The Effect of Pestivirus on the *in vitro* Development of Bovine Morula and Blastocysts

Kafi*[1], M., McGowan, M.R.[2] and Young, M.[3]

1. Clinical Sciences Dept., School of Veterinary Medicine, Shiraz University, P.O.Box 71345, Shiraz, Iran

2. Royal Veterinary College, University of London, North Mymms, AL9 7TA, UK

3. School of Veterinary Medicine, University of Queensland, 4070 QLD, Australia

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Summary

The *in vitro* effect of a non-cytopathogenic (nCP) pestivirus type 1 isolate on the development of bovine embryos was determined. In experiment 1, forty *in vivo*-derived blastocysts were randomly assigned to a control group which was not exposed to bovine pestivirus and a treated group which was inoculated with the virus (2.9 to 4.4$\log_{10}TCID_{50}$/50µl) each of 20. In experiment 2, *in vitro* derived morula (n=18) were randomly divided into a control group (n=8) and a treated group (as above; n=10). All embryos were cultured and their morphologic appearance was assessed at 24 and 48h after the commencement of culture. Results obtained from experiment 1 showed no significant difference ($p<0.05$) in the rate of hatching between the control and treated groups (80 vs 85%, respectively). Also, no significant difference ($p<0.05$) was observed in the rate of blastocyst formation when *in vitro* produced morula were exposed to pestivirus during a 48h culture period. One surprising observation in this study was a non-significant enhanced embryonic development in the pestivirus-exposed embryos comparing to the control non-exposed embryos. Results of the present study showed no decreasing effect on the overall rate of *in vitro* embryo development in the presence of nCP bovine pestivirus.

Key words: pestivirus, embryos, *in vitro* culture, cattle

Introduction

Bovine pestivirus is a widespread pathogen among world cattle population (Kafi

*Author for correspondence. E-mail: Kafi@shirazu.ac.ir

1998). This infection has also long been recognized as a threat to Iranian cattle industry (Mirshamsy *et al* 1967, Sedighi-Nezhad 1996). The virus exists as two biotypes non-cytopathogenic (nCP) and cytopathogenic (CP). Both nCP and CP viruses can be sub-divided into two genetic groups genotypes 1 and 2 (Donis 1995). The virus has a high disseminating potential due to the high titers in most reproductive tissues (Bielanski *et al* 1993, Brownlie *et al* 1997). Although there is evidence to suggest that CP pestivirus can adversely affect the integrity of the uterus (Archbald *et al* 1979) or the embryo (Grahn *et al* 1984, Brock & Stringfellow 1993), the data to elucidate the pathogenesis of conception failure in cattle transiently infected with a nCP pestivirus are equivocal (Bielanski & Hare 1988, Bielanski & Dubuc 1995, Booth *et al* 1998). Virak *et al* (1988) and McGowan *et al* (1993) recorded a reduction in conception rate in cattle underwent nCP pestivirus infection at approximately the time of insemination. Specific research on pestivirus-bovine embryo interaction is still required to explain the pathogenesis of the lower conception rate in pestivirus infected cows. The aim of the present experiment was to determine the effect of nCP type 1 pestvirus on the hatchability of *in vivo* derived bovine embryos and the *in vitro* development of *in vitro* derived bovine morula.

Materials and Methods

***In vivo* production of embryos.** The oestrous cycle of eight Holstein heifers was synchronized using two intramuscularly injections of $PGF_2\alpha$ 11 days apart. Heifers received a total dose of 30mg FSH-P (Schering Animal Health, USA) over 4 days commencing on day 10±2 after the synchronized oestrus. Luteolysis was induced by administration of $PGF_2\alpha$ at 48h after the initial FSH-P injection. The heifers were artificially inseminated twice (approximately 12h apart) at 8-12h after the onset of standing heat. Ova/embryos were collected by flushing the uterine horns with Dolbecco's phosphate buffered saline solution (PBSS) supplemented with 2% fetal calf serum and antibiotics (100U of penicillinG, 100μg streptomycin, 2.5μg

amphotericinB/ml). Embryos were examined at 100X magnification, using phase contrast microscopy to determine morphologic classification. Recovered ova/embryos were classified according to Elsden (1980). A total of 53 ova/embryo were collected. Forty morphologically normal embryos in blasocyst stage were identified and used for experiment 1.

***In vitro* production of embryos.** *In vitro* maturation, fertilization, and *in vitro* culture of zygotes were carried out as previously described (Kafi *et al* 2002). The semen used for *in vitro* insemination of the oocytes was from a proved pestivirus free bull with a high fertility.

***In vitro* embryo culture.** The embryo culture medium used in this experiment was a modification of the synthetic oviductal fluid (SOF) developed by Tervit *et al* (1972). It was composed of 99.7mM sodium chloride, 7.16mM potassium chloride, 1.2mM potassium dihydrogen orthophosphate, 49mM magnesium chloride, 5.5mM sodium lactate, 1.5mM glucose, 25mM sodium bicarbonate, 0.33mM pyruvate, 1.71mM calcium dihydrochloride and 1mM L-glutamine supplemented by 8% BSA (Pentex, Miles, USA) and 2% essential amino acid and 1% non-essential amino acid (ICN, USA). In experiment 1, the blastocysts were washed three times in SOF medium and then divided into two control and pestivirus-exposed groups, each of 20. Five morphologically normal blastocysts were randomly selected and placed in each droplet of control media or virus inoculated droplets. The blastocysts were then incubated under mineral oil in an atmosphere of 88%N_2 and 7%CO_2 at 39°C. The numbers of blastocysts, which developed to a morphologically normal expanded blastocyst, hatching or hatched blastocysts and number of degenerate embryos were recorded. Eighteen morula were selected in good morphologic quality for experiment 2. They were randomly placed in each droplet of control culture (n=8) media or virus inoculated droplets (n=10). The morula were then incubated under mineral oil and cultured for a 48h culture period as described in experiment 1. The morphologic appearances of morula were assessed at 24 and 48h of culture.

Virus and virus exposure. An Australian isolate of nCP type 1 bovine pestivirus was used. The titer 5.5-7$\log_{10}$TCID$_{50}$/ml of the virus determined by a microtitration method was inoculated at the time of commencement of *in vitro* blastocysts culture. To each 45µl droplet of culture media, 5µl of either inoculated media or virus was added and the droplets were then covered with mineral oil. The titer of the virus in the embryo culture droplets ranged from 2.9 to 4.4$\log_{10}$ TCID$_{50}$/50µl. The prepared droplets were placed in a CO_2 incubator for 2h for equilibration before commencing the *in vitro* culture.

Virus titration and serology. Samples from the control and treatment droplets of SOF before and after 48h *in vitro* culture were taken and frozen in cryovials and stored at -196°C until thawed for virus isolation. Cell cultures were prepared as described elsewhere (Kirkland *et al* 1991) except that the virus was isolated on monolayers of either secondary bovine testis cells (passage 5) or secondary fetal bovine kidney cells (passage 6). Stock cells for medium supplementation were obtained from animals in a closed, bovine pestivirus free donor herd. Samples were assayed for the presence of virus by direct inoculation of microtiter plate cultures at seeding. After 5 days, formalin fixed cells (Potter *et al* 1984) were stained by immunoperoxidase technique to detect nCP-bovine pestivirus, using a monoclonal antibody with strong reactivity against bovine pestivirus group antigens. This was followed by a peroxidase-conjugated anti-mouse IgG. Virus titers were determined by quantal assay using the Poisson distribution. Samples from SOF and fetal calf serum were tested for the presence of antibody against pestivirus. All serology tests were performed by Dr. P.Kirkland at Elizabeth MacArthur agricultural Institute, Camden, NSW, Australia. Virus neutralisation test (VNT) was used to detect pestivirus antibody. The VNT was reassessed after immunoperoxidase staining and employed homologous noncytpathogenic virus that was originally isolated from and animal in the closed herd of origin of the PI cow used in this experiment.

Statistical analysis. The endpoints for this experiment included the proportions of

the formed or hatched blastocysts. Odds ratio analysis was carried out to estimate the risk of developmental competence of pestivirus exposed blastocysts. A standard chi-squared test or a Fisher exact test was used to assess the statistical differences between the experimental groups. Differences at a probability of <0.05 were considered significant.

Results

In vitro development of morula and blastocysts. Table 1 presents the results of blastocyst culture. 45% (9/20) of control pestivirus non-exposed blastocysts and 55% (11/20) of pestivirus exposed blastocysts hatched after 24h culture period, respectively (p>0.05). Overall, there was no significant difference (p>0.05) between the control and the treatment groups in the hatching rate of blastocysts after 48h culture (80% versus 85%, respectively).

Table 1. *A composite summary of the effect of bovine pestivirus on the hatching rate of in vivo derived blastocysts*

Group	No. cultured blastocysts	No.(%) hatched blastocysts 24h.	48h.	No. degenerate	Virus titer after 48h. culture ($log_{10}TCID_{50}$/ml)
Control	20	9 (45%)	16 (80%)	3	-
Treatment	20	11 (55%)	17 (85%)	2	4.68

The percentage of hatching and hatched blastocysts was non-significantly higher in the virus-exposed group than the blastocysts in the control group after 24h of culture period (20, 45 and 30, 55%, respectively). Overall, three (15%) blastocysts in control group showed signs of degeneration after 24h of culture and none of them hatched by 48h of culture, while only two (10%) pestivirus-exposed blastocysts became degenerate by 48h of culture. Odds ratio analysis showed no significant difference in risk of hatching in pestivirus exposed blastocysts and control non-exposed blastocysts (P>0.05, Odds ratio=1.25). At 24h culture in experiment 2, the morula in the control group had developed to five early-blastocysts, two mid-

blastocysts and one degenerating morula whereas the pestivirus-exposed morula had developed to two early blastocysts, six mid-blastocysts, one late-blastocysts and one degenerated morula. After 48h of culture, a total of two mid-blastocysts, three late-blastocysts had been formed and three degenerate embryos were observed in the control group whereas of ten morula cultured in the present of pestivirus, one had developed to the mid-blastocyst stage, six to the late-blastocyst stage, one was in the hatching blastocyst stage and two were degenerated. The difference in the number of morula developed to mid-blastocyst stage after 24h culture was significant between the control and pestivirus-exposed morula ($p<0.05$).

Virus isolation and serology. Bovine pestivirus was isolated from samples of the SOF media of each treatment culture dish after 48h culture period. The virus titer was 4.02-5.34$\log_{10}$$TCID_{50}$/ml. It was not isolated from any samples of culture media of the control culture dishes. Serological results showed no presence of antibody against pestivirus in SOF culture media and fetal calf serum samples.

Discussion

The method to investigate the effect of bovine pestivirus in this study was an *in vitro* approach. This approach created the possibility to perform the experiment in a more controllable condition and to determine the direct effect of pestivirus on the process of morula and blastocyst development.

Singh *et al* (1982), Potter *et al* (1984) and Zurovac *et al* (1994) observed an absence or a significant reduction of infective dose of pestivirus in their culture media 24h after inoculation. This was probably due to the presence of antibody against the virus in the fetal calf serum that used in culture media or unsuitable culture condition. Results of virus isolation in the present study showed that despite a reduction in the virus titer throughout the 48h culture period, there was still a significant titer of pestivirus throughout the 48h period of *in vitro* culture of blastocysts in the treatment group. In addition it has been reported (Booth *et al*

1999) that as little as 5-min exposure can result in pestivirus contamination of the embryo. The results of the serological tests did not show the presence of antibody against pestivirus in SOF culture media and fetal calf serum samples. No pestivirus was detected in the SOF culture media before commencing the present experiments and 48h after culture in the control group. This implies that there has not been any inadvertent virus contamination of the *in vitro* culture.

The results of the experiments 1 and 2 show that pestivirus has no adverse *in vitro* effect on the rate of blastocyst formation or the rate of hatching of *in vivo* derived blastocysts, respectively. This is consistent with the previous findings (Bielanski & Hare 1988, Brock & Stringfellow 1993) in that *in vitro* exposure of *in vivo* derived embryos to nCP pestivirus does not result in embryonic death. In the study by Brock and Stringfellow (1993) seven-day-old bovine embryos were obtained from superovulated cows, hatched *in vitro* and then the selected embryos exposed to a CP or nCP strain of bovine pestivirus and then cultured for up to 14 days. It was observed that the infection of post–hatching embryos with the CP strain of pestivirus could cause death of the embryo. However, no cythopathic effects were evident in the hatched embryos exposed to the nCP strain of pestivirus. Taking the results of the present study and the above-mentioned studies it can be concluded that the nCP biotype of pestivirus has not a direct detrimental effect on the developmental competence of blastocysts. Therefore, the cause(s) of the conception failure in cows undergoing a transient pestivirus infection around the time of blastocyst hatching can particularly be attributed to other factors such as inadequate luteal function (McGowan *et al* 2003) and/or altered endometrial sensitivity to oxytocin (Fray *et al* 1999). Our observations in experiment 2 are consistent with those of Zurovac *et al* (1994) and Stringfellow *et al* (1997) but are contrary to those of previous reports (Guerin *et al* 1992, Allietta *et al* 1995). These differences could possibly be explained by the number of embryos cultured, the number of replicates, by different *in vitro* production system protocols, different experimental design or

by different viral strains. Kafi *et al* (2002) reported a non-significant higher rate of cleavage in pestivirus-exposed *in vitro* derived zygotes. However, only 3.6% of these cleaved zygotes developed to blastocyst stage. The difference in the rate of blastocysts formation between the present experiment and our previous study (Kafi *et al* 2002) can be explained by different period of *in vitro* culture.

One interesting observation in the present experiments was that the blastocyst development was faster in pestvirus-exposed group. The presence of pestivirus tended to slightly increase the rate of the development of blastocysts in the current study particularly in experiment 2. Such an enhancement in embryonic development has also been observed by Booth *et al* (1998). This observation was ascribed either to a direct effect altering the embryo metabolism or an indirect effect on the metabolism of the infected granulosa co-culture cells. The later explanation seems to be questionable, as the blastocysts developed in the current experiment were cultured in a medium free of co-culture cells. Therefore the capability of pestivirus to penetrate the zona pellucida and replicating in the embryoinc cells may be a more resoenable explanation. This explanation creates questions as whether the zona pellucida is an efficient barrier to prevent the entry of pestiviruses.

In conclusion, non-cytopathogenic bovine pestivirus has no adverse effect on the process of *in vitro* development of either *in vivo* or *in vitro* derived embryos. The observations described above opens new areas of research on pestivirus–ova/embryo interactions i.e. to identify the localization of pestivirus in the *in vitro* produced embryos, to determine if the virus can penetrate the zona pellucida and replicate in embryonic cells and to identify the mechanisms by which pestivirus improves embryonic development. More study with a higher sample size is required to elucidate the mechanisms involved in pestivirus-ova/embryo interaction.

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