Effect of Fluorescent Mercury Light Irradiation on In Vitro and In Vivo Development of Mouse Oocytes after Parthenogenetic Activation or Sperm Microinjection

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Abstract. The detection of specific cellular components using fluorescent agents such as green fluorescent protein (GFP), red fluorescent protein or Hoechst dyes provides a powerful tool for studying cell biology. However, specimens must be exposed to high-intensity light, which might cause cellular damage. Here, we exposed mouse metaphase stage (MII) oocytes to fluorescent mercury vapor light at three wavelengths (539 nm, 488 nm and 341 nm) to determine the maximum exposure time that would avoid damage. When oocytes were activated parthenogenetically after exposure to these wavelengths for more than 20 min, 5 min or 4 sec, respectively, the percentages of dead oocytes after activation increased, and none of the surviving embryos developed to blastocysts. However, embryos fertilized by intracytoplasmic sperm injection (ICSI) were more tolerant to light damage, even though the quality of blastocysts, judged by cell number and cell allocation to the inner cell mass and trophectoderm measured by immunostaining for Oct4 and Cdx2, was reduced as exposure times increased. Live, healthy offspring were obtained when these exposed embryos were transferred into recipient pseudopregnant females at the 2-cell stage. In addition, MII oocytes collected from GFP-expressing transgenic mice after 5 min of irradiation with 488-nm light were also able to develop to full term following ICSI. Thus, we determined the safe period of exposure to several wavelengths for oocyte manipulation or observation that would permit subsequent development.

Key words: Embryo development, Exposure time, Fluorescent light

Received: February 2, 2011
Accepted: April 22, 2011
Published online in J-STAGE: June 3, 2011
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Recent advances in fluorescence microscopy and the development of novel fluorescent dyes have provided the ability to detect specific cellular components. Cell labeling using agents such as red fluorescence protein (RFP), green fluorescent protein (GFP) and Hoechst 33342 has provided a powerful set of tools for studying cell biology, such as observing chromosomal dynamics during embryonic cell division [1].

In nuclear transfer (NT) protocols, enucleated oocytes are the most common recipients, and one standard method for oocyte enucleation is aspiration of the metaphase II (MII) plate and spindle by micromanipulation [2, 3]. However, especially in porcine or bovine oocytes, the nucleus cannot be clearly distinguished from the cytoplasm using phase contrast or differential interference microscopy because of lipid droplets [4]. Therefore, enucleation of these oocytes is usually performed under visualizing the MII by staining the DNA with Hoechst 33342 and excitation of the dye using fluorescent high energy light. In nature, mammalian oocytes and embryos are never exposed to sunlight or strong artificial light. Therefore, they are sensitive to fluorescent or even room-light wavelengths [5, 6]; this phenomenon is referred to as phototoxicity. Maturation-promoting factor kinase degradation and pronuclear formation were compromised after irradiation of the MII plate in bovine oocytes. This treatment may further compromise the developmental potential of the NT-generated embryo because of the harmful effects of ultraviolet (UV) irradiation on the oocyte membrane and intracellular components [7–9].

If embryonic development is compromised by light exposure, the results probably do not reflect biological truth. To minimize phototoxicity, Yamagata et al. developed a new imaging system [10], in which samples are exposed to as low an intensity of light as possible and the fluorescent signals are collected at maximum efficiency using an ultrasensitive camera. Although this system allows observation of preimplantation embryo development with minimum damage, these instruments are unfortunately very expensive. For embryology using routine fluorescence microscopy, it is very important to know the exact toxicity of the light to minimize any effects on embryo development.

In this study, we used the mouse as a model of a domestic animal. Although there is some difference between the mouse and other animals, such as the concentration of lipid droplets in the oocyte, the mouse is highly specialized experimental animal, and the results from commons strains of mouse are more solid compared with those from domestic animals. Furthermore, it is very important to examine the full-term development of irradiated oocytes because developmental ability to the blastocysts stage is not enough to judge the damage to oocytes, and the mouse is best
animal for such studies. The mouse MII oocytes were exposed to fluorescent mercury light using three wavelengths for different periods, and the maximum exposure time that would avoid damage to the oocytes was determined by repeating embryo transfer experiments. Here, the full-term developmental abilities of irradiated oocytes were examined by ICSI instead of somatic cell NT because the offspring rate of NT embryos was too low to compare the oocyte damage between different period of irradiation. ICSI can mimic nuclear injection into oocytes, and parthenogenetic activation can mimic activation of an NT oocyte. Moreover, to examine the effects of combinations of fluorescent dyes and light irradiation, we compared BDF1-strain mouse MII oocytes with BDF1 GFP-expressing transgenic (Tg) mouse MII oocytes.

Materials and Methods

Animals

B6D2F1 (C57BL/6×DBA/2) mice with or without the gene for GFP, aged 8–10 weeks, were used to prepare oocytes and spermatozoa. The surrogate pseudopregnant females were ICR-strain mice mated with vasectomized males of the same strain. All animals were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan), and all animal experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the RIKEN Center for Developmental Biology.

Collection of oocytes

Mature oocytes were collected from the oviducts of 8–10-week-old female mice that had been induced to superovulate with 5 IU pregnant mare serum gonadotropin (PMSG; Teikokuzoki, Tokyo, Japan) followed by 5 IU human chorionic gonadotropin (hCG, Teikokuzoki) 48 h later. Cumulus–oocyte complexes (COCs) were collected from the oviducts approximately 16 h after hCG injection. After collection, COCs were placed in HEPES-buffered CZB medium (H-CZB) and treated with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich, St Louis, MO, USA). After several minutes, the cumulus-free oocytes were washed twice and then moved to a droplet of KSOM medium (Specialty Media, Phillipsburg, NJ, USA) for culture.

UV irradiation

For irradiation, three kinds of mirror units were used, U-MWIG3, U-MNIBA2 and U-MWU2 (Olympus, Tokyo, Japan), whose excitation wavelengths were 530–550 nm, 470–490 nm and 330–385 nm, respectively. Oocyte irradiation was performed in H-CZB medium in a Falcon Petri Dish, 100×15 mm style, with a 20× objective for up to 30 min, 20 min and 10 sec, respectively. Oocyte irradiation was performed in H-CZB medium in a Falcon Petri Dish, 100×15 mm style, with a 20× objective for up to 30 min, 20 min and 10 sec, respectively. After irradiation, the power of the mercury vapor light was adjusted to 100 mW using a U-MWIG3 mirror unit with a 20× objective, measured using a TB-200 power meter (Yokogawa Electric, Tokyo, Japan).

Parthenogenetically activated oocytes

Oocytes were activated with 10 mM SrCl2 for 6 h in Ca2+-free H-CZB containing 5 μg cytochalasin B [11]. After activation, the number of dead oocytes that lost their oolemma was counted, and the living oocytes were cultured in KSOM at 37°C under 5% CO2 in air for 96 h.

Intracytoplasmic sperm injection (ICSI) and embryo transfer

ICSI was performed as described previously [12]. About 1 μl of the sperm suspension was mixed with a drop of polyvinylpyrrolidone solution (Irvine Scientific, Santa Ana, CA, USA). The sperm head was separated from the tail by the application of several piezo pulses (PrimeTech, Tokyo, Japan), and the head was then injected into the oocytes. The oocytes were then cultured in KSOM medium for preimplantation development or embryo transfer.

To produce offspring, 2-cell stage embryos were transferred into the oviducts of pseudopregnant ICR-strain female mice at 0.5 days post coitum (dpc), which had been mated with a vasectomized male the night before transfer. At 18.5 dpc, the offspring were delivered by Caesarean section.

Immunofluorescence

Blastocysts were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min, washed twice with 1% bovine serum albumin (BSA) in PBS, transferred into 1% BSA–PBS con-
containing 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) and incubated overnight at 4°C. The blastocysts were then washed twice with 1% BSA–PBS and incubated with the primary antibody overnight at 4°C. The primary antibodies used were rabbit polyclonal anti-Oct3/4 (1:100 dilution; Santa Cruz Biotechnology, Tokyo, Japan) and Cdx2 (1:100 dilution; BioGenex, San Ramon, CA, USA). After the blastocysts had been washed twice in 1% BSA–PBS, they were incubated for 1 h with antibodies, Alexa Fluor 488-labeled goat anti-mouse immunoglobulin (Ig) G and Alexa Fluor 546-labeled goat anti-rabbit IgG (1:200 dilution; Molecular Probes, Eugene, OR, USA). The blastocysts were washed again in 1% BSA–PBS, and their DNA was stained with 2 μg/ml of 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Finally, the embryos were washed and observed with a confocal scanning laser microscope (FV-1000, Olympus). Oct3/4 and Cdx2 are markers of the inner cell mass (ICM) and trophectoderm (TE), respectively.

Fig. 2. Effects of mercury vapor fluorescent light irradiation on in vitro development. Meiosis (M) stage II oocytes were irradiated to the light with the wavelength of 539 nm (A), 488 nm (B) or 341 nm (C). Blastocyst formation rates following parthenogenetic activation or intracytoplasmic sperm injection (ICSI) were examined on day 4. (D) After irradiation with 539 nm for 20 min, 488 nm for 5 min or 341 nm for 4 sec, ICSI-generated embryos developed to the blastocyst stage, although parthenotes did not. Statistical differences between the control and the irradiated group within each period are indicated by *P<0.05.
DAMAGE OF FLUORESCENT LIGHT IRRADIATION

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EVALUATION OF BLASTOCYSTS AFTER IMMUNOSTAINING

Blastocysts were classified into four types, type I – type IV, according to the criteria established by Kishigami et al. [13] (Fig. 1). Briefly, separation of the first two lineages — TE and ICM — is a crucial event in the development of an early embryo [14]. The best quality blastocysts that expressed Oct4/3 and Cdx2 in the ICM and TE, respectively, were classified as type I. Blastocysts in which Cdx2 was expressed in the nuclei of the TE and Oct3/4 was expressed in the nuclei of both the TE and ICM were classified as Type II. Blastocysts that had lost the ICM and in which Oct3/4 and Cdx2 were co-localized in the nuclei of TE-like cells were classified as Type III. Blastocysts that express only Oct3/4 were classified as Type IV. In terms of cell number, nuclei expressing only Oct3/4 were counted as ICM in type II blastocysts. In type III and IV, all cells were counted as TE.

STATISTICAL ANALYSIS

Blastocyst formation and oocyte survival rates were evaluated using Chi-squared tests. The blastocyst formation rate was compared between control and fluorescent mercury light exposed oocytes. Cell numbers of blastocysts were analyzed using analysis of variance (ANOVA), followed by Bonferroni’s protected least significant difference test.

RESULTS

In vitro development of parthenogenetically activated or ICSI-generated embryos after exposure to fluorescent mercury vapor light at various wavelengths

As shown in Fig. 2, blastocyst formation rates in parthenogenetic embryos were gradually decreased when using 539 nm (Fig. 2A), 488 nm (Fig. 2B) or 341 nm (Fig. 2C) mirror systems, and no embryos developed after 20 min, 5 min or 4 sec exposure, respectively. In contrast, in ICSI-generated embryos, the blastocyst formation rates were not significantly decreased when exposed to mercury vapor light for the same times. These results indicate that ICSI-generated embryos were more tolerant of fluorescent mercury light damage at the MII stage than were parthenogenetic embryos.

Quality of blastocysts derived from MII oocytes after exposure to fluorescent mercury vapor light of various wavelengths following ICSI

The quality of ICSI-generated blastocysts was evaluated based on the cell number and patterns of Oct4 and Cdx2 expression (Fig. 3). As shown in Fig. 4, the percentages of type I blastocysts (A–C) and total and TE cell numbers (D–F) were decreased after longer exposures to fluorescent mercury light. Especially in oocytes exposed to light with 341 nm wavelength for more than 2 sec, the total and TE cell numbers were significantly lower than in the controls or the oocytes exposed to 341-nm light for 1 sec (P<0.05). In addition, an increase in time of exposure to 341-nm light caused a gradual decrease in viable oocytes after activation or ICSI (Fig. 5), but other wavelengths did not produce this damage. This indicates that shorter wavelength light was more harmful to the oolemma.

Full-term development of embryos after exposure to various fluorescent mercury vapor light wavelengths following ICSI

Following the results of in vitro development, we tried to confirm the maximum exposure time that would permit full-term development. MII oocytes were exposed to fluorescent mercury vapor light with 539 nm for <10, 15, 20 and 30 min, 488 nm for <3, 4–5 and 10–20 min or 341 nm for <4, 6 and 10 sec. Control oocytes were not exposed to fluorescent mercury light. As shown in Table 1, live and healthy offspring were obtained after exposure with the U-MWIG3 system for 20 min, exposure with the U-MNIBA2 for 5 min or exposure with the U-MWU2 system for 6 sec. Although mean body weights and placental weights did not change depending on the exposure times at any wavelength, the percentages of embryos implanted and offspring were decreased after longer exposures with the U-MWU2 system. In addition, to assess the effects of fluorescent dye, GFP-Tg BDF1 mouse MII oocytes were exposed to mercury vapor light, and full-term development was examined following ICSI. Of the offspring, 61.5% expressed the GFP gene, and the percentage of offspring derived from GFP-Tg irradiated oocytes per embryo transfer was not decreased significantly compared with those derived from BDF1 irradiated oocytes (Table 1).
Discussion

The results of this study provide information on the effect of mercury vapor light irradiation on MII oocytes and the maximum exposure time that can be used to avoid damage. First, ICSI-generated embryos showed more tolerance to fluorescent light than parthenogenetic embryos. Second, the percentage of dead oocytes with ruptured ooplasm after activation was increased with longer irradiation times. Finally, MII oocytes exposed to 539-nm light for 20 min, 488-nm light for 5 min or 341-nm light for 6 sec were able...
Damage of fluorescent light irradiation

Interestingly, irradiated oocytes could develop to the blastocyst stage following ICSI even though they stopped developing before the blastocyst stage following parthenogenetic activation. It is likely that the decrease in developmental potential of parthenogenetic embryos after irradiation was caused by damage to the maternal nuclear DNA and/or cytoplasmic organelles by absorbance of excessive fluorescent light. Organelles are so abundant in the oocyte that presumably some of them can avoid fluorescent light phototoxicity.

In the NT experiments, even if oocytes were irradiated by fluorescent mercury light for enucleation, the damaged maternal DNA was presumably exchanged with undamaged somatic cell DNA, so some of the NT-generated cloned embryos could develop to full term without rescue of damaged organelles. However, we noticed that longer irradiation weakens the oolemma and that oocytes often died not only after injection but also after SrCl2 treatment. It is reported that irradiated oocytes produce high levels of mitochondrial activity [8]. This indicates the need for increased energy in repairing membranes, intracellular phototoxicity or perhaps both. The weakened membrane poses a problem for several types of experiments, including micromanipulation of oocytes. Without enough strength, the membrane will break and the oocytes will die after NT, the procedure for which contains microinjection that is performed under the same conditions as ICSI and SrCl2 treatment that is performed under the same conditions as parthenogenetic activation; however, the membrane can recover to some extent [12]. When fluorescent mercury light-exposed oocytes are used for micromanipulation, they should be left at room temperature a little longer than usual to allow recovery.

To determine the quality of fluorescent light-irradiated oocytes, we analyzed the lineage regulators Cdx2 and Oct4 at the blastocyst stage and classified the blastocysts according to expression patterns. Separation of the TE and ICM lineages is a crucial event in the development of the early embryo [14]. As the fluorescent mercury light exposure time increased, the percentage of type I (best-quality blastocysts) decreased and the percentage of other types (blastocysts composed of cells that did not differentiate to ICM or TE completely) increased. The damage to the nuclear DNA prevents the proper development of the fluorescent light-exposed oocytes.

In conclusion, our mouse experiments showed that exposure of oocytes to fluorescent mercury vapor light caused detrimental effects on in vitro and full-term development. Care must be taken when oocytes are irradiated to the light with a wavelength of 539 nm for more than 20 min, 488 nm for more than 5 min or 341 nm for more than 6 sec. At the same time, irradiation with longer wavelengths causes less damage to the oocyte’s developmental potential. The period of tolerance against each wavelength probably differs between species. However, our mouse results will help to determine the maximum tolerance period for oocytes of other species.
Acknowledgments

We thank Y Sakaide, T Oyanagi and S Wakayama for assistance in preparing this manuscript. We are grateful to the Laboratory for Animal Resources and Genetic Engineering for housing the mice. Financial support for this research was provided by a Grant-in-Aid for Scientific Research in Priority Areas (15080211, to TW).

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Table 1. Full term development of oocytes exposed to mercury light following ICSI

| Filter | Type of strain | Exposure time | No. used oocytes | No. survived oocytes (%) | No. with PN formation (%) | No. 2-cell embryos (%) |
|--------|----------------|---------------|-----------------|-------------------------|--------------------------|-----------------------|
| Control BDF1 | - | 135 | 113 (83.7) | 111 (98.2) | 101 (91.0) |
| U-MWIG3 BDF1 | < 10 min | 67 | 37 (55.2) | 36 (97.3) | 35 (97.2) |
| | 15 min | 46 | 20 (43.5) | 20 (100.0) | 20 (100.0) |
| | 20 min | 53 | 30 (56.6) | 30 (100.0) | 30 (100.0) |
| | 30 min | 24 | 16 (66.7) | 16 (100.0) | 12 (75.0) |
| U-MNIBA2 BDF1 | < 3 min | 185 | 158 (85.4) | 155 (98.1) | 152 (98.1) |
| | 4–5 min | 165 | 88 (53.3) | 84 (95.5) | 81 (96.4) |
| | 10–20 min | 98 | 24 (24.5) | 19 (79.2) | 0 (0.0) |
| BDF1-GFP | 3 min | 52 | 29 (55.8) | 29 (100.0) | 27 (93.1) |
| | 4 min | 73 | 41 (56.2) | 41 (100.0) | 41 (100.0) |
| | 5 min | 58 | 25 (43.1) | 24 (96.0) | 23 (95.8) |
| U-MWU2 BDF1 | < 4 sec | 63 | 24 (38.1) | 23 (95.8) | 21 (91.3) |
| | 6 sec | 59 | 33 (55.9) | 33 (100.0) | 29 (87.9) |
| | 10 sec | 54 | 14 (25.9) | 13 (92.9) | 3 (23.1) |

Filter | Type of strain | No. embryos transferred (recipients) | No. embryos implanted (%) | No. offspring (%) | Mean body weight (g) | Mean placental weight (g) |
|--------|----------------|-------------------------------|--------------------------|-----------------|---------------------|--------------------------|
| Control BDF1 | 101 (8) | 76 (75.2) | 51 (50.5) | 1.66 ± 0.17 | 0.12 ± 0.02 |
| U-MWIG3 BDF1 | 35 (2) | 24 (68.6) | 10 (28.6) | 1.69 ± 0.13 | 0.14 ± 0.03 |
| | 20 (2) | 11 (55.0) | 7 (35.0) | 1.49 ± 0.23 | 0.14 ± 0.02 |
| | 30 (2) | 23 (76.7) | 6 (20.0) | 1.65 ± 0.14 | 0.13 ± 0.02 |
| | 12 (1) | 0 (0.0) | 0 (0.0) | n.a. | n.a. |
| U-MNIBA2 BDF1 | 145 (11) | 96 (66.2) | 57 (39.3) | 1.67 ± 0.13 | 0.14 ± 0.03 |
| | 80 (6) | 50 (62.5) | 2 (32.5) | 1.67 ± 0.18 | 0.15 ± 0.03 |
| BDF1-GFP | 27 (2) | 20 (74.1) | 6 (22.2) | 1.70 ± 0.15 | 0.13 ± 0.05 |
| | 40 (3) | 16 (40.0) | 5 (12.5) | 1.70 ± 0.18 | 0.18 ± 0.06 |
| | 23 (2) | 13 (56.5) | 5 (21.7) | 1.68 ± 0.14 | 0.14 ± 0.05 |
| U-MWU2 BDF1 | 20 (1) | 17 (85.0) | 8 (40.0) | 1.43 ± 0.40 | 0.12 ± 0.04 |
| | 29 (2) | 12 (41.4) | 8 (27.6) | 1.72 ± 0.09 | 0.15 ± 0.03 |
| | 3 (1) | 0 (0.0) | 0 (0.0) | n.a. | n.a. |

1 Based on the number of surviving oocytes. 2 Based on the number of pronuclear embryos. 3 Based on the number of transferred embryos. 4 n.a. means not available.
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