—Review—

The inability of human sperm with chromosomal abnormalities to penetrate the oocyte in assisted reproductive technology (ART): risk factors and the role of seminal plasma

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Abstract: The combination of ICSI and a sperm chromosome assay provides a useful opportunity to directly investigate relationships between chromosomal aberrations and head morphology, motility or *in vitro* aging. An increase of aneuploidy was identified in sperm with large heads. Structural chromosome aberrations were frequently found in elongated heads. These types of sperm should therefore be avoided for ICSI and can easily be identified under a microscope. In sperm with normal heads, the chromosomal aberration rate was initially low but increased gradually after removal of the seminal plasma (3.3% to 15.9%); in immotile sperm, the increase of DNA damage was more pronounced (4.5% to 48.1%). Therefore, there are motile and immotile sperm populations in human ejaculates that are potentially vulnerable to *in vitro* culture conditions. Since these sperm can maintain DNA integrity in the seminal plasma for at least 3 h, and even after cryopreservation, the seminal plasma appears to be an efficient suppressor of DNA damage. We report here our attempts at using ICSI using sperm stored in the seminal plasma (SP-ICSI) to treat infertile patients with normozoospermia and provide data to confirm the safety of this technique. For poor quality semen, SP-ICSI may be more effective in achieving a successful outcome.
Key words: Human sperm, Chromosome aberrations, Amorphous, Immotile, *In vitro* aging

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Introduction

In 1983, Mikamo and Kamiguchi [1] combined the gradual fixation-air drying method, which is a very effective technique for the preparation of chromosomal specimens from oocytes, and interspecific *in vitro* fertilization (IVF) between human sperm and zona-free hamster oocytes [2], which permitted the analysis of the chromosomes in individual human sperm. Since then, these authors have published a series of papers relating to the characteristics of chromosomal abnormalities in human sperm. This work demonstrated that the incidence of structural chromosome aberration is very high in human sperm, with a mean incidence of 14%, and shows significant individual variation [3]. This was considered by Edmonds *et al.* [4] to underlie the low outcome rate in human reproduction. However, the precise mechanisms underlying the high incidence of DNA damage in human sperm has yet to be elucidated. This study was conducted after obtaining the ethical approval of our institute.

The development of intracytoplasmic sperm injection (ICSI) has created a fundamental new problem in how we should select a sperm for ICSI, particularly in terms of selecting sperm with good genetic integrity. However, human sperm chromosome assays are not able to assess genetic integrity for ICSI because it is impossible to determine the precise features of how sperm penetrate an oocyte. To resolve this issue, this paper describes our attempts to analyze chromosomes of individual sperm by ICSI into mouse oocytes with a piezo-micromanipulator,

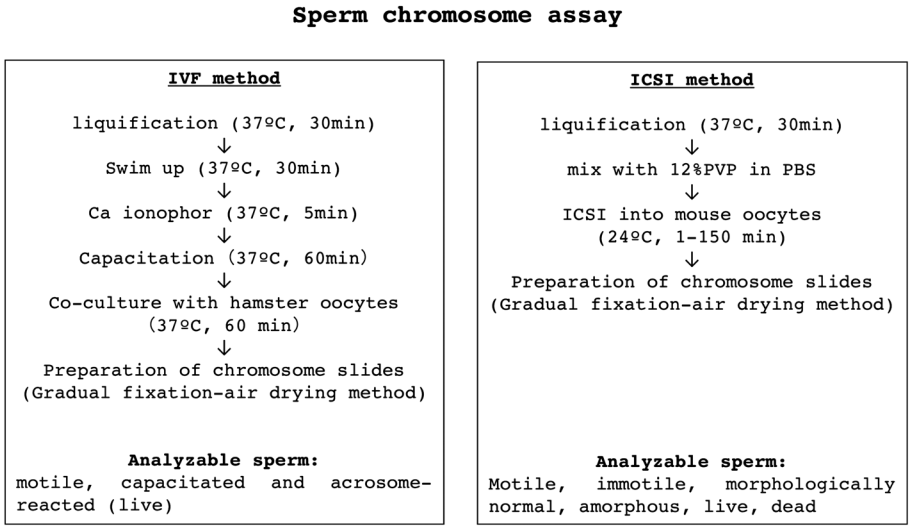

Fig. 1. Two methods to analyze sperm chromosomes. There are two methods that can be used for the analysis of human sperm chromosomes with IVF or ICSI. The IVF method requires sperm to be capacitated and then penetrated into zone-free hamster oocytes prior to chromosome analysis; two and a half hours are necessary for the sperm preparation involved. With the ICSI method, sperm can be injected into mouse oocytes soon after liquefaction.

a technique originally developed by Kimura *et al.* [5]. This paper also describes our results pertaining to human sperm chromosomes. Fig. 1 shows the differences between the sperm chromosome assay when used with IVF or ICSI; ICSI allows us to study the relationship between chromosomes and sperm morphology, motility and viability.

Sperm Head Abnormality

By injecting sperm with a particular feature into mouse oocytes, it is possible to investigate the associated genetic risk. We compared the risk of structural and numerical chromosome aberrations between motile sperm with aberrant and normal heads from normozoospermia donors [6] (Fig. 2 and 3).

Small heads

The incidence of numerical chromosome aberrations did not differ between sperm with small heads (<4 μm in length and <3 μm in width) and those with normal heads which were present in semen from a normozoospermic donor; this suggests that small heads do not result from loss of chromosomes (hypoploidy). This result is inconsistent with globozoospermia patients who show a significant increase of sperm aneuploidy when analyzed by multi-color FISH analysis [7, 8]. Since these studies evaluated a sperm population without taking into account motility, morphology and viability, there is a possibility that the genetic risk associated with round sperm is overestimated on account of contamination by dead and amorphous sperm. On the other hand, the incidence of structural chromosome aberrations was slightly lower in sperm with small heads than with normal heads. Sperm with a small nucleus, containing tightly condensed DNA, may be therefore be resistant to *in vitro* environments responsible for DNA damage.

Large heads

No increase in the incidence of structural chromosome aberrations, which are caused by single- and double-stranded DNA damage, was found in sperm with large heads (>5 μm in length and >3 μm in width). On the other hand, sperm with large heads were associated with a risk of diploidy. We have experienced a case of a patient in which all sperm in the ejaculate had a diploid large head. In such patients, chromosome analysis will be required prior to ICSI. However, the risk was very low in the normozoospermic donors we examined, and accordingly, the total incidence of chromosome aberrations did not differ between sperm with large and normal heads. Therefore, sperm with large heads appear to represent an alternative to sperm with normal heads for ICSI, depending upon the particular circumstances of the case involved. It is not clear how frequent diploid large-headed sperm are spontaneously produced in the testis of hu-

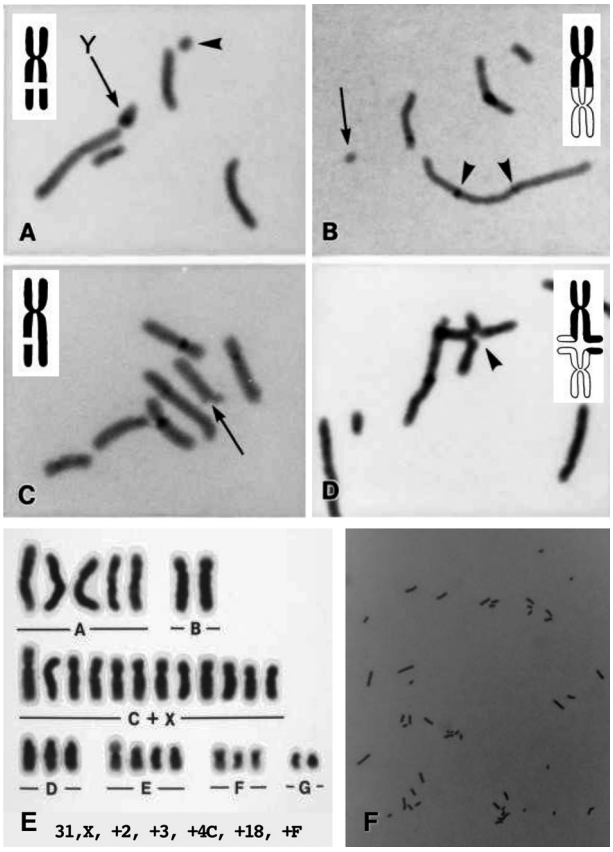

Fig. 2. Types of chromosome abnormality in human sperm. Giemsa-stained human sperm chromosomes formed in murine oocytes around 20 hours after fertilization. A-D shows 4 types of structural chromosome aberrations resulting from DNA breaks. A, Chromosome-type breakage. The arrowhead shows a chromosome fragment caused by a double-strand DNA break. B, Chromosome-type exchange. A dicentric chromosome, which has two centromeres (arrowheads), results from the reconstruction of two chromosomes with a double-strand DNA break. C, Chromatid-type break. A chromatid break (arrow) is derived from a single-strand DNA break that is not repaired. D, Chromatid-type exchange. The arrowhead shows a chromosome reconstructed with two chromosomes having single-strand DNA break. E, Aneuploidy. Thirty-one chromosomes were found in a sperm with a large head. F, Diploidy. Forty-six chromosomes were found in a sperm with a large head. The images in B, E and F were reproduced from our previous paper [6].

mans. Fortunately, this type of aberrant sperm is not predominant in normozoospermic semen and can be easily discriminated from the normal-headed sperm under a comparably low magnification (×200).

Elongated heads

Despite the fact that elongated- and large-headed sperm are the same length, structural chromosomal aberrations were found in 33.3% of the elongated sperm heads (length >5 μm), and their DNA was shredded. This inconsistency between the elongated and large heads highlights the possibility that sperm DNA is packed in normal heads in a manner that retains the nuclear length/width ratio within the normal range (approximately 5:3) and thus avoids collateral damage. Although it is not clear why the DNA of elongated sperm is severely damaged, it has been reported that endogenous DNA nick formation gradually increases during spermiogenesis but is undetectable in mature sperm [9]. Therefore, in the elongated sperm head, DNA nicks may remain, even after the completion of spermiogenesis. Head length is a crucial factor in avoiding fertilization of an oocyte with genetically abnormal sperm. Later in this paper, we provide further evidence to support this fact in infertile men by TUNEL assay [10, 11]. Given the above, elongated sperm are not recommended, even for ICSI treatment. Genetic alteration resulting from a DNA break (s) appears to be maintained and is heritable; this differs from cases of aneuploidy, in which aberrant embryos are mostly aborted during early pregnancy.

Pointed heads

Pointed heads is a morphological abnormality in the acrosomal region, which can be barely distinguished at a magnification of ×400 in normozoospermic sperm. While there was not any significant structural or numerical chromosome aberrations in sperm with this abnormality, we observed a significant reduction (10%) of the oocyte activation rate in mouse oocytes following ICSI. We did not expect that minor acrosomal abnormalities would influence oocyte activation and thus prevent the sperm nucleus from decondensing, because factors responsible for the induction of oocyte activation are localized in the equatorial region of human sperm. Although there is a possibility that human sperm activate mouse and human oocytes in different manners, it is very important to carefully evaluate the morphology of sperm destined for ICSI at a magnification of ×400.

Sperm Head Size

In our previous data (Fig. 3), we noticed that the incidence of structural chromosomal aberrations was slightly lower in sperm with small heads (6.3%) compared with those with normal heads (8.6%), suggesting the possibility that the DNA of sperm with tightly condensed heads is

not comparably damaged.

Therefore, to minimize the risk of choosing sperm with DNA fragmentation for ICSI, the most preferable sperm head size was determined by comparing the frequency of

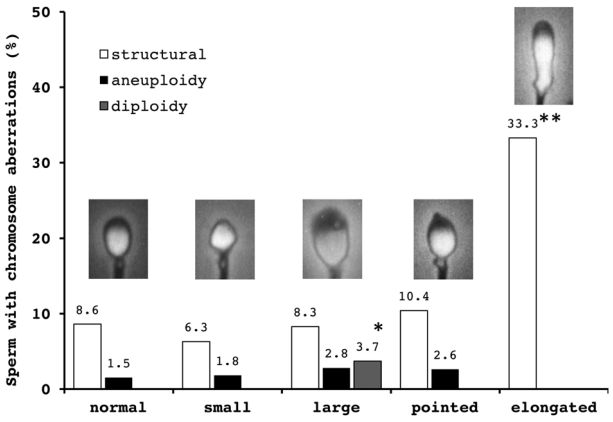

Fig. 3. Relationship between sperm head morphology and chromosome aberrations. The incidence of structural chromosome abnormality, aneuploidy and diploidy was compared among sperm with normal, small, large, pointed and elongated heads obtained from normozoospermic males. *Significant at the $P<0.05$ level. Images of the sperm heads are reproduced from our previous paper [6].

TUNEL-positive cells [11, 12] among 9 groups which were classified based on the length and width of the sperm head (Fig. 4). In 7 normozoospermic sperm samples, the width and length of sperm heads were measured for 100 individual sperm stained with Giemsa stain (G) and sperm that were TUNEL-positive (T). In all samples, 50% or more of the sperm population consisted of normal (N), narrow (Na) and small heads (S). In five cases (donors 1, 2, 3, 5 and 6), the populations of sperm with narrow and normal heads were similar. In three cases (donors 5, 6 and 7), small-headed sperm were predominant. All samples were determined to be normozoospermic by routine microscopic investigations carried out in our hospital laboratory. However, data relating to sperm head sizes measured using video scaling on a PC monitor suggests that many sperm with a slightly narrow head were classified into the normal sperm population. Sperm with DNA fragmentation were mostly those with normal or larger heads (groups E, L, Sh and W) and TUNEL-positive sperm with narrow (Na) or small heads (S) were rarely found. Amorphous sperm can be identified easily under a microscope and thus can be avoided for ICSI. Therefore, we should pay attention to the fact that the risk of selecting a DNA-damaged sperm from the population of sperm with a normal head is higher than from popula-

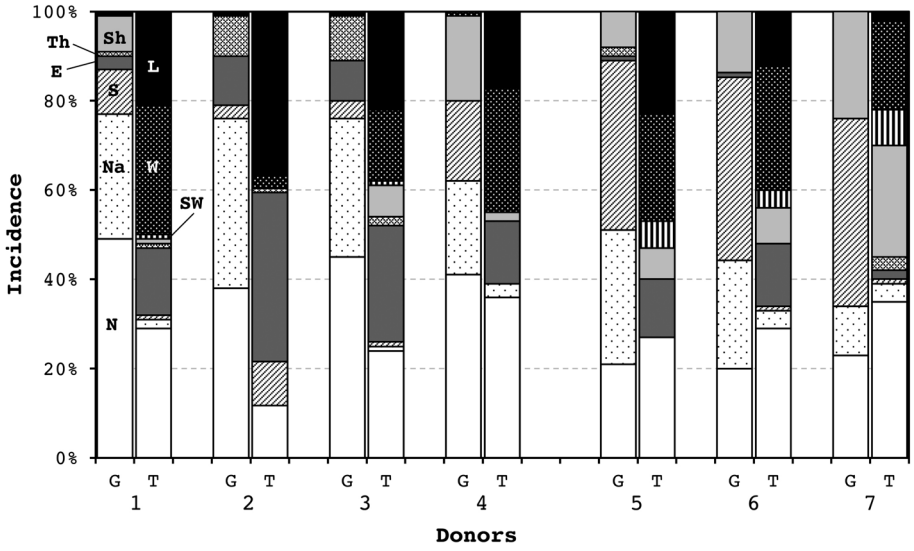

Fig. 4. The relationship between sperm head sizes and DNA fragmentation. In semen samples from 7 normozoospermic IVF patients, we compared the head sizes of 100 sperm stained with Giemsa's solution (G) and 100 DNA-damaged sperm detected by TUNEL assay (T). N, normal (based on WHO criteria [13]); Na, narrow (<2.5 μm in width); S, small (<4 μm in length and <2.5 μm in width); E, elongated (>5 μm in length); Sh, short (<4 μm in length); W, wide (>3.5 μm in width); L, large (>5 μm in length and >3.5 μm in width); Th, Thin (>5 μm in length and <2.5 μm in width); SW, short and wide (<4 μm in length and >3.5 μm in width).

tions with narrow or small heads. This difference in risk is approximately fivefold [11]. The application of narrow- or small-headed sperm in ICSI may reduce the risk of pre- and postimplantation embryo loss caused by sperm chromosomal damage. DNA integrity may be maintained in sperm nuclei packaged more tightly with protamine.

In Vitro Aging

Munne and Estop [14] previously reported that oocyte development is reduced after the penetration of sperm stored *in vitro* for more than 6 h, suggesting that sperm DNA are gradually damaged over time *in vitro*. Therefore, sperm aging appears to be recognized as a factor that prevents the successful treatment of infertile males by IVF. However, *in vitro* culture is a necessary requirement for sperm to induce "capacitation," a vital physiological change required to penetrate oocytes; accordingly, sperm aging appears to be accepted as an inevitable risk. For the same reason, the sperm chromosome assay for IVF cannot assess sperm DNA damage caused in the few hours that it undergoes sperm capacitation. This limitation was removed after Kimura *et al.* [5] applied a piezo-micromanipulator to carry out ICSI into mouse oocytes. Since then, we have successfully utilized this device for the human sperm chromosome assay with ICSI into mouse oocytes [15], and the resultant data has allowed us to investigate the effect of short-term (a few hours) *in vitro* aging on sperm DNA. The results of this analysis are shown in Fig. 5. The incidence of structural chromosome aberrations did not differ between motile and morphologically-normal sperm that penetrated murine oocytes by either IVF and ICSI during 2.5 h after ejaculation (10.6% *versus* 8.6%). However, when the aberration rates were recalculated for every 30 mins of prior incubation for ICSI, we identified a remarkable increase of the aberration rate at 30 and 120 mins (Fig. 5B). This data shows that there are three types of motile normal sperm with genetic fragility in ejaculated semen. The first is the sperm in which DNA was damaged in the testis or the epididymis. These are detectable using the sperm chromosome assay 30 min after sperm preparation and accounted for 3.3% of normal motile sperm. The second is the sperm in which DNA was newly fragmented at 30 min after sperm preparation; this population increases the population of sperm with DNA fragmentation to approximately 10%. This phenomenon is caused by the removal of seminal plasma or exposure to culture medium. Evidence for this is also described in the section relating to seminal plasma herein. The third group is sperm in which DNA was fragmented at more than

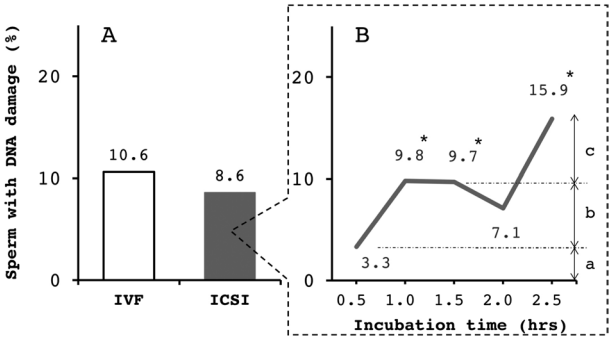

Fig. 5. Time-dependent increase of structural chromosome aberration in motile and morphologically normal human sperm. A, The total incidence of DNA-damaged sperm in IVF and ICSI methods in a normozoospermic donor. B, Alterations of the aberrant sperm incidence. a, sperm DNA initially damaged; b, sperm DNA damage resulting from potential membrane damage; c, sperm DNA damage newly produced *in vitro*. *Significant at the $P < 0.05$ level.

2 h after sperm preparation, creating a second peak of the DNA fragmentation rate (15.9%). This phenomenon could be akin to the process of "*in vitro* aging" previously reported for murine sperm, which resulted from the effect of active oxygen species produced by sperm movement [16]. The individual variation in DNA damage which was demonstrated by the sperm chromosome assay with IVF may arise from the difference in sperm sensitivity to the removal of seminal plasma and short-term *in vitro* aging. This result provides us with valuable information indicating that sperm preparation protocols generally used in the IVF laboratories are not necessarily suitable for sperm applied for ICSI.

Immotile Sperm

In cases of severe asthenozoospermia, embryologists may consider the use of immotile sperm with normal heads for ICSI. In general, the hypoosmotic swelling test (HOST) is the recommended method with which to distinguish live cells, which involves the swelling of sperm in a hypoosmotic solution.

A combination of ICSI and the sperm chromosome assay enables us to analyze the genetic integrity of immotile sperm. Previously, an author of this study revealed a mechanism for producing DNA damage in immotile sperm [17]. Surprisingly, of the immotile sperm directly selected from normozoospermic donor ejaculates, only 4.5% were genetically abnormal, although 48% were dead cells whose nuclei were stained red with 0.1%

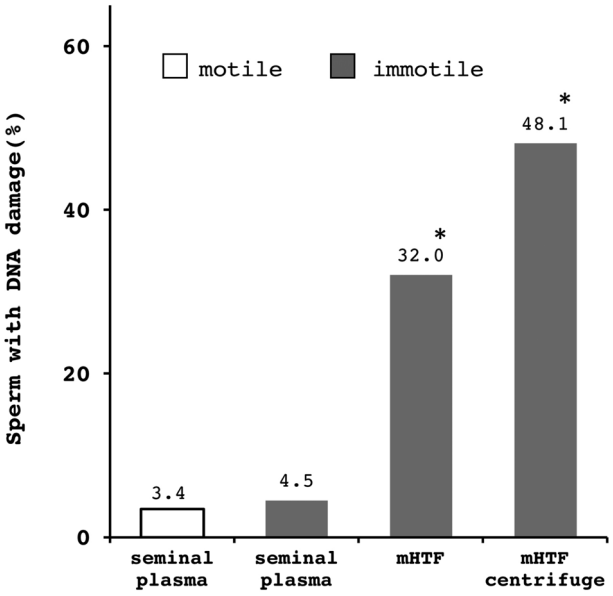

Fig. 6. An increase of DNA damage in immotile normal-shaped sperm. Initially, almost all immotile sperm were genetically normal (95.5%). In some of these, however, DNA was quickly damaged by the culture medium and centrifugation. *Significant at the $P<0.05$ level.

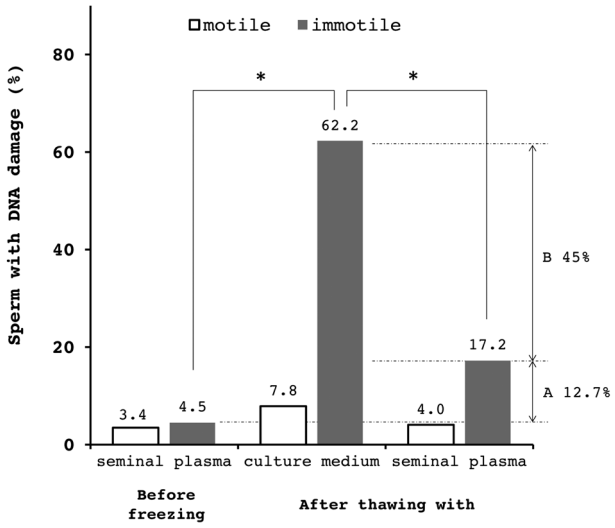

Fig. 7. Inhibitory effect of seminal plasma upon DNA damage in immotile normal-shaped sperm. The seminal plasma completely, or efficiently, inhibited DNA damage in motile or immotile sperm, respectively. A, sperm with DNA directly damaged by ice crystals; B, sperm with DNA damaged by the thawing process due to potential membrane damage. *Significant at the $P<0.05$ level.

eosin-Y solution due to the poor integrity of the plasma membrane. In contrast, when immotile sperm were transferred and stored in several types of culture media for approximately 2 h, the incidence of structural chromosome aberration increased up to 32%. Moreover, centrifugation with a culture medium, a technique generally performed to remove seminal plasma in ART, resulted in DNA damage in 48.1% of immotile sperm (Fig. 6). This result shows that both dead and live immotile sperm mostly maintain their DNA integrity in seminal plasma, although the DNA of dead sperm is vulnerable to the culture media used to remove the seminal plasma. The osmotic pressure of seminal plasma is higher (over 300 mOsm) than those (270–280 mOsm) of commercial media generally used for ART. It is likely that sperm with poor membrane integrity cannot absorb the influx of water and ions required to equilibrate the difference in osmotic pressure, thus resulting in DNA damage. This is supported by the proven fact that the influx of sodium ions caused DNA lesions in mouse sperm when their plasma membranes were artificially damaged by sonication [18]. Accordingly, in cases of severe asthenozoospermia, the live/dead sperm ratio provides an indicator for predicting the results of subsequent ART treatment. If the live sperm ratio is high, HOST is efficient in selecting genetically normal sperm. In contrast, when almost all sperm are dead, the use of HOST does not make sense because the hypotonic solution quickly destroys their DNA.

Efficiency of the Seminal Plasma

As mentioned above, our data showed that culture media induces DNA damage in both motile and immotile sperm. This means that the seminal plasma is an excellent preservative material that can prevent sperm DNA damage. In the seminal plasma, the incidence of structural chromosome aberrations in the motile and immotile sperm remained less than 5% for at least 3 h after ejaculation (Fig. 6). Therefore, an attempt was made to confirm whether DNA damage caused by freezing and thawing (F/T) operations is suppressed within the seminal plasma [17]. Cryopreserved semen samples were thawed and then centrifuged with a culture medium or seminal plasma. The results of our chromosomal analysis are shown in Fig. 7. The incidence of DNA damage in the F/T immotile sperm increased to 62.2% after thawing in culture medium and was higher than with fresh immotile sperm (Fig. 5, 48.1%). Therefore, 14.2% (62.2–48.1%) of F/T immotile sperm appears to be injured accidentally by the formation of ice crystals during the freezing process. In contrast, the seminal plasma significantly suppressed DNA damage and rescued 45% (62.2–17.2%) of the F/T immotile sperm, although DNA received irreversible inju-

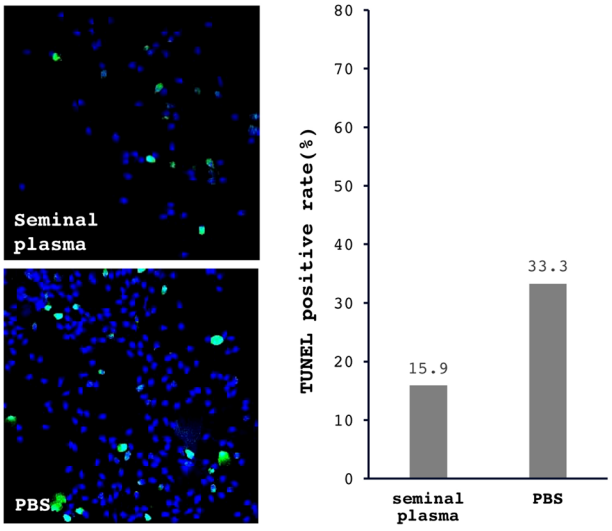

Fig. 8. The incidence of TUNEL-positive sperm in an IVF patient sperm sample before and after the removal of the seminal plasma.

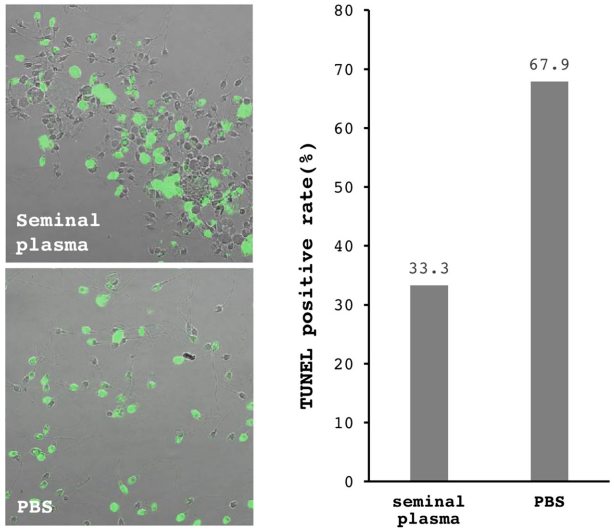

Fig. 9. The incidence of TUNEL-positive sperm in an ICSI patient who experienced repeated failures of ICSI treatment. It was difficult to find genetically normal sperm after the removal of seminal plasma.

Table 1. The success rate of SP-ICSI and ICSI

	SP-ICSI	ICSI	
Oocytes penetrated	59	61	
Oocyte fertilized	36/59 (61.0%)	38/61 (62.3%)	NS
Excellent quality embryos at day 3	25/28 (89.3%)	25/27 (92.6%)	NS
Embryos transferred or cryopreserved	15/28 (53.6%)	15/27 (55.6%)	NS
Fresh ET cycles	7	7	
Pregnancy rate	3 (42.9%)	2 (28.6%)	NS

ry due to ice crystals in 12.7% of the F/T immotile sperm (17.2–4.5%). The rates of DNA-damaged immotile sperm caused by ice crystals were very similar when compared between the medium and seminal plasma groups. The above indicates that immotile sperm samples after the F/T operation consist of cells whose DNA and plasma membrane were intact and cells with intact DNA and a fragile plasma membrane. Thus, the seminal plasma protects the fragile plasma membrane, which probably allows water and ions to influx and injure sperm DNA.

The effect of the seminal plasma and culture media upon sperm DNA damage shown in this review appears to affect other sperm DNA assays, such as TUNEL, HALO and Acridine orange tests. A typical sperm sample presented with an increase in the number of TUNEL-positive cells before and after the suspension of sperm in PBS, as shown in Fig. 8. When a denaturing process with acetic acid is additionally combined, the rate of TUNEL-positive cells increases further. Therefore, it is important to consider the difference between initial and potential DNA damage when applying cytogenetic sperm analysis.

Intracytoplasmic Injection of Sperm Selected from the Seminal Plasma

Based on the effect of seminal plasma upon sperm DNA, ICSI of sperm selected from the seminal plasma (abbreviated as SP-ICSI) was attempted in the hospital of our institute. The results are shown in Table 1. In the SP-ICSI group, sperm selected from normozoospermic patient ejaculates were transferred into a 7% PVP droplet to remove the seminal plasma by gentle pipetting for a few seconds and then injected into oocytes. In the normozoospermic patient ejaculates used, we found no difference in either oocyte development or implantation when compared between conventional ICSI and SP-ICSI groups, suggesting that SP-ICSI is as safe as conventional ICSI and that sperm washing and swim-up proce-

dures are not necessary. For infertile patients with poor semen quality, SP-ICSI will be more effective, although more data is required to confirm this. We have experienced the case of a severe asthenozoospermic patient who produced only short-tailed sperm and the case of a normozoospermic patient who has repeatedly received ART treatments; both were able to obtain normal cleavage embryos using SP-ICSI. We also found via the live/dead sperm test and TUNEL assay that most sperm from these patients were damaged after sperm washing (Fig. 9).

Sperm Vacuoles

Since the ICSI of sperm which are morphologically selected under very high magnification (IMSI, ×6,600 magnification) was introduced [19–21], increasing attention has been focused upon the relationship between large vacuoles on the sperm heads and ICSI outcome. During IMSI, sperm are selected based on several factors determined by the motile sperm organelle morphology examination (MSOME), and accordingly, it is not clear what factor is mostly responsible for ICSI outcome. However, these IMSI studies were misleading if large sperm head vacuoles caused a significant decrease of ICSI outcome, although amorphous sperm with large vacuoles were actually used in the study. As shown above, it has already become clear that amorphous sperm heads strongly correlate to DNA damage [6, 22]. Therefore, we conducted a sperm chromosome assay and TUNEL assay [10] to examine the potential influence of large vacuoles. The above indicates that a large vacuole found in motile and oval-shaped sperm did not relate to an increase in the rate of DNA fragmentation in the normozoospermic IVF or ICSI patients examined. We also noticed that such large vacuoles are visible at x400 magnification, which is the standard lens combination equipped on the inverted microscopes generally used for ICSI. In addition, a few small vacuoles were found in more than 90% of normal sperm, whereas the aberration rates shown by TUNEL and chromosome assays were very low (3.5% and 4.1%, respectively). Therefore, we cannot see the advantage of spending a long time looking for sperm head vacuoles under very high magnifications in semen samples from normozoospermic patients. Our data indicated the possibility that SP-ICSI is highly effective in improving ICSI outcome in severely infertile male patients.

Conclusion

Using the sperm chromosome assay with ICSI revealed detailed characteristics of DNA damage in human sperm. Our results seem to lead to the conclusion that sperm of any type of semen quality should be stored in seminal plasma to maintain DNA integrity prior to ICSI. On the other hand, there is also an advantage of using SP-ICSI to reduce processing times and the use of consumables for sperm preparation. As a next step, it is necessary to demonstrate the advantages of using narrow sperm over normal sperm in clinical applications.

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