Development of golden hamster embryos effectively produced by injection of sperm heads sonicated in Tris-HCl buffer with EGTA

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Abstract
Purpose: To investigate the effects of sperm treatment medium—TCM199 or EGTA in Tris-HCl buffer (TBS + EGTA)—for sonication of frozen-thawed hamster spermatozoa in terms of sperm chromosome integrity and development of hamster oocytes injected with the sperm heads (ICSI).

Methods: Frozen-thawed hamster spermatozoa were separated into heads and tails by sonication in TCM199 or TBS + EGTA. Sperm heads were injected into mouse oocytes to assess hamster sperm chromosomes. We further compared the development of hamster ICSI embryos produced by injecting sonicated sperm heads in TCM199 vs TBS + EGTA.

Results: Sperm chromosome integrity was greater following sonication of frozen-thawed hamster spermatozoa in TBS + EGTA than in TCM199 (89.7% vs 69.0%). Embryonic development was improved following hamster oocyte injection with sperm heads sonicated in TBS + EGTA compared to in TCM199 (8-cell: 84.1% vs 65.4%; morula: 78.4% vs 43.2%; blastocyst: 42.0% vs 17.3%). Gene expression of zygotic genome activation in 2-cell embryos was significantly higher with TBS + EGTA than with TCM199. We transferred 43 morulae/blasto cysts from the TBS + EGTA group to foster mothers, and 4 (9.3%) developed into live offspring.

Conclusion: These results showed that the rapid injection of hamster sperm heads separated by sonication in TBS + EGTA effectively produced more ICSI embryos during a short time.

KEYWORDS
Hamster, intracytoplasmic sperm injection, live offspring, sonication, sperm chromosome

1 | INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a method of producing fertilized oocytes. The first application of ICSI in mammals was performed with golden hamster in 1976. Since then, ICSI has successfully been performed to produce live offspring in many species, including humans. In human infertility clinics, ICSI is a powerful tool to overcome male infertility and produce fertilized oocyte.

We previously reported the first birth of live offspring following ICSI in golden hamsters. Shortly thereafter, Haigo et al demonstrated that injection of hamster round spermatids into mature oocytes can produce live offspring. Later, Muneto and Horiuchi...
reported that injection of oocytes with freeze-dried spermatozoa produced hamster embryos that could develop into live offspring after embryo transfer. However, only a low percentage of ICSI embryos exhibit in vitro and in vivo development.

Hamster oocytes are sensitive to short-wave visible light emitted from ordinary fluorescence light sources, such that oocytes injected with spermatozoa under fluorescence light never develop beyond the 2-cell stage.\(^5\)\(^7\)\(^9\) In hamster zygotes, fluorescent light reportedly increases reactive oxygen species (ROS).\(^9\)

Moreover, intracytoplasmic injection of fresh sperm heads with intact acrosomes dissolves the cytoplasm of hamster oocytes, leading to oocyte death.\(^4\)\(^10\) Successful hamster ICSI requires the removal of acrosomes from spermatozoa. After injection of hamster or rabbit acrosome-intact sperm heads into mouse oocytes, extrusion of the second polar body is followed by degeneration of the mouse oocyte cytoplasm, and cleavage never occurs.\(^10\) On the other hand, when a single acrosome-intact spermatozoon is injected during human and mouse ICSI, the released acrosome enzymes do not cause any serious problem.\(^11\)

Another complicating factor is that golden hamster oocytes show a high rate of spontaneous activation.\(^12\)\(^13\)\(^14\) Therefore, in hamsters, ICSI should be performed as soon as possible after ovulated oocyte collection. Yamauchi et al\(^4\) produced fertilized eggs by injecting acrosome-free sperm heads that were separated from frozen-thawed spermatozoa using a piezo pulse. However, compared to mouse spermatozoa, hamster spermatozoa show a relatively strong "neck" connection between the head and midpiece, such that it takes several minutes to detach sperm heads using a piezo pulse.

Sonication is an easy method for separating sperm heads from tails prior to ICSI. In early studies of hamster ICSI, Yanagida et al and Katayose et al used sperm heads detached by sonication.\(^15\)\(^16\) They observed pronuclear formation following sperm head injection after heating or freeze-drying, but did not demonstrate the developmental competence of zygotes following sonicated sperm head injection. In contrast, Kuretake et al\(^17\) performed mouse ICSI and showed higher percentages of blastocysts and normal offspring development following intracytoplasmic injection of sperm heads that were sonicated in nuclear isolation medium (NIM; a K-rich and EDTA-containing medium). Tateno et al demonstrated that sonication per se is not deleterious to sperm chromosomes, but that sperm suspension medium is easily damaged by sonication and chromosomal abnormalities can be caused by activation of Ca\(^2+\)-dependent DNase adjacent to the sperm chromosomes.\(^18\)

The solution used to freeze-dry spermatozoa without chromosomal aberrations is also an attractive choice of solution for sonication. Suspension of mouse spermatozoa in a simple Tris-HCl-buffered solution containing 50 mM EGTA and 50 mM NaCl enables maintenance of chromosome integrity during freeze-drying or freezing without cryoprotection.\(^19\) Furthermore, Kusakabe et al\(^20\) showed that prior incubation of mouse spermatozoa in Tris-HCl-buffered solution containing only 50 mM EGTA (without NaCl) increases the chromosomal normality after freeze-drying and that these spermatozoa can support embryo development. However, it is presently unclear whether this solution can reduce chromosomal aberrations in sperm heads separated by sonication.

In the present study, we aimed to examine how the solution used during frozen-thawing and sonication influenced hamster spermatozoa integrity, and the development of hamster zygotes produced by injecting sonicated sperm heads into oocytes. We also analyzed the expression of maternal effect genes (MEGs) and zygotic gene activation (ZGA) genes in the ICSI embryos.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and animals

Inorganic salts were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Nacalai Tesque Inc (Kyoto, Japan). Organic reagents were purchased from Sigma-Aldrich unless otherwise stated.

Golden hamsters (Mesocricetus auratus) with brown coats and black eyes were purchased from Japan SLC Inc (Shizuoka, Japan). Mature females of 2-4 months of age were used for the collection of unfertilized oocytes. Mature males of 2-5 months of age were used for the collection of motile spermatozoa. All hamsters were fed a standard diet ad libitum and maintained in a temperature- and light-controlled room at 25°C, with a 14-hour light/10-hour dark cycle (light starting at 06:00 hours). The experiments were approved by the Committee for Ethics on Animal Experiments of the Prefectural University of Hiroshima, Japan (16SA002).

### 2.2 | Oocyte collection

Golden hamster females were induced to superovulate via an im injection of 20 IU eCG (Asuka Pharmaceuticals, Tokyo, Japan) during the morning of the day of postestrus discharge.\(^21\) At 56 hours after eCG injection, the hamsters received an im injection of 20 IU hCG (Asuka Pharmaceuticals, Tokyo, Japan).\(^22\) At approximately 15 hours hCG injection, mature unfertilized oocytes were collected from oviducts and were freed from cumulus cells by a 1-minute treatment with 250 IU/mL hyaluronidase in M2 medium.\(^23\) Next, the oocytes were rinsed with TCM199 TE and stored in fresh medium. All experiments were performed in a dark room with a small table light, and red filters were used on the microscope light source, as previously reported.\(^4\)\(^6\)

### 2.3 | Sperm preparation

From mature male hamster (2-5 months of age), a dense sperm mass was collected from the cauda epididymis. A small drop of this sperm mass was placed in a 3.5-mL dish containing 5 mL of TCM199 supplemented with 0.1% bovine serum albumin (Sigma-Aldrich; A6003). Spermatozoa were allowed to swim through this medium for 5 minutes at 37.5°C, and then 700 μL aliquots of the medium were dispensed into 1.5-mL centrifuge tubes. The collected sperm were washed by centrifugal separation and preserved with 50 mM EGTA.
Control sperm samples were stored in TCM199 supplemented with bovine serum albumin (1 mg/mL). As a negative control, we used TBS containing 2 mM CaCl₂ (TBS + Ca), which is the same concentration as in TCM199. Sperm samples were frozen at −196°C with liquid nitrogen. Then, the frozen sperm were thawed in a water bath at 37°C and sonicated in the experimental or control medium using an ultra homogenizer (VP-52; TITEC, Japan) for 1 minute just prior to ICSI. After the spermatozoa were frozen-thawed and sonicated, they were immobilized, they lost their acrosomes, and the sperm heads and tails were separated.

2.4 Chromosomal analysis of hamster spermatozoa by injection into mouse oocytes

To analyze the sperm chromosomes, we performed intracytoplasmic injection of hamster spermatozoa into mouse oocytes, following the method described by Kishikawa et al.²⁴ B6D2F1 mouse females (CLEA Japan, Tokyo) at 8-12 weeks of age were each injected with 5 IU eCG followed by 5 IU hCG 48 later. Oocytes were collected from oviducts between 15 and 16 hours after hCG injection. They were freed from cumulus cells by a 1-minute treatment with 250 IU/mL hyaluronidase in M2 medium. These oocytes were kept in Global Total medium (Astec, Fukuoka, Japan) at 37°C for 30 minutes before sperm injection.

About 5 hours after hamster sperm injection, mouse oocytes exhibiting two well-developed pronuclei and a distinct second polar body were transferred to Global Total medium containing 0.006 µM hyaluronidase in M2 medium. These oocytes were kept in Global Total medium (Astec, Fukuoka, Japan) at 37°C for 30 minutes before sperm injection.

A table showing quantitative PCR primer sequences is included:

| Gene | Primers (5’→3’) | Product size (bp) | Annealing temperature (°C) |
|------|----------------|------------------|---------------------------|
| Mater | CCGGCTTACAAAAGCTCAAG | 128 | 60 |
| Npm2 | CAGCCCTCAGGACAAAGAGTC | 108 | 60 |
| Hsf1 | TCTACTGGTGCAAGTCGAAC | 154 | 60 |
| Hspa1a | CGACCTGAAACAAGACATCA | 213 | 60 |
| Myc | CGACTCCACAGCTTCTCCTC | 179 | 60 |
| Hdac1 | CGATGACTCAACTTATGGCT | 134 | 60 |
| 18s | ATGGCCGTTTCTTATGCTTG | 217 | 60 |

2.5 Microinjection of sperm heads into oocytes

One drop (15 µL) of sonicated sperm solution was thoroughly mixed with 30 µL of Ca- and Mg-free M2 medium containing 12% (w/v) polyvinylpyrrolidone (PVP; MP Biochemicals, OH, USA). This sperm mixture was then transferred to the micromanipulation chamber on the microscope stage. A sperm head was drawn into a pipette and injected into an oocyte under TCM199TE.⁴⁻⁶

2.6 Embryo culture

Sperm-injected oocytes were incubated for about 6 hours in 40 µL droplets of modified Hamster Embryo Culture Medium-9 (mHECM-9) supplemented with 5% fetal bovine serum (HyClone, UT, USA), under mineral oil and kept at 37.5°C in an atmosphere of 10% CO₂, 10% O₂, and 80% N₂. The oocytes were then examined using an inverted microscope (Diaphot TMD, Diaphot 300; Nikon, Tokyo, Japan) equipped with Hoffman modulation contrast optics.²⁶ An oocyte exhibiting two distinct pronuclei and a clearly visible second polar body was considered normally fertilized. At 24 hours after sperm injection, all fertilized oocytes were transferred into 40 µL droplets of mHECM-9 supplemented with 0.5 mg/mL human serum albumin (Sigma-Aldrich; A-1653), and cultured for 72 hours under the same gas mixture.
2.7 Quantitative RT-PCR analysis in hamster embryos

At 6 hours post-ICSI, 24 of pronuclear embryos and, at 44 hours post-ICSI, 23 of 2-cell embryos from the TCM199 or TBS + EGTA, respectively, were washed in PBS and collected in a small volume (~1 µL) of PBS as each sample. Total RNA was extracted from these embryos using the PureLink RNA Mini Kit (Invitrogen by Thermo Fisher Scientific, CA, USA) following the instruction manual. Extracted RNA was dissolved in 30 µL RNase-free water.

Total RNA samples were reverse-transcribed into cDNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, ABgene, UK) following the instruction manual. Gene transcripts were quantified in three replicates by quantitative RT-PCR with specific primers (Table 1) using an Agilent Technologies Stratagene Mx3000P with Brilliant III (Agilent Technologies, CA, USA). The total reaction volume was 25 µL and contained Brilliant III, forward and reverse primers, and cDNA template. Reaction conditions were as follows: 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 20 seconds at 60°C. Data were normalized to the expression level of the 18s, ribosomal RNA gene.

2.8 Embryo transfer

About 78 hours after ICSI, we randomly selected 43 morulae and blastocysts for transferred into recipient albino females that had been naturally mated with albino males 3 days previously. We transferred 4-7 morulae/blastocysts into the uterus of each recipient hamster. The mothers were allowed to deliver and raise their own pups (albino) as well as the foster pups (brown coat and black eyes).4-6,27

2.9 Statistical analysis

The chi-square test was used to compare fertilization and embryo development rates, and chromosome analysis results. Quantitative PCR data were compared using Student’s t test. Differences between means were evaluated using GraphPad PRISM 5 (GraphPad Software, La Jolla, CA, USA). Differences were considered to be significant at P < 0.05.

3 RESULTS

3.1 Chromosome analysis of hamster spermatozoa after sonication in TCM199 or TBS + EGTA

Table 2 summarizes the results of chromosomal analysis of hamster spermatozoa following injection into mouse oocytes. The percentage of normal karyotypes was significantly higher in spermatozoa that were frozen-stored and sonicated in TBS + EGTA (89.7%) than in TCM199 (69.0%) or TBS + Ca (68.0%; P < 0.05). Chromosome-type breaks were the chromosome aberration most commonly observed after sonication (Figure 1A,B).

3.2 In vitro development of hamster embryos after injecting sonicated sperm heads into oocytes

Table 3 summarizes the results regarding the in vitro development of hamster embryos produced by microinjection of sperm heads that were sonicated in TCM199 or TBS + EGTA. The percentages of normally fertilized oocytes and 2-cell embryos did not significantly differ between the TCM199 and TBS + EGTA conditions. However, compared to TCM199, TBS + EGTA yielded significantly higher percentages of 8-cell embryos (84.1% vs 65.4%), morulae (78.4% vs 43.2%), and blastocysts (42.0% vs 17.3%).

3.3 Gene expression of MEGs (Mater, Npm2, and Hsf1) in pronuclear embryos and ZGA (Hspa1a, Myc, and Hdac1) in 2-cell embryos

Figure 2 shows the differences in expression of MEGs and ZGA genes between ICSI embryos in TCM199 and TBS + EGTA. Expression of maternal effect genes (Mater, Npm2, and Hsf1) in pronuclear embryos did not significantly differ between TCM199 and TBS + EGTA. In contrast, the marker genes for zygotic genome activation showed significantly upregulated the transcription levels in 2-cell embryos with TBS + EGTA compared to TCM199.

| Medium used for storage and sonication | No. of zygotes analyzed | No. (%) of oocytes with normal sperm chromosomes | No. of spermatozoa with chromosome aberrations |
|----------------------------------------|-------------------------|-----------------------------------------------|---------------------------------------------|
|                                        |                         |                                               | Structural | Aneuploidy |
| TCM199                                 | 58                      | 40 (69.0)b                                  | 18      | 0 |
| TBS + EGTA                             | 58                      | 52 (89.7)a                                  | 6       | 0 |
| TBS + Ca                               | 50                      | 34 (68.0)b                                  | 16      | 0 |

TBS + EGTA, 100 mM Tris-HCl-buffered solution +50 mM EGTA; TBS + Ca, 100 mM Tris-HCl-buffered solution +2 mM CaCl₂.
Significantly different values (P < 0.05) between different superscripts. Percentages of oocytes with normal sperm chromosomes were analyzed by Fisher’s exact probability test and chi-square test.

 STRUCTURAL CHROMOSOME ABERRATIONS INCLUDED CHROMOSOME/CHROMATID-TYPE BREAKS AND EXCHANGES.
3.4 Full-term development of embryos fertilized by injection of sperm heads sonicated in TBS + EGTA

Table 4 summarizes the result of embryo transfer. A total of 43 embryos derived from the injection of sperm heads sonicated in TBS + EGTA were transferred to recipients, of which 4 (9.3%) developed into live young. Figure 3 shows a recipient mother that gave birth to one pup (brown fur) that developed from the oocytes fertilized by injection of sonicated sperm heads in TBS + EGTA.

4 DISCUSSION

Our present results showed that TBS + EGTA was effective for cryopreservation and sonication of hamster spermatozoa, enabling the injection of sperm heads with a higher proportion of normal chromosomes (Table 2). The percentages of 8-cell embryos, morulae, and blastocysts were significantly higher in TBS + EGTA than in TCM199 (Table 3). Furthermore, after transfer of 43 ICSI embryos, 4 developed into live offspring (Table 4). These results indicated that the use of TBS + EGTA for cryopreservation and sonication was effective for maintaining sperm chromosomal normality, and supported the development of zygotes fertilized by sperm head injection into oocytes.

The incidence of structural chromosome aberrations in hamster spermatozoa was significantly reduced when the spermatozoa were frozen-stored and sonicated in TBS + EGTA compared to in TCM199. Moreover, sonication in TBS + Ca (TBS containing 2 mM calcium chloride which is the same concentration as in TCM199) led to a significantly increased incidence of sperm chromosome aberrations. The percentages of chromosome aberrations were similar between TCM199 and TBS + Ca. These findings suggest that the presence of calcium ions during spermatozoa sonication activates DNase in sperm heads, thus increasing structural chromosome aberration. Since divalent cations are essential for DNase activity, chelating agents (e.g., EGTA) would suppress their activity.28

To analyze hamster sperm chromosomes, we performed xenogeneic injection of hamster sperm heads separated by sonication into mouse oocytes. This method of interspecies sperm chromosome analysis using mouse oocytes is systematic, and the procedure is completely controlled.18,24,25 The xenogeneic system makes it easy to identify the hamster sperm chromosomes. However, future studies should include homogeneous sperm chromosome analysis using hamster oocytes to determine the relationship between sperm chromosome normality and ICSI embryo development.

The sonication solution contained 50 mM EGTA to prevent chromosome breakage by DNase in the sperm heads. Prior to sperm head injection, the EGTA solution with sperm heads was mixed and diluted by one-third with 12% PVP solution (final volume ~17 mM). With an estimated injection volume of ~1 pL, the injected amount of EGTA is estimated to be ~17 fmol. Changes in the intercellular calcium concentration underlie the initiation, progression, and completion of fertilization.29 In our present study, hamster ICSI with EGTA did not disturb pronuclear formation, nor did the amount of EGTA injected into the ooplasm with the sperm head seem to affect the fertilization process. However, the present data are insufficient to confirm the minimal EGTA concentration for sonication and sperm injection, and further investigations are needed to optimize ICSI.

TABLE 3 In vitro development of hamster oocytes injected with sperm heads separated by sonication in TCM199 or TBS + EGTA

| Medium used for storage and sonication | No. of oocytes injected (no. of replicates) | No. of oocytes with 2PN (%) | No. (%) of embryos developed to ≥2-cell | ≥8-cell | ≥Morulae | Blastocysts |
|---------------------------------------|------------------------------------------|---------------------------|--------------------------------------|---------|----------|------------|
| TCM199                                | 81 (4)                                   | 75 (92.6)a                 | 72 (88.9)a                           | 53 (65.4)a | 35 (43.2)a | 14 (17.3)a  |
| TBS + EGTA                            | 88 (4)                                   | 80 (90.9)a                 | 80 (90.9)a                           | 74 (84.1)b | 69 (78.4)b | 37 (42.0)b  |

TBS + EGTA, 100 mM Tris-HCl-buffered solution +50 mM EGTA; 2PN, two well-developed pronuclei. Significantly different values (P < 0.05) between different superscripts within the same column. Percentage of embryo development was analyzed by chi-square test.

FIGURE 1 Chromosome spreads of hamster spermatozoa, prepared by sonicating the spermatozoa in TCM199 or TBS + EGTA, and then injecting sperm heads into mouse oocytes. Representative normal (A) and abnormal (B) hamster sperm chromosomes are shown. Arrows indicate chromosome fragments. n = 22 normal, n > 22 abnormal. Bars = 10 μm.
Mouse and hamster embryonic genome activation occur at the 2-cell embryo stage. Bovine embryonic genome is activated at the 8-cell embryo stage, and embryo arrest reportedly occurs mainly during embryonic gene activation. ZGA marker genes, and the gene expression in 2-cell embryos derived from ICSI is significantly lower than in vivo. The histone deacetylase gene Hdac1 is one factor that controls transcription, and Hdac1 knockout leads to arrested embryonic development in mice.
higher quality hamster ICSI embryos, we must improve the manipulation of sonicated sperm heads to enable smooth fertilization, optimize the culture medium for ICSI embryos, and regulate spontaneous oocyte activation.

In conclusion, here we found that the use of TBS + EGTA for sonication of hamster frozen-thawed spermatozoa reduced sperm chromosome aberrations. Upon injecting hamster oocytes with sonicated sperm heads having less chromosome aberrations, we found that the ZGA gene expression at the two-cell stage was relatively higher than with TCM199. Moreover, the ICSI zygotes produced using TBS + EGTA developed to morulae and blastocysts at a higher rate compared to with TCM199. Finally, ICSI embryos produced with TBS + EGTA successfully developed to live offspring after embryo transfer. Overall, these results suggest that rapid ICSI using sperm heads separated by sonication in TBS + EGTA leads to increased chromosome normality and effective production of live offspring.

DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. Human rights statement and informed consent: This article does not contain any study with human participants that has been performed by any of the authors. Animal studies: All the experiments in this research were approved by the Committee for Ethics on Animal Experiments of the Prefectural University of Hiroshima, Japan (16SA002).

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How to cite this article: Morishita N, Ochi M, Horiuchi T. Development of golden hamster embryos effectively produced by injection of sperm heads sonicated in Tris-HCl buffer with EGTA. Reprod Med Biol. 2019;18:83-90. https://doi.org/10.1002/rmb2.12253