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Risk of chromosomal aberration in spermatozoa during intracytoplasmic sperm injection

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Abstract. Intracytoplasmic sperm injection (ICSI) has become critical for the treatment of severe male infertility. The principal feature of ICSI is the direct injection of spermatozoon into an oocyte, which facilitates the production of fertilized embryos regardless of semen characteristics, such as sperm concentration and motility. However, the chromosomal integrity of ICSI zygotes is degraded compared to that of zygotes obtained via *in vitro* fertilization. This chromosomal damage may occur due to the injection of non-capacitated, acrosome-intact spermatozoa, which never enter the oocytes under natural fertilization. Furthermore, it is possible that the *in vitro* incubation and pre-treatment of spermatozoa during ICSI results in DNA damage. Chromosomal aberrations in embryos induce early pregnancy losses. However, these issues may be overcome by embryo production using gametes with guaranteed chromosomal integrity. Because conventional chromosome analysis requires fixing cells to obtain the chromosome spreads, embryos cannot be produced using the nucleus that has been analyzed. On the other hand, genome cloning using androgenic or gynogenic embryos provides an additional nucleus for chromosome analysis following embryo production. Thus, this review aims to highlight the hazardous nature of chromosomal aberrations in sperm during ICSI and to introduce a method for the prezygotic examination for chromosomal aberrations.
Key words: Chromosomal aberration, Diagnosis, Intracytoplasmic sperm injection, Spermatozoa

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Since the first report of a healthy baby being obtained via *in vitro* fertilized (IVF) embryos [1], assisted reproductive technology (ART) has advanced greatly and spread globally. It has now become possible to manipulate gametes *in vitro* and produce embryos in various species. In the early to mid-90s, successful embryo production after intracytoplasmic sperm injection (ICSI) was reported in bovines [2], mice [3], and humans [4]. A significant benefit of ICSI is the ability to produce embryos regardless of sperm motility and sperm count. Therefore, ICSI is imperative for the treatment of male infertility, allowing infertile couples to have children. The number of treatment cycles in which embryos are produced via ICSI continues to increase in human ART [5]. However, in many countries, the frequency of birth from *in vitro*-produced embryos does not exceed 30% [5] and the situation is similar in farm animals. The main cause of embryo loss may be chromosomal aberrations in the embryos, because these damaged embryos might be produced from gametes with chromosomal damage. Alternatively, chromosomal aberrations are also generated in a part of the blastomere during cleavage [6, 7] owing to chromosome non-disjunction. In ICSI, spermatozoa for injection are picked by an operator based on ambiguous criteria, which include sperm morphology and motility. During the injection procedure, acrosomal membranes and enzymes are frequently injected

with the spermatozoa into oocytes; however, they never penetrate the plasma membrane of oocytes during conventional fertilization. Large amounts of acrosomal enzymes disturb the progression of fertilization [8, 9]. Moreover, a number of embryos produced by ICSI have been reported to contain chromosomal aberrations [10–12]. Additionally, there is an increased risk of producing aneuploid embryos, in which the second polar body is not normally extracted due to the destruction of the metaphase II (MII) spindle during sperm injection [13, 14]. Therefore, fertilization under these "unnatural" conditions may induce the production of embryos with damaged chromosomes, leading to the loss of embryos.

It was recently reported that spermatogenesis was achieved *in vitro* using organ culture in a mouse model [15–18]. Furthermore, successful gamete production from pluripotent stem cells was also reported in spermatozoa [19] and oocytes [20, 21]. These advancements represent a viable option for providing gametes as genetic resources for the field of reproductive biology. However, it is necessary to fully understand whether the produced gametes possess genetic/chromosomal integrity.

Thus, this review focuses on the risk of chromosomal aberration in spermatozoa that potentially exists with the ICSI and introduces a technique for prezygotically analyzing the chromosomes of gametes before embryo production.

Characteristic Changes in the Sperm Nucleus during ICSI

At the spermatid stage, DNA is associated with protamines instead of histones [22]. An acrosomal cap also appears in an adjacent area of the spermatid nucleus. Thereafter, a flagellum grows to function

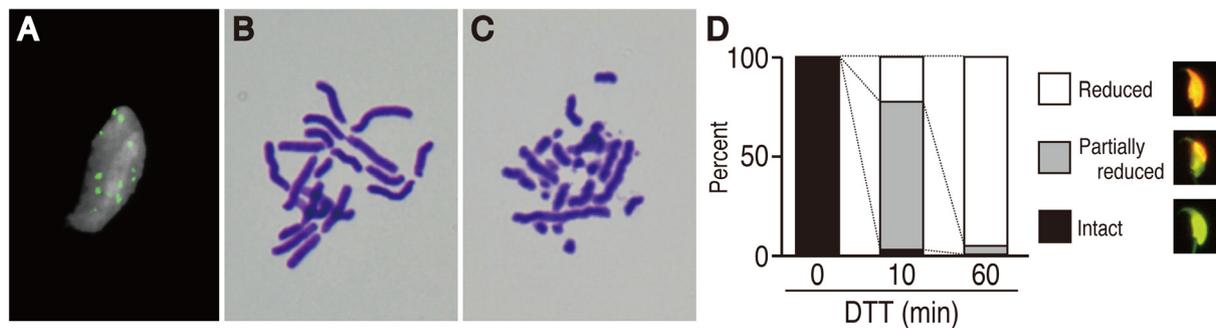Correspondence: H Watanabe (e-mail: h-wata@asahikawa-med.ac.jp)

Fig. 1. Sperm chromatin remodeling and paternal chromosome spreads. (A) γH2AX foci (green) of the decondensed sperm head (white) at 90 min after intracytoplasmic sperm injection (ICSI). Before ICSI, spermatozoa were exposed to 4 Gy γ-rays to induce DNA damage. (B) Normal chromosome spreads of ICSI zygotes (n = 20). (C) Paternal chromosome spread with numerous chromosome breaks. The chromosome spread was obtained from ICSI zygotes injected with dithiothreitol (DTT)-treated spermatozoa (5 mM, for 60 min). (D) Treatment time-dependent changes in the disulfide bonds in DTT-treated spermatozoa (5 mM, for 0, 10, and 60 min). When spermatozoa were treated with DTT for 10 min, 74% of them had a sperm head with partially reduced disulfide bonds. Spermatozoa were stained with acridine orange; the region in which disulfide bonds were reduced is indicated in red (adapted from Watanabe *et al*. [33]).

as the tail of the sperm, and almost all of the cytoplasm is extruded from the cell, differentiating it into a spermatozoon. The cross-links formed by protamine disulfide bonds are accompanied by the passage of the sperm from the caput to the cauda epididymis [23–25]. This unique form confers a significant advantage when spermatozoa migrate through the female genital tract. On the other hand, nucleic activities such as DNA synthesis and repair are markedly restrained. If the spermatozoa acquire DNA damage, then the damage accumulates in their nucleus and is carried into oocytes through fertilization.

Spermatozoa undergo morphological changes in the cytoplasm of oocytes via a process called chromatin remodeling. Sperm nuclei decondense, recondense, and finally form male pronuclei. Nuclear decondensation is accompanied by the replacement of protamines with histones, following the reduction of disulfide bonds by the action of ooplasmic glutathione. This event begins about 30 min after ICSI [26, 27]. Decondensation of the sperm chromatin allows contact with ooplasmic DNA repair enzymes. In fact, foci of phosphorylated histone (γH2AX), a marker of DNA double-strand breaks, were found in mouse zygotes 80 min after IVF [28] and 90 min after ICSI (Fig. 1A). Additionally, androgenic 1-cell embryos were used to determine that sperm chromatin remodeling is catalyzed by an ooplasmic topoisomerase II that cleaves and rejoins double-stranded DNA to remake the topological state [29]. When the androgenic 1-cell embryos were exposed to topoisomerase II inhibitors during sperm chromatin remodeling, at least half of the embryos displayed structural chromosomal aberrations at the first mitotic metaphase. Particularly, exposure to the inhibitor 2–4 h after sperm injection drastically increased the chances of chromosomal aberrations to approximately 99%. Thus, the early stage of sperm chromatin remodeling is a crucial period to not only achieve fertilization but also ensure genetic normality.

Risk of Chromosomal Aberrations in Spermatozoa Present in ICSI

During the ICSI procedure, sperm pretreatment is often performed to mimic the conditions of natural fertilization and to support the progression of fertilization events. For example, induction of sperm capacitation [30] or the acrosome reaction [9, 31–33] prior to ICSI was shown to improve the fertilization and/or development of the resultant zygotes. On the other hand, studies on mouse models revealed that the chromosomal integrity of zygotes derived from ICSI without any pretreatment of spermatozoa was impaired in comparison with that of zygotes derived from conventional IVF [12, 34, 35]. The damage was reduced by injecting spermatozoa cultured with bovine serum albumin or methyl-β-cyclodextrin [12, 35], which triggered sperm capacitation due to cholesterol efflux on the plasma membrane [36, 37]. Similarly, the frequency of chromosomal aberrations originating from sperm was low when spermatozoa were treated with calcium ionophore A23187 to induce the acrosome reaction [35]. These findings suggested that injection of non-capacitated and acrosome-intact spermatozoa resulted in the generation of chromosome-damaged embryos.

To reduce the risk of chromosomal aberrations during the ICSI procedure, it is likely important that sperm capacitation and the acrosome reaction are artificially induced in the appropriate medium before use. The incidence of chromosomal aberrations in ICSI zygotes derived from spermatozoa incubated in TYH (Toyoda-Yokoyama-Hosi; [38]) medium (commonly used for sperm capacitation) was shown to decrease to the same level as that in IVF zygotes [12]. However, there was no reduction in chromosomal aberrations in ICSI zygotes when spermatozoa were incubated in CZB (Chatot-Ziomek-Bavister; [39]) medium (commonly used for embryo culture) [12]. *In vitro* incubation of spermatozoa in HEPES-buffered and phosphate-buffered media was shown to significantly increase chromosomal aberrations in the subsequent ICSI zygotes; this increase was found to be directly correlated with the duration of sperm incubation [12, 33, 34]. These results suggested that the medium components and ion balance affected chromosomal integrity during the incubation of spermatozoa and that excessive sperm pretreatment generated DNA lesions in spermatozoa and impaired the genetic integrity of the subsequent zygotes. The detrimental effect of *in vitro* incubation on sperm

DNA lesions might be caused by endogenous nucleases released from membrane-damaged spermatozoa [40]. It is possible that the incubation of sperm nuclei in HEPES-buffered or phosphate-buffered media altered the structure of sperm chromatin, leading to incomplete chromatin remodeling by ooplasmic topoisomerase II. Meanwhile, it was reported that testicular spermatozoa were unaffected by long *in vitro* incubations in these media [41]. Although this resistance to DNA damage may arise from minor cross-links by protamine disulfide bonds in immature (testicular) spermatozoa, inconsistent results have been obtained in mature (cauda epididymal) spermatozoa with artificially reduced protamine disulfide bonds, as discussed below.

Dithiothreitol (DTT) has been frequently used for the pretreatment of mature spermatozoa to reduce protamine disulfide bonds to thiol groups [42–47], as one of the main causes of unsuccessful fertilization is the failure of sperm nuclear decondensation or male pronuclear formation post-ICSI [48, 49]. However, some studies have reported that DTT treatment is not always effective for embryo development [50, 51], implying that sperm pretreatment with DTT induces chromosomal damage. Although DTT is a non-DNA-interacting agent, excessive exposure of mouse spermatozoa to DTT (e.g., 5 mM DTT for 60 min) induced severe DNA damage in paternal pronuclei; many chromosomal breaks were found in some metaphase spreads [33] (Fig. 1B and C). Szczygiel and Ward [52] also reported that the majority of spermatozoa treated with DTT (2 mM) and Triton X-100 (0.5%) for 15 min had chromosomal aberrations. It was demonstrated that the weakly protaminated human spermatozoa had DNA lesions [53–55]. Mouse spermatozoa freeze-dried in DTT exhibited decreased chromosomal integrity in ICSI zygotes [56]. In contrast, the rate of chromosomal aberrations decreased in testicular spermatozoa freeze-dried with a thiol-oxidizing agent [56]. It seems that there is a close relation between the number of protamine disulfide bonds and chromosomal stability in mature spermatozoa. However, the characteristic features of chromosomal stability in immature spermatozoa, which possess minor cross-links by protamine disulfide bonds, remain to be fully elucidated.

It was reported that spermatozoa treated with DTT and Triton X-100 began to decondense along the whole length of the dorsal side of the sperm head, whereas sperm decondensation in non-treated spermatozoa began at the basal side of the sperm head [27]. In agreement with this result, protamine disulfide bonds were initially reduced at the dorsal side of the sperm head post DTT treatment (Fig. 1D). Because treatment with DTT at an adequate concentration and duration is effective in improving fertilization after ICSI, the relationship between chromosomal damage and unusual sperm nuclear decondensation should be clarified.

The developmental competence of chromosome-damaged embryos depends on the degree of damage. When the chromosomal damage is minimal, the embryos can develop to term. However, embryos with moderate chromosomal damage are lost early in pregnancy, whereas severe damage does not support embryo development up to the blastocyst stage. When chromosomal damage in spermatozoa was generated by exposure to γ-ray irradiation (5 Gy), the developmental competence of the embryos to the blastocyst stage decreased to approximately half of that of the control (non-irradiated) group, and the rates of blastocyst formation were found to be negatively correlated with the radiation dose [57, 58]. On the other hand, chromosomal damage in spermatozoa derived from excessive DTT treatment did not affect fertilizability and developmental competence to the blastocyst stage but decreased fetal development [33]. This result suggested that the quality of embryos could not be predicted precisely by their developmental competence, at least up to the blastocyst stage. Therefore, methods to identify embryos without any genetic damage need to be established.

Prezygotic Detection of Chromosomal Aberrations in Gametes

Chromosome analysis is classically performed at the first mitotic metaphase of 1-cell embryos to determine their genetic integrity and/or constitution. In this stage, paternal and maternal nuclei in an embryo can be separately analyzed [59]. More recently, molecular diagnostic methods such as comparative genomic hybridization have been predominately used for the selection of competent embryos, especially in human ART (reviewed in Harper and SenGupta [60]). However, this method is invasive because a part of the embryo (polar bodies and blastomeres) is used for the analysis. When the embryos are determined to be chromosomally aberrant, there is currently no way to rescue the embryo from the genetic defect. The genetic constitution of an oocyte can be easily predicted from that of a polar body, which is a sister cell of the oocyte. In contrast, it is not possible to estimate the genetic constitution of spermatozoa because no counterpart cells are available during sperm chromosome analysis.

Because genetic analysis requires the killing (or fixing) of cells, it is difficult to produce embryos using the analyzed gametes. Successful prezygotic chromosome examination through genome cloning of gametes has been reported [61, 62], as shown in Fig. 2. Genome cloning was efficiently performed using haploid androgenic and gynogenic 2-cell embryos for both spermatozoa [63–66] and oocytes [67] (Fig. 2A), respectively, thereby allowing the use of one sister blastomere for chromosome analysis (Fig. 2B) and the other for embryo production (Fig. 2C). The sister blastomeres of androgenic and gynogenic 2-cell embryos enabled chromosome analysis because the nuclei of the 2-cell embryos were in a synchronized and the comparatively longer G2 stage of the cell cycle. A good chromosome spread could be rapidly obtained from the G2 nucleus by premature chromosome condensation (PCC) induced by cell fusion with an unfertilized MII oocyte [68] or exposure to calyculin A, a specific inhibitor of type 1 and 2A protein phosphatases [69, 70]. Unfortunately, androgenic and gynogenic 4-cell embryos are not available for chromosome analysis using PCC because the cell cycles of the blastomeres are asynchronous [61]. Blastomeres of androgenic and gynogenic 2-cell embryos can be used as the genetic resource to produce biparental embryos [66, 67, 71, 72]. When blastomeres of androgenic and gynogenic 2-cell embryos were fused, chromosomes from each blastomere were assigned to the same metaphase plate and distributed normally to the daughter cells [62]. This prezygotic chromosome examination using androgenic and gynogenic 2-cell embryos is the only method to directly/simultaneously evaluate the chromosomal integrity of both gametes and the developmental competence of the resultant embryos.

Prezygotic chromosome examination enables the elimination of embryos with chromosomal aberrations inherited from both
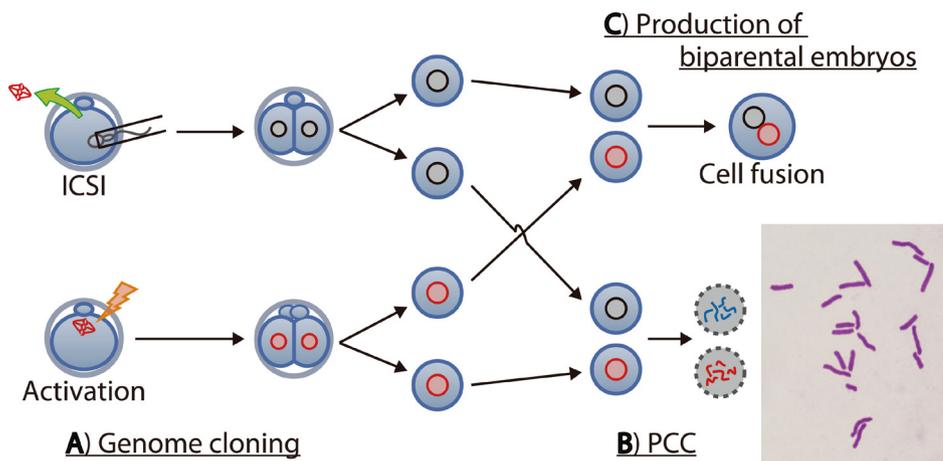
Fig. 2. A flowchart of prezygotic chromosome examination. (A) Genome cloning of gametes using androgenic and gynogenic embryos. (B) Rapid visualization of chromosomes using premature chromosome condensation (PCC). PCC was induced by the fusion of a blastomere with a fresh metaphase II oocyte or by the exposure of a blastomere to calyculin A. (C) Production of biparental diploid embryos. These embryos were capable of developing to term. These procedures enable the prezygotic analysis of the chromosomal integrity of gametes and the production of embryos from gametes with a known chromosomal constitution (revised from Watanabe *et al.* [61, 62]).

gametes. Vichera *et al.* [66, 67] reported the successful selection of bovine embryos expressing a transgene using the blastomeres of androgenic or gynogenic embryos, followed by the reconstruction of biparental embryos. Prezygotic examination of the genetic constitution of zygotes theoretically enables the production of "genetically designed" embryos for animal breeding. However, many problems regarding the safety and ethical aspects need to be resolved before its application in human ART.

Conclusions

Spermatozoa acquire DNA damage from various factors even before ejaculation [73]. Once the spermatozoa are extracted to the culture media, further damages accumulate in the DNA without being repaired. Unfortunately, DNA damage in spermatozoa due to culture media (i.e., medium components and ion balance) is unavoidable, suggesting that the currently used media require improvement. During the ICSI procedure, injection of spermatozoa in an "unnatural" condition (non-capacitated and acrosome-intact) is one way through which embryos with chromosomal aberrations are generated. It is clear that even non-DNA-interacting agents, such as DTT, can cause breaks in sperm DNA. These damages may be induced by the unusual remodeling of sperm chromatin. However, how the sperm DNA is damaged remains to be determined. Notably, embryos with chromosomal aberrations can develop to the blastocyst stage with acceptable frequency, suggesting that the fate of an embryo cannot be predicted by its developmental competence at least up to the blastocyst stage. Given these issues, it is essential that gametes with chromosomal aberrations be excluded before embryo production. Prezygotic chromosome examination is a powerful tool to produce embryos without any chromosomal damage and to elucidate the relationship between embryo development and chromosomal damage.

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