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Lack of Transmission of Mouse Minute Virus (MMV) from In Vitro-Produced Embryos to Recipients and Pups Due to the Presence of Cumulus Cells During the In Vitro Fertilization Process

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ABSTRACT

The risk of transmission of mouse minute virus (MMV) to recipients of murine embryos arising from in vitro fertilization (IVF) of cumulus-enclosed oocytes (CEOs) or without cumulus cells (CDOs) in the presence of MMV-exposed (10^4 $TCID_{50}$ [mean tissue culture infective dose]/ml MMVp [prototype strain of MMV]) spermatozoa was evaluated. Also, the time after embryo transfer to detection of MMV antibody and the presence of MMV DNA in the mesenteric lymph nodes of recipients and pups were investigated. All mice were MMV free, but two seropositive recipients and four seropositive pups were found in the group with CDOs. With regard to the CEOs, two of 11 holding drops and five of 11 groups of embryos were MMV positive using PCR, while neither holding drops nor embryos carried infectious MMVp, as evidenced by the in vitro infectivity assay. From IVF with CDOs, five of 14 holding drops and four of nine groups of embryos were MMV positive, while one of 14 holding drops and no embryos carried infectious MMVp. When 10^5 cumulus cells were analyzed 5 h after exposure to 10^4 $TCID_{50}$/ml MMVp, cells had an average titer of 10^4 $TCID_{50}$/ml MMVp. The present data show that, in contrast to CDOs, 2-cell embryos from CEOs did not transmit infectious MMVp to the holding drops and to recipients. This observation is due to the presence of cumulus cells during the IVF process that reduce entry of MMV into the zona pellucida and absorb some of the virus. These data further confirm the efficacy of the IVF procedure in producing embryos that are free of infectious virus, leading to virus-free seronegative recipients and rederived pups.

assisted reproductive technology, embryo, in vitro fertilization, mouse, mouse minute virus

INTRODUCTION

In contemporary mouse facilities, the mouse germ line is usually archived by cryopreservation of spermatozoa [1] and embryos [2, 3]. When cryopreservation of spermatozoa is performed, the mouse line or strain is then rederived by performing in vitro fertilization and embryo transfer (IVF-ET) [4, 5]. The health of recipients is monitored, and provided that they are negative for relevant microorganisms, their progeny are released into full-barrier holding areas.

Mouse minute virus (MMV) is a nonenveloped linear positive-strand DNA virus of the Parvoviridae family having a diameter of approximately 20 nm [6]. The virus is highly contagious, but the outcome of natural infection in immunocompetent mice is essentially asymptomatic. Breeding mice become infected during the second and third months of life [7]. During acute infection, virus replication occurs in the small intestine and the lymphatic organs. The virus can persist in the mesenteric lymph nodes for a long period. It is transmitted primarily by urinary and/or fecal excretion. Contaminated food and bedding [7] also have a role, as the virus is highly resistant to environmental factors and even to a number of disinfectants [8, 9].

The intact zona pellucida (ZP) is regarded as a mechanism in preventing pathogen transmission during ET [4, 10–16]. Furthermore, the International Embryo Transfer Society recommends that embryos be washed 10 times before transfer to recipients [17]. For cryopreservation of sperm, the caudae epididymides and vasa deferentia together with their blood vessels are removed from the mouse and torn to allow release of the spermatozoa. Thus, if males are infected with MMV, the possibility exists that this virus will also be present in the sperm suspension. In the fertilization dish, MMV may become attached directly to the cumulus cells and/or the oocytes. Furthermore, because spermatozoa penetrate the ZP to enable fertilization, they may also serve as vectors for transmission of MMV into the oocyte. The presence of MMV on or within the in vitro-produced embryos even after washing may lead to MMV-positive recipients and pups.

In vivo-produced embryos that had been exposed in vitro to a minimum of 10^4 $TCID_{50}$ [mean tissue culture infective dose]/ml MMVp [prototype strain of MMV] and washed 10 times led to seropositive recipients [18]. In contrast, when IVF was performed with CEOs and cryopreserved spermatozoa in the presence of 10^4 $TCID_{50}$/ml MMVp, recipients and pups were MMV free and seronegative for MMV. This difference in results may be due to the presence of cumulus cells in the IVF procedure that may have prevented entry of virus to the ZP, and/or the cumulus cells may have absorbed virus.

To further extend previous studies and to determine the reason for the lack of MMV antibody in the recipients and pups after IVF in the presence of MMVp, the objective of the present study was to determine whether the presence of MMVp in the IVF procedure with cumulus-denuded oocytes (CDOs) leads to attachment of this virus to the resulting embryos and if washing the embryos 10 times leads to its removal and absence

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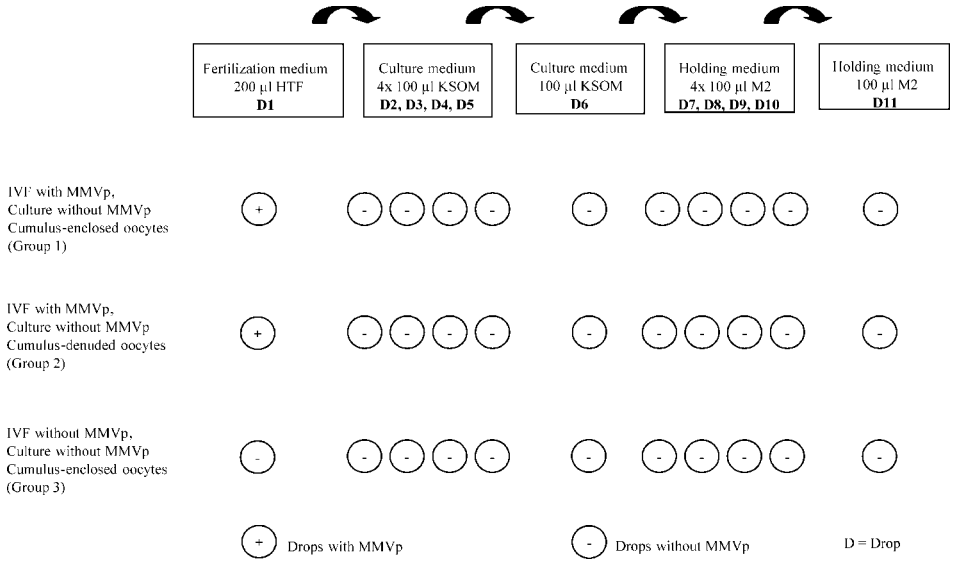

FIG. 1. The experimental design for IVF and culture in the present study. Oocytes were fertilized in the fertilization medium (HTF), washed four times in the culture medium (KSOM), and cultured overnight in KSOM, and the 2-cell embryos were washed four times in holding medium M2 and kept in M2 before ET to suitable recipients. The CEOs (group 1), CDOs (group 2), and spermatozoa were exposed to MMVp in HTF only and transferred after a standard washing procedure with 10 washing steps in virus-free KSOM and M2. The CEOs and spermatozoa were also exposed to virus-free HTF, KSOM, and M2 (group 3).

of MMV antibodies in recipients and rederived pups. In addition, the ability of the cumulus cells to uptake MMVp was investigated.

MATERIALS AND METHODS

Propagation of MMV

The MMVp stock (VR-1346), its producer cell line A9 (CCL-1.4), and L929 cells (CCL-1) were obtained from the American Type Culture Collection (Manassas, VA). A9 cells were used for propagation, while L929 cells were used for titration. A9 and L929 cell cultures were maintained in Dulbecco minimum essential medium (DMEM) supplemented with 4.5 g/L D-glucose/L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (L929 in 5% FCS). Propagation of the MMVp stock was performed in 75-cm^2 cell culture flasks (Nunc, Roskilde, DK) at 37°C using 5% CO_2 in a humidified atmosphere. Cultures of permissive cells were infected with MMV for 1 h, followed by removal of the virus suspension and replacement with 10 ml of cell culture medium. After 5 days of culture, MMVp-infected permissive cells were freeze-thawed three times to allow release of virus, centrifugation was performed at $3000 \times g$ for 5 min to separate the virus from cell debris, and the supernatant was passed through a Minisart filter (0.2 µm; Sartorius, Göttingen, Germany). For virus titration, L929 cells were seeded in 96-well plates at a concentration of 3×10^3/well, cultured overnight, and infected with 100 µl of serial 10-fold dilutions of the virus suspension. The cytopathic effect, observed as detachment of cells, was determined on the sixth day of culture. The $TCID_{50}$ for the viral stock was calculated according to the Spearman-Kaerber method [19, 20]. The MMVp stock used in this study had a titer of 10^6 $TCID_{50}$/ml and was stored at −80°C until used.

Mice and Husbandry

Inbred C3HeB/FeJ mice were bred in a full-barrier unit at the Helmholtz Center Munich. Breeding colonies were kept in filter-topped type II Makrolon (Bayer MaterialScience, Pittsburgh, PA) cages at a temperature of 20–24°C and a humidity of 50%–60% with 20 air exchanges per hour and a 12L:12D cycle. Wood shavings (3/4; Rettenmaier, Rosenberg, Germany) were provided as bedding. Mice were fed a standardized mouse diet (1314; Altromin, Lage, Germany) and were provided drinking water ad libitum.

Before entering a mouse room, staff wore a clean suit and disposable gloves, bonnets, and face masks. During routine weekly changes of cages, including lids, wire bars, and water bottles in class II laminar flow changing stations, mice were transferred to new cages using forceps padded with silicone tubing. Forceps were disinfected after each cage change with 70% ethanol. All materials were autoclaved before use.

Microbiological examination was performed every 6 wk using male Crl:CD1(Icr) sentinels. Aliquots of approximately 5 cm^3 of soiled bedding were taken from each used cage on a rack. A mixture of these aliquots was pooled with an equivalent amount of new sterile bedding and distributed to the sentinel cage of the same rack in weekly intervals over a period of 12 wk. The serological examinations were performed as recommended by the Federation of European Laboratory Animal Science Associations [21], with the addition of Leptospira serogroups (ballum, canicola, hebdomadis, and icterohaemorrhagiae), K virus, lactate dehydrogenase virus, polyoma virus, mouse thymic virus, hantaviruses [22], and murine norovirus. The mice were found to be consistently negative for all of these infectious agents, including MMV.

All animal studies were approved by the Helmholtz Center Munich Institutional Animal Care and Use Committee. They were also approved by the Government of Upper Bavaria, Germany (211-2531–8/02).

Preparation of Sperm Samples

Three-month-old C3HeB/FeJ males were killed by cervical dislocation. Both caudae epididymides and vasa deferentia were torn with sterile injection needles (Braun, Melsungen, Germany) in a culture dish with 220 ml of human tubal fluid (HTF) [23], and the spermatozoa were allowed to disperse for 5 min at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator. The dish was gently shaken until a homogeneous sperm suspension was obtained. A volume of 3 µl of spermatozoa was placed in 197-µl drops of pre-equilibrated virus-free or virus-containing medium under embryo-tested mineral oil (Sigma-Aldrich, Taufkirchen, Germany). The 200-µl HTF drops with spermatozoa were incubated for 30 min at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator and were used for IVF (fertilization drops). The motility and progression of the sperm were determined using a Hamilton Thorne (Beverly, MA) IVOS computerized semen analyzer as previously described [5]. The concentration of the spermatozoa in the HTF drops ranged from 19 to 86×10^6/ml; the ranges of motility and progression were 60%–83% and 26%–50%, respectively.

IVF and Washing Procedure

Inbred C3HeB/FeJ female mice aged 21–28 days were superovulated by i.p. injection of 5 IU of equine chorionic gonadotropin (Intergonan 1000; Intervet, Unterschleißheim, Germany) at 1600 h, followed by 5 IU of human chorionic gonadotropin (Ovogest 1500; Intervet) 50 h later. Mice were killed by cervical dislocation 13–14 h after the human chorionic gonadotropin injection, and the oviducts were removed. The cumulus-oocyte complexes were isolated from the oviducts in a 500-µl HTF drop under mineral oil. The experimental design for IVF and culture in the present study is shown in Figure 1. Oocytes and spermatozoa were coincubated in the fertilization medium (HTF) for 5 h at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator. Then, the oocytes from each fertilization drop were washed through each of four 100-µl drops of potassium simplex optimized medium (KSOM) [24] (Powdered Media Kit; Metachem Diagnostics Ltd., Northampton, United Kingdom) and were cultured overnight in the fifth 100-µl drop of KSOM under mineral oil. Twenty-four hours after commencement of the IVF procedure, the number of morphologically intact 2-cell embryos having blastomeres of approximately the same size and the number of 1-cell embryos and dead and fragmented oocytes were counted. The 2-cell embryos

were washed four times in the holding medium (M2) and were kept in M2 before ET to suitable recipients. Oocytes with cumulus cells (group 1 containing CEOs) or without cumulus cells (group 2 containing CDOs) and spermatozoa were exposed to MMVp in HTF only. To produce CDOs, the CEOs were treated with 300 μg/ml hyaluronidase (type IV-S; Sigma-Aldrich, St. Louis, MO) for 2–3 min to remove the cumulus cells. The CDOs were then washed four times in 0.5 ml of the HTF medium. The CEOs and spermatozoa were also exposed to virus-free HTF, KSOM, and M2 (group 3). The CDOs or CEOs from five females were coincubated with spermatozoa in a fertilization drop. The cleavage rate was calculated as the percentage of 2-cell embryos obtained from the total number of intact oocytes placed in a fertilization dish. A maximum of 80–100 embryos from different fertilization dishes was placed in the fifth 100-μl drop of M2 holding medium (D11 sample). In the IVF procedure, the oocytes and embryos were washed 10 times through the corresponding media. Thus, from fertilization to ET, 11 sequential drops were obtained, and this is referred to herein as a "set" of drops. A new sterile micropipette was used between each IVF dish. The dilution factor between washing drops was approximately 1:100. The maximum number of oocytes washed with the same pipette was 100.

Embryo Transfer

Nine to 10 embryos from each experimental group were transferred into each oviduct of Day 0.5 pseudopregnant Crl:CD1(Icr) recipients as previously described [25]. Negative control embryos were transferred before experimental embryos. The average volume of medium, including embryos transferred, was 0.2 μl per recipient. Surgery was performed under general anesthesia with appropriate analgesic support as previously described [25]. After ET, the recipients were housed singly in individually ventilated cages (IVCs). Pups were weaned at age 3 wk and were housed singly in IVCs.

Serological Analysis

To determine if the recipients seroconverted, blood was collected from the tail vein on Days 14, 21, 28, and 42 after ET. Sera from progeny were prepared on Day 42 and Day 63 after ET and were analyzed. Sera were diluted 1:60 in the serum diluent, which is composed of 99 ml of PBS (Oxoid, Hants, United Kingdom) containing 5 g of nonfat dry bovine milk (Marvel dried skimmed milk; Premier International Foods Ltd., Spalding, United Kingdom), 5 mg of gentamycin sulphate (Sigma-Aldrich, Dorset, United Kingdom), and 33 μl of Antifoam A concentrate (Sigma-Aldrich, Dorset, United Kingdom). Sera were tested for specific antibodies (IgG whole molecule) to MMV via an ELISA using control non-viral-coated and viral-coated plates and negative and positive serum (Charles River Laboratories, Wilmington, MA). The optical density (OD) was read at 492 nm with a Multiskan ELISA plate reader (Thermo Life Sciences, Hampshire, United Kingdom). The final OD for the sample is calculated by subtracting the control well OD from the test well OD. Serological results for MMV were positive when the final OD was greater than 0.399.

Virological Examination of Washing Drops and Embryos

The used media were stored in sterile tubes at −80°C until analyzed. In total, sequential drops from 40 and 18 sets of IVF and culture dishes from the CDO and CEO groups, respectively, were analyzed. All drops were diluted 1:10 with DMEM and 10% FCS and were tested for the presence of MMVp by PCR and for virus infectivity by in vitro infectivity assay (IVIA) in cell culture using the protocols previously described [18, 26]. DMEM containing 10% FCS was used as the diluent to facilitate cell culture.

Briefly, for the IVIA, L929 cells were seeded in 96-well plates at a concentration of 3×10^3/well and cultured overnight. After removal of the culture medium, samples were run in duplicate by inoculating each of two wells with 100 μl of each dilution. MMVp-specific cytopathic effects, observed as detachment of cells, were determined on the sixth day of culture.

For PCR, 200 μl of the diluted drops was analyzed. Total DNA from each diluted drop was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers used were those designed by Bootz et al. [27] as follows: 5′-GAGCGCCATCTAGT GAGC-3′ (forward) and 5′-ATTTGCCTGTGCTGGCTG-3′ (reverse), yielding a 483-base pair (bp) product. A double-distilled water sample served as a negative PCR control. The PCR was performed in a total volume of 20 μl using Taq DNA polymerase (Qiagen) for 40 cycles in a thermocycler (Biometra; Biomedizinische Analytik GmbH, Göttingen, Germany). Denaturation was performed at 94°C for 4 min. Each cycle consisted of 94°C (30 sec), 55°C (30 sec), and 72°C (30 sec). The last cycle was followed by a 7-min extension period at 72°C. The PCR products (10 μl) were mixed with 2 μl of loading buffer (MBI Fermentas, St. Leon-Rot, Germany), electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

In addition, groups of embryos from IVF with CDOs (n = 3, 4, 6, 10, 20, 20, 20, 20, 20) or with CEOs (n = 4, 4, 7, 10, 10, 10, 20, 20, 20, 20, 20) were analyzed for the presence of MMVp using PCR and IVIA. Before DNA extraction and PCR as already described for the media, embryos were frozen and thawed three times to allow release of the virus.

PCR Analysis of the Mesenteric Lymph Nodes from Recipients and Pups

In addition to serology, the mesenteric lymph nodes from the recipients and pups were examined for the presence of MMV by PCR as already described. The mesenteric lymph nodes were collected on Day 42 from recipients and from pups on Day 42 or Day 63 after ET. DNA was extracted using the QIAamp DNA mini kit according to the manufacturer's instructions.

Uptake of MMVp by Cumulus Cells

Immediately after removal by enzymatic digestion, the cumulus cells were taken up in 1 ml of HTF, centrifuged at $1200 \times g$ for 5 min, and washed two times in 1 ml of HTF. A total of 1×10^5 cumulus cells from approximately five females, corresponding to the number of cells in one fertilization dish, was coincubated in the MMVp containing 200-μl fertilization drops (0.02 $TCID_{50}$ per cell) for 5 h in the incubator under the same conditions as for IVF. The cells were then centrifuged, and the supernatant was removed. The latter was diluted 1:10 in DMEM with 10% FCS (Seromed; Biochrom, Berlin, Germany) before titration by IVIA as already described. The cell pellet was taken up in fresh DMEM with 10% FCS and washed two times in DMEM before titration by IVIA as already described. In addition, the same experiment was performed with L929 cells, which are permissive for MMVp, to compare viral uptake with that of the cumulus cells. Five and four replications were performed for the cumulus cells and the L929 cells, respectively.

Scanning Electron Microscopy of Oocytes and 2-Cell Embryos

To compare the ZP of CDOs and CEOs 5 h after coincubation of the gametes, a minimum of four oocytes from each group was examined using scanning electron microscopy. In addition, CDOs and 2-cell embryos from each experimental group were fixed immediately after removal of the cumulus cells and on the day of ET, respectively, and a minimum of four each was also examined using scanning electron microscopy. The oocytes and embryos were washed in PBS (pH 7.4) and prefixed in 1% glutaraldehyde in PBS overnight. The samples were dehydrated in a graded-ethanol, critical-point-dried, and sputter-coated series (K575; Emitech Ltd., Ashford, United Kingdom) with 5 nm of platinum. Coated samples were examined in a field emission scanning electron microscope (JSM-6300F; JEOL, Tokyo, Japan) with accelerating voltages of 5 kV in secondary electron mode.

Statistical Analysis

The differences between experimental groups with regard to the reproductive data and the data generated by PCR analysis of the washing drops and embryos were compared using Fisher exact test. Because of missing effective observations (values with 0), no p values were computed for data generated using serology (see Table 2) and IVIA (see Table 3). The global level of significance was chosen to be 0.05. All statistical analyses were performed using SAS (*SAS/STAT User's Guide*. Version 9.1. Cary, NC: SAS Institute Inc.; 2003).

RESULTS

Reproductive Data

The percentages of intact oocytes from the total number (intact, degenerated, and fragmented) collected in group 1, group 2, and group 3 were 83% (888/1066), 85% (1872/2194), and 92% (393/429), respectively (data not shown). The cleavage rates and reproductive data after transfer of IVF embryos for the three groups investigated are given in Table 1. The cleavage rates, defined as the percentage of 2-cell embryos developing from the number of intact oocytes coincubated, were 51%, 27%, and 41% for group 1, group 2, and group 3,

TABLE 1. Cleavage rate and reproductive data after transfer of embryos from in vitro fertilization with gametes exposed to 10^4 $TCID_{50}$/ml MMVp.

Experimental group	No. of two-cell embryos/ no. of oocytes coincubated (%)	No. of recipients littering/ no. of recipients (%)	No. of live young/ no. of 2-cell embryos transferred (%)
IVF with CEOs and MMVp (group 1)	453/888 (51)[a]	5/6 (83)[a]	25/116 (22)[a]
IVF with CDOs and MMVp (group 2)	499/1872 (27)[b]	7/15 (47)[a]	28/289 (10)[b]
IVF with CEOs and without MMVp (group 3)	162/393 (41)[ab]	4/6 (67)[a]	24/116 (21)[a]

[a,b] Values with different superscript letters in the same column differ significantly, $P < 0.05$; Fischer exact test.

respectively. Significant differences in the cleavage rates were observed between group 1 and group 2 ($p < 0.05$). The percentages of recipients from group 1, group 2, and group 3 that littered were 83%, 47%, and 67%, respectively ($p > 0.05$). With regard to group 1, group 2, and group 3, the percentages of live pups born from the number of embryos that were transferred were 22%, 10%, and 21% from 116, 289, and 116 embryos, respectively. Significant differences in the number of pups born were observed between group 1 and group 2 and between group 2 and group 3 ($p < 0.05$). No significant differences were found between group 1 and group 3 ($p > 0.05$).

Serological Data

With regard to group 1, none of the six recipients or their pups were seropositive for MMVp throughout the experimental period. From 15 recipients in group 2, two were seropositive between Day 21 and Day 42. From one of the two seropositive recipients in group 2, four pups were seropositive for MMVp on Day 42, whose mesenteric lymph nodes (in addition to those from 11 other pups) were collected for PCR analysis. None of the remaining 13 pups had anti-MMV antibody on Day 63. In group 3, all six recipients and their pups were seronegative throughout the study. The serological data are summarized in Table 2.

PCR Analysis of the Mesenteric Lymph Nodes from Recipients and Pups

The mesenteric lymph nodes from recipients and pups were analyzed by PCR. The mesenteric lymph nodes from all recipients from group 1 (n = 6), group 2 (n = 15), and group 3 (n = 6) and from all pups on Day 42 (n = 11, n = 15, and n = 12, respectively) and on Day 63 (n = 14, n = 13, and n = 13, respectively) were shown to be free of MMV by PCR analysis.

Virological Examination of Washing Fluids, Remaining Media, and Embryos

Figure 2 shows the data for detection of MMVp using PCR (black bars) and the IVIA (grey bars) in group 1 (A) and group 2 (B). Representative PCR photographs of the position of the 483-bp bands are shown in Figure 3. From the PCR testing of drops from a total of 18 fertilization dishes from group 1, the HTF medium (D1) and drops two to six (D2–D6) were positive in 18 cases. In D7, the number of positive drops decreased to six (Fig. 2A), whereas one drop was positive in D8. In D9 and D10, none of the drops were positive. When embryos were held before transfer (D11), MMVp was detected in two of 11 drops (Table 3). Five of 11 groups of embryos tested positive for MMV (Table 3).

The IVIA showed that the HTF medium (D1) and D2 from all 18 dishes were positive for MMVp. The numbers of dishes that were positive for MMVp in D3, D4, D5, D6, and D7–D10 were 16, 9, 3, 1, and 0, respectively (Fig. 2A). When embryos were held before transfer (D11), MMVp was detected in none of 11 drops analyzed (Table 3). The embryos tested negative for MMVp (Table 3).

From the PCR testing of drops from 40 fertilization dishes from group 2, the HTF medium (D1) and drops two to four (D2–D4) were positive in 40 cases (Fig. 2B). In D5, D6, D7, and D8, the number of positive drops decreased to 38, 37, 17, and 3, respectively. In D9 and D10, none of the drops were positive. When embryos were held before transfer (D11), MMVp was detected in five of 14 drops (Table 3). Four of nine groups of embryos tested positive for MMV (Table 3).

The IVIA showed that the HTF medium (D1) and D2 from all 40 dishes were positive for MMVp (Fig. 2B). The numbers of dishes that were positive for MMVp in D3, D4, D5, D6, and D7–D10 were 39, 28, 9, 3, and 0, respectively (Fig. 2B). When embryos were held before transfer (D11), MMVp was detected in one of 14 drops analyzed. The embryos tested negative for MMVp (Table 3).

TABLE 2. Seroconversion of recipients and progeny after in vitro fertilization and transfer of 2-cell embryos into Day 0.5 pseudopregnant recipients following exposure of gametes to 10^4 $TCID_{50}$/ml MMVp.

	No. of seropositive mice/no. of mice*					
	Recipients				Progeny	
Experimental group	Day 14	Day 21	Day 28	Day 42	Day 42	Day 63
IVF with CEOs and MMVp (group 1)	0/6	0/6	0/6	0/6	0/25[†]	0/14
IVF with CDOs and MMVp (group 2)	0/15	2/15	2/15	2/15	4/28[‡]	0/13
IVF with CEOs and without MMVp (group 3)	0/6	0/6	0/6	0/6	0/25[§]	0/13

* No *P* values were computed for serological data due to missing effective observations.
† Eleven pups were used for PCR analysis.
‡ Fifteen pups, including the four seropositive pups, were used for PCR analysis.
§ Twelve pups were used for PCR analysis.

TABLE 3. Detection of MMVp by PCR and in vitro infectivity assay of the holding medium (D11) and groups of embryos after in vitro fertilization with gametes exposed to 10^4 $TCID_{50}$/ml MMVp.

	PCR		In vitro infectivity assay*	
Sample analyzed	IVF with CEOs and MMVp (group 1)	IVF with CDOs and MMVp (group 2)	IVF with CEOs and MMVp (group 1)	IVF with CDOs and MMVp (group 2)
D11	2/11[a]	5/14[a]	0/11	1/14
Embryos	5/11[†a]	4/9[‡a]	0/11[†]	0/9[‡]

* No *P* values were computed for in vitro infectivity assay data due to missing effective observations.
† Groups of 4–20 embryos were analyzed.
‡ Groups of 3–20 embryos were analyzed.
[a] Values with the same superscript letter in the same row do not differ significantly, $P > 0.05$; Fischer exact test.

Statistical analysis of data from the washing drops and embryos was performed. The results did not reveal significant differences between group 1 and group 2 ($p > 0.05$).

Uptake and Replication of MMVp by Cumulus Cells

Titration of the cumulus cells and L929 cells and their supernatants was performed to determine virus uptake during a 5-h period corresponding to the duration of incubation of the oocytes and spermatozoa. The results from five replications with the cumulus cells and four replications with the L929 cells are given in Table 4. The data show that the cumulus cells and their supernatants had average titers of $10^{3.9}$ and 10^4 $TCID_{50}$/ml, respectively. The L929 cells and their supernatants had titers of $10^{3.1}$ and $10^{4.4}$ $TCID_{50}$/ml, respectively.

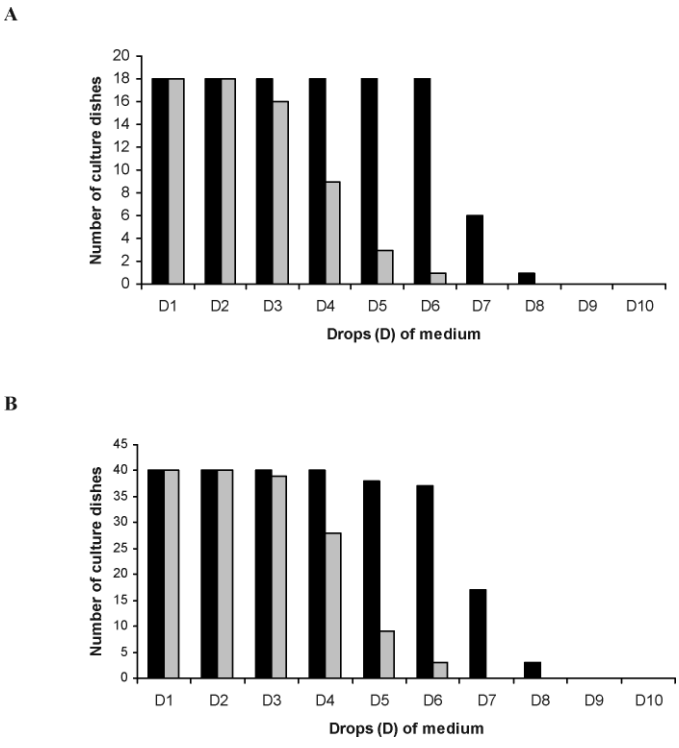

FIG. 2. Number of media drops (D) that were positive for MMVp by PCR (black bars) and by IVIA (grey bars), in which the fertilization dishes contained 10^4 $TCID_{50}$ MMVp/ml HTF (D1). D2–D5: Washing steps with KSOM. D6: Overnight culture in KSOM. D7–D10: Washing steps with M2. In (**A**) and (**B**), 18 and 40 sets of dishes were analyzed, respectively. There were no significant differences between group 1 and group 2 with regard to the PCR and IVIA data from the washing drops ($p > 0.05$, Fisher exact test).

Scanning Electron Microscopy

Scanning electron microscopy of oocytes and 2-cell embryos was performed to determine if there were morphological changes in the surface of the ZP that may have influenced uptake and transmission of MMV. Scanning electron microscopy showed that the ZP of the CDOs had fewer pores immediately after removal of the cumulus cells with hyaluronidase (Fig. 4A). However, no differences in the ZP of CDOs (Fig. 4B) and CEOs (Fig. 4C) were observed 5 h after coincubation with the spermatozoa. With regard to the 2-cell embryos, the surface of the ZP was generally smoother than that of the oocytes (Fig. 4, D–F). Also, the surface of the ZP of 2-cell embryos originating from CEOs fertilized in the presence of MMV (Fig. 4F) was smoother than that of those produced from CDOs fertilized in the presence of MMVp (Fig. 4E) and CEOs fertilized in the absence of MMVp (Fig. 4D).

DISCUSSION

In vivo-produced embryos that had been exposed in vitro to 10^4 $TCID_{50}$/ml MMVp and washed 10 times led to seropositive recipients after ET [18]. However, when CEOs were fertilized in vitro with cryopreserved spermatozoa in the presence of 10^4 $TCID_{50}$/ml MMVp and the embryos were washed 10 times, all recipients were seronegative [28]. The lack of transmission of infectious MMVp by IVF embryos may be due to the cumulus cells surrounding the oocytes, which may have prevented entry of MMVp to the ZP, and/or they may have absorbed the virus.

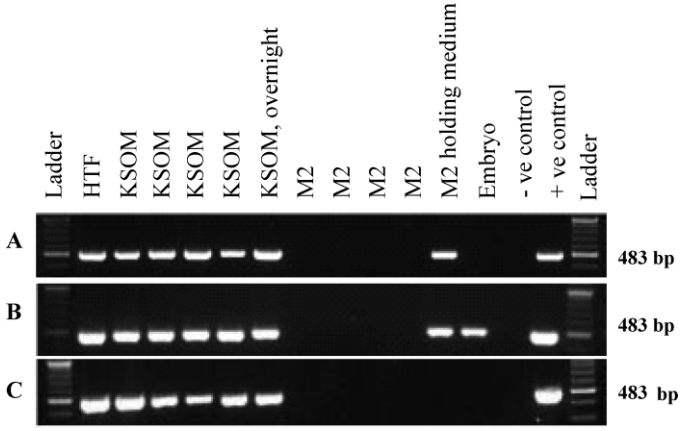

FIG. 3. Representative PCR photographs showing the positions of the 483-bp bands obtained by analysis of the washing drops and embryos. **A**) Washing drops are positive up to D6, D11 is positive, and embryos are negative. **B**) Washing drops are positive up to D6, and D11 and embryos are positive. **C**) Washing drops are positive up to D6, and D11 and embryos are negative. – ve, negative control; + ve, positive control.

TABLE 4. Titration results ($TCID_{50}$/ml) of cumulus cells, L929 cells, and their supernatants after exposure of cells to 10^4 $TCID_{50}$/ml for five hours.

	Cumulus cells		L929 cells	
Replication no.	10^5 Cells	Supernatant	10^5 Cells	Supernatant
1	$10^{4.6}$	$10^{4.3}$	$10^{2.8}$	$10^{4.6}$
2	$10^{3.6}$	$10^{3.8}$	$10^{3.5}$	$10^{4.5}$
3	$10^{3.6}$	$10^{3.5}$	$10^{3.1}$	$10^{3.8}$
4	$10^{4.0}$	$10^{4.1}$	$10^{3.0}$	$10^{4.6}$
5	$10^{3.5}$	$10^{4.5}$	ND*	ND*
Average of all replications	$10^{3.9}$	$10^{4.0}$	$10^{3.1}$	$10^{4.4}$

* ND, not determined.

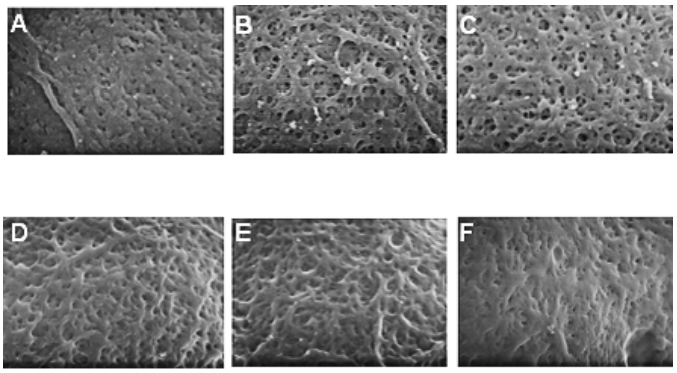

FIG. 4. Scanning electron micrographs of oocytes (**A**–**C**) and 2-cell embryos derived from IVF (**D**–**F**). **A**) The CDOs before IVF. **B**) The CDOs 5 h after coincubation with the spermatozoa in the presence of MMVp. **C**) The CEOs 5 h after coincubation with the spermatozoa in the presence of MMVp. **D**) Two-cell embryos derived from CEOs in the absence of MMVp (negative control). **E**) Two-cell embryos derived from CDOs in the presence of MMVp. **F**) Two-cell embryos derived from CEOs in the presence of MMVp. Bar = 1 μm.

In the present study, we investigated the risk of transmission of MMVp to recipients and their pups by embryos arising from IVF of CEOs and CDOs with MMV-exposed spermatozoa. Our data show that, in contrast to CDOs, 2-cell embryos from CEOs did not transmit infectious MMVp to the holding drops or to recipients and pups most likely due to the presence of cumulus cells during the IVF procedure. Furthermore, for IVF with CEOs in the presence of MMVp, washing the embryos 10 times is sufficient to remove the viral load to a noninfectious nonimmunogenic level, confirming previous results [28]. Key factors contributing to the reduction of the viral load are the use of new pipettes for each fertilization or culture dish and each washing drop and the fact that a maximum of 80–100 oocytes or embryos was contained in each dish.

The C3HeB/FeJ strain of mice was chosen for this study because it is used to produce mutant mice and can be successfully archived by cryopreservation of spermatozoa and revitalized via IVF-ET [1]. Crl:CD1(Icr) mice are typically used as ET recipients at many facilities. With respect to the virus, we used the MMVp to extend the results of previous studies [18, 26, 28], thereby enabling comparison of data.

The PCR analysis used in the present study, which also detects noninfectious virus to a limit of detection of 10^{-6} $TCID_{50}$, showed that there was a continuous reduction in the viral load in the IVF procedure with both CEOs and CDOs until drop nine, when no MMVp DNA was detected. The IVIA, which detects infectious virus, showed that there was a steep decrease in the number of dishes containing infectious viruses until drop seven, when MMVp was not detected. In the groups with both CEOs and CDOs, drops nine and 10 tested negative for MMVp using both PCR and IVIA. Although some of the holding drops and some of the embryos contained MMV DNA in both groups, infectious virus was found in only one of 14 groups of embryos derived from CDOs using the IVIA. Previous work in our laboratory [18] showed that pups obtain maternal antibodies when the recipients seroconvert by Day 14 after ET, which dwindled by Day 133 after ET. As evidenced by the presence of MMV antibodies in two recipients and four pups on Day 21 and Day 42 of testing, respectively, embryos derived from CDOs served as vectors of MMVp. Vertical transmission of MMVp was not observed because all recipients and pups were free of MMV DNA, as demonstrated by PCR of mesenteric lymph nodes. The latter observation confirms previous studies [18, 28, 29].

During development of the oocyte, the cumulus cells are connected with the oolema by cytoplasmic filaments penetrating the ZP. When the cumulus cells are removed from the ZP, micropores of approximately 140-1000 nm in the ZP of murine embryos [30] arise. During the standard IVF process with CEOs, cumulus cells are progressively dispersed from the oocytes, and not all are removed by the time the oocytes are taken from the fertilization drop and washed in preparation for culture. Therefore, the number of micropores in the ZP of CEOs is not expected to be as high as that found in CDOs, thus allowing entrapment of a lower quantity of the virus from the fertilization media. Furthermore, the cumulus cells can absorb some of the virus. Such a situation may be conducive to adequate removal of the virus by washing to a noninfectious nonimunogenic dose for the ET recipients, as demonstrated in the present study.

In vivo, cumulus cells have a role in the prevention of dissemination of pathogens to the oocyte itself. Previous studies [31–34] show that, in different species, the cumulus cells themselves carried virus, but the oocytes were virus free. Removal of the cumulus cells from the oocytes led to mouse parvovirus (MPV)-free oocytes [35]. In the present study, exposure of cumulus cells to MMVp for 5 h and titration of cells and supernatants showed that the cumulus cells were positive for MMVp, which may be on and/or within the cells themselves. Their viral titers were similar to those of the permissive L929 cells. These results strongly suggest that the cumulus cells reduce MMV transmission during the IVF procedure both by blocking entry of the virus to the oocyte itself and by uptaking some of the virus from the media.

In the present study, IVF was performed with oocytes from pathogen-free mice. During routine rederivation work, the health status of a mouse room is known if health monitoring is performed regularly, but that of particular mouse colonies may not be known. Therefore, special care has to be taken because oocytes may originate from virus-positive mice, and their cumulus cells may serve as vectors of viruses, thereby transmitting them to the developing embryo during the IVF procedure and ultimately to recipients, which could seroconvert. The higher risk of transmission of another parvovirus (MPV) by embryos originating from infected females compared with those from infected males has been recently documented in detail [29]. This knowledge emphasizes the fact that oocytes for IVF should be collected from pathogen-free mice, and stringent hygienic measures should be implemented, including recipients being kept in IVCs and their being screened before release of the rederived pups [36].

The cumulus cells surrounding the oocyte are embedded in an extracellular matrix rich in hyaluronic acid. The spermato-

zoa produce hyaluronidase, allowing them to pass through the cumulus cell layers [37, 38]. Capacitation describes changes that occur, allowing the spermatozoa to undergo the acrosome reaction. The latter refers to a change in membrane structure occurring when the spermatozoa have passed through the cumulus cells and have established contact with the ZP. Only capacitated sperm will bind tightly to the ZP [37, 38], leading to fertilization. Although no significant differences were found in the number of recipients littering in the present study, the fertilization rate of 27% of CDOs in the presence of MMVp was significantly lower than that of CEOs in the presence of MMVp (51%). Likewise, the number of live pups born in group 2 was significantly lower than that in group 1. There are conflicting reports on the success of IVF with CDOs vs. with CEOs. The lower cleavage rates and development with CDOs in the present study confirm previous results with oocytes from mice, cattle, and humans [39–43], while other researchers observed no significant differences in fertilization using murine CDOs or CEOs [44, 45]. It was reported that cumulus cells improve the fertilization rate by providing a capacitation-inducing mechanism [40, 42] or increase the contact area between spermatozoa and the oocyte, thus allowing more spermatozoa to become attached [41, 42]. In addition, they may assist acrosome-reacting or acrosome-reacted spermatozoa in penetrating the ZP, as oocyte movement is greatly reduced by the cumulus cells. However, when the cumulus cells are absent, the oocytes migrate due to the force of sperm motility, reducing sperm penetration into the ZP [46]. Furthermore, the cumulus cells may prevent spontaneous zona hardening [47, 48], which leads to decreased fertilization rates.

To determine whether denudation of cumulus cells by hyaluronidase affected the surface of the ZP, which could ultimately affect viral uptake, scanning electron microscopy was performed on oocytes and 2-cell embryos. The present data initially reflect slight differences in the ZP of CDOs, characterized by fewer pores, immediately after cumulus cell removal, which were not observed 5 h later. The surface of the ZP of the 2-cell embryos was generally smoother than that of the oocytes. The surface of the ZP of 2-cell embryos originating from CEOs fertilized in the presence of MMVp was less porous than that of the 2-cell embryos originating from CEOs fertilized in the absence of MMVp and the 2-cell embryos produced from CDOs fertilized in the presence of MMVp. It should be noted that all oocytes and embryos from the different groups were processed and analyzed in parallel. The unexpected observation of a smoother ZP may be due to more spermatozoa passing through the cumulus cells and becoming attached to the ZP. A higher number of spermatozoa should lead to a higher level of inherent hyaluronidase in the fertilization dishes. However, the number of spermatozoa attaching to the oocytes, the number of sites of spermatozoa attachment, and the amount of hyaluronidase in the fertilization dishes were not determined in the present study.

Taken together, our findings confirm that embryos arising from IVF of CEOs with MMVp-exposed spermatozoa did not lead to seropositive recipients and pups, in contrast to IVF of CDOs. As such, the risk of transmission of MMVp by in vitro-produced embryos to recipients during ET appears to be very low or nonexistent due to the presence of cumulus cells around the oocytes during the IVF procedure.

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REFERENCES

1. Marschall S, Huffstadt U, Balling R, Hrabe de Angelis M. Reliable recovery of inbred mouse lines using cryopreserved spermatozoa. Mamm Genome 1999; 10:773–776.
2. Glenister PH, Whittingham DG, Wood MJ. Genome cryopreservation: a valuable contribution to mammalian genetic research. Genet Res 1990; 56: 253–258.
3. Shaw JM, Nakagata N. Cryopreservation of transgenic mouse lines. Methods Mol Biol 2002; 180:207–228.
4. Suzuki H, Yorozu K, Watanabe T, Nakura M, Adachi J. Rederivation of mice by means of in vitro fertilization and embryo transfer. Exp Anim 1996; 45:33–38.
5. Sztein JM, Farley JS, Mobraaten LE. In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod 2000; 63:1774–1780.
6. Crawford LV. A minute virus of mice. Virology 1966; 29:605–612.
7. Parker JC, Collins MJ Jr, Cross SS, Rowe WP. Minute virus of mice, II: prevalence, epidemiology, and occurrence as a contaminant of transplanted tumors. J Natl Cancer Inst 1970; 45:305–310.
8. Harris RE, Coleman PH, Morahan PS. Stability of minute virus of mice to chemical and physical agents. Appl Microbiol 1974; 28:351–354.
9. Boschetti N, Wyss K, Mischler A, Hostettler T, Kempf C. Stability of minute virus of mice against temperature and sodium hydroxide. Biologicals 2003; 31:181–185.
10. Neighbour PA. Studies on the susceptibility of the mouse preimplantation embryo to infection with cytomegalovirus. J Reprod Fertil 1978; 54:15–20.
11. Reetz IC, Wullenweber-Schmidt M, Kraft V, Hedrich HJ. Rederivation of inbred strains of mice by means of embryo transfer. Lab Anim Sci 1988; 38:696–701.
12. Carthew P, Wood MJ, Kirby C. Pathogenicity of mouse hepatitis virus for preimplantation mouse embryos. J Reprod Fertil 1985; 73:207–213.
13. Zeichner SL, Plotkin SA. Mechanisms and pathways of congenital infections. Clin Perinatol 1988; 15:163–188.
14. Vanroose G, Nauwynck H, Van Soom A, Vanopdenbosch E, de Kruif A. Replication of cytopathic and noncytopathic bovine viral diarrhea virus in zona-free and zona-intact in vitro-produced bovine embryos and the effect on embryo quality. Biol Reprod 1998; 58:857–866.
15. Vanroose G, Nauwynck H, Van Soom A, Vanopdenbosch E, de Kruif A. Effect of bovine herpesvirus-1 or bovine viral diarrhea virus on development of in vitro-produced bovine embryos. Mol Reprod Dev 1999; 54:255–263.
16. Van Keuren ML, Saunders TL. Rederivation of transgenic and gene-targeted mice by embryo transfer. Transgenic Res 2004; 13:363–371.
17. Stringfellow DA, Seidel SM (eds.). Manual of the International Embryo Transfer Society. Savoy: International Embryo Transfer Society; 1998.
18. Mahabir E, Bulian D, Needham J, Mayer A, Mateusen B, Soom AV, Nauwynck H, Schmidt J. Transmission of mouse minute virus (MMV) but not mouse hepatitis virus (MHV) following embryo transfer with experimentally exposed in vivo-derived embryos. Biol Reprod 2007; 76: 189–197.
19. Spearman C. The method of 'right and wrong cases' (constant stimuli) without Gauss's formulae. Br J Psychol 1908; 2:227–242.
20. Kaerber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Pathol Pharmakol 1931; 162:480–483.
21. Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, Illgen-Wilcke B. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim 2002; 36: 20–42.
22. Kraft V, Blanchet HM, Boot R, Deeny A, Hansen AK, Hem A, van Herck H, Kunstyr I, Needham JR, Nicklas W, Perrot A, Rehbinder C, et al. Recommendations for the health monitoring of mouse, rat, hamster, guineapig and rabbit breeding colonies: report of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Animal Health accepted by the FELASA Board of Management November 1992. Lab Anim 1994; 28:1–12.
23. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985; 44:493–498.
24. Lawitts JA, Biggers JD. Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol Reprod 1991; 45:245–251.
25. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the mouse embryo. New York: Cold Spring Harbor Laboratory Press; 2003.
26. Mahabir E, Jacobsen K, Brielmeier M, Peters D, Needham J, Schmidt J. Mouse antibody production test: can we do without it? J Virol Methods 2004; 120:239–245.

27. Bootz F, Sieber I, Popovic D, Tischhauser M, Homberger FR. Comparison of the sensitivity of in vivo antibody production tests with in vitro PCR-based methods to detect infectious contamination of biological materials. Lab Anim 2003; 37:341–351.
28. Mahabir E, Bulian D, Schmoller R, Needham J, Schmidt J. Production of virus-free seronegative pups from murine embryos arising from in vitro fertilization with mouse minute virus-exposed spermatozoa. Biol Reprod 2008; 78:53–58.
29. Besselsen DG, Romero-Aleshire MJ, Munger SJ, Marcus EC, Henderson KS, Wagner AM. Embryo transfer rederivation of C.B-17/Icr-Prkdc(scid) mice experimentally infected with mouse parvovirus 1. Comp Med 2008; 58:353–359.
30. Nogues C, Ponsa M, Vidal F, Boada M, Egozcue J. Effects of aging on the zona pellucida surface of mouse oocytes. J In Vitro Fert Embryo Transf 1988; 5:225–229.
31. Cortez Romero C, Fieni F, Roux C, Russo P, Guibert JM, Guiguen F, Chebloune Y, Pepin M, Pellerin JL. Detection of ovine lentivirus in the cumulus cells, but not in the oocytes or follicular fluid, of naturally infected sheep. Theriogenology 2006; 66:1131–1139.
32. Ali Al Ahmad MZ, Fieni F, Martignat L, Chatagnon G, Baril G, Bouvier F, Chebloune Y. Proviral DNA of caprine arthritis encephalitis virus (CAEV) is detected in cumulus oophorus cells but not in oocytes from naturally infected goats. Theriogenology 2005; 64:1656–1666.
33. Fray MD, Prentice H, Clarke MC, Charleston B. Immunohistochemical evidence for the localization of bovine viral diarrhea virus, a single-stranded RNA virus, in ovarian oocytes in the cow. Vet Pathol 1998; 35: 253–259.
34. Tsuboi T, Kanazawa Y, Syoji T, Tokuhisa S. Growth activity of bovid herpesvirus 1 in bovine follicular oocytes with cumulus cells. J Vet Med Sci 1992; 54:1179–1181.
35. Agca Y, Bauer BA, Johnson DK, Critser JK, Riley LK. Detection of mouse parvovirus in Mus musculus gametes, embryos, and ovarian tissues by polymerase chain reaction assay. Comp Med 2007; 57:51–56.
36. Mahabir E, Bauer B, Schmidt J. Rodent and germplasm trafficking: risks of microbial contamination in a high-tech biomedical world. ILAR J 2008; 49:347–355.
37. Joyce CL, Mack SR, Anderson RA, Zaneveld LJ. Effect of hyaluronidase, beta-glucuronidase and beta-N-acetylglucosaminidase inhibitors on sperm penetration of the mouse oocyte. Biol Reprod 1986; 35:336–346.
38. Lin Y, Mahan K, Lathrop WF, Myles DG, Primakoff P. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. J Cell Biol 1994; 125:1157–1163.
39. Cross PC, Brinster RL. In vitro development of mouse oocytes. Biol Reprod 1970; 3:298–307.
40. Pavlok A, McLaren A. The role of cumulus cells and the zona pellucida in fertilization of mouse eggs in vitro. J Reprod Fertil 1972; 29:91–97.
41. Stanger JD, Quinn P. Fertilization of cumulus-free, zona-intact mouse ova in vitro at high and low sperm concentrations. Gamete Res 1982; 5:61–70.
42. Cox JF, Hormazabal J, Santa Maria A. Effect of the cumulus on in vitro fertilization of bovine matured oocytes. Theriogenology 1993; 40:1259–1267.
43. Tao T, Robichaud A, Nadeau S, Savoie R, Gallant B, Ouellette R. Effect of cumulus cell removal on the fertilization and the day 3 embryo quality in human IVF. In: Grundy K (ed.), Proceedings of the 18th World Congress on Fertility and Sterility (IFFS 2004), Montreal, Canada 23–28 May 2004. Amsterdam: Elsevier; 2004:135–138. International Congress Series 1271.
44. Hashlamoun LA, Killian GJ. Effects of timing of ovum recovery, cumulus cells, sperm preincubation time, and pH on in vitro fertilization in C57BL/6 mice. Arch Androl 1985; 15:159–171.
45. Vergara GJ, Irwin MH, Moffatt RJ, Pinkert CA. In vitro fertilization in mice: strain differences in response to superovulation protocols and effect of cumulus cell removal. Theriogenology 1997; 47:1245–1252.
46. Kim E, Yamashita M, Kimura M, Honda A, Kashiwabara S, Baba T. Sperm penetration through cumulus mass and zona pellucida. Int J Dev Biol 2008; 52:677–682.
47. Vanderhyden BC, Armstrong DT. Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. Biol Reprod 1989; 40:720–728.
48. Galeati G, Modina S, Lauria A, Mattioli M. Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution. Mol Reprod Dev 1991; 29:40–46.