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## THE EFFECTIVENESS OF TRYPSIN TREATMENT TO REMOVE SENDAI VIRUS ADHERING TO THE ZONA PELLUCIDA OF MOUSE PREIMPLANTATION EMBRYOS

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The standard washing and trypsin treatment procedures to remove viruses adhering to the zona pellucida (ZP) were evaluated. Mouse embryos at the early blastocyst stage were exposed to Sendai virus, and then washed or treated with trypsin. Even after washing or trypsin treatment, Sendai virus was detected in the twelfth and final wash. The virus was still shown to adhere to the ZP by immunofluorescence assay. The embryos developed into expanded blastocysts following 24 hours of *in vitro* culture. Viral antigen was clearly demonstrated in the cells forming the expanded blastocysts, indicating that viral replication occurred in these cells. The present results suggest that the standard washing or trypsin treatment are not sufficient to remove Sendai virus adhering to the ZP of mouse embryos.

Key words: mouse embryo, Sendai virus, washing, trypsin treatment

### INTRODUCTION

The transmission of infectious viral diseases through embryos is possible if the virus is carried in the gamete or if the developing embryo is infected with virus that is present in the reproductive tract of the mother. Embryo transfer is now being used as a technique for rederivation of inbred strains of mice or as a means of disease control<sup>10</sup>. Thus, the success of embryo transfer for disease control depends on the virus not being transmitted via the gamete or not infecting the embryo prior to collection for transfer to recipient animals<sup>13</sup>.

There has been limited study regarding the interaction of viral pathogens with preimplantation embryos. Few viruses have been implicated in the infection of gametes and it is most likely that infections would come from the uterine environment<sup>5</sup>. It was observed that the ZP acted as an effective barrier against infection of embryos when viruses are not capable of penetrating the ZP. However, some viruses can

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penetrate or adhere to the ZP<sup>8,20</sup>.

Sendai virus is one of the most common viruses detected in mouse breeding colonies<sup>3</sup>. The mouse is particularly susceptible to the virus infection resulting in severe respiratory disease<sup>7</sup>. Evidence of intrauterine transmission has been reported<sup>21</sup>. In vitro studies of embryos exposed to Sendai virus showed that the embryonic cells were susceptible to the infection<sup>2</sup>. The virus did not penetrate the ZP but was capable of adhering to the ZP at the blastocyst stage<sup>8</sup>. The International Embryo Transfer Society recommended the standard procedures of washing and trypsin treatment to remove virus adhering to the ZP<sup>14</sup>.

The objective of the present study is to determine whether Sendai virus can be removed or eliminated from the ZP by washing or trypsin treatment, and the effect on development of embryos was examined.

#### MATERIALS AND METHODS

##### *Virus and Antiserum*

MN strain of Sendai virus (parainfluenza type 1) was propagated in 10-day-old embryonated chicken eggs for 2 days at 35°C. The virus infectivity titer of the allantoic fluid was  $10^{8.7}$  EID<sub>50</sub> / 0.1 ml. Antiserum to Sendai virus was prepared in a rabbit by two intramuscular injections of partially purified virus with Freund's adjuvant. Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG was purchased (Cappel, PA).

##### *Embryo Collection*

Four to six-week-old ICR mice were superovulated by intraperitoneal injection with 5 IU of pregnant mare gonadotrophin (PMSG, Serotrophin; Teikoku-zoki, Japan), followed 48 hours later by human chorionic gonadotrophin (hCG, Gonatrophin, Teikoku-zoki, Japan). The mice were mated, and vaginal plugs were checked on the following morning (Day 1). On Day 4, or 92 hours post hCG, the mice were sacrificed, and embryos at the early blastocyst stage were flushed from the uterine horns using 0.01M phosphate buffered saline (PBS, pH 7.2) supplemented with 10 % calf serum (Gibco Lab., Life Technology, NY). The embryos were washed and pooled in sterile plastic dishes (Nunclon, Denmark) until used.

##### *In vitro exposure of embryos to Sendai virus*

Groups of 10 to 20 embryos were exposed to 25  $\mu$ l Sendai virus stock in 100  $\mu$ l of culture medium (CM1) or Whitten's Medium supplemented with 3 % bovine serum albumin with antibiotics added, embedded in liquid paraffin and incubated at 37°C in 5 % CO<sub>2</sub> in air. After 1 hour of exposure to Sendai virus, the embryos were transferred to fresh culture medium (CM2) and cultured for 24 hours to allow further development. At the end of this period, CM1 and CM2 were kept at -80°C for titration of viral infectivity.

### *Washing procedure*

Prior to transfer of the embryos to fresh culture medium, the embryos were washed<sup>14</sup>. Two ml of supplemented culture medium with antibiotics dispensed into each well of a 12-well cell culture plate (Costar, Cambridge, MA) comprised the postexposure sequential washes. Using separate sterile micropipettes between washes, embryos were washed in groups of 10 to 20. The embryos were gently agitated before being moved to the next well to ensure adequate washing. The embryo-containing portions of medium in micropipettes never exceeded 20  $\mu$ l. After washing, the embryos were transferred to culture medium to allow further development.

### *Trypsin treatment*

A combined washing and trypsin treatment were carried out in accordance to the standard procedure set by the International Embryo Transfer Society<sup>14</sup>. Briefly, embryos were transferred through five washes of PBS without  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  but with antibiotics and 0.04 % bovine serum albumin. The embryos were then exposed through two washes of 0.25 % trypsin, pH 7.6 (Gibco Lab., Life Technology, NY), for a total time of 60 seconds. After trypsin treatment, the embryos were transferred through five washes of PBS containing  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ , antibiotics and 2 % calf serum.

### *Titration of Viral Infectivity*

A tenfold serial dilution of each wash and culture media was inoculated into the allantoic cavity of four 10-day-old chicken embryonated eggs, and the allantoic fluids were assayed for the presence of the virus by hemagglutination test. The  $\text{EID}_{50}$  was calculated by the Reed and Muench formula. After the culture period, the embryos were examined for the presence of viral antigens by indirect immunofluorescence assay (IFA)<sup>7</sup>. Data were analyzed by the Chi-square test.

## RESULTS

Two hundred and ten excellent zona-intact embryos at the early blastocyst stage were assigned to 3 groups for in vitro exposure to Sendai virus. Group 1 comprised the embryos that were not washed nor treated with trypsin. Groups 2 and 3 constituted the washing and trypsin-treated groups of embryos, respectively. Control embryos were cultured in the virus-free medium.

The results of in vitro development and IFA of early blastocysts after exposure to Sendai virus are shown in Table 1. In terms of development to expanded blastocyst stage, no significant differences were noted between the 3 groups that were exposed to Sendai virus, washed and treated with trypsin. It was shown that trypsin treatment had no detrimental effect on the embryonic survival of embryos in vitro considering that the embryos developed to expanded blastocyst when further cultured.

After the culture period, the embryos were examined for the presence of viral

Table 1. In vitro development and IFA of embryos exposed to Sendai virus after 24-hour culture

Group No.**	No. of embryos examined	No. (%) developed expanded blastocyst	No. (%) positive for IFA
1	72	60 (83.3)	40 (55.0)
2	73	63 (86.3)	34* (48.5)
3	65	57 (87.6)	31 (47.6)
Control	75	70 (93.3)	0 (0)

\* Three embryos were lost during IFA.

\*\* Group 1, embryos were neither washed nor treated with trypsin.

Group 2, embryos were washed.

Group 3, embryos were washed and treated with trypsin.

Control, embryos were cultured in virus-free medium.

antigen by IFA. Control embryos appeared bluish in coloration under the fluorescence microscope (Fig. 1). On the other hand, embryos exposed to Sendai virus showed specific immunofluorescence in the ZP indicating the adherence of Sendai virus (Fig. 2). Blastomeres protruding from the ZP have bright greenish illumination, suggesting that viral replication has occurred. Embryos that were washed (Fig. 3) or treated with trypsin (Fig. 4) showed positive immunofluorescence in the ZP and blastomeres.

Virus titration in the washing medium revealed that Sendai virus was detected until the last sequential wash in Groups 2 and 3. Titer of the virus in the washing medium gradually decreased. A small amount of virus was detected in the culture medium after washing and trypsin treatment.

#### DISCUSSION

Sendai virus has been shown to adhere to the ZP of mouse embryos at the early blastocyst stage<sup>8</sup>. Its adherence might lead to infection of the embryo as it emerges through the ZP. Washing or trypsin treatment is now being recommended to remove adherent viruses<sup>14</sup>. The present results have shown that this may not be effective to remove Sendai virus adhering to the ZP.

Washing or treating embryos with trypsin prior to embryo transfer have been used to control disease transmission through embryo transfer. It has been shown that trypsin treatment was effective in inactivating / removing infectious bovine rhinotracheitis virus, vesicular stomatitis virus, hog cholera virus and bovine herpesvirus-4 that adhered to the ZP<sup>4,18,19,20</sup>. On the other hand, trypsin was not effective in removing African swine fever virus, swine vesicular disease virus, foot and mouth disease virus and *mycoplasma bovis genitalium* and *mycoplasma bovis*<sup>11,15,16,17</sup>.

Specific immunofluorescence was clearly demonstrated in both the ZP and the blastomeres after 24 hours of culture. This finding may indicate that Sendai virus had

penetrated the ZP. Although the embryos were found to be infected, the embryos developed into expanded blastocysts.

The mechanism of attachment of Sendai virus to the ZP is not known as there is no available data that can explain the association of this virus with the ZP. However, evidence of this work implies that there is an apparent strong attachment which may be attributed to the presence of receptors in the ZP to Sendai virus.

In the study of viruses in tissue culture cells, it has been shown that paramyxovirions possess two glycoprotein spikes protruding from the envelope. The hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins are responsible for the attachment of the virus to cell surface receptors and fusion of the viral and cellular membranes<sup>6)</sup>. Trypsinization enhances the infectivity of these viruses by promoting cleavage of F protein molecules<sup>9,12)</sup>. Hence, trypsin treatment of embryos exposed to Sendai virus may have caused some changes on the cell surface structure of the ZP, thereby allowing the virus to penetrate to the ZP.

In conclusion, the present study indicates that washing and trypsin treatment did not have any detrimental effect on the embryonic survival of embryos in vitro. This procedure is not capable of eliminating or removing adherent Sendai virus in the ZP. Studies must be done using other nonproteolytic enzymes or to develop effective methods to clear or inactivate Sendai virus from exposed embryos. Some investigations have been done to use antimicrobial substances for disinfection of embryos<sup>1)</sup>. It should be noted that Sendai virus has not been reported to be transmitted via embryo transfer. However, to determine if a hazard exists, further studies are necessary to define the potential for the occurrence of in vivo exposures to Sendai virus.

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**PLATE****EXPLANATION OF FIGURES**

- Fig. 1** Blastocyst stage mouse embryo showing negative immunofluorescence by IFA, control embryo. ( $\times 400$ )
- Fig. 2** A hatching blastocyst from embryo exposed to Sendai virus. Note the positive immunofluorescence in the ZP and blastomeres. ( $\times 400$ )
- Fig. 3** Blastocyst exposed to Sendai virus and then washed. Positive immunofluorescence was shown in the ZP and blastomeres. ( $\times 400$ )
- Fig. 4** A trypsin-treated blastocyst after exposure to Sendai virus. Note the presence of specific immunofluorescence in the ZP and blastomeres. ( $\times 400$ )

PLATE

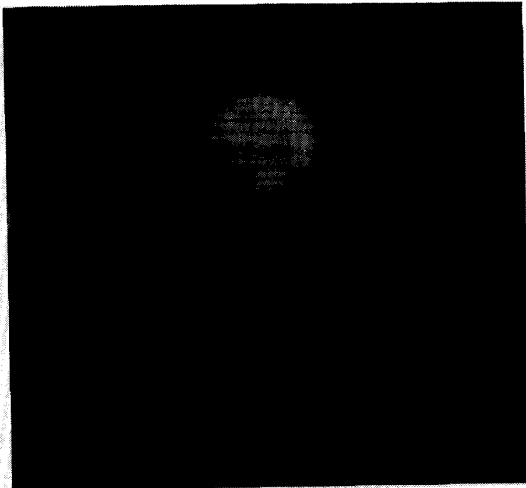


Fig. 1



Fig. 2



Fig. 3

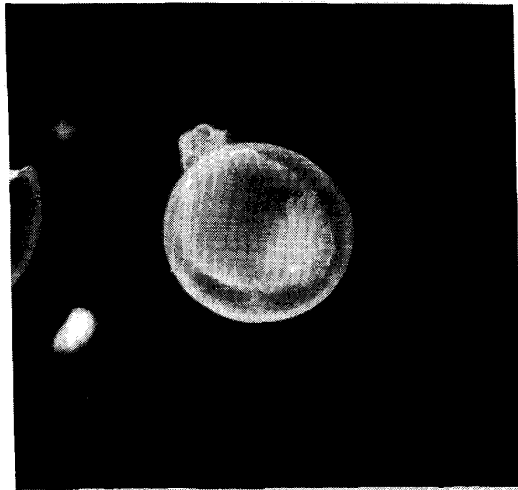


Fig. 4