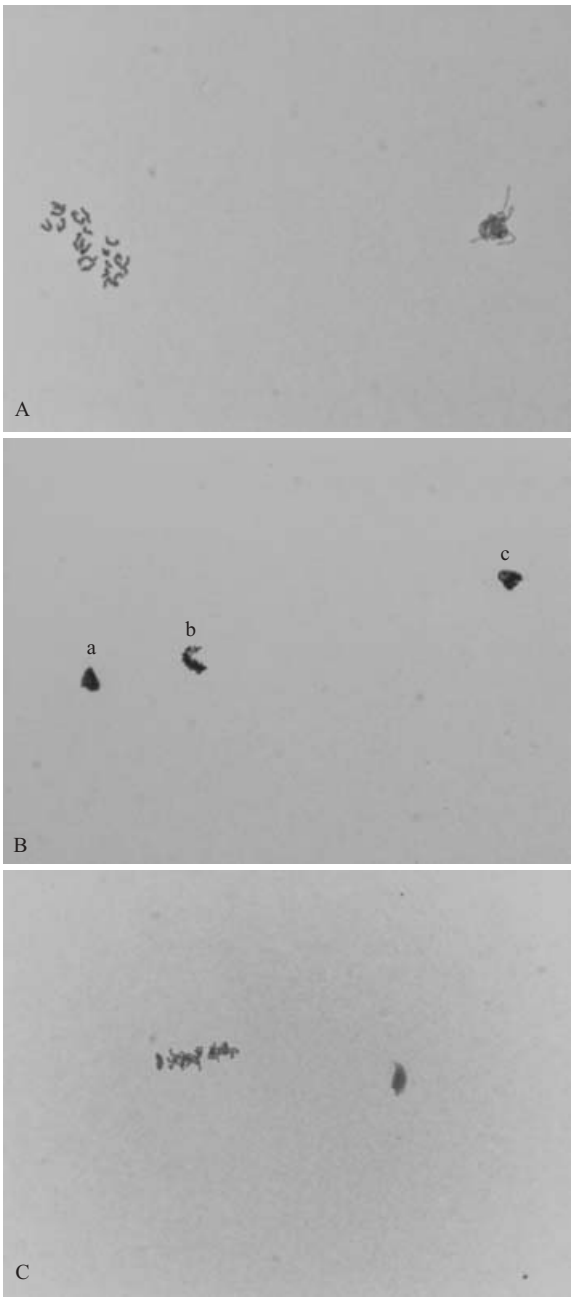

Figure 1 Cytological preparations of non-activated or incompletely activated oocytes 6 h after sperm injection. (*A*) Oocyte injected with a heated sperm head. The oocyte nucleus (left) remains in metaphase II, and the sperm chromatin (right) undergoes partial premature chromosome condensation. (*B*) Oocyte injected with an ethanol-treated spermatozoon. Because the oocyte did not enter interphase following extrusion of the second polar body, there are the chromatin mass of the second polar body (a), a cluster of metaphase III (monad) chromosomes (b) and a decondensed sperm nucleus (c). (*C*) Oocyte injected with a NaClO-treated sperm head. There is a metaphase II chromosome set of oocytes (left) and a condensed sperm head (right).

the CZB medium and sperm heads remained intact (Fig. 1C). Although the oocytes successfully activated and formed a female pronucleus following the 2 h incubation in Sr^{2+}-CZB medium, the sperm chromatin remained condensed.

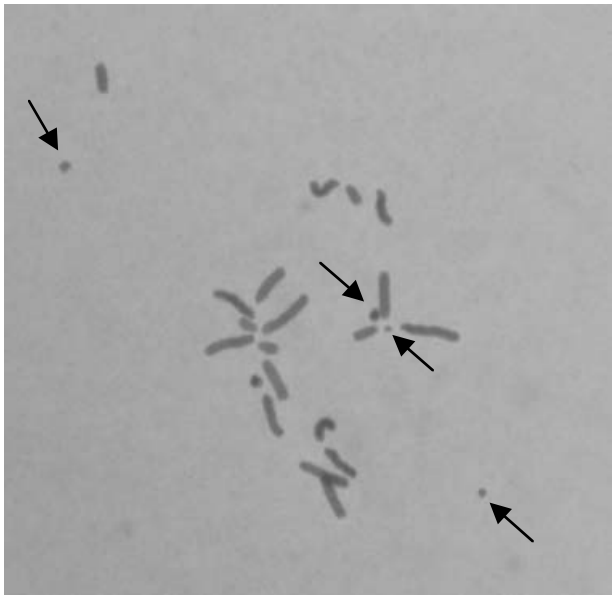

Figure 2 Multiple chromosome fragments (arrows) in a male pronucleus of a 1-cell zygote derived from a heated sperm head.

Chromosome analysis

Based on these results, chromosome analysis was conducted on the heated spermatozoa and ethanol-treated spermatozoa (Table 2). When heated sperm heads were used, the incidence of diploid zygotes with structural chromosome aberrations significantly increased (45.6% vs 5.5% in the control). Many of the abnormal zygotes had multiple chromosome fragments (Fig. 2). When ethanol-treated spermatozoa were used, 91.2% of the zygotes had severe structural chromosome damage. The injection of heated and ethanol-treated spermatozoa bore no relation to the generation of aneuploidy and triploidy in the zygotes.

Discussion

The present results revealed that mouse oocytes were not activated by the injection of spermatozoa heated at 56 °C for 30 min, indicating that sperm-borne oocyte activating factor(s) (SOAF) are completely destroyed or inactivated by heat treatment in mouse spermatozoa. Cozzi *et al*. (2001) have also reported evidence supporting the fact that fertilization does not occur after the microinjection of mouse spermatozoa heated at 56 °C for 30 min unless activation is artificially induced. Furthermore, SOAF has been shown to be heat-sensitive (Perry *et al*., 1999). In contrast, Hoshi *et al*. (1992) reported that fertilization of rabbit eggs injected

https://doi.org/10.1017/S0967199404002989 Published online by Cambridge University Press

Table 2 Chromosome analysis of 1-cell zygotes derived from heated or ethanol-treated spermatozoa

Treatments	No. of zygotes analysed	Normal (%)	Chromosome aberrations (%)			
			Structural	Aneuploidy	Triploidy	Total
Control (I)	109	99 (90.8)	6 (5.5)[a]	3 (2.8)	1* (0.9)	10 (9.2)[a]
Heated	182	98 (53.8)	83 (45.6)[b]	0	1* (0.5)	84 (46.2)[b]
Control (II)	57	50 (87.7)	6 (10.5)[c]	1* (1.8)	0	7 (12.3)[c]
Ethanol	57	5 (8.8)	52 (91.2)[d]	0	0	52 (91.2)[d]

[a,b,c,d], significantly different, $p < 0.0001$ (chi-square test).

* These cases are accompanied by structural chromosome aberrations.

with heated spermatozoa (60 °C for 30 min) occurred at a success rate of 43% without artificial stimulation. The discrepancy among these studies may be due to species-specific differences. Treatment with 70% ethanol for 24 h at −20 °C made mouse spermatozoa completely incapable of activating oocytes (Tateno *et al*., 1998). However, the present study found that approximately 40% of the spermatozoa treated with 50% ethanol for 10 min at RT maintained the capability of activating oocytes, suggesting that SOAF is not completely destroyed or inactivated by brief treatment with ethanol at a low concentration. Oocytes injected with NaClO-treated sperm heads were never activated. Furthermore, sperm chromatin never decondensed in the ooplasm. NaClO is an oxidizing agent, so SOAF and protamines were completely denatured through oxidization.

As opposed to the capability of activating oocytes, zygotes derived from spermatozoa treated with 50% ethanol for 10 min at RT had an extremely high aberration rate (91.2%). Thus, the clinical use of ethanol-treated spermatozoa in assisted reproduction would be impractical. Interestingly, the aberration rate was not significantly different ($p = 0.342$, chi-square test) from that (81.3%: 26/32) in zygotes derived from spermatozoa stored in 70% ethanol for 24 h at −20 °C (Tateno *et al*., 1998). A recent study has shown that the remodelling of sperm chromatin during fertilization is accompanied by formation of transient DNA cleavage mediated by ooplasmic topoisomerase II (Tateno & Kamiguchi, 2004). When the enzyme is incapable of acting on sperm chromatin altered by ethanol, a large number of DNA strand breaks would be secondarily generated. This may be the reason why severe structural chromosome aberrations were induced in ethanol-treated spermatozoa.

A significant increase in the incidence of structural chromosome aberrations was also found in zygotes derived from heated sperm heads. However, it should be noted that about 54% of the zygotes had a normal chromosome complement. This result is not necessarily a poor one, and we feel that analysing the chromosomes of heated human spermatozoa warrants further study. In addition, it has been reported that mouse spermatozoa heated under the same conditions (56 °C for 30 min) can support full embryonic development (Cozzi *et al*., 2001). If chromatin damage caused by heat treatment could be reduced by some type of pretreatment, or if such damaged spermatozoa could in some way be separated before ICSI, heat treatment could be used as a means of removing HIV from infected semen in assisted reproductive technology.

Acknowledgements

The authors thank Dr Hiromi Komiya for helpful discussion.

References

Baccetti, B., Benedetto, A., Burrini, A.G., *et al*. (1994). HIV-particles in spermatozoa of patients with AIDS and their transfer into the oocyte. *J. Cell Biol*. **127**, 903–914.

Ball, M.J. (1987). Effect of two disinfectant treatments on laboratory analyses. *J. Soc. Med*. **80**, 482–484.

Bedford, J.M. & Calvin, H.I. (1974). The occurrence and possible functional significance of 'SS' crosslinks in sperm heads, with particular reference of eutherian mammals. *J. Exp. Zool*. **188**, 137–156.

Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. & Torres, I. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *J. Reprod. Fertil*. **86**, 679–688.

Cozzi, J., Monier-Gavelle, F., Lièvre, N., Bomsel, M. & Wolf, J.P. (2001). Mouse offspring after microinjection of heated spermatozoa. *Biol. Reprod*. **65**, 1518–1521.

Hanabusa, H., Kuji, N., Kato, S., Tagami, H., Kaneko, S., Tanaka, H. & Yoshimura, Y. (2000). An evaluation of semen processing methods for eliminating HIV-1. *AIDS* **14**, 1611–1616.

Hoshi, K., Yazawa, H., Yanagida, K. & Sato, A. (1992). Microinsemination of rabbit oocytes with heat treated sperm: embryonic development. *Arch. Androl*. **29**, 233–237.

Katayose, H., Matsuda, J. & Yanagimachi, R. (1992). The ability of dehydrated hamster and human sperm nuclei to develop into pronuclei. *Biol. Reprod*. **47**, 277–284.

Kimura, Y. & Yanagimachi, R. (1995). Intracytoplasmic sperm injection in the mouse. *Biol. Reprod*. **52**, 709–720.

Lee, C., Mazrimas, J. & Balhorn, R. (1991). Analysis of protamines isolated sperm heads. *Mol. Reprod. Dev*. **30**, 154–158.

Mikamo, K. & Kamiguchi, Y. (1983). A new assessment system for chromosomal mutagenicity using oocytes and early zygotes of the Chinese hamster. In *Radiation-Induced Chromosome Damage in Man* (ed. T. Ishihara & M.S. Sasaki), pp. 411–432. New York: Alan R. Liss.

Perry, A.C., Wakayama, T. & Yanagimachi, R. (1999). A novel transcomplementation assay suggests full mammalian oocyte activation is coordinately initiated by multiple, sub-membrane sperm components. *Biol. Reprod*. **60**, 747–755.

Politch, J.A., Xu, C., Tucker, L. & Anderson, D. (2004). Separation of human immunodeficiency virus type 1 from motile sperm by the double tube gradient method versus other methods. *Fertil. Steril*. **81**, 440–447.

Sambrook, J., Fritsch, E.F. & Maniatas, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Semprini, A.E., Fiore, S. & Pardi, G. (1997). Reproductive counselling for HIV-discordant couples. *Lancet*. **349**, 1401–1402.

Tateno, H. & Kamiguchi, Y. (2004). Chromosome analysis of mouse one-cell androgenones derived from a sperm nucleus exposed to topoisomerase II inhibitors at pre- and post-fertilization stages. *Mutation Res*. **556**, 117–126.

Tateno, H., Wakayama, T., Ward, W.S. & Yanagimachi, R. (1998). Can alcohol retain the reproductive and genetic potential of sperm nuclei? Chromosome analysis of mouse spermatozoa stored in alcohol. *Zygote* **6**, 233–238.

Yanagida, K., Yanagimachi, R., Perreault, S.D. & Kleinfeld, R.G. (1991). Thermostability of sperm nuclei assessed by microinjection into hamster oocytes. *Biol. Reprod.* **44**, 440–447.