INTERACTION OF VIRUSES WITH PORCINE EMBRYOS

Proefschrift ter verkrijging van de graad van Doctor in de Diergeneeskundige Wetenschappen (PhD) aan de Faculteit Diergeneeskunde, Universiteit Gent

door

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Promotoren: Prof. Dr. A. Van Soom, Prof. Dr. D. Maes, Prof. Dr. H.J. Nauwynck
TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CHAPTER 1: INTRODUCTION........................................................................................................... 9
1.1. Methods of assessing the quality and/or viability of in vivo-derived porcine embryos .... 9
1.2. The effects of infections with porcine circovirus type 2, pseudorabies virus and porcine reproductive and respiratory syndrome virus on reproduction ............................ 12
1.3. Sources of viral infection for embryos .................................................................................. 20
1.4. Virus interactions with in vivo-derived porcine embryos ................................................ 23

CHAPTER 2: AIMS OF THE STUDY............................................................................................... 37

CHAPTER 3: EMBRYO QUALITY OF IN-VIVO DERIVED PORCINE EMBRYOS.......................... 43
3.1. Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal caser scanning microscopic study ............... 45

CHAPTER 4: INTERACTION OF VIRUSES WITH IN-VIVO DERIVED PORCINE EMBRYOS................................................................. 77
4.1. Susceptibility of pig embryos to porcine circovirus type 2 infection ............................. 79
4.2. Receptor-determined susceptibility of preimplantation embryos to pseudorabies virus and porcine reproductive and respiratory syndrome virus ............................ 99
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR</td>
<td>Apoptotic Cell Ratio</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens Junction</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Aujeszky virus</td>
</tr>
<tr>
<td>BVDV</td>
<td>Bovine Viral Diarrhoea Virus</td>
</tr>
<tr>
<td>CM</td>
<td>Compact Morula</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Dpi</td>
<td>Day Post Insemination</td>
</tr>
<tr>
<td>EAV</td>
<td>Equine Arteritis Virus</td>
</tr>
<tr>
<td>EB</td>
<td>Early Blastocyst</td>
</tr>
<tr>
<td>ECG</td>
<td>Equine Chorionic Gonadotropin</td>
</tr>
<tr>
<td>EGA</td>
<td>Embryonic Genome Activation</td>
</tr>
<tr>
<td>EM</td>
<td>Early Morula</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis Virus</td>
</tr>
<tr>
<td>ET</td>
<td>Embryo Transfer</td>
</tr>
<tr>
<td>ExB</td>
<td>Expanded Blastocyst</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence In Situ Hybridisation</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FP</td>
<td>Fragmentation Pattern</td>
</tr>
<tr>
<td>HB</td>
<td>Hatched Blastocyst</td>
</tr>
<tr>
<td>HCG</td>
<td>Human Chorionic Gonadotropin</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post insemination</td>
</tr>
<tr>
<td>HveA</td>
<td>Herpesvirus entry mediator A</td>
</tr>
<tr>
<td>HveC</td>
<td>Herpesvirus entry mediator C</td>
</tr>
<tr>
<td>HVEM</td>
<td>Herpes Virus Entry Mediator</td>
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<tr>
<td>ICM</td>
<td>Inner Cell Mass</td>
</tr>
<tr>
<td>IDH</td>
<td>Isocitrate Dehydrogenase</td>
</tr>
<tr>
<td>IETS</td>
<td>International Embryo Transfer Society</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IgG1</td>
<td>Immunoglobulin G1</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IIF</td>
<td>Indirect Immunofluorescence</td>
</tr>
<tr>
<td>IPMA</td>
<td>Immunoperoxidase Monolayer Assay</td>
</tr>
<tr>
<td>ISH</td>
<td>In Situ Hybridization</td>
</tr>
<tr>
<td>IVF</td>
<td><em>in vitro</em> fertilisation</td>
</tr>
<tr>
<td>LDV</td>
<td>lactate dehydrogenase-elevating virus</td>
</tr>
<tr>
<td>LV</td>
<td>Lelystad Virus</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>NCSU-23</td>
<td>North Carolina State University-23</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Porcine Circovirus</td>
</tr>
<tr>
<td>PCV2</td>
<td>Porcine Circovirus type 2</td>
</tr>
<tr>
<td>PDNS</td>
<td>Porcine Dermatitis Nephropathy Syndrome</td>
</tr>
<tr>
<td>PED</td>
<td>Preimplantation Development</td>
</tr>
<tr>
<td>PERV</td>
<td>Porcine Endogenous Retroviruses</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PK-15</td>
<td>Porcine Kidney cell line 15</td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant Mare Serum Gonadotropin</td>
</tr>
<tr>
<td>PMWS</td>
<td>Postweaning Multisystemic Wasting Syndrome</td>
</tr>
<tr>
<td>PPV</td>
<td>Porcine Parvovirus</td>
</tr>
<tr>
<td>PRDC</td>
<td>Porcine Dermatitis Nephropathy Syndrome</td>
</tr>
<tr>
<td>PRRSV</td>
<td>Porcine Reproductive and Respiratory Syndrome Virus</td>
</tr>
<tr>
<td>PRV</td>
<td>Pseudorabies Virus</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl Pyrrolidone</td>
</tr>
<tr>
<td>PVRL1</td>
<td>Poliovirus Receptor-Related 1</td>
</tr>
<tr>
<td>PZP</td>
<td>Porcine Zona Pellucida</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo Nucleic Acid</td>
</tr>
<tr>
<td>SHFV</td>
<td>Simian Hemorrhagic Fever Virus</td>
</tr>
<tr>
<td>SLBP</td>
<td>Stem-Loop Binding Protein</td>
</tr>
<tr>
<td>TCID50</td>
<td>Tissue Culture Infectious Dose 50</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junctions</td>
</tr>
<tr>
<td>TRIS</td>
<td>Trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labelling</td>
</tr>
<tr>
<td>ZP</td>
<td>Zona Pellucida</td>
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CHAPTER 1:

INTRODUCTION
CHAPTER 1: INTRODUCTION

The studies undertaken for this thesis were carried out because the mechanisms and causes of early embryonic loss in pigs are still poorly understood. There is a need to improve methods for selecting viable embryos prior to their transfer into recipients; so, in the first part of the thesis I explored how embryo quality and viability can be assessed. Subsequently, in the remainder of the thesis, I addressed some questions about how porcine circovirus type 2 (PCV2), pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) interact with early embryos and affect their viability. I have chosen these viruses out of several viruses that can reach the reproductive tract of sows (and therefore might interact with early embryos) since they cause diseases of a high economic impact in the European and worldwide swine industry. The work builds on a foundation of earlier research that is summarised in the following review of the scientific literature.

1.1. Methods of assessing the quality and/or viability of in vivo-derived porcine embryos.

Embryo quality assessment is necessary to be able to select embryos that have the highest capability of sustaining pregnancy after embryo transplantation. The quality of embryos can be assessed by means of either invasive or non-invasive techniques. The main disadvantage of using invasive methods is the fact that the embryo may be lost for transfer. Up to now, embryo morphology as a non-invasive way of quality control remains the method of choice to select viable embryos for transfer. The morphological evaluation of porcine in vivo-derived embryos has primarily been based on studies in bovine embryos (Lindner and Wright (1983)) where colour and texture of blastomeres, presence or absence of fragmentation, presence of uneven cleavage and degree of compaction were the main quality parameters. In porcine embryos, the diameter of the embryo at the blastocyst stage has been correlated with
pregnancy rates (Hazeleger and Kemp, 1999). Higher pregnancy rates were observed when
day 5 blastocysts with a diameter >159 µm were transferred. Another non-invasive way of
embryo quality control that has been used for porcine embryos is the evaluation of embryo
metabolism. Swain et al. (2002) detected that the Krebs cycle metabolism of pyruvate,
glutamine and glucose was higher in high quality in vivo-derived embryos than in in vitro-
derived porcine embryos with a lower embryo quality. In other mammalian species, the
timing of early embryo development is also an important non-invasive indicator of viability
(Van Soom et al., 1992; Grisart et al., 1994; Gonzales et al., 1995; Holm et al., 1998;
Lonergan et al., 1999). However, data on timing of cleavage for in vivo-derived porcine
embryos under in vitro conditions are lacking.

Several invasive techniques of assessing the quality of porcine embryos have been
described. Staining of the embryo to evaluate cell number, cell allocation, cell death and
mitosis has been used repeatedly to evaluate the quality of pig embryos. The allocation of a
sufficient number of cells to the inner cell mass and to the trophectoderm is important for
embryo survival, and can be determined by means of differential staining. In in vivo-derived
expanded pig blastocysts, about 17 to 23% of the total cell number is composed of the inner
cell mass (Rath et al., 1995; De La Fuente and King, 1997; Machaty et al., 1998). Another
way to evaluate the quality of an embryo is to assess the number of apoptotic cells (Hardy et
al., 1989). In a viable embryo, there is a balance between cell division and cell death.
Apoptosis or programmed cell death is a natural defense mechanism during mammalian
embryo development by which unwanted, damaged or potential harmful cells are eliminated.
Although apoptosis is characterized by typical morphological cell features such as membrane
blebbing and cytoplasmic fragmentation (Hardy and Spanos, 2002), non-invasive techniques
to evaluate apoptotic cells in embryos are not yet available. If we could detect a correlation
between fragmentation and apoptosis, then the occurrence of fragmentation could be used as a
non-invasive marker of apoptosis in embryos. Up to now, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), an invasive technique that allows the detection of nuclear DNA fragmentation, is mostly used to evaluate apoptosis in porcine embryos (Long et al., 1998; Hao et al., 2003; Pomar et al., 2004).

Other more complex, and invasive methods that can be used to gain information on porcine embryo quality include electron microscopy (Hyttel and Niemann, 1990; Hyttel et al., 2000), embryonic gene expression to select marker genes for normal development (Bjerregaard et al., 2004) and karyotyping using fluorescence in situ hybridisation (FISH) to identify chromosome abnormalities in embryos (Long and Williams, 1982; van der Hoeven et al., 1985).

Reliable non-invasive methods for embryo quality assessment are imperative when pig embryos need to be selected for transfer. Viable embryos and embryos which are not contaminated with pathogens yield the highest chance for a successful pregnancy. However, several porcine viruses may be found in the genital tract of sows and consequently, may come into contact with developing embryos. In some cases, viral infection can be detected by morphological changes in the embryos, in other cases, the infection may proceed inconspicuously.
Chapter 1: Introduction

1.2. The effects of infection with porcine circovirus type 2, pseudorabies virus and porcine reproductive and respiratory syndrome virus on reproduction in sows

1.2.1. Porcine circovirus type 2

1.2.1.1. Virus characteristics

The porcine circoviruses (PCV) are members of the family Circoviridae, a family composed of the smallest non-enveloped, single-stranded, circular DNA viruses (Tischer et al., 1982). Porcine circovirus was first described as a non-cytopathogenic, picornavirus-like contaminant in the porcine kidney cell line, PK-15 (Tischer et al., 1982). In the late 1990’s, the worldwide emergence of an apparently novel circovirus was associated with ‘postweaning multisystemic wasting syndrome’ (PMWS) (Allan and Ellis, 2000). The nucleotide sequence analysis of the PCV isolates from diseased pigs worldwide showed that they formed a genetically closely related group (>96% homology), but they were significantly different from the PCV contaminant in PK-15 cells (Meehan et al., 1998; Morozov et al., 1998). Therefore, this new PCV was designated as porcine circovirus type 2 (PCV2), whereas the original PCV contaminant in PK-15 cells was designated porcine circovirus type 1 (PCV1) (Allan and Ellis, 2000). Since the identification of PCV2 and its association with PMWS, also other potentially associated disease syndromes such as porcine dermatitis nephropathy syndrome (PDNS) (Rosell et al., 2000), reproductive failure (West et al., 1999), porcine respiratory disease complex (PRDC) (Allan and Ellis, 2000) and congenital tremor (Stevenson et al., 2001) have been described. From these latter postulated disease manifestations, PMWS and reproductive failure have been accepted to be PCV2-related.
1.2.1.2. Reproductive failure associated with porcine circovirus type 2 infection

Reproductive failure due to PCV2 infection was firstly demonstrated under natural conditions. The first report described a field case in a Canadian 450-sow herd that recently had been populated with first-parity-sows and where an increase in late-abortion and stillborn piglets was detected (West et al., 1999). One of the aborted foetuses had myocarditis lesions and immunohistochemical staining revealed abundant presence of PCV2-antigens in those lesions. Samples examined for other viral reproductive pathogens such as porcine parvovirus (PPV), PRRSV, encephalomyocarditis virus (EMCV) and enterovirus were negative. The authors concluded that infection with PCV2 was the cause of the reproductive failure. A second Canadian field report followed, describing an outbreak of PCV2-related reproductive disease in a newly-established 3000-sow herd (O’Connor et al., 2001). At first farrowing, an increase in the proportion of stillbirths (8%), mummified foetuses (15%), pre-weaning mortality (11%) and late-term abortions were observed. PCV2 antigens were demonstrated in myocardial lesions and virus was isolated out of affected hearts and pooled lung and spleen tissue of stillborns and some neonatal pigs. However, since in some of the dead foetuses also PPV, PRRSV or EMCV infections were detected, the exact role of PCV2 on this problem farm was uncertain. A case description in a 25-sow specific-pathogen-free, Danish herd by Ladekjaer-Mikkelsen et al. (2001) was the first European report on PCV2-related reproductive failure. In that herd, PCV2 antigens and antibodies were detected in the offspring of a sow that gave birth to 7 dead foetuses, 1 mummified, and 1 stillborn piglet after 121 days of pregnancy. All other 24 sows present in that herd seroconverted against PCV2 during that same period.

Farnham et al. (2003) described the association of PCV2 infection and reproductive problems on 3 American farms experiencing more than 10% mummified fetuses and stillborn piglets for a period of more than 6 months. From a total of 171 serum samples from stillborn
foetuses, 28 had PCV2 antibodies (titers ≥1:16) of which 13 were found to contain PCV2 DNA by polymerase chain reaction (PCR) and of which 9 yielded PCV2 on virus isolation. These case studies indicate that PCV2-related reproductive failure mainly occurs when serologically negative gilts or sows undergo a primo-infection with PCV2.

To gain more insight on the importance of PCV2 as a cause of reproductive failure under field circumstances, routine diagnostic materials from cases of abortion and stillborn piglets were retrospectively examined in several countries. Bogdan et al. (2001) did not find PCV2 using PCR and immunohistochemistry in 29 submitted cases of reproductive failure from Canadian swine herds. Segalés et al. (2002) only found one PCV2 positive foetus by in situ hybridisation (ISH) on a total of 195 aborted foetuses and stillborn piglets that originated from 125 Spanish herds. In another Spanish study by Maldonado et al. (2005), also only one PCV2 positive sample out of 100 different cases of late-term abortion and premature farrowing was detected. The aborted foetus did not show any of the characteristic myocarditis lesions that are normally associated with foetal PCV2 infections (West et al., 1999). Also, no PCV2 nucleic acid was detected by ISH in the myocardial or liver tissue of that PCR positive foetus. In a South-Korean study by Kim et al. (2004), 46 (13%) out of a total of 350 samples from aborted foetuses and stillborn piglets were positive for PCV2 by PCR. In that study, samples from aborted foetuses of all stages of gestation (D0-D30, D30-D70, D70-D114) and from stillborn piglets were analyzed. Foetal tissues were also subjected to ISH and immunohistochemistry for PCV2. PCV2 was detected in all gestation periods, with a gradual increase of positive results in later stages of gestation. In a German retrospective study by Ritzmann et al. (2005), an even higher percentage (27%, 57/210) of PCR positive results for PCV2 was detected. Samples were taken from aborted or mummified foetuses, stillborn and nonviable neonatal piglets of several farms with reproductive problems, and only a PCR assay was used to analyze the samples for PCV2. The results of the retrospective analyses of foetal
tissues indicate that vertical transmission of PCV2 occurs under field circumstances but whether the presence of PCV2 always plays a causal role in the reproductive failure remains uncertain. From the abovementioned studies, it is clear that significant differences are observed in the prevalence of PCV2. The reasons for these differences are unclear but they can be related to the characteristics of the diagnostic methods used, to regional differences in the prevalence or importance of PCV2-related reproductive failure, or to the fact that the occurrence of PCV2-associated reproductive failure varies between years.

Under experimental circumstances, the susceptibility of foetuses at different stages of development for PCV2 infection was investigated using intra-foetal inoculation by Sanchez et al. (2001). PCV2 replicated in foetuses that were inoculated at 57, 75 and 95 days of gestation, but gross lesions (hemorrhages and congestion of internal organs suggestive of heart failure) were only observed in the foetuses inoculated at 57 days of gestation. The foetal heart displayed the highest levels of virus replication and served as the main target of PCV2 during foetal life. In a similar experiment performed by Yoon et al. (2004), foetuses of mid- and late-gestation were inoculated with PCV2. Also here, PCV2 replication in cardiac myocytes leading to foetal death was described.

Recently, transplacental spread of PCV2 from the sow to the foeti was shown after intranasal inoculation of sows serologically negative for PCV2 at 92 days of gestation (Park et al., 2005). The PCV2-inoculated sows showed abortion and premature farrowing, with PCV2 replicating primarily in cells of the macrophage lineage. This difference in target cells in comparison with the findings of Sanchez et al. (2001) may be due to the difference in inoculation route between the experiments or may depend on the viral strain or the duration of the infection.

In an experimental model, mimicking the effects of using PCV2-contaminated semen, Cariolet et al. (2002) were also able to cause a vertical transmission of PCV2 after intra-
uterine insemination of virus-spiked semen. Despite the early stage of gestation at which exposure to the virus occurred (i.e. via the semen), replication of the virus appears to have been delayed until the fetal stages of development when it led to fetal death and mummification.

1.2.2. Pseudorabies virus (PRV)

1.2.2.1. Virus characteristics

Pseudorabies virus is also called Aujeszky’s disease virus and was first described by Aladar Aujeszky in 1902 (Aujeszky, 1902). The virus belongs to the alphaherpesvirus subfamily of the *Herpesviridae*. It is a double-stranded DNA virus with an envelope. Like all alphaherpesviruses, the virus is able to establish a life-long, dormant state of infection in neurons of the peripheral nervous system. This dormant state of infection is called latency and specific stimuli may cause the virus to reactivate from latency (Roizman, 1996). The disease manifestations of PRV are dependent on the strain of virus, the infectious dose and the age of the pig. In suckling and weaned piglets nervous signs are most commonly observed following PRV infection, while respiratory signs are seen in finisher pigs and adult swine. Reproductive failure, including abortion, is the most prominent clinical feature of PRV infection in pregnant sows.

1.2.2.2. Reproductive failure associated with PRV infection

Field studies show that sows primarily develop respiratory signs after PRV infection. Infection of sows during the first 35 days of pregnancy may lead to resorption of embryos and return to oestrus of the sow. If infection occurs in the second or third trimester of pregnancy, it usually leads to abortion or to the birth of dead or weak piglets. Pathologic and viral investigations were performed on foetal placentas and on aborted foetuses associated with
naturally occurring pseudorabies in swine (Hsu et al., 1980). Of the 13 fetal placentas examined, 7 (53.8%) had various degrees of necrotizing placentitis. Numerous viral particles, ultrastructurally indistinguishable from herpesvirus, were observed by electron microscopy in the affected chorionic membrane. Large aggregates of herpesvirus virions were demonstrated in the nucleus and cytoplasm of degenerated trophoblast and mesenchymal cells. Of the 23 aborted foetuses examined, 22 (95.6%) had typical areas with coagulative necrosis in the liver, spleen, adrenal glands, and visceral lymph nodes. Inclusions similar to those in the chorionic placenta were observed in the parenchymal cells of those organs, on the margins of the necrotic areas. PRV was isolated from various organs of the aborted foetuses. The authors concluded that the placental lesions due to the PRV infection were the main cause of the reproductive problems. In a experimental study by Bolin et al. (1985), PRV was inoculated into the uterus of 15 gilts within 6 hours after natural breeding. None of the gilts became pregnant which indicated that PRV infection affected fertilization and/or conception. PRV was isolated from the reproductive tract up to day 14. Lesions in the reproductive tract consisted of lymphohistiocytic vaginitis and endometritis, and lymphoplasmacytic aggregates in the corpora lutea. Multiple ulcers were seen in the vagina or endometrium of several gilts.

1.2.3. Porcine Reproductive and Respiratory Syndrome Virus

1.2.3.1. Virus characteristics

Porcine reproductive and respiratory syndrome virus (PRRSV) was first described in the USA in 1987 (Keffaber, 1989) causing late-term reproductive failure, delayed return to oestrus, stillborn and weak pigs and severe pneumonia in neonatal pigs. Subsequently, the disease spread rapidly throughout Europe. The etiologic agent was first isolated in pulmonary macrophage cultures by Wensvoort et al. (1991) and it was designated Lelystad virus (LV). Soon thereafter, American researchers were able to isolate a virus out of an established cell
line and named it VR-2332 (Benfield et al., 1992; Collins et al., 1992). Research revealed that the two viruses were sufficiently related to be considered strains from PRRSV. However, there were genetically and antigenically differences which led to the establishment of two distinct genotypes with LV being the prototype of the European isolates and with VR-2332 being the prototype for the American isolates. PRRSV is a member of the genus Arterivirus in the family of the *Arteriviridae*. This family *Arteriviridae* also includes equine arteritis virus (EAV), mouse lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992; Meulenberg et al., 1993). PRRSV is a circular, single-stranded, enveloped RNA-virus with a diameter of 50-65 nm (Benfield et al., 1992; Wensvoort et al., 1992; Dea et al., 1995).

1.2.3.2. Reproductive failure associated with porcine reproductive and respiratory syndrome virus infection

Field studies have shown that an infection of susceptible pregnant sows with PRRSV may cause a variety of clinical presentations ranging from sporadic abortions to abortion storms (Albina et al., 1994). However, most epizootics of PRRSV-induced reproductive failure are characterized by late-term abortions, early farrowings, stillborn piglets, and weak and unthrifty newborn pigs (Keffaber, 1989; Meldrum, 1991; Wensvoort et al., 1991; Hopper et al., 1992). Mid-gestation abortion or irregular returns to oestrus are less frequently reported in infected pig herds (Christianson et al., 1993).

Experimental data on intra-uterine or intranasal and intravenous PRRSV inoculation at the day of insemination did not show any effect on fertilization and conception rates (Lager et al., 1996; Prieto et al., 1996a; Prieto et al., 1997). However, as a result of these inoculations at the time of conception, a transplacental infection of a small proportion (6-16%) of the embryos was detected around the 20th day of gestation by Prieto et al. (1997).
The effect of a PRRSV infection in the first trimester (D1-D38) of gestation was investigated in 2 experimental studies. Mengeling et al. (1994) found that an intravenous PRRSV inoculation of 3 gilts at the 30\textsuperscript{th} day of gestation did not lead to a transplacental infection of embryos. However, in a study by Prieto et al. (1996a), 18\% (2/11) of the sows that were inoculated intranasally at D7, D14 or D21 experienced a transplacental infection with 2.7\% (4/149) of the live embryos being PRRSV positive. Experimental infections of sows in mid-gestation (D39-D72) were performed in 2 studies. Christianson et al. (1993) found that in 1 out of 8 sows inoculated with PRRSV intranasally between D45-D50, a vertical transmission occurred leading to 2\% (2/99) PRRSV-positive fetuses. An intravenous inoculation with PRRSV at D50 or D70 of gestation resulted in a transplacental infection in 2 out of 6 sows, in which virus was isolated in 6\% (3/50) of the foetuses.

Experimental exposure of pregnant sows to PRRSV around the 90\textsuperscript{th} day of gestation typically results in the classical PRRSV symptoms such as early farrowings and late-term abortions (Terpstra et al., 1991, Christianson et al., 1992; Mengeling et al., 1994). The incidence of transplacental spread in those sows is close to 100\% with the proportion of PRRSV-infected foetuses or newborn piglets reaching up to 75\% (Mengeling et al., 1994).

These experimental data clearly show that exposure of pregnant sows to PRRSV in late gestation results in a higher incidence of transplacental infection in comparison to infection in early and mid-gestation. The reasons for this gestation stage-dependent difference in efficiency of vertical virus transmission are currently unknown. Intra-amnion and intramuscular inoculation experiments have shown that foetuses from the 34\textsuperscript{th} day throughout the end of gestation are susceptible to a PRRSV infection (Christianson et al., 1993; Lager and Mengeling, 1995). However, there is an age-related difference in onset of mortality. Intra-foetal inoculation during the first half of gestation leads to virus replication without formation of gross lesions, whereas intra-foetal inoculation in the second half of gestation may result in...
foetal death within days (Lager and Mengeling, 1995). The increase in the likelihood of transplacental infection with PRRSV might be related to the maternal-foetal capillary interrelationship of the placenta that becomes more intense in late gestation.

In conclusion, experimental data show that PRRSV inoculation of susceptible sows at the onset of gestation has no effect on conception and fertilisation rates, although it can result in a transplacental infection around the 20\textsuperscript{th} day of pregnancy. An infection in early and mid-gestation can also induce a vertical transmission of PRRSV although the likelihood and incidence of this foetal infection is markedly lower than in sows infected in late-gestation.

1.3. Sources of viral infection for embryos.

For pathogen transmission by the transfer of \textit{in vivo}-derived embryos, a sequence of events has to occur, (1) exposure to the pathogen, (2) continued association of the pathogen with the embryos, (3) maintenance of infectivity of the pathogen throughout embryo manipulation and processing and (4) delivery of an infective dose of pathogen to a susceptible recipient (Stringfellow and Givens, 2000). Embryos can get exposed to all kinds of viruses but viruses with a tropism for the reproductive tract bear the highest risk for attaining the embryos. In swine, there are several viruses including porcine parvovirus, swine vesicular disease virus, bovine viral diarrhea virus, encephalomyocarditis virus, classical swine fever virus, PCV2, PRV and PRRSV that can reach the reproductive tract of sows.

Embryos can get exposed to pathogens either via their gametes, or via the environment. In an embryonic infection through gametes the pathogen resides in the oocyte that is fertilized or is carried into the ovum by an infected spermatozoon. Porcine endogenous retroviruses (PERV) are present in the genomic DNA of all pig cells including spermatozoa and oocytes (Patience et al., 1997). The PERV survives as a part of the host genome rather than as an infectious agent and vertical transmission occurs in a manner analogous to a Mendelian trait.
Chapter 1: Introduction

An infection with PERV is normally not pathogenic in its natural host the pig. Whether its presence in spermatozoa and oocytes can have any effect on embryos after fertilization is not yet determined. However, there are some concerns about a possible re-activation of PERV after cell nuclear transfer (Thibier, 2001). To our knowledge, no other viruses that can reside inside an oocyte e.g. exogenous retroviruses are described in the pig. Another possibility is that some viruses may be able to attach to or integrate into spermatozoa and can be carried into the oocyte at fertilization. This has been described in in vitro models for Simian virus 40 and rabbit spermatozoa (Brackett et al., 1971), and for Sendai virus, influenza virus and Semliki Forest virus exposed to bull spermatozoa (Nussbaum et al., 1993). Under in vitro and in vivo circumstances this phenomenon has been described for human immunodeficiency virus type 1 (Baccetti et al., 1994). Experimental data on other human viruses that have the capability of entering spermatozoa such as hepatitis B virus and human papilloma virus also suggest that these viruses can be carried into the oocyte at fertilization (Ali et al., 2006; Chan, 1992). In domestic livestock, such a carry-over of viruses into the oocyte through spermatozoa has never been described. The possible risks of getting virus infected in vivo-derived embryos by using virus-contaminated semen in cattle were thoroughly reviewed by Wrathall et al. (2006).

For pigs, the 3 pathogens investigated in this manuscript (PCV2, PRV, PRRSV) can be detected in semen of clinical and subclinical infected boars.

PCV2 has been detected by PCR in semen from naturally infected boars (Hamel et al., 2000; Kim et al., 2003) and in semen from boars that were intranasally inoculated (Larochelle et al., 2000). Shedding in the semen was observed between 5 and 47 days post infection. The seminal plasma is usually contaminated but PCV2 is also frequently detected in the sperm and non-sperm cell fractions (Hamel et al., 2000; Kim et al., 2003). These data indicate that PCV2
can be intermittently excreted by infected boars, and suggest that semen may be a significant vehicle for the virus.

Infection of boars with PRV may cause testicular degeneration and transient elevation in sperm abnormalities (Hall et al., 1984). The virus can be irregularly isolated either from urine, preputial membranes or semen after both natural (Medveczky et al., 1981) and experimental infection (Vannier and Gueguen, 1979). Very high viral concentrations (from $10^{3.7}$ to $10^{9}$ TCID$_{50}$/mL) have been reported in semen. Virus excretion is not strictly associated with clinical disease or with a reduction in semen quality, and recrudescence of boars latently infected with PRV constitutes a permanent risk. Sows inseminated with contaminated semen show seroconversion, and may suffer from vaginitis, endometritis and embryonic mortality.

For PRV, semen becomes contaminated mainly by virus replication in the serosa of the genital organs of the boar and not by virus replication in germinal epithelia (Miry et al., 1987). There are conflicting data on the ability of PRRSV to replicate in cells of the spermatic epithelium of the seminiferous tubules. In ejaculates, PRRSV was detected by IHC and ISH in abnormal cells of spermiogenic origin and in multinucleated giant cells (Sur et al., 1997). In contrast, Prieto and Castro (2005) detected PRRSV mainly in monocytes and macrophages of the seminal plasma. The duration of shedding in semen samples of experimentally infected boars varies widely, ranging from 2 (Shin et al., 1997) to 92 (Christopher-Hennings et al., 1995) days post-infection. This marked variability may be due to different factors including individual boar variation, virus strain, and technique used for detection of the virus (i.e. virus isolation, PCR, swine bioassay).

Transmission of the pathogen by semen to the sow has been clearly proven for PRRS virus after experimental inoculation of boars (Yaeger et al., 1993), and after experimental inoculation of semen (Swenson et al., 1994; Prieto et al., 1997). This implies that contaminated semen does indeed constitute a serious risk for transmission, but it does not
mean that transmission of these pathogens to the sow by AI will consistently occur (e.g. PRRSV) (Yaeger et al., 1993; Swenson et al., 1994).

Also the embryo’s environment, being the Fallopian tube and uterine environment could be sources for any viral infection. There are two possible routes for a viral contamination of the genital tract. In the first model, infectious virus is present in the blood as a consequence of a primary infection of the sow. In such a viraemic sow, the virus can spread through maternal tissues and may reach embryos. A second possible way of viral contamination of the environment of embryos is via virus-contaminated semen. Viruses present in boar semen are frequently detected in the sperm plasm and the non-spermatozoon cell fraction. When these viruses are resistant (e.g. PCV2) or can infect genital epithelia (e.g. PRV) they may become a source of contamination for embryos.

1.4. Virus interactions with in vivo-derived embryos

There are several reasons for investigating porcine embryo-pathogen interactions. First of all, with the recent advent of successful cryopreservation techniques for porcine embryos (reviewed by Martinat-Botté et al., 2006) and the establishment of practical non-surgical embryo transfer techniques (Hazeleger et al., 2001; Ducro-Steverink et al., 2004), important advances for the commercial application of porcine embryo transfer have been achieved. However, from an epidemiological point of view, the worldwide implementation of porcine embryo transfer techniques might form a potential risk of spreading pathogens.

In order to circumvent associated biohazards of porcine embryo transfer, information on the interaction between pathogens and embryos is necessary. The pathogens of interest are those that are shed in reproductive tissues, enabling an interaction with embryos. To date, a limited number of studies have investigated the risk of infection and disease transmission through porcine embryos.
Chapter 1: Introduction

There are 4 models for examining embryo-pathogen interactions: (1) *in vitro* virus exposure – *in vitro* follow up, (2) *in vitro* virus exposure – *in vivo* follow up, (3) *in vivo* virus exposure – *in vitro* follow up, (4) *in vivo* virus exposure – *in vivo* follow up. The pros and cons of each of these models will now be considered briefly.

1.4.1. *In vitro* virus exposure – *in vitro* follow up model

This model is most frequently used to investigate embryo-pathogen interactions. In this model, embryos are exposed *in vitro* to viruses, washed or trypsin treated and assayed for the presence of virus through an *in vitro* assay. Unless co-culture following artificial exposure is performed (Givens *et al.*, 1999), insights dealing with the possibilities of virus transmission are limited using this model. In swine, this approach has been used to investigate the interaction of porcine parvovirus with mainly zona pellucida (ZP) intact embryos (Wrathall and Mengeling, 1979b; Bane *et al.*, 1990), PRV (Bolin *et al.*, 1981), foot-and-mouth disease virus (Singh *et al.*, 1986), swine vesicular disease virus (Singh *et al.*, 1987), vesicular stomatitis virus (Singh and Thomas, 1987), classical swine fever virus (Schuurmann *et al.*, 2005), and PRRSV (Prieto *et al.*, 1996b). In all but one study (PRRSV study from Prieto *et al.*, 1996b), the *in vitro* assays even after washing procedures, revealed the presence of infective virus associated with some of the embryos. Except for one case, where authors claimed the presence of infective porcine parvovirus within blastomeres of ZP intact embryos (Bane *et al.*, 1990), viruses were not able to cross the ZP but were detected in the network of the ZP. This “stickiness” of the ZP seems to be typical for porcine embryos and is different from that in other species such as ruminants where washing procedures are in most cases able to free embryos of viruses after *in vitro* incubation (Stringfellow and Givens, 2000). Possible causes for the “stickiness” of porcine ZPs may be related to the physical characteristics of the network of pores and meshes or to the presence of carbohydrates and glycoproteins that may
function as receptors for viruses. Whether these virus particles that are stuck to the ZP are able to infect the hatching blastocyst or to infect the recipient sow can be examined in artificial exposure – *in vivo* follow up models.

There has been only a limited amount of research on the interaction of viruses with porcine embryonic cells using the *in vitro* exposure - *in vitro* follow up model. Although the risk of disease transmission restricts the use of embryos for transfer to those with an intact ZP, there remains at least a theoretical risk that viruses [or pathogens] could get into contact with blastomeres because any kind of embryo handling can damage the ZP. Also the development of new reproductive technologies in swine such as intracytoplasmic sperm injection (ICSI), blastomere biopsy and cryopreservation augment the probability of viruses passing the ZP. The main reason why the embryo transfer industry is interested to investigate virus infectivity for blastomeres is because they need to know whether such a viral infection can affect morphological appearance and/or further embryonic development. A worst case scenario consists of an infected embryo that does not express any morphological or developmental deficiencies, but is able to transmit viruses.

Only three studies investigated the susceptibility of *in vivo* derived porcine embryonic cells for viral infection either by virus incubation after pronase treatment (Bolin et al., 1981) or by sub-zonal virus microinjection (Bane et al., 1990; Prieto et al., 1996b). The results of these studies showed that blastomeres of embryonic stages before the 16-cell stage were refractory both to a PRV infection (Bolin et al., 1981) and a PRRSV infection (Prieto et al., 1996b). Bane et al. (1990) detected parvovirus DNA in trophectoderm cells after subzonal micro-injection and concluded that embryonic cells were susceptible to a parvovirus infection.
1.4.2. *In vitro* virus exposure – *in vivo* follow up model

In this model embryos are exposed to viruses *in vitro*, washed according to the IETS protocol (See Table 1, adapted from Stringfellow, 1998) and transferred to uninfected recipients. Here, embryos are generally exposed to much higher levels of virus *in vitro* than is likely to occur under *in vivo* conditions, thus maximizing the chance of virus transmission. Conclusions can be drawn from the serological and/or viral status of recipient animals and their offspring. For porcine embryos, this kind of embryo transfer model has been used for porcine parvovirus (Wrathall et al., 1979) and PRV (Bolin et al., 1982). Under these extreme conditions, both viruses were able to infect recipient animals and their offspring after embryo transfer.

**Table 1. Requirements for proper washing of embryos.**

<table>
<thead>
<tr>
<th>Washing procedures according to IETS manual</th>
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<tbody>
<tr>
<td>Only embryos from a single donor are washed together.</td>
</tr>
<tr>
<td>Ten or fewer embryos are washed at one time.</td>
</tr>
<tr>
<td>Only zona pellucida-intact embryos are washed.</td>
</tr>
<tr>
<td>Only embryos free of adherent material are washed.</td>
</tr>
<tr>
<td>Embryos are washed thoroughly at least ten times.</td>
</tr>
<tr>
<td>A new sterile micropipet is used between wash steps.</td>
</tr>
<tr>
<td>Each wash is at least a 100-fold dilution of the previous wash.</td>
</tr>
</tbody>
</table>

1.4.3. *In vivo* virus exposure – *in vitro* follow up model

Using this approach, embryos of seropositive or experimentally infected sows are analysed for the presence of virus. This model investigates the possibility of vertical transmission from the sow to the embryos. The transmission can be caused by an infection of female gametes or by the presence of infective viruses in the lumen or cells of the Fallopian tube or uterus. Studies have been conducted for porcine parvovirus (Gradil et al., 1994), PRV
(Bolin and Bolin, 1984), PRRSV (Randall et al., 1999) and PCV2 (Bielanski et al., 2004).
Porcine parvovirus, PRV and PCV2 were found in association with embryos from the infected or seropositive donors, whereas PRRSV could not be detected in embryos using this \textit{in vivo} exposure embryo model.

1.4.4. \textit{In vivo} virus exposure – \textit{in vivo} follow up model

Embryos of seropositive or experimentally infected donors are collected, washed following the IETS protocol (Stringfellow, 1998) and transferred to uninfected recipients. This model still overestimates the risk of virus transmission through embryo transfer but comes closest to what might be expected for porcine embryo transfer using healthy donor sows. In experiments where donor sows were infected with swine vesicular disease (Singh et al., 1987) and PRRSV (Randall et al., 1999; Smits et al., 2002) recipients and offspring remained disease-free. PRV was the only virus that was transmitted to recipients and offspring when embryo transfers were performed using embryos from infected donors. The donors were infected by depositing a very high dosage of virus ($10^8$ TCID$_{50}$/ml) by the intrauterine and intranasal route. When embryos from PRV infected donors were trypsin treated, there was no virus transmission to recipients or offspring (Bolin et al., 1982).
REFERENCES


Chapter 1: Introduction


CHAPTER 2

AIMS
Due to recent progress in techniques such as embryo cryopreservation and non-surgical embryo transfer, most obstacles to implementation of embryo transfer as a practical tool for exchanging germplasm in swine have disappeared. In order to obtain acceptable pregnancy rates it is desirable to select only top quality embryos for transfer. To date, research regarding embryo quality has mainly focused on human, murine or bovine embryos, and objective non-invasive parameters for porcine embryo quality assessment are lacking.

Porcine embryo transfer has a great potential for the future of the swine industry because it is regarded as a safe way of transferring genetics. However, to meet expectations, more research on porcine embryo-pathogen interactions is needed. Because embryo transfer is only allowed with zona pellucida (ZP) intact embryos, most research has focused on the interaction between ZP intact embryos and viruses. However, with the introduction of new techniques that can harm the barrier function of the ZP such as intracytoplasmic sperm injection, blastomere biopsy and embryo cryopreservation, the susceptibility for and the effects of a virus infection in embryonic cells have to be elucidated.

The major aims of this thesis were therefore first to define parameters for evaluating porcine, in vivo derived embryos and second to investigate the interaction between porcine preimplantation embryos and porcine viruses that can induce reproductive failure in pigs, namely porcine circovirus type 2 (PCV2), pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV).

The objectives of this thesis can therefore be specified as follows:

- To set a standard for kinetics of development of in vivo-derived embryos that can be used as a reference for in vitro-produced or nuclear transfer pig embryos.
• To predict the developmental competence of *in vivo*-derived porcine embryos by relating non-invasive parameters of embryo quality such as timing of development and embryo morphology with invasive techniques, such as evaluation of apoptosis.

• To determine whether PCV2, PRV and PRRSV are able to replicate in the cells of *in vivo*-derived preimplantation embryos.

• To investigate possible differences in virus replication between ZP-intact and ZP-free embryos for PCV2, PRV and PRRSV.

• To compare viral susceptibility of porcine embryonic cells at different stages of development and to relate it with the expression of specific virus receptors.

To determine the effects of a PCV2 infection after embryo transfer during the first 3 weeks of development.
CHAPTER 3

EMBRYO QUALITY OF IN-VIVO DERIVED PORCINE EMBRYOS
CHAPTER 3.1:

PORCINE EMBRYO DEVELOPMENT AND FRAGMENTATION AND THEIR RELATION TO APOPTOTIC MARKERS: A CINEMATOGRAPHIC AND CONFOCAL LASER SCANNING MICROSCOPIC STUDY

Adapted from: Reproduction 2005, 129(4):443-452

ABSTRACT

Porcine embryo selection prior to transfer is mainly influenced by morphological criteria. However, the relationship between embryonic morphology, developmental potential and cell death by apoptosis in porcine embryos is still unclear. The aim of this study was to establish embryo quality parameters for in vivo fertilised porcine embryos based on timing of development in vitro, embryo morphology and the presence of apoptosis. The kinetics of development and morphological parameters were investigated in a time-lapse cinematographic experiment. Possible links between embryo morphology and apoptosis were examined via a confocal laser scanning experiment, analysing nuclear changes, annexin V and terminal dUTP nick-end labelling. The timing of early cleavages was firmly linked to embryo developmental competence in vitro. Attainment of at least the 5-cell stage before 77 hours post insemination and attainment of the morula stage before 102 hours post insemination significantly increased the odds for reaching the early blastocyst stage. Overall, a negative effect of fragmentation percentage and fragmentation pattern on subsequent embryonic development was observed, but the developmental potential of embryos experiencing slight fragmentation (0-5%) was not different from embryos without fragmentation. Correlations
detected between developmental arrest and fragmentation, and fragmentation and apoptosis were 0.60 and 0.87 (P<0.05), respectively. Only a minority of the embryos arrested at the 1- to the 4-cell stage displayed biochemical characteristics of apoptosis. Consequentially, a significant correlation (0.57) between developmental arrest and apoptosis could only be established for embryos arrested after embryonic genome activation.

INTRODUCTION

Quality assessment of preimplantation embryos relies heavily on non-invasive techniques based on morphological criteria. One of these techniques is timing of development, which has been linked to in vitro blastocyst formation for hamster (Gonzales et al. 1995) and bovine (Van Soom et al. 1992; Grisart et al. 1994; Holm et al. 1998; Lonergan et al. 1999) embryos. Furthermore, it has been shown in hamster (McKiernan & Bavister 1999) bovine (Hasler 1998) and human (Racowsky et al. 2000) embryos that slow-cleaving embryos are less viable, i.e. have a lower foetal development ratio after transplantation, in comparison to fast-cleaving embryos. Despite the relative abundance of kinetics data in other species, data on timing of development have not been established yet for porcine embryos.

Another important parameter for mammalian embryo quality is the assessment of blastomere fragmentation (Lindner & Wright 1983; Antczak & Van Blerkom 1999). Cellular fragmentation is a common feature during early development of mammalian embryos (Van Blerkom et al. 2001), but is generally considered as indicative of poor embryo quality both in bovine (Lindner & Wright 1983) and human (Puissant et al. 1987). Moreover, human embryos with a substantial amount of cellular fragmentation have a markedly reduced implantation rate (Ziebe et al. 1997; Ebner et al. 2001). For human in vitro fertilised (IVF) embryos, not only degrees (in percentage) but also distinct patterns of fragmentation, which
are correlated with in vitro development, have been defined (Warner et al. 1998a; Alikani et al. 1999). Similar data in pig embryos are presently lacking.

Despite the importance of fragmentation as a morphological indicator of embryonic viability, the origin of fragmentation is still unclear. Since fragments resemble apoptotic bodies seen in other cell types (Hardy 1999), it seems obvious to investigate whether fragmentation can be used as a non-invasive marker of the occurrence of apoptosis in the embryo. However, the relationship between fragmentation and apoptosis in embryos is not clear. In human embryology, Jurisicova et al. (1996) proposed that these fragments represented apoptotic bodies, but failure of the majority of the fragmented embryos to show either in situ terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) or annexin V labelling led Antczak and Van Blerkom (1999) to postulate that fragmentation was not related to apoptosis. Apoptotic nuclei have been detected in embryos from many mammalian species including the pig (Long et al. 1998; Hao et al. 2003; Rubio Pomar et al. 2004). Apoptosis is a natural process during mammalian preimplantation development which could involve elimination of unwanted or damaged cells, but its role in preimplantation embryonic development is not well characterized.

The aim of this study was to investigate the developmental competence of porcine embryos by looking for associations between timing of development, embryo morphology characteristics and occurrence of apoptosis. We chose to use in vivo fertilised pig embryos because such embryos have been produced under optimal conditions and their development may be considered as a gold standard for in vitro embryo development in pigs.
MATERIALS AND METHODS

**Collection of in vivo fertilised porcine embryos**

A total of 29 multiparous sows, *Sus scrofa*, (Rattlerow-Seghers) were used. The sows were superovulated using eCG (Folligon® 1500 IU im) three days after weaning, followed by hCG (Chorulon® 1500 IU im) 72 h later. They were fixed-time inseminated with boar semen of proven fertility 24 h after hCG administration and were slaughtered 45 h post insemination. The reproductive tracts were removed and transported to the laboratory in a pre-warmed box (39°C) within 30 min after slaughter. Each oviduct was flushed with 15ml of pre-warmed Hepes-buffered North Carolina State University-23 (NCSU-23) medium to collect the embryos. After 10 washings with Hepes-buffered NCSU-23, embryos were cultured in NCSU-23 (Petters & Wells 1993) at 39°C.

**Cinematography of pig embryos**

To perform time-lapse cinematography, in vivo derived pig embryos were incubated in a culture dish placed in a small chamber on the plate of an inverted microscope (Zeiss). The chamber was regularly flushed with a humidified and warmed gas mixture consisting of 5% CO₂, 5% O₂, and 90% N₂. To maintain a constant temperature of 39°C, a Plexiglas box was adapted to fit onto the microscope and connected to a heating system controlled by a temperature probe. The recording equipment consisted of a colour video camera KY-F55E from JVC and two computers. The first computer synchronized the lighting of the lamp and the shooting. The second digitized and recorded the frames with the program Perception Video Recorder (Alpha M). One image was recorded every 240 seconds (4 min) (Lequarre *et al.* 2003). The development of the embryos was filmed starting from 48 hours post insemination (hpi) (3 hours after collection) for 8 consecutive days at magnification 100. Only embryos visible in the camera field throughout the complete culture period were examined.
Evaluation of embryonic morphology

Morphology of cleaving embryos was evaluated every 4 minutes starting from 48 hpi in experiment 1 and at Day 7 post insemination in experiment 2. The degree of fragmentation was first expressed as a percentage and defined as the embryonic volume occupied by anucleate cytoplasmic fragments (Puissant et al. 1987). In addition, a fragmentation pattern was defined based on the spatial distribution and relative size of the fragments as described previously for human embryos (Alikani et al. 1999) (Figure 1.). Embryos exhibiting minimal fragments, usually in association with a single blastomere were designated as fragmentation pattern 1 (FP1). Fragmentation pattern 2 (FP2) was used to characterize highly localized fragments that often appear in a cluster or a column. In pattern 3 fragmentation (FP3), many small fragments could be seen throughout the cleavage cavity and perivitelline space. Pattern 4 embryos (FP4) also had many large fragments that were randomly distributed and always associated with uneven cells.

Figure 1. Four distinct patterns of fragmentation in porcine morulae: (A) fragmentation pattern 1 (FP1), (B) fragmentation pattern 2 (FP2), (C) fragmentation pattern 3 (FP3), (D) fragmentation pattern 4 (FP4).
Evaluation of apoptosis by means of propidium iodide, annexin V and TUNEL assay

Propidium iodide (PI) and annexin V staining (Vybrant™ Apoptosis Assay kit #3, Molecular Probes, Eugene, Oregon, USA) of living embryos was performed to determine the cell membrane integrity and the presence of phosphatidylserine residues on the outer surface of the plasma membrane, respectively. Positive Day 7 control embryos were incubated for 12 h with 1µM staurosporine to induce apoptosis. Day 7 embryos were first washed for 5 minutes in annexin binding buffer at 37°C and incubated for 15 minutes in the presence of FITC conjugate of annexin V (25µl/ml) and PI solution (3µg/ml) according to the manufacturer’s recommendations for the Vybrant™ Apoptosis Assay kit #3. Then embryos were washed for 5 minutes in PBS and transferred to a drop of pre-warmed PBS (37°C) on a microscopic slide and examined by scanning laser confocal microscopy. Positive labelling for annexin V on the outer surface membrane was observed as bright yellow staining. In preparation for TUNEL, the embryos analyzed for annexin V labelling were fixed in 4% paraformaldehyde in PBS and washed for at least 12 hours in polyvinyl pyrrolidone (PVP) solution (1mg PVP/ml phosphate buffered saline). After washing, they were permeabilized with 0.5% Triton X-100 in PBS for 20 minutes and washed again in PVP solution. Positive and negative controls were treated with DNase (50 Units/ml in PBS) for 1 h at 37°C to ensure detection of strand breaks by TUNEL (In Situ Cell Death Detection kit, Boehringer, Mannheim, Germany). After three washings in PVP solution, positive controls and samples were incubated in fluorescein-dUTP and terminal deoxynucleotidyl transferase for 1 h at 37°C in the dark. Negative controls were incubated in nucleotide mixture only in the absence of transferase. After again three washings in PVP solution, controls and samples were incubated in RNase A (50µg/ml in PBS) for 1 h at room temperature. The nuclei were then counterstained with 0.5% PI for 1 h at room temperature. Subsequently, the slides were three times washed with PVP solution and embryos were mounted in glycerol with 1,4-
diazabicyclo (2.2.2) octane (25mg/ml). Samples were examined by laser scanning confocal microscopy. TUNEL-positive nuclei appeared bright yellowish-green, whereas the PI staining allowed a rapid identification, localization and quantification of normal, fragmented or condensed nuclei as defined previously by Gjørret et al. (2003).

**Confocal laser scanning microscopy**

Double stained samples were examined with a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany) linked to a Leica DM IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). An Argon laser was used to excite FITC (488 nm) and PI (586 nm) fluorochromes. Total embryo height was evaluated and sections were made at 3µm intervals. Analysis of the images was performed with Leica confocal software.

**Experimental design**

Experiment 1: Cinematographic analysis of porcine embryonic morphology and development

In vivo fertilised pig embryos (n=86) obtained 48 hpi were cultured in groups of 25 in 50µl NCSU23 under oil at 39°C in 5% CO₂, 5% O₂, and 90% N₂. These embryos were subjected to cinematographic analysis. Embryos of the same donors that were simultaneously cultured in a classic incubator served as a control (n=103). The blastocyst yield of these control embryos was 64.1% after 8 days of in vitro culture.

The length of the first cell cycle could not be measured as the exact timing of conception was unknown. Movies were analyzed by assessing, for each embryo, the time of appearance of 3-cell, 4-cell, 5-cell, 6-cell, 7-cell, 8-cell, early morula, compacted morula, early blastocyst, expanded blastocyst and hatched blastocyst stages. Beyond the 8-cell stage, it was not possible to count or extrapolate cell numbers accurately on the basis of cleavage
observations. An embryo was defined as an early morula after it contained a “cell ball” of small distinguishable blastomeres (Lindner & Wright 1983). A compacted morula was defined as the stage at which blastomeres had coalesced to form a smooth, compact cell mass. Cavitation was estimated by the first appearance of a stable confluent blastocoel. Blastocyst expansion was defined by the increase in diameter of the zona pellucida (ZP). Cracking of the ZP of an expanded blastocyst was described as hatching.

Experiment 2: Evaluation of the relationship between embryonic morphology and apoptotic markers

In vivo fertilised porcine embryos (n=132) obtained 48 h post insemination were cultured in groups of 25 in 500µl NCSU23 at 39°C in 5% CO₂ in air. At Day 7 post insemination, the embryonic cleavage stage, degree and pattern of fragmentation were assessed by differential interference contrast microscopy (Olympus IX70) at a total magnification of 300. Embryos that had not reached the blastocyst stage at Day 7 post insemination were defined as arrested embryos. Such embryos were divided in two groups, embryos that stopped cleaving before transition of maternal to the embryonic genome (at the 4-cell stage in porcine embryos) (Jarrell et al. 1991; Schoenbeck et al. 1992; Viuff et al. 2002) and embryos that arrested during the 5-cell to the morula stage period.

Subsequently, all embryos were stained with annexin V and TUNEL assay and analyzed using confocal laser scanning microscopy. An embryonic cell was categorized as apoptotic if (i) the cell had nuclear morphological characteristics of apoptosis such as fragmentation or condensation; (ii) the cell membrane was annexin V positive and, (iii) the nucleus of the cell was TUNEL labelled. Based on this definition, an apoptotic cell ratio (ACR) was determined as the percentage of apoptotic cells per embryo.
Statistical analysis

The time that an embryo remained in a particular cell stage was defined as the time from appearance of that stage to the first appearance of one or more additional cells. To study the 4-cell lag phase phenomenon, the duration of each cell stage was compared with the duration of the 4-cell stage by the general linear model, using only those embryos for which the two relevant duration times were available.

The effect of asynchronous cleavage and fragmentation on further development of the embryos was studied by the Cox model with last stage attained as response variable and asynchronous cleavage as time-varying covariate. Asynchronous cleavage was introduced as a binary variable (yes/no), whereas for fragmentation different aspects were considered. First, fragmentation was investigated as a binary variable (yes/no) and next as a categorical variable with 4 categories: no fragmentation, 0-5% fragmentation, 5-15% and >15%. Additionally, the fragmentation pattern was investigated as a categorical variable with categories: no fragmentation, and fragmentation pattern 1, 2, 3 and 4. Time points at which embryos attained a specified cell stage with the highest significant odds of becoming blastocyst in later embryonic development were calculated using Chi-square analysis. The standard statistical analysis for binary data is based on the odds ratio. The analysis of such data is based on odds and not on probabilities (Hosmer & Lemeshow 1989). The standard statistical analysis for time-to-event or survival data is based on the proportional hazards model and the corresponding measure, the hazard ratio. The hazard is a conditional probability: given the event did not take place up to a certain moment, what is the probability it occurs that moment (Collett 1994).

Differences in ratios of fragmented or apoptotic embryos were analyzed by Chi-square tests or when small numbers were involved Fisher’s exact tests were used. Logistic regression
with the embryo as a random factor was used to compare average fragmentation %, and average ACR. Correlation analysis between embryonic arrest, fragmentation and apoptosis was performed by Spearman’s rank test. Statistical significance was assumed at $P<0.05$. The statistical analyses were performed using SAS version 8.
RESULTS

*Cinematographic analysis of porcine embryonic morphology and development*

(*Experiment 1*)

Five movies were analyzed including a total of 86 embryos. At recovery, 3 one-cell, 28 two-cell, 10 three-cell, 42 four-cell and 3 more than five-cell embryos were retrieved. The descriptive developmental kinetics of the cell stages are given in Table 1. The developmental capacity of embryos cultured in the time-lapse culture system was comparable to that in a classic incubator (n=103) since the blastocyst rate was 67.4% and 64.1% in the two systems, respectively.

Table 1. Descriptive developmental kinetics of porcine embryos in a cinematographic time-lapse system.

<table>
<thead>
<tr>
<th>Cell stage</th>
<th>Mean time (hours ± SEM) post insemination to reach cell stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-cell</td>
<td>55.61 ± 2.42</td>
</tr>
<tr>
<td>4-cell</td>
<td>57.41 ± 2.36</td>
</tr>
<tr>
<td>5-cell</td>
<td>85.64 ± 1.73</td>
</tr>
<tr>
<td>6-cell</td>
<td>87.83 ± 1.71</td>
</tr>
<tr>
<td>7-cell</td>
<td>89.81 ± 1.96</td>
</tr>
<tr>
<td>8-cell</td>
<td>92.78 ± 1.54</td>
</tr>
<tr>
<td>Early morula</td>
<td>103.43 ± 1.43</td>
</tr>
<tr>
<td>Compacted morula</td>
<td>112.30 ± 2.41</td>
</tr>
<tr>
<td>Early blastocyst</td>
<td>135.77 ± 2.94</td>
</tr>
<tr>
<td>Expanded blastocyst</td>
<td>148.34 ± 3.10</td>
</tr>
<tr>
<td>Hatching blastocyst</td>
<td>161.50 ± 9.57</td>
</tr>
<tr>
<td>Hatched blastocyst</td>
<td>176.04 ± 24.57</td>
</tr>
</tbody>
</table>
Cinematographic analysis revealed that the 4-cell stage (average duration 38.20 h) lasted longer (P<0.01) than the 8-cell, early morula, compacted morula and early blastocyst stage, although all embryos resumed division regardless of the duration of the 4-cell stage. Three out of the 32 (9.4%) embryos with measurable third cell cycle (from 4-cell to 5-cell stage) were passing through the 4-cell stage in less than 15 h. All of these reached the early blastocyst stage, while the early blastocyst percentage of embryos with a 4-cell lag phase was 93.1% (27/29).

The asynchrony between blastomere cleavages increased at the third cell cycle. The time interval between the appearance of 3-cell and 4-cell stage was on average $2.3 \pm 5.4$ h (n=25) whereas the blastomeres of 4-cell stage embryos cleaved asynchronously with an average of $9.2 \pm 10.8$ h (n=55) interval between the first and the last blastomere cleavage. No significant effects of these asynchronous cleavages on further development or blastocyst formation were detected (P=0.43).

Extrusion of blastomeres was recorded in 24.4% (21/86) of the embryos. The highest frequency was observed at the morula stages (11/21=52.4%) followed by the 5-cell to the 8-cell stages (6/21=28.6%). Extrusion of blastomeres also had no negative influence on further embryonic development. In 76.2% (16/21) of the cases, this asymmetry of the embryo disappeared during a later cell cycle due to remerging of the extruded blastomeres with the embryonic mass.

In general, fast cleaving embryos reached the blastocyst stage at higher frequencies than slower cleaving embryos. In Figure 2, a difference in time between insemination and first appearance of the 5- to 8-cell stages was detected for embryos that stopped developing at the morula stage in comparison to embryos that reached the blastocyst stage (P=0.019). A retrospective analysis of the cleavage data revealed an optimal cleavage pattern for embryos with in vitro blastulation capacity at a given cell stage. Embryos attaining at least the 5-cell
stage before 77 hpi, had a better odds of reaching the blastocyst stage (estimated odds ratio (OR) = 9.95, P=0.031) than embryos that reached these cell stages at a later time point (P<0.05). Another selection criterion for blastocyst formation was attainment of the early morula stage before 102 hpi (OR=4.29) (P=0.019).

Figure 2. The developmental kinetics of embryos that arrested at the morula stage in comparison with the development of embryos that reached the blastocyst stage.

(*)Significant difference within a stage of development with P<0.05.

Stages of development: 3-4c = 3- to 4-cell stage; 5-8c = 5- to 8-cell stage; EM = early morula; CM = compacted morula; EB = early blastocyst; ExB = expanded blastocyst; HchB = hatching blastocyst; HB = hatched blastocyst.
Time-lapse recordings showed 2 different kinds of fragmentation: static fragments detached from blastomeres (n=2) and fragments that moved in concert with adjacent blastomeres (n=54). These latter fragments often changed location and size during further development. An apparent disappearance and reoccurrence of fragmentation was also a common feature of these embryos.

Overall, a negative effect of fragmentation percentage on subsequent embryonic development was detected. The hazard for not reaching the next embryonic stage was 11.8 times higher for embryos with >15% fragmentation (n=10) compared to embryos with a fragmentation percentage of ≤15% (n=76) (P<0.0001). The effect on subsequent embryonic development of embryos experiencing slight fragmentation (0-5%) (n=34) was not different from embryos without fragmentation (n=30) (hazard ratio=0.89, P=0.891).

The pattern of fragmentation was also associated with subsequent embryonic development. Fragmentation pattern 1 (n=45) and FP=2 or 3 (n=7) had detrimental effects on subsequent embryo cleavage since they had a hazard ratio of 3.1 (P=0.011) and 20.5 (P<0.0001), respectively in comparison to embryos without fragmentation. Furthermore, FP2 or 3 embryos had a lower developmental potential than FP1 embryos with an estimated hazard ratio of 6.5 (P=0.007).

**Evaluation of the relationship between embryonic morphology and apoptotic markers**

*(Experiment 2)*

Of the 132 embryos included in this experiment, 61 (46%) arrested during the in vitro culture period, and 71 (54%) embryos reached the blastocyst stage at Day 7 post insemination (dpi). A small proportion (8/61, 13%) of the arrested embryos stopped cleaving before or at the 4-cell stage. More arrested embryos were fragmented compared to embryos that reached the blastocyst stage at 7 dpi (P<0.05). Also, the average fragmentation percentage was higher
for arrested embryos compared to blastocyst stage embryos at Day 7 post insemination (P<0.05) (Table 2). The correlation detected between developmental arrest and fragmentation was 0.60 (P<0.05).

None of the embryos without fragmentation had cells categorized as apoptotic, whereas 50 out of 55 embryos with fragmentation possessed apoptotic cells. The percentage of embryos with apoptotic cells was higher for embryos arrested during the 5-cell to the morula stage compared to embryos that arrested before or at the 4-cell stage and embryos with blastocyst development at Day 7 post insemination (P<0.05). The average ACR of embryos arrested at the 5-cell to the morula stage was higher compared to the average ACR of blastocysts at 7 dpi (P<0.05) (Table 2). The correlation detected between the developmental arrest during the 5-cell to the morula stage period and apoptosis was 0.57 (P<0.01).

Table 2. Assessment of fragmentation % and apoptosis in arrested and blastocyst stage embryos (n=132) by annexin V, TUNEL assay and nuclear morphology analysis at Day 7 post insemination.

<table>
<thead>
<tr>
<th>Stage of development at Day 7 post insemination</th>
<th>% (ratio) of embryos with fragmentation</th>
<th>Average fragmentation % ± SEM</th>
<th>% (ratio) of embryos with apoptosis</th>
<th>Average ACR (%) ± SEM of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrested 1- to 4-cell</td>
<td>50.0&lt;sup&gt;a&lt;/sup&gt; (4/8)</td>
<td>10.0&lt;sup&gt;a&lt;/sup&gt; ± 4.2</td>
<td>25.0&lt;sup&gt;a&lt;/sup&gt; (2/8)</td>
<td>8.3&lt;sup&gt;ab&lt;/sup&gt; ± 5.5</td>
</tr>
<tr>
<td>Arrested 5-cell to morula</td>
<td>77.4&lt;sup&gt;a&lt;/sup&gt; (41/53)</td>
<td>10.8&lt;sup&gt;a&lt;/sup&gt; ± 1.2</td>
<td>71.7&lt;sup&gt;b&lt;/sup&gt; (38/53)</td>
<td>16.1&lt;sup&gt;b&lt;/sup&gt; ± 2.2</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>14.1&lt;sup&gt;b&lt;/sup&gt; (10/71)</td>
<td>2.5&lt;sup&gt;b&lt;/sup&gt; ± 1.0</td>
<td>14.1&lt;sup&gt;a&lt;/sup&gt; (10/71)</td>
<td>3.4&lt;sup&gt;a&lt;/sup&gt; ± 1.2</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Within a column, values with a different superscript differ significantly (P<0.05).

As shown in Tables 3 and 4, the percentages as well as the pattern of embryo fragmentation were both associated with the apoptotic cell ratio (P<0.05). For both fragmentation assessments a significant difference in apoptosis was detected between
embryos without and with fragmentation. Embryos experiencing slight fragmentation (0-5%) had a lower average ACR than embryos with a fragmentation percentage of >5% (P<0.05) (Table 3). The correlation detected between fragmentation and apoptosis was 0.87 (P<0.05).

Table 3: The relationship between fragmentation percentage and average apoptotic cell ratio (ACR) in Day 7 cultured pig embryos (n=132).

<table>
<thead>
<tr>
<th>Fragmentation percentage</th>
<th>N° of embryos</th>
<th>% (ratio) of embryos with apoptosis</th>
<th>Average ACR (%) ± SE of embryos with apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>77</td>
<td>0.0 (^a) (0/77)</td>
<td>0.0 (^a) ± 0</td>
</tr>
<tr>
<td>0-5%</td>
<td>11</td>
<td>90.9 (^b) (10/11)</td>
<td>12.7 (^b) ± 2.0</td>
</tr>
<tr>
<td>5-15%</td>
<td>27</td>
<td>94.7 (^b) (26/27)</td>
<td>28.8 (^c) ± 3.5</td>
</tr>
<tr>
<td>&gt;15%</td>
<td>17</td>
<td>82.3 (^b) (14/17)</td>
<td>23.2 (^c) ± 3.4</td>
</tr>
</tbody>
</table>

\(^a,b,c\) Within a column, values with a different superscript differ significantly (P<0.05).

Table 4: The relationship between fragmentation pattern and ACR (average apoptotic cell ratio) in Day 7 cultured pig embryos (n=132).

<table>
<thead>
<tr>
<th>Fragmentation pattern</th>
<th>N° of embryos</th>
<th>% (ratio) of embryos with apoptosis</th>
<th>Average ACR (%) ± SE of embryos with apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No fragmentation</td>
<td>77</td>
<td>0.0 (^a) (0/77)</td>
<td>0.0 (^a) ± 0</td>
</tr>
<tr>
<td>FP1</td>
<td>31</td>
<td>96.8 (^b) (30/31)</td>
<td>22.3 (^b) ± 2.9</td>
</tr>
<tr>
<td>FP2</td>
<td>10</td>
<td>80.0 (^b) (8/10)</td>
<td>17.8 (^b) ± 4.2</td>
</tr>
<tr>
<td>FP3</td>
<td>13</td>
<td>84.6 (^b) (11/13)</td>
<td>19.6 (^b) ± 4.3</td>
</tr>
<tr>
<td>FP4</td>
<td>1</td>
<td>100.0 (^b) (1/1)</td>
<td>37.9 (^b) ± 0</td>
</tr>
</tbody>
</table>

\(^a,b\) Within a column, values with a different superscript differ significantly (P<0.05).
Occurrences of biochemical cell changes indicative for apoptosis of fragmented embryos are presented in figure 3.

Figure 3. Differential interference contrast microscopic and confocal laser scanning images of fragmented in vivo fertilised and in vitro cultured porcine embryos stained with propidium iodide, annexin V and TUNEL (bar = 40.00µm).

A-B-C: Embryos under differential interference contrast microscopy, A’-B’-C’: Confocal images of live embryos stained with propidium iodide and annexin V, A’’-B’’-C’’: Confocal images of fixed embryos stained with propidium iodide and TUNEL, A-A’’: (A) Image of an arrested morula stage embryo with <5% fragmentation. (A’) Annexin V labelling surrounding one blastomere (a’), also annexin V positive labelling was detected surrounding fragmentation (b). (A’’) The nucleus of the blastomere with annexin V labelling in A’ is condensed and displays TUNEL labelling (a’’), B-B’’: (B) Image of an arrested morula stage embryo with 5-
15% fragmentation. (B’) One blastomere (c’) is labeled with annexin V but the nucleus is also propidium iodide positive, indicating that the blastomere is undergoing necrotic cell death. Three other blastomeres (d’, e’, f’) have annexin V labelling without a propidium positive nucleus. (B’’) Note in the blastomere c’’ TUNEL labelling is not only confined to the nucleus, but diffusely stains the blastomere which indicates necrosis of that blastomere. The nuclei of the blastomeres with annexin V labelling in B’ are condensed and display TUNEL labelling (d’’, e’’, f’’), C-C’’': (C) Image of a highly fragmented embryo (>15%) with a blastocoele. (C’-C’’’) A lot of the blastomeres show simultaneous annexin V and TUNEL labelling.
DISCUSSION

The present study was performed to establish guidelines and standards for evaluating the quality of porcine embryos. The in vitro developmental kinetics of in vivo fertilised porcine embryos as defined in the first experiment can be used as a reference for further embryological cellular and molecular studies of porcine embryos. At the same time, the kinetics of embryonic fragmentation in developing pig embryos was described. Timing of embryo development is a rapid, simple, accurate and non-invasive way to evaluate embryos. Using time-lapse cinematography, a high degree of precision on the measurements of development timing is established and it captures all morphological characteristics at a light microscope level. The ability to form a blastocoel cavity is probably the best morphological indicator of the developmental competence of a preimplantation embryo (Bavister 1995) but the relationship between developmental competence and viability is more complex and can only be established using embryo transfer experiments.

In the present study, high developmental rates to the blastocyst stage (67.4% and 64.1%) were obtained after in vitro culture in contrast to the blastocyst rates (about 25%) which are usually found after culture of IVF pig embryos (Abeydeera 2001). This is not surprising, since also in other species such as cattle substantial differences in morula-blastocyst rates have been described after culture of in vivo vs. in vitro produced embryos, which are probably due to the inferior conditions of maturation and fertilisation to which IVF embryos have been exposed (Van Soom & de Kruif 1992).

In our study, pig embryos showed a 4-cell lag phase which lasted on average 38.2 h, which is comparable to findings by Anderson et al. (1999) who found an average 4-cell stage length between 38 and 44 h for in vitro cultured porcine embryos. For in vivo developing pig embryos, the 4-cell stage lasts between 20 to 24 h (Hunter 1974; Flint 1981) which is
characteristically shorter than findings of in vitro cultured embryos (Bavister 1995). The 4-cell lag phase is likely attributed to imperfections of in vitro culture conditions. Possible causes for this delay are transition of maternal to zygotic control of embryonic development which takes place at the 4-cell stage for porcine embryos (Jarrell et al. 1991; Schoenbeck et al. 1992; Viuff et al. 2002), change in metabolism and needs of embryos (Schultz et al. 1993), inadequate energy supply by the medium and/or effects of the production of free radicals (Jarrell et al. 1991).

The average time needed to cleave from the 3 to the 4-cell stage was 2.3 h, but increased to 9.2 h for the 5 to the 8-cell stage. For bovine embryos, a comparable time interval of 9.2 h was detected between 9 to the 16-cell stage (Holm et al. 1998). An increase in asynchrony at these two species specific cleavage stages can be related to the transition of maternal to zygotic control that takes place at the cell stage prior to these cleavage stages (4-cell stage for porcine and 8-cell stage for bovine embryos). Human embryos with unevenly sized blastomeres have a lower pregnancy and implantation rate (Hardarson et al. 2001). Also, asymmetry in bovine early embryonic stages is regarded as a characteristic of poor embryo quality (Lindner & Wright 1983), but its impact on viability is uncertain. In the present study, no effect of asynchronous cleavage or blastomere extrusion on blastulation was detected. Cleavage of extruded blastomeres ceased, but in most of the embryos (76%), the asymmetry disappeared by reabsorbing the extruded blastomeres in the embryonic mass during later cleavage divisions.

We could demonstrate that also in pig embryos, the time needed to reach the third cell cycle and the early morula stage was inversely correlated with the probability of blastulation \( P<0.05 \). Embryos which failed to reach the blastocyst stage needed on average 6.25 h and 5.44 h more to reach the third cell cycle and early morula stage, respectively, compared to embryos that completed blastocyst development. These findings are in agreement with earlier
studies that correlate cleavage kinetics with blastocyst development in bovine (Van Soom et al. 1992; Grisart et al. 1994; Holm et al. 1998; Lonergan et al. 1999) and in hamster embryos (McKiernan & Bavister 1994; Gonzales et al. 2001). By analogy with studies in other species, reference time points for in vitro development in hpi were calculated. For the specific culture system used, attainment of at least the 5-cell stage before 77 hpi and attainment of the early morula stage before 102 hpi increased the odds for reaching the early blastocyst stage to 995% and 429%, respectively, compared to embryos that reached these cell stages at a later time point (P<0.05). The reason why faster cleaving embryos are more capable of developing is not known, but there are a number of factors that can influence in vitro cleavage rate such as ooplasm quality, culture medium, environment and several genetic factors. The preimplantation development (PED) gene in mouse embryos has a remarkable regulatory function on the timing of embryo development (Warner et al. 1998b) and potential human and bovine homologues of this PED gene have been identified (Cao et al. 1999; Fair et al. 2004). Further are paternal influences on the S- and G1-phase of zygotes (Eid et al. 1994; Comizzoli et al. 2000), aberrant maternal inherited cytoplasm (Liu & Keef 2000; Meirelles et al. 2004) and sex differences of mouse and bovine embryos related to cleavage rate (Tsunoda et al. 1985; Mittwoch 1989; Yadav et al. 1993). Chromosome abnormalities have also been shown to influence early development (Kawarsky et al. 1996; Viuff et al. 2001) but more research is necessary to clarify their impact.

Embryonic fragmentation was next to the timing of development as the most important morphological parameters analyzed in the cinematographic experiment. The time-lapse recordings clearly showed that porcine embryo fragmentation is a dynamic feature in which the location and size of fragmentation can vary in time. These findings are in accordance with observations of fragmentation in human IVF embryos (Van Blerkom et al. 2001). Overall, a negative effect of fragmentation on subsequent embryonic development was
observed, but fragmentation *per se* is not an absolute determinant of developmental incompetence. As described for human IVF embryos (Alikani *et al.* 2000), the results of the time-lapse experiment show that the developmental potential of slightly fragmented (0-5%) embryos was not different from embryos without fragmentation, indicating that minor fragmentation in porcine embryos may be normal. However, fragmentation exceeding 15% of the embryonic volume had a significant adverse effect on subsequent development with an estimated hazard ratio of 11.8. The distribution and relative size of the fragments also had a significant impact on embryo developmental potential. The presence of localized fragments which appeared to have resulted from complete fragmentation of one or more blastomeres (FP 2) or small, scattered fragments distributed all over the embryonic cell mass (FP 3) posed the most serious threat on further embryonic development. There are several possible explanations for the negative effects of marked fragmentation on embryonic viability. In human IVF embryos, extensive fragmentation has been associated with a higher incidence of chromosomal abnormalities in less viable embryos (Pellestor *et al.* 1994). Furthermore, according to Antczak and Van Blerkom (1999) fragmentation can result in a depletion of cortically positioned regulatory proteins resulting in a compromising effect on embryo cleavage. Fragments may also interfere with normal cell-to-cell contact between blastomeres, or induce degenerative processes in adjacent blastomeres (Alikani *et al.* 1999).

Because fragmentation is one of the hallmarks of programmed cell death or apoptosis (Hardy 1999), it could also be used as a non-invasive marker of embryonic apoptosis. Nevertheless, in (human) embryology the relationship between fragmentation and apoptosis has been the subject of controversy. In studies using arrested fragmented early cleavage embryos, morphological and biochemical (TUNEL and annexin V staining) characteristics of apoptosis were detected (Jurisicova *et al.* 1996; Levy *et al.* 1998). However, in a study by Antczak and Van Blerkom (1999), a majority of fragmented developing embryos did not
show either TUNEL or annexin V labelling, leading to their conclusion that fragmentation was not correlated with apoptosis.

In the present study, only cells that concurrently displayed nuclear fragmentation or condensation, positive annexin V staining of the cell membrane and TUNEL positive nuclei were categorized as apoptotic. Nuclear fragmentation and condensation are morphological key elements of apoptosis and are necessary to confirm biochemical assessments of apoptosis (Hardy 1999; Gjørret et al. 2003). Annexin V has a specific and high affinity for phosphatidylserine that redistributes to the outer leaflet of the cell membrane in an apoptotic cell (Martin et al. 1995). By using a propidium iodide staining to assess membrane permeability in addition to the annexin V, it is possible to distinguish apoptosis from necrosis (Levy et al. 1998; van den Eijnde et al. 1997). TUNEL allows the assessment of another classic feature of apoptosis namely nuclear DNA fragmentation (Gavrieli et al. 1992). Using these conservative criteria for apoptosis, the incidence of false positive results due to necrosis, misinterpretation of prophase nuclei or nuclear fragmentation independent of apoptosis should be reduced to a minimum. Because the annexin V staining is characteristic for early apoptosis (Martin et al. 1995), false negative results could have occurred only in 2 embryos where nuclei were fragmented and the annexin V staining was positive, but where no TUNEL signal was detected. The average ACR of Day 7 blastocysts (3.4%) in our study was higher than the average ACR estimated for in vivo embryos flushed at Day 4 (0.4%) (Rubio Pomar et al. 2004) but numerically lower than the average ACR of in vitro produced Day 7 blastocysts (4.9%) (Hao et al. 2003). These results indicate that apoptosis is a natural process of porcine preimplantation embryo development that is increased by suboptimal in vitro culture conditions.

Following an analysis for apoptosis of arrested (n=61) and non-arrested (n=71) porcine preimplantation stage embryos, significant correlations between developmental arrest,
fragmentation and apoptosis were detected. Seventy-two percent of the arrested embryos showed signs of cytoplasmic fragmentation, which is comparable to the 89% found in arrested, in vitro produced human embryos (Jurisicova et al. 1996). A majority (89%) of these fragmented, arrested embryos showed biochemical evidence of apoptosis. This is in accordance with the finding of Hardy (1999) that a prolonged culture of arrested embryos can trigger the apoptotic machinery. In the present study, a correlation between arrested development and apoptosis was only detected for embryos arrested at 5-cell to morula stage, but not for embryos arrested before or at the 4-cell stage. Only 2 out of 8 embryos arrested at the 1 to the 4-cell stage showed biochemical characteristics of apoptosis. This indicates that embryonic arrest is associated with apoptosis in a stage-specific manner in which the apoptotic cascade for embryos arrested before embryonic genome activation is induced at a lesser extent.

In the current study, a strong direct correlation of 0.87 between fragmentation and apoptosis was detected following the analysis of in vivo fertilised, in vitro cultured embryos with biochemical apoptotic markers. This is in accordance with a recent study by Hao et al. (2003) suggesting that cytoplasmic fragmentation is a typical morphological feature of porcine IVF and nuclear transfer embryos undergoing apoptosis. However, not all fragmented embryos displayed positive apoptotic markers, indicating that not all fragmentation is related to apoptosis. As stated by others, fragmentation can also be caused by instability of the microfilament network (Antczak & Van Blerkom 1999), low levels of ATP (Van Blerkom et al. 1995), chromosomal abnormalities (Munné & Cohen 1993) and necrotic processes (Jurisicova et al. 1996). The percentage of embryos with >5% fragmentation (33.3%) was comparable to the percentage detected in the study of Hao et al. (2003) using in vitro cultured IVF embryos (35.2%), suggesting that in vitro culture conditions have a major influence on embryo fragmentation. The result of the time-lapse experiment showed that slight
fragmentation (0-5%) did not affect subsequent embryonic development. Furthermore, the average ACR of embryos with slight fragmentation was significant lower than the average ACR of embryos with >5% fragmentation. It seems therefore that embryonic cell death by apoptosis affects the developmental potential of porcine embryos only when it crosses a certain threshold ACR value. Further research will focus on determining this threshold value for porcine embryos.

In summary, using in vitro the time-lapse system it was shown that in vivo fertilised porcine embryos that reached the blastocyst stage cleaved faster than embryos whose development ceased at the morula stages. On this basis, kinetic selection criteria for porcine embryos with blastulation capacity were defined under the specific culture conditions used. In addition, a negative effect of fragmentation on subsequent embryonic development was detected. Strong significant correlations between developmental arrest and fragmentation, and fragmentation and apoptosis were observed, whereas a significant correlation between developmental arrest and apoptosis could only be established after embryonic genome activation.

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CHAPTER 4

INTERACTION OF VIRUSES WITH IN-VIVO DERIVED PORCINE EMBRYOS
CHAPTER 4.1.: 

SUSCEPTIBILITY OF PIG EMBRYOS TO PORCINE CIRCOVIRUS TYPE 2 INFECTION

Adapted from: Theriogenology 2004, 61(1): 91-101

ABSTRACT

The aim of the present study was to determine if porcine circovirus type 2 (PCV2) is able to infect embryonic cells of in vivo-produced porcine embryos with and without zona pellucida (ZP). Zona pellucida-intact and ZP-free morulae (6 d post insemination) and early blastocyst (7 d post insemination), and hatched blastocysts (8 d post insemination) were exposed to 10^{5.0}\text{TCID}_{50} PCV2 per ml (strain 1121, 5^{th} passage PK15). At 48 h post incubation, the percentage of infected embryos and the percentage of viral antigen positive cells per embryo were determined by indirect immunofluorescence. Significantly different percentages of infected embryos were detected: 15% for ZP-free morulae, 50% for ZP-free early blastocysts and 100% for hatched blastocysts. The percentage of cells that expressed viral antigens was similar for the three stages of development. PCV2 exposure did not affect the in vitro development of the embryos during the 48 h study period. All ZP-intact embryos remained negative for viral antigens. In an additional experiment the diameter of the channels in the porcine ZP was determined. After incubation of early blastocysts with fluorescent microspheres of three different sizes, beads with a diameter of 20 nm and beads with a diameter of 26 nm crossed the zona whereas beads with a diameter of 200 nm did not.

In conclusion, it can be stated that PCV2 is able to replicate in in vivo-produced ZP-free morulae and blastocysts and that the susceptibility increases during development. The ZP
forms a barrier to PCV2 infection, but based on the size of the channels in the ZP the possibility that PCV2 particles cross the ZP cannot be excluded.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, single-stranded deoxyribonucleic acid (DNA) virus, recognized as the causal agent of Porcine Post-Weaning Multisystemic Wasting Syndrome (PMWS) (Allan and Ellis, 2000). The syndrome is clinically characterized by wasting, paleness of the skin and increased mortality in weaned pigs (Harding and Clark, 1997). Recently, PCV2-infection has been associated with reproductive failure. Several reports have described the isolation of PCV2 from aborted fetuses originating from recently infected herds (West et al., 1999; Ladekjær-Mikkelsen et al., 2001; O’Connor et al., 2001).

In general, infection of oocytes, embryos or fetuses can be established either by viremia followed by a transplacental spread or by contaminated/infected gametes. To date, evidence for a transplacental spread by PCV2 has not been found (Cariolet et al., 2002). Research on the existence of contaminated/infected porcine female gametes has not been done yet. A few studies have investigated the role of semen (Larochelle et al., 2000, Kim et al., 2001). Using PCR, Larochelle and co-workers (Larochelle et al., 2000) demonstrated the presence of PCV2 DNA in boar semen. Cariolet and co-workers (Cariolet et al., 2002) developed a model that simulates the effects of PCV2-contaminated semen. After intra-uterine administration of PCV2 at insemination in specific pathogen-free sows, replication of PCV2 in fetuses was demonstrated. This indicates that PCV2-contaminated semen can be the virus source for embryos or fetuses. To our knowledge, information on the interaction between PCV2 and preimplantation stage embryos is not available. Therefore the objectives of the present study were (i) to determine if PCV2 is able to replicate in embryonic cells of porcine embryos
collected at different stages of development and if so, (ii) to examine whether the zona pellucida (ZP) may serve as a physical barrier for PCV2.

MATERIALS AND METHODS

Collection of in vivo produced porcine embryos

A total of 30 multiparous, PCV2-immune sows (Rattlerow-Seghers sows) were used in the present study. The sows were superovulated using PMSG (Folligon® 1500 IU im) three days after weaning, followed by hCG (Chorulon® 1500 IU im) 72 h later. The sows were fixed-time inseminated with boar semen of proven fertility 24 h after hCG administration. In experiments 1 and 3, the sows were slaughtered 48 h post insemination. In experiment 2, the sows were slaughtered 192 h post insemination. The reproductive tracts were removed and transported to the laboratory in a pre-warmed box (39 °C) within 30 min after slaughter. For experiments 1 and 3, each oviduct was flushed with 15 ml of pre-warmed Hepes-buffered North Carolina State University-23 (NCSU-23) medium (Petters and Wells, 1993). For experiment 2, the uterus was flushed with 500 ml of pre-warmed Hepes-buffered NCSU-23. After 10 washings (Stringfellow, 1998) with Hepes-buffered NCSU-23, they were cultured in NCSU-23 (Petters and Wells, 1993) at 39 °C in 5% CO₂ in air until they reached the morula or the blastocyst stage (experiments 1 and 3).

Porcine circovirus type 2 (PCV2) exposure

Porcine circovirus type 2 strain 1121 was used for inoculation. It was originally obtained from a pool of lungs, liver and spleen of dead fetuses/piglets at partum and further passaged five times in PK-15 cells (West et al., 1999). The embryos were placed in 1 ml of minimum essential medium (MEM), containing 10⁵.0 50% tissue culture infectious doses (TCID₅₀) and incubated for 1 h at 39 °C in an atmosphere of 5% CO₂ in air. Control embryos were
incubated under the same circumstances in NCSU-23 without PCV2. After 1 h of exposure, all embryos were washed 5 times in Hepes-buffered NCSU-23 medium. Embryos were further cultured in NCSU-23 medium for 48 h at 39 °C in an atmosphere of 5% CO₂ in air.

Detection of viral antigen expression

The ratio of infected embryos and the ratio of PCV2-antigen positive cells per infected embryo were determined by indirect immunofluorescence (IF). The embryos were fixed in 4% paraformaldehyde at room temperature for 30 min. After fixation, the embryos were washed 3 times in polyvinyl pyrrolidone (PVP) solution (1 mg PVP/ml phosphate buffered saline). The embryos were treated with 0.5% Triton X-100 (in PVP solution) for 1 h. After three 5 min washes in PVP solution, embryos and controls were incubated for 1 min at 39 °C in 10% goat serum. Next, the embryos and the controls were incubated for 1 h at 39 °C with a 1:100 dilution of PCV2 monoclonal antibody (F217) (McNeilly et al., 2001), followed by a 1:100 dilution of goat-anti-mouse FITC (Molecular Probes, USA). Nuclear staining by Hoechst 33342 was performed to assess the embryonic cell number (Ebert et al., 1985). Briefly, the embryos were incubated in 2 ml Hoechst 33342 solution (10 µl/ml Hoechst 33342 in ethanol 96%) for 10 min at 4 °C. After mounting the embryos in glycerol with 1,4-diazabicyclo (2.2.2) octane (25mg/ml), the number of fluorescent cells was counted in each embryo by means of a Leica DM/RBE fluorescence microscope at a magnification of 400 x.

Virus titration

PCV2 was titrated in the 5th washing fluid after inoculation and in the NCSU-23 medium 48 h after inoculation. Tenfold dilutions of the wash and culture medium were made in PBS. Fifty µl of each dilution was inoculated into 96-well microtitre plates containing semi-confluent monolayers of PCV2-free PK/15 cells. The plates were examined for PCV2 three days later
by immunoperoxidase staining using a hyperimmune PCV2 serum. Virus titres were
determined using the method of Reed and Muench (1938).

Experimental design

**Experiment 1: Susceptibility of zona pellucida (ZP)-intact and ZP-free morulae and early blastocysts to PCV2-infection**

Cleavage stage embryos (n = 254) were harvested from 26 sows at 2 d post insemination. They were cultured for 4 or 5 days until they reached the morula (n = 80) or early blastocyst stage (n = 88), respectively. Fifty-two and 50 of the morulae and early blastocysts, respectively, were incubated in 2.5% protease (Sigma P-6911) in HEPES-buffered NCSU-23 during approximately 3 min (Menino and Wright, 1983). As a result, 40 morulae and 40 early blastocysts were made ZP-free. After a recovery period of 12 h in NCSU-23 at 39 °C in 5% CO₂ in air, ZP-intact morulae (n = 14) and blastocysts (n = 19), and ZP-free morulae (n = 20) and blastocysts (n = 20) were exposed separately to \(10^{5.0}\) TCID₅₀ PCV2 per ml (1121, 5th passage PK15) for 1 h. Zona-intact and zona-free control embryos (n = 33 and n = 40, respectively) were incubated under the same circumstances in NCSU-23 but without PCV2. At 0, 24, and 48 h after virus incubation, embryos were evaluated for morphology and development. Forty-eight hours after incubation, embryos were fixed and stained for PCV2 by indirect IF as described above.

**Experiment 2: Susceptibility of hatched blastocysts to PCV2 infection**

Hatched blastocysts (n = 11) were recovered from the uterus of 3 PCV2-immune sows at 8 d post insemination. After being cultured for 12 h in NCSU-23 at 39 °C in 5% CO₂ in air, 8 embryos were incubated with \(10^{5.0}\) TCID₅₀ PCV2 per ml (1121, 5th passage PK/15) for 1 h. Control embryos (n = 3) were incubated under the same circumstances in NCSU-23 but
without PCV2. Embryos were fixed and stained for PCV2 by indirect IF as described above, 48 h after inoculation.

**Experiment 3: Permeability of porcine ZP to fluorescent particles**

Fluorescent microspheres (Fluospheres®, Molecular Probes) were used to investigate the permeability of the porcine ZP for particles with physical dimensions comparable to or larger than PCV2 (17 nm) (Allan et al., 1994). Three types of fluorescent microspheres were used: (i) crimson red fluorescent microspheres with a diameter of 200 nm (F-8806; excitation/emission maximum of 625/645), (ii) yellow-green fluorescent microspheres with a diameter of 26 nm (F-8787; excitation/emission maximum of 505/515), (iii) blue fluorescent microspheres with a diameter of 20 nm (F-8781; excitation/emission maximum of 365/415). Six ZP-intact early blastocysts were simultaneously exposed to $10^{5.0}$ particles per ml of microspheres of the three different sizes in one single dish. Two ZP-intact early blastocysts were used as negative controls and cultured in NCSU-23 medium without microspheres. The localization of the microspheres was visualized by a Bio-Rad Radiance 2100 Blue Laser Diode BLDTM (Bio-Rad House, Hertfordshire, UK) linked to a Nikon Diaphot 300 inverted microscope (Nikon Corporation, Tokyo, Japan). Green Helium Neon laser light was used to excite crimson red fluorescence (543 nm line) and Argon 4-line laser light was used to excite yellow-green fluorescence (488 nm line) of the microspheres. Blue Diode laser light was used to excite the blue fluorescent microspheres (405 nm line). Images were collected electronically using software provided by the manufacturer.

**Statistical analysis**

The number of infected embryos and differences in rates of development were analysed using Chi-square analysis. Fisher’s exact tests were applied when small numbers were involved.
Logistic regression was used to compare the average ratio of antigen-positive cells in infected embryos for the developmental stages. Variables were considered to be significant at a 0.05 level (two-sided). The statistical analyses were performed using SPSS (SPSS 10, SPSS Inc. Illinois 60611, USA, 1999).

RESULTS

Experiment 1: Susceptibility of ZP-intact and ZP-free morulae and early blastocysts to PCV2-infection

Viral antigen expression 48 h after PCV2 inoculation

The percentage of infected ZP-free embryos and the average ratio of antigen-positive cells in the infected embryos are summarized in Table 1. All ZP-intact and non-inoculated control embryos were negative for viral antigens. Significantly more ZP-free early blastocysts (50%) became infected than ZP-free morulae (15%) \((P < 0.05)\). Only 5.6 and 6.1% of embryonic cells expressed viral antigens in inoculated early blastocysts and morulae, respectively \((P > 0.05)\). Both nuclear and cytoplasmic stainings were found.
Table 1. Susceptibility of zona pellucida (ZP)-intact and ZP-free morulae and blastocysts to PCV2-infection.

<table>
<thead>
<tr>
<th>Stage of development at inoculation</th>
<th>Presence of ZP</th>
<th>Number of inoculated embryos</th>
<th>Number (%) of infected embryos at 48 h post inoculation</th>
<th>Average ratio (% ± SD) of antigen-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morula</td>
<td>Yes</td>
<td>14</td>
<td>0&lt;sup&gt;a&lt;/sup&gt; (0)</td>
<td>0.0/19.3 (0.0)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>20</td>
<td>3&lt;sup&gt;a&lt;/sup&gt; (15)</td>
<td>1.3/21.6 (6.1 ± 2.9)</td>
</tr>
<tr>
<td>Early Blastocyst</td>
<td>Yes</td>
<td>19</td>
<td>0&lt;sup&gt;a&lt;/sup&gt; (0)</td>
<td>0.0/35.2 (0.0)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>20</td>
<td>10&lt;sup&gt;b&lt;/sup&gt; (50)</td>
<td>2.1/37.6 (5.6 ± 3.2)</td>
</tr>
<tr>
<td>Hatched Blastocyst</td>
<td>No</td>
<td>8</td>
<td>8&lt;sup&gt;c&lt;/sup&gt; (100)</td>
<td>23.9/390.0 (6.1 ± 2.8)</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Columns with different superscripts differ significantly (<i>P</i> < 0.05).

Effect of PCV2 exposure on the development of ZP-intact and ZP-free embryos in vitro

To determine whether exposure of embryos to PCV2 had an influence on further embryonic development, we compared it to the development of non-inoculated controls. No significant difference in percentage of embryos reaching the blastocyst stage was detected for embryos inoculated at the morula stage in comparison to non-inoculated controls (Table 2).
Table 2. In vitro development of zona pellucida (ZP)-intact and ZP-free morulae during a period of 48 h following exposure to PCV2.

<table>
<thead>
<tr>
<th>Presence of ZP</th>
<th>PCV2 inoculation</th>
<th>Number of morulae</th>
<th>In vitro development to blastocyst, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>14</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>14</td>
<td>7 (50)</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>20</td>
<td>7 (35)</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>20</td>
<td>9 (45)</td>
</tr>
</tbody>
</table>

Also, no significant difference in percentage of embryos reaching the expanded blastocyst stage was detected for embryos inoculated at the blastocyst stage in comparison to non-inoculated controls (Table 3).

Table 3. In vitro development of zona pellucida (ZP)-intact and ZP-free early blastocysts during a period of 48 h following exposure to PCV2.

<table>
<thead>
<tr>
<th>Presence of ZP</th>
<th>PCV2 inoculation</th>
<th>Number of early blastocysts</th>
<th>In vitro development to expanded blastocyst, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>19</td>
<td>12 (63)</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>19</td>
<td>13 (68)</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>20</td>
<td>10 (50)</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>20</td>
<td>8 (40)</td>
</tr>
</tbody>
</table>
Experiment 2: Susceptibility of hatched blastocysts to PCV2 infection.

PCV2-positive cells were detected in all hatched blastocysts 48 h post inoculation (Figure 1). Both nuclear and cytoplasmic stainings were found.

Figure 1. Detection of porcine circovirus type 2 (PCV2) viral antigens in in vivo-produced hatched blastocyst 48 h after incubation with PCV2 strain 1121 (5th passage PK/15) by indirect immunofluorescence technique. The objective used was 10x and the zoom factor was 3.5.
The percentage of infected hatched blastocysts was significantly different from the percentage of infected embryos originating from embryos exposed to PCV2 at the morula or early blastocyst stage (Table 1). Non-inoculated control embryos were negative for viral antigens. Extracellular virus was not detected in the culture medium 48 h post inoculation.

_Virus titration of fifth washing fluid and culture medium of experiment 1 and 2 at 48h after inoculation_

Residual virus (1.3 log10 TCID$_{50}$/ml) was detected in the 5$^{th}$ washing fluid in one replicate of ZP-intact morula and blastocyst stage. Extracellular virus was not detected in the NCSU-23 culture medium 48 h after inoculation.
Experiment 3: Permeability of porcine ZP to fluorescent particles

After incubation of the embryos in a suspension of microspheres for 24 h, beads with a size of 20 nm and 26 nm crossed the ZP and were detected inside the ZP in all 6 incubated blastocysts (Figure 2). Beads with a size of 200 nm in diameter were deposited at the outer layers of the ZP.

Figure 2. Confocal laser scanning microscopy image of a porcine blastocyst: crimson red fluorescent microspheres with a diameter of 200 nm, yellow-green fluorescent microspheres with a diameter of 26 nm and blue fluorescent microspheres with a diameter of 20 nm. The objective used was 10x and the zoom factor was 3.5.
DISCUSSION

This study demonstrated that (i) ZP-free porcine morulae and blastocysts are susceptible to PCV2, with the more advanced embryonic stages being more susceptible, and that (ii) ZP-intact embryos are resistant to PCV2-infection.

A number of studies have described the in vitro interaction of pathogens with porcine embryos, but only two dealt with the susceptibility of blastomeres. Bolin and co-workers (1981) did not detect infected blastomeres 48 h after inoculation of ZP-free 2- to 16-cell stage embryos with pseudorabies virus (PrV) and therefore concluded that the 2- to 16-cell stage embryos were refractory to the virus. Prieto and co-workers (Prieto et al, 1996) used another model to investigate the susceptibility of blastomeres for porcine reproductive and respiratory syndrome virus (PRRSV). Seventy-two hours after microinjecting 10 to 20 TCID$_{50}$ into intact 4- to 16-cell stage embryos, PRRSV was not detected in association with the embryos. In contrast to PrV and PRRSV, it was shown in the present study that PCV2 can replicate in morulae, early blastocysts and hatched blastocysts. A possible explanation for the differences in susceptibility of embryonic stages to viral pathogens may be the variation in expression of cell surface receptors for different viruses and/or an intrinsic resistance of embryos (Vanroose et al., 1998).

In contrast to the ZP-free embryos, PCV2-antigen positive embryonic cells were not detected in ZP-intact embryos 48 h post inoculation, showing that the porcine ZP forms a protective barrier against PCV2 infection. Most of the porcine viruses adhere to, but cannot penetrate through the ZP of porcine embryos (Bolin et al., 1983; Singh et al., 1984; Singh and Thomas, 1987; Singh, 1987; Dulac and Singh, 1988; Bane et al., 1990). The basis for this barrier may be the architecture of the ZP. The results of our third experiment demonstrated that beads with diameter of 20 and 26 nm were able to cross the ZP, whereas beads of 200 nm were detected only within the outer fourth part of the ZP. This finding indicates that the network of pores in
the ZP of in vivo produced blastocysts can be crossed by an inert particle, which has a size comparable to that of PCV2. Whether this is also the case for the virus particle itself, remains to be determined, but was not obvious from our infection experiments. Only once, transzonal infection of porcine ZP-intact embryos by a pathogen has been reported (Bane et al., 1990). By means of transmission electron microscopy, porcine parvovirus, a single stranded DNA virus of 20 nm in diameter, which is comparable to the physical dimension of PCV2 was detected in embryonic cells of ZP-intact embryos after incubation with the virus (Bane et al., 1990). The possible sites of virus entry are the sperm tracks (Bolin et al., 1983) and the pores in the ZP (Vanroose et al., 2000). The diameter of the channels in the ZP of different species decreases centripetally (Vanroose et al., 2000; Dudkiewicz and Williams, 1977; Keefe et al., 1997). Why PCV2 did not pass the ZP to infect the susceptible embryos in our study is not clear but may be due to a specific attachment of PCV2 to the ZP or due to the shorter period of incubation of the virus compared to that of the fluorobeads. It is recognized that viruses tend to adhere to the porcine ZP more easily than to the ZP of other species, but little is known about the basis for this species dependent difference in tenacity (Wrathall and Sutmöller, 1998). The stickiness of the porcine ZP can be a possible explanation of detection of extra-cellular virus in the 5th washing fluid in one replicate of ZP-intact morulae and blastocysts. Adhering viruses represent a risk during embryo transfer since they may cause infection of the recipient (Wrathall and Mengeling, 1979a; Medveczky et al, 1996).

PCV2 replication in ZP-free embryos did not affect embryonic development during the 48 h observation period. This may be due to the relatively small number of PCV2-infected embryonic cells (± 6%). The effect of virus replication on development of the embryo has been documented with embryos of other species. Some viruses, such as blue tongue virus, bovine herpes virus type 1 and cytopathogenic bovine viral diarrhoea virus (BVDV) have harmful effects on early embryonic development (Bowen et al, 1982; Vanroose et al., 1997;
Vanroose et al., 1998), whereas cytomegalovirus, noncytopathic BVDV and caprine arthritis-encephalitis virus have no effect on development (Neighbour, 1978; Vanroose et al., 1998; Lamara et al., 2002). These contrasting results may be explained by differences in the fraction of embryonic cells allowing viral replication and/or by differences in virulence of the viruses.

So far, evidence of PCV2 infections of embryonic stages under field circumstances has not been reported. Because PCV2 is difficult to inactivate (Allan et al., 1994), early cleavage stages could theoretically be infected several days after insemination with contaminated semen. However, whether semen contains infectious virus, has not been demonstrated yet. Although Larochelle and co-workers (2000) detected PCV2 genetic material in the semen of recently infected seronegative boars, they did not detect the presence of infectious virus. Whether PCV2 is able to cause a transplacental infection of embryos in non-immune sows has also not been proven (Pensaert et al., 2004). Further, since most pigs are infection-immune at the time of breeding (Labarque et al, 2000), the chance that infectious PCV2 is shed in semen of the boar or causes a transplacental spread in the sow will be low in the field. More research is necessary to answer all these questions.

In conclusion, this study showed that in vivo-produced porcine morula and blastocyst stage embryos are susceptible to PCV2 infection. The ZP acts as a barrier to the virus, but based on the size of the channels in the ZP, it cannot be excluded that small PCV2 particles may reach the embryonic cells.
REFERENCES


Chapter 4: Interaction of viruses with in-vivo derived porcine embryos


CHAPTER 4.2.: RECEPTOR-DETERMINED SUSCEPTIBILITY OF PREIMPLANTATION EMBRYOS TO PSEUDORABIES VIRUS AND PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Adapted from: Biology of Reproduction, 2007, 76(3):415-423

ABSTRACT

In the present study, the interaction of embryos with pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) was determined by viral antigen detection after in vitro virus exposure and by investigating the expression of virus receptors, namely poliovirus receptor-related 1 (PVRL1; formerly known as nectin 1) for PRV and sialoadhesin for PRRSV.

Embryonic cells of zona pellucida intact embryos incubated with PRV remained negative for viral antigens. Also, PRV incubation of protease treated zona pellucida free embryos did not lead to antigen positive embryos because the protease disrupted the expression of PRVL 1.

Starting from the 5-cell stage onward, viral antigen positive cells were detected after subzonal microinjection of PRV. At this stage, the first foci of PVRL 1, also a known cell adhesion molecule, were detected. At the expanded blastocyst stage, a lining pattern of PVRL 1 in the apico-lateral border of trophectoderm cells is present, whereas the expression in the inner cell mass is low. Furthermore, PVRL 1 specific monoclonal antibody CK41 significantly blocked PRV infection of trophectoderm cells, while the infection of the inner cell mass was only partly inhibited. Preimplantation embryos up to the hatched blastocysts stage remained refractory to PRRSV infection and no expression of sialoadhesin was detected. We conclude
that the use of protease to investigate the virus embryo interaction can lead to misinterpretation of results. Results also show that blastomeres of 5-cell embryos up to the hatched blastocysts can get infected with PRV, but there is no risk of a PRRSV infection.

INTRODUCTION

Due to the recent progress in non-surgical embryo transfer (ET) in pigs (Ducro-Steverink et al., 2004) and cryopreservation of porcine embryos (Cuello et al., 2005), most obstacles for the implementation of commercial porcine ET have disappeared. It is generally accepted that the risk of pathogen transmission by ET is lower than by trading animals or semen. However, there are still some sanitary risks inherent to embryo transfers that warrant more research on embryo-pathogen interaction. Until now, most studies on embryo-virus interactions have been performed with embryos which are surrounded by the zona pellucida (ZP) (Bolin et al., 1983; Singh and Thomas, 1987; Bane et al., 1990) and the ZP is considered to be a firm physical and chemical barrier against pathogens (Van Roose et al., 2000). Whether its barrier function remains guaranteed under all circumstances is not clear. Some of the smallest viruses seem to be able to trespass the ZP in mouse (Gwatkin, 1967) and perhaps in pig embryos (Bane et al., 1990). Embryo handling in itself, or routinely used techniques such as intracytoplasmic sperm injection, blastomere biopsy and embryo cryopreservation may induce ZP damage (Van Den Abbeel and Van Steirteghem, 2000) and form ports for viral entry. Under these conditions, every virus may reach embryonic cells. For that reason, it is necessary to have better insights in the susceptibility of these cells to infection with viruses with a tropism for the reproductive tract. In pigs, two of such viruses are pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV). These viruses, originating from blood or semen, may get in contact with embryos and as such may represent a sanitary risk for the international trade in porcine embryos.
Pseudorabies virus (PRV), a member of the α-herpesvirinae causes return to oestrus, abortion or birth of death or weak piglets in sows. In vitro inoculation of ZP intact embryos with PRV (Bolin et al., 1983; Singh and Thomas, 1987) and embryo transfer experiments (Bolin et al., 1982; Medveczky et al., 1996) showed that PRV can be associated with embryos. Using electron microscopy, Bolin et al. (1983) observed that PRV particles remain limited to the outer surface of the ZP. In an in vitro PRV inoculation experiment with ZP free, 2- to 16-cell stage embryos, blastomeres were not infected 48h after inoculation. Therefore, Bolin et al. (1981) concluded that 2- to 16-cell stage embryos were refractory to PRV.

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the Arteriviridae, causes reduced conception rates, late-term abortion and an increase in dead/mummified foetuses. Incubation and microinjection of 4- to 16-cell stage embryos with PRRSV had no effect on further embryonic development and PRRSV was not detected in association with the embryos. Prieto et al. (1996) concluded that 4- to 16-cell stage embryos were not susceptible to PRRSV infection.

Insusceptibility of cells to infection is determined by several factors. Absence of one or more virus receptors in the plasma membrane disables the virus to enter the cell and is as such one of the most important resistance factors. Nectin-1 has been shown to be a major receptor for PRV. An interaction between the envelope glycoprotein gD and nectin-1 enables the virus to enter mammalian cells (Geraghty et al., 1998; Milne et al., 2001). Physiologically, nectin-1 has been characterized as a Ca\(^{2+}\)-independent immunoglobulin-like cell-cell adhesion molecule in cadherin-based adherens junctions (AJ). It is expressed in a variety of cells, most notably in epithelial cells and neurons (Geraghty et al., 1998; Takai and Nakanishi, 2003). Porcine sialoadhesin has recently been demonstrated to act as an internalization receptor for PRRSV (Vanderheijden et al., 2003). Porcine sialoadhesin is a member of the sialic acid-
binding immunoglobulin-like lectins expressed on specific subsets of macrophages (Crocker and Gordon, 1986).

The specific objectives of the present experiments were: (i) to determine the susceptibility of different stages of preimplantation embryos to PRV and PRRSV, and (ii) to relate the susceptibility with specific viral receptor expression.

MATERIALS AND METHODS

Media and reagents
Dulbecco’s PBS was obtained from GIBCO-BRL Life Technologies (Merelbeke, Belgium). Fetal calf serum (FCS) was purchased from Biochrom AG (Berlin, Germany). Propidium iodide was purchased from Molecular Probes (Leiden, The Netherlands). The other components were bought from Sigma (Bornem, Belgium). All the media were put through a sterile 0.22 µm filter (Millipore Corporation, New Bedford, USA).

Embryo collection, culture and manipulation
A total of 50 multiparous culling-sows (Rattlerow-Seghers sows, Buggenhout, Belgium) were used in the present study. The sows were superovulated using 1500 IU eCG im (Folligon®, Intervet, Boxmeer, The Netherlands) three days after weaning, followed by 1500 IU hCG im (Chorulon®, Intervet, Boxmeer, The Netherlands) 72 h later. The sows were fixed-time inseminated with boar semen of proven fertility 24 h after hCG administration. They were slaughtered either 48 h post insemination to generate 2-cell stage embryos, or 144 h after insemination to obtain hatched blastocysts. The oviducts of the sows slaughtered 48 h post insemination were flushed with 15 ml of pre-warmed Dulbecco’s PBS supplemented with 0.5% FCS. After 10 washings (Stringfellow, 1998) with NCSU-23 (Petters and Wells, 1993)
under oil, embryos were cultured in NCSU-23 at 39 °C in 5% CO\textsubscript{2} in air. Two-cell (48 h after insemination), four-cell (57 h after insemination), five- to eight-cell (77-86 h after insemination), morula (102 h after insemination) and early blastocyst (135 h after insemination) stage embryos were identified using morphological criteria (Mateusen et al, 2005). To get ZP free morulae and blastocysts, embryos were incubated in 2.5% protease (Sigma P-6911) in NCSU-23 during approximately 2 min at 37°C (Menino and Wright, 1983). In the group of the sows that were slaughtered 144 h post insemination, the proximal third of both uterine horns was clamped, removed and subsequently flushed with 150 ml of pre-warmed Dulbecco’s PBS supplemented with 0.5% FCS. After washing procedures, expanded blastocysts were cultured for 24 h in NCSU-23 with 2% FCS until reaching the hatched blastocyst stages based on morphological criteria.

**Virus preparation**

Pseudorabies strain 89V87 and PRRSV strain Lelystad Virus (LV) were used for inoculation. Pseudorabies strain (89V87) (Nauwynck and Pensaert, 1992) was passaged two times on PK-15 cells leading to a stock concentration of $10^{9.0}$ TCID\textsubscript{50} per ml. The Lelystad Virus strain was isolated from aborted fetuses (Wensvoort et al., 1991) and passaged 13 times on porcine alveolar macrophages resulting in a stock virus titer of $10^{6.9}$ TCID\textsubscript{50} per ml. To carry out the subzonal microinjections, the PRRSV stock had to be concentrated. Therefore, the viral stock was ultracentrifuged at 100,000 g at 4°C for 3 h. Then the PRRSV titer was adjusted to $10^{8.7}$ TCID\textsubscript{50} per ml, using the method of Reed and Muench (1938).

**Virus exposure**

Zona pellucida intact and ZP free preimplantation embryos were incubated with $10^{5.0}$ TCID\textsubscript{50} PRV (89V87) or PRRSV (LV) per ml for 1 h. Control embryos were incubated under the
same circumstances in NCSU-23 but without virus. Subzonal virus microinjection of pig embryos was carried out using an inverted Leica microscope at a magnification of x400, in microdrops of NCSU-23 medium covered with mineral oil in Petri dishes. Embryos were microinjected with $10^{3.0}$ TCID$_{50}$ PRV or PRRSV suspended in 1 nl of PBS. Control embryos were microinjected under the same circumstances with PBS. To validate the subzonal microinjection model, $10^{3.0}$ TCID$_{50}$ PRV or PRRSV suspended in 1 nl of PBS was injected close to cells of permissive cell lines (PK-15 cells and macrophages, respectively).

After virus exposure, all embryos were washed 10 times in NCSU23 under oil. At 0, 24, and 48 h after virus incubation, embryos were evaluated for morphology and development. Embryonic development was determined by investigating the rate of embryos attaining the next developmental stage. Impaired in vitro development was defined as embryos not reaching the next developmental stage. Forty-eight hours after incubation, embryos were collected and examined for viral antigen.

**Viral antigen expression**

The ratio of infected embryos and the ratio of viral antigen positive cells per infected embryo were determined by immunofluorescence staining. The embryos were fixed in 4% paraformaldehyde at room temperature for 1 h. After fixation, the embryos were washed 3 times in polyvinyl pyrrolidone (PVP) solution (1 mg PVP/ml PBS). The embryos were treated with 0.5% Triton X-100 (in PVP solution) for 1 h. After three 5 min washes in PVP solution, embryos and controls were incubated for 1 min at 39 °C in 10% goat serum. Next, the embryos and the controls were incubated for 1 h at 39 °C with 1:100 dilution of monoclonal antibodies against PRV (1C11), or against PRRSV (A27). After washing thoroughly in PVP solution, embryos were transferred to a 1:100 FITC-conjugated goat anti-mouse IgG for 1 h at 39 °C. Nuclear staining by propidium iodide was performed to assess the embryonic cell
number. After mounting the embryos in glycerol with 1,4-diazabicyclo (2.2.2) octane (25mg/ml), the number of fluorescent cells was counted in each embryo at a magnification of 400 x with a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany) linked to a Leica DM IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). An Argon laser was used to excite FITC (488 nm) and PI (586 nm) fluorochromes. Total embryo height was evaluated and sections were made at 3µm intervals. Analysis of the images was performed with Leica confocal software.

Distribution of nectin-1 and sialoadhesin

Staged embryos were incubated in Ca\(^{2+}\)-free medium for 30 min before fixation in PBS containing 1% paraformaldehyde. After fixation, the embryos were thoroughly washed in PVP solution and treated with 0.5% Triton X-100 (in PVP solution) for 1 h. After three 5 min washes in PVP solution; embryos were stained for 1 h with either monoclonal antibody CK41 (1:100) (provided by C. Krummenacher, G. Cohen and R. Eisenberg, Univ. of Pennsylvania), directed against nectin-1 (HveC), or with monoclonal antibody 41D3 (1:100), directed against porcine sialoadhesin (Duan et al., 1997). The isotype-matched (immunoglobulin G1) monoclonal antibody 13D12 (1:100), directed against pseudorabies virus glycoprotein gD (Nauwynck and Pensaert, 1995) was used as a control. After 3 wash steps in PVP solution, embryos were labeled with a 1:200 dilution of FITC-labeled goat-anti-mouse secondary antibody. Nuclear staining by propidium iodide was performed to assess the embryonic cell number. After mounting in glycerol with 1,4-diazabicyclo (2.2.2) octane (25mg/ml), embryos were analyzed using confocal laser scanning microscopy at a magnification of 400 x or 630 x.
PRV blocking assay using monoclonal antibody CK41

Various concentrations of CK41 were added to hatched blastocysts cultured in Dulbecco’s PBS with 2% FCS and held at 15 °C during 1 h. The isotype-matched (Ig G1) monoclonal antibody 41D3 (10 µg/ml), directed against sialoadhesin (Duan et al, 1997) was used as a control. Subsequently, embryos were incubated in NCSU23 with various concentrations of CK41 and $10^5$ TCID$_{50}$ PRV (strain 89V87) per ml for 1 h at 39 °C in 5% CO$_2$ in air. Afterwards, embryos were washed 10 times in NCSU23 under oil and cultured in NCSU23 with 2% FCS for 12 h. After fixation in 4% paraformaldehyde, viral infection was assessed by investigating viral antigen expression using immunofluorescent confocal laser scanning microscopy.

Statistical analysis

The number of infected embryos and differences in rates of development were analyzed using Chi-square analysis. Fisher’s exact tests were applied when small numbers were involved. Logistic regression was used to compare the average ratio of antigen-positive cells in infected embryos for the developmental stages and univariate analysis of variance with replicate as random variable was used to analyze nectin-1 expression and PRV infection in the PRV blocking assay. Variables were considered to be significant at a 0.05 level (two-sided). The statistical analyses were performed using SPSS (SPSS 12, SPSS Inc. Illinois 60611, USA, 2001).
RESULTS

Susceptibility of preimplantation embryos to PRV (Table 1)

*Virus exposure of ZP intact and ZP free embryos by incubation with PRV.* For all ZP intact embryos incubated with PRV, embryonic cells were negative for viral antigens after a 48 h embryo culture period, and further embryonic development was not different from negative control embryos. PRV-positive cells were also not detected after the incubation of PRV with preimplantation embryos that were made ZP free using 2.5% protease before virus incubation. Incubation of hatched blastocysts with PRV led to an infection of the embryonic cells and had a detrimental effect on further embryonic development.

*Virus exposure of ZP intact embryos by subzonal microinjection of PRV (Table 1, Figure 1).*

Embryos up to the 4 cell stage were negative for viral antigen 48 h post microinjection. Zona pellucida intact 5-8 cell embryos, morulae and blastocysts were infected 48 h after subzonal microinjection with PRV and their further embryonic development was significantly lower compared to that of control embryos injected with PBS.
Table 1. Effects of Pseudorabies virus (PRV (89V87)) exposure on preimplantation embryos assessed 48 h after virus exposure.

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Treatment</th>
<th>Virus exposure</th>
<th>Number of embryos</th>
<th>Number of infected embryos (%)</th>
<th>Average ratio of antigen positive cells</th>
<th>Impaired in vitro development, n (%)</th>
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<tr>
<td>2- to 4-cell stage embryos</td>
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<td>None</td>
<td>30</td>
<td>0 (0)</td>
<td>0/4.7</td>
<td>5 (16.67)</td>
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<tr>
<td></td>
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</tr>
<tr>
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</tr>
<tr>
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<td>0 (0)</td>
<td>0/4.4</td>
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<td>5- to 8-cell stage embryos</td>
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<td>0/107.9</td>
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<td>34* (85)</td>
<td>90.7/90.7*</td>
<td>37* (92.5)</td>
</tr>
</tbody>
</table>

( )Data marked with an asterisk differ significantly with from data in the same column without an asterisk (P<0.05)
Figure 1.

Detection of pseudorabies viral antigens by indirect immunofluorescence in preimplantation stage embryos 48 h after subzonal microinjection with $10^{3.0} \text{TCID}_{50}$ PRV (89V87) (A, B, C, D, E), and 48 h after incubation of hatched blastocysts with $10^{5.0} \text{TCID}_{50}$ PRV per ml (F). Viral glycoproteins in green and nuclei in red.

A: PRV microinjected as a 2-cell embryo; B: PRV microinjected as a 4-cell embryo; C: PRV microinjected as a 8-cell embryo; D: PRV microinjected as a young morula (9 cells); E: PRV microinjected as a young blastocyst (22 cells); F: PRV incubation as a hatched blastocyst (92 cells).
The incubation or microinjection of ZP intact and ZP free preimplantation embryos with PRRSV did not lead to virus infection, nor was there any effect on further embryonic development.

Table 2. Effects of porcine reproductive and respiratory syndrome virus (PRRSV (LV)) exposure on preimplantation embryos, assessed 48 h after virus exposure.

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Treatment</th>
<th>Virus exposure</th>
<th>Number of embryos</th>
<th>Number of infected embryos (%)</th>
<th>Average ratio of antigen positive cells</th>
<th>Impaired in vitro development, n (%)</th>
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<tr>
<td>2- to 4-cell stage embryos</td>
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<tr>
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<td>0/4.4</td>
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<td>0/121.2</td>
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</table>
Presence and distribution of nectin-1 and sialoadhesin in preimplantation embryos (Figure 2 and 3)

To investigate whether the susceptibility/resistance of embryos to a viral infection was consistent with the presence/absence of virus receptors, immunofluorescent stainings were performed to visualize receptors for PRV (nectin-1) and PRRSV (sialoadhesin). In ZP intact embryos, the ZP always showed foci of intense nectin-1 staining (arrowhead in Figure 2, A). The first distinct nectin-1 staining of embryonic cells occurred in the pre-compact 5- to 8-cell stages of development. The nectin-1 expression was diffusely distributed throughout the cells, being more concentrated in a scattered pattern of punctuate foci on the cell membrane (arrowheads in Figure 2, B'''' and C''''', which represent enlargements of the boxed areas in Figures 2, B” and C”') up to the young blastocyst stages. However, in addition to the diffuse cytoplasmic signal, the expanded and hatched blastocyst stages displayed a clear lining pattern of nectin-1 staining (arrowheads in Figure 2, D’’’’ and E’’’’, which represent enlargements of the boxed areas in Figures 2, D” and E”’) at the apico-lateral border of some trophectoderm cells (arrow in Figure 3, A’’’). This lining pattern, localized at presumed sites of tight junctions (TJ), was expressed in mural (adjacent to the blastocoelic cavity) trophectoderm cells in expanded blastocyst (Figure 2, D’’’), and in mural and polar (adjacent to the ICM) trophectoderm cells in hatched blastocyst (Figure 3). Only weak signals were detected at contact sites within the inner cell mass (ICM) (Figure 3). Having demonstrated that protease treatment of morulae and blastocysts led to a refractory state for PRV infection, the nectin-1 expression in protease treated embryos was also determined and no positive signals could be detected (Figure 2, F’’’’).

Sialoadhesin staining was never observed in preimplantation embryos up to the hatched blastocyst stage (data not shown, n=20 per stage of development in three replicates). Also, the
isotype-matched 13D12 staining, which was used as a negative control, did not give a signal in the preimplantation stage embryos.
Representative confocal laser scanning images of nectin-1 expression in porcine preimplantation embryos. (A-F) Immunofluorescent images of embryos stained with CK41 (Mab against nectin-1, green). (A’-F’) Immunofluorescent images of embryos stained with propidium iodide (red). (A’’-F’’) Overlay images of embryos stained with CK41 (green) and propidium iodide (red). (A’’’-F’’’) Enlargements of the box areas in A’’-F’’. (A-A’’) 2-cell
embryo; (B-B’’) 6-cell embryo; (C-C’’) compacted morula; (D-D’’) expanded blastocyst; (E-E’’) hatched blastocyst; (F-F’’) pronase (2.5%) treated blastocyst.

Magnification = 400x; bar = 20µm.

Figure 3.

Detailed representative confocal laser scanning images of nectin-1 expression in a porcine hatched blastocyst. (A and B) Immunofluorescent images of embryonic cells stained with CK41 (Mab against nectin-1, green). (A’ and B’) Immunofluorescent images of embryonic cells stained with propidium iodide (red). (A’’ and B’’’) Overlay images of embryonic cells stained with CK41 (green) and propidium iodide (red). (A-A’’) Detail images of inner cell mass cells and surrounding trophectoderm cells. (B-B’’) Detail images of trophectoderm cells. Magnification = 630x; bar = 20µm. ICM: inner cell mass cell; Tr: trophectoderm cell
PRV blocking assay using monoclonal antibody CK41 (Figure 4 and 5)

To examine if PRV uses nectin-1 to infect embryonic cells, a blocking assay was performed in hatched blastocysts using monoclonal antibody (MAb) CK41 in different concentrations. MAb CK 41 reduced PRV infection in the trophectoderm cells clearly in a dose-dependent manner; up to 98% at a concentration of 10 µg/ml. Also, CK41 addition resulted in a trend of reduced PRV infection in the ICM cells but the differences between the different concentrations were not significant. Addition of the isotype matched control monoclonal antibody 41D3 at a concentration of 10 µg/ml had no effect on PRV infection.
Figure 4.

Representative confocal laser scanning images of PRV infected hatched blastocysts after a nectin-1 mediated PRV blocking assay with anti-nectin-1 Mab CK41. (A-E)

Immunofluorescent images of viral glycoprotein (green) distribution in embryos. (A’-E’)

Immunofluorescent images of embryos stained with propidium iodide (red). (A’’-E’’)

Overlay images of embryos stained for viral glycoprotein (green) and propidium iodide (red).
(A-A’’) embryos preincubated with 0µg/ml CK41; (B-B’’) embryos preincubated with 0.1µg/ml CK41; (C-C’’) embryos preincubated with 1µg/ml CK41; (D-D’’) embryos preincubated with 10µg/ml CK41; (E-E’’) negative control embryo.

Magnification = 400x; bar = 20µm.

Figure 5. Blocking results of nectin-1 mediated PRV infection in hatched blastocysts with anti-nectin-1 Mab CK41. Embryos were incubated for 1 h at 15°C in different concentrations of Mab CK41. Subsequently, embryos were incubated for 1 h with $10^5$ TCID$_{50}$ PRV per ml NCSU23. After a 12 h culture period in NCSU23, the average ratio (%) of PRV-positive cells in trophectoderm (dark grey bars) and in inner cell mass (light grey bars) was determined by confocal microscopy. A significant difference (P<0.05) was detected between bars with different superscript (abc) (n=5 per concentration of Mab CK41 in 3 replicates).
DISCUSSION

In the present study it has been shown that embryonic cells are refractory to PRV infection during the first cleavage stages. Starting from the 5-cell stage, they become susceptible. This coincides with the cellular expression of a known PRV entry receptor, nectin-1. All preimplantation stages up to the hatched blastocyst remain resistant to PRRSV. The absence of sialoadhesin, the internalization receptor for PRRSV, is likely to be of major importance for this refractory state of embryos.

In analogy with the results of Bolin et al. (1981), our data indicate that the porcine ZP forms a protective barrier against PRV infection. Pseudorabies virus positive embryonic cells were never detected in ZP-intact embryos incubated with PRV, whereas subzonal microinjection in embryos starting from the 5-cell stage led to a PRV infection. Important to mention here, were the differences in the results of the microinjection experiments compared to the results obtained by PRV incubation of ZP-free embryos after protease treatment. In one way or another, protease treatment made blastomeres refractory to a PRV infection. This likely explains why Bolin et al. (1981), who also used protease to remove the ZP from pig embryos, found that porcine embryonic cells up to the 16-cell stage were refractory to a PRV infection. In the present study, an explanation was found for this contradiction. It was shown that protease treatment caused a disruption of the dimensional structure of nectin-1, since the conformation-dependent MAb CK41 (Krummenacher et al., 2000) was not able to bind to the V-like ectodomain of nectin-1. The finding that enzymes such as proteases have an effect on viral receptors has its repercussion on previous data regarding virus susceptibility of embryonic cells. The present results of the subzonal microinjection experiments indicate that this technique is the method of choice to circumvent the use of protease for investigating the interaction between pathogens and embryonic cells.
Based on the results of the in vitro embryo-PRRSV experiments we can conclude that preimplantation stages of development up to the hatched blastocyst stage are refractory to a PRRSV infection. Because it has been shown that a transplacental infection of embryos can be established at a 20 day pregnancy (Prieto et al., 1996), further research is needed to investigate at which developmental stage pig embryos become susceptible to a PRRSV infection.

Nectin-1 is a known entry receptor for α-herpes viruses including herpes simplex viruses type 1 and 2, bovine herpes virus type 1 and PRV (Geraghty et al, 1998) and it is also a molecule that plays an important role in cell adhesion and in a variety of cell-cell junctions such as AJ and TJ (Sakisaka and Takai, 2004). These AJ and TJ only become fully functional at embryo compaction (Watson et al., 2004; Fleming et al., 2000). In a mouse embryo model, the expression of nectin-2 was undetectable before compaction (Thomas et al., 2004). However, in the present study the appearance of nectin-1 expression coincided with the development of pre-compact 5- to 8-cell stage embryos. This latter finding is in agreement with data from murine embryos where other AJ components such as E-cadherin, α- and β-catenins and vezatin are already present in early pre-compaction stages of development (Hyenne et al., 2005). The nectin-1 distribution in blastomeres is also similar as described for nectin-2 and these other AJ components, namely diffusely distributed in the blastomeres, being enriched at cell-cell contact sites. At the expanded blastocyst stage, the first continuous staining of nectin-1 in the cell membrane was detected in the apico-lateral border of mural trophectoderm cells (Figure 2, D’’). This nectin-1 expression may contribute to the formation of the tight junction seal surrounding the blastocoel cavity that controls paracellular movement of water and by this also controls the expansion of the blastocyst (Watson and Barcroft, 2001). In the hatched blastocyst stage, the nectin-1 expression was continuous in all (mural and polar) trophectoderm cells (Figure 2, E’’). This might be related to a higher need of cell adhesion in
hatched embryos to keep embryonic cells together without ZP in processes such as the elongation, spacing and migration. Nectin-1 expression in the undifferentiated cells of the ICM of blastocysts was low. The first tissue to differentiate during mammalian development is the trophectoderm epithelium and since nectin-1 is predominantly expressed in tissues of epithelial origin, it makes sense that expression is situated in the trophectoderm rather than in the undifferentiated cells of the ICM. Moreover, PRV entry in trophectoderm cells could significantly be blocked (up to 98%) in a dose-dependent way by MAb CK41, whereas the PRV infection of ICM cells could only partially (block up to 47%) be prevented by adding MAb CK41. The finding that the ICM cells that are enclosed by trophectoderm cells could be infected with PRV also forms an indirect proof for the existence of substantial gaps in the polar trophectoderm cells in 8 day embryos described by Barends et al. (1989). PRV blocking assays with CK41 in other cell types also showed variable results. In a CHO K1-derived cell line that stably expresses porcine nectin-1, PRV entry was blocked, whereas entry of PRV in PK15 cells was unaffected by CK41 (Milne et al., 2001). A possible explanation for this difference in blocking capability of CK41 may be that PK15 and ICM cells express other PRV entry receptors, whereas trophectoderm and CHO K1-derived cells do not express those receptors. Up to now, 5 α-herpesvirus receptors have been identified: namely HveA (HVEM) which has no entry activity for PRV, nectin-1, nectin-2, HveD (CD155) and 3-O-sulfated heparan sulfate (Montgomery et al., 1996; Cocchi et al., 1998; Warner et al., 1998; Shukla et al, 1999). From these entry receptors, the expression of nectin-2 in the ICM has already been described in a murine embryo model (Thomas et al., 2004). Further research will show whether nectin-2 or possibly another receptor, may be responsible for the nectin-1 independent way of PRV entry in porcine ICM cells.

In the present study, an innovative way of investigating virus-embryo interaction was applied, linking the results of in vitro virus exposure with virus receptor expression. Based on the
results, we can conclude that the use of proteases to remove the ZP and establish contact between blastomeres and viruses can affect virus receptor expression and may lead to wrong conclusions. The findings of the virus incubation experiments confirm the hypothesis that an intact ZP forms a sufficient barrier against most pathogens. Exposure of embryonic cells to PRV revealed that blastomeres from the 5-cell stage onwards are susceptible to a PRV infection. Furthermore, the expression patterns of nectin-1 in blastomeres of preimplantation embryos were in accordance with this finding. Based on the results of the PRRSV exposure and sialoadhesin expression of embryos we can conclude that blastomeres of preimplantation embryos up to the hatched blastocyst stage are refractory to a PRRSV infection. By looking into the distribution of nectin-1 in expanded and hatched blastocyst, a difference in the expression of nectin-1 in the trophectoderm and ICM cells was detected. Also, the results of the blocking assay using MAb CK41 suggest that ICM cells can use a different PRV entry receptor than nectin-1. Further research will focus on the effects this difference can have on virus replication in embryonic cells.

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CHAPTER 5

PORCINE CIRCOVIRUS TYPE 2

DURING EARLY PREGNANCY
EFFECT OF A PORCINE CIRCOVIRUS TYPE 2 INFECTION ON EMBRYOS DURING EARLY PREGNANCY

ABSTRACT

The aim of the present study was to assess the effects of porcine circovirus type 2 (PCV2) on porcine embryos and their recipient sows during the first 21 days of pregnancy. Hatched blastocysts exposed to $10^{5.0} \text{TCID}_{50}$ PCV2 per ml (strain 1121, fifth passage PK15) and negative control embryos were transferred to PCV2-immune sows at the 7th day of the cycle. Two weeks after transfer (D21), the recipient sows were euthanized and embryos were recovered. They were assessed macroscopically for viability and examined for viral antigen-positive cells by immunoperoxidase staining. The embryonic survival rate of the PCV2-exposed embryos (6.4%, 7 viable embryos out of 110 transferred) was significantly lower than the survival rate of the negative control embryos (65.4%, 34 viable embryos out of 52 transferred). All of the non-viable PCV2-exposed embryos (n=9) displayed immunohistochemical positive signals for PCV2-antigen in degenerated tissues. In the PCV2-exposed embryos that were categorized as viable at D21, small clusters (n=4) or no PCV2-positive cells (n=3) were detected. The pregnancy results of the recipient sows that received PCV2-exposed embryos (1/5) were considerably different from the negative control recipients (2/2), with 3 out of five sows displaying a regular return to oestrus.

In conclusion, it can be stated that PCV2 can replicate in embryos and might lead to embryonic death. In a small proportion of embryos, PCV2 exposure does not have a detrimental effect on embryo development before D21.
INTRODUCTION

Porcine circovirus type 2 (PCV2) is a widespread, circular, single-stranded DNA virus, recognized as the causal agent of Post-Weaning Multisystemic Wasting Syndrome (PMWS) (Allan and Ellis, 2000). This syndrome, associated with substantial economic losses in the pig industry worldwide, is clinically characterized by wasting, paleness of the skin and increased mortality in weaned pigs. PCV2 infection of pregnant sows can also cause fetal death leading to mummification or late-term abortion. Several field reports have described the detection of PCV2-antigens mainly in heart lesions of aborted or mummified fetuses from swine herds that were recently primary infected with PCV2 (West et al., 1999; Ladekjær-Mikkelsen et al., 2001; O’Connor et al., 2001; Farnham et al., 2003). Intra-fetal inoculation experiments with PCV2 in different fetal stages of development confirmed its pathogenic capacity in fetal tissues (Sanchez et al., 2001; Sanchez et al., 2003; Yoon et al., 2004). Recently, transplacental spread of PCV2 leading to abortion was also experimentally induced after intranasal inoculation of sows serologically negative for PCV2 at 92 days of gestation (Park et al., 2005).

In general, next to transplacental spread after viraemia, viral contamination of female or male gametes can also induce a vertical transmission of virus. For PCV2, using nested PCR assays, viral DNA was demonstrated in 11% of the cumulus oocyte complexes of sows with PCV2-antibodies (Bielanski et al., 2004) and intermittently in semen samples of experimentally and naturally infected boars (Larochelle et al., 2000; Kim et al., 2003; McIntosh et al., 2006. Research on the infectivity of these contaminated gametes is limited. In an experimental model, mimicking the effects of using PCV2-contaminated semen, Cariolet et al. (2002) were able to cause a vertical transmission of PCV2 after intra-uterine insemination of virus-spiked semen. This resulted in virus replication in fetal stages of development leading to fetal death.
and mummification. Previous work of our group has shown that the zona pellucida forms a barrier against PCV2 infection. Post hatching, PCV2 can infect porcine blastomeres after in vitro virus incubation. However, no influence on in vitro embryo development and no degenerative processes could be detected during a 48 h period following virus inoculation (Mateusen et al., 2004). This raises concerns for the risk of virus transmission through embryo transfer since morphological criteria that are used to select transferable porcine embryos cannot distinguish PCV2-infected from PCV2-free morulae and blastocysts. To date, it is unknown which effects PCV2 infection of embryos may have further on during pregnancy. The aims of this study were to investigate the effect of transferring PCV2-exposed porcine embryos on (i) viability and extent of infection of the embryos at 14 days after transfer and, (ii) pregnancy results of sows.
MATERIALS AND METHODS

Collection of in vivo derived porcine embryos

A total of 25 multiparous (parity 2 to 5), PCV2-infection immune sows (Rattlerow-Seghers sows) were used in the present study. The sows were superovulated using PMSG (Folligon® 1500 IU im) three days after weaning, followed by hCG (Chorulon® 1500 IU im) 72 h later. The sows were fixed-time inseminated with boar semen of Piétrain boars of proven fertility, 24 h after hCG administration and slaughtered 6 days post insemination. The reproductive tracts were removed and transported to the laboratory in a pre-warmed box (39°C) within 30 min after slaughter. The uterus was flushed with 500 ml of Dulbecco’s PBS supplemented with 2% foetal bovine serum (FBS). After 10 washings (Stringfellow, 1998), blastocysts (n=235) were cultured in NCSU-23 (Petters and Wells, 1993) supplemented with 2% foetal bovine serum (FBS) at 39°C in 5% CO₂ in air for 24 h until they reached the hatched blastocyst stage (n=212).

Porcine circovirus type 2 (PCV2) exposure

PCV2 strain 1121 was used for inoculation. It was originally obtained from a pool of lungs, liver and spleen of dead fetuses/piglets at partum (West et al., 1999) and further passaged five times in PK-15 cells. The hatched blastocysts were placed in 1 ml of minimum essential medium (MEM), containing 10⁵.⁰ tissue culture infectious doses 50% end point (TCID₅₀) and incubated for 1 h at 39 °C in an atmosphere of 5% CO₂ in air. Negative control embryos were incubated under the same circumstances in MEM without PCV2. After 1 h of exposure, all embryos were washed 10 times in NCSU-23 medium and divided at random in groups of approximately 23 embryos which were put into straws. As positive controls, embryos incubated with PCV2 were cultured at 39°C in 5% CO₂ in air for 48 h, fixed in 4%
paraformaldehyde and examined for PCV2-antigens using indirect immunofluorescence (IIF) (Mateusen et al., 2004).

**Experimental design**

Hatched blastocysts (n=182) were surgically transferred as described by Hancock and Howell (1962) on the 7th day of the cycle to a total of 8 synchronised PCV2-infection immune sows. Donor and recipient sows had been vaccinated against porcine parvovirus, *Erisipelothrix rhusiopathiae* and pseudorabies virus. On average twenty-three embryos were transferred per sow. Five recipient sows received hatched blastocysts that were PCV2-exposed, whereas 3 control sows received negative control embryos. After the embryo transfer, sows were clinically evaluated (general condition, rectal temperature and appetite) on a daily basis for 14 consecutive days after which the sows were euthanized and their genital tracts examined. Uteri were opened and embryos were collected and scored macroscopically. Embryonic remnants lacking the normal morphologic structure of a porcine embryo 21 days after insemination were categorized as non-viable. A sow was defined as being pregnant when viable embryos were recovered from the uterus. An embryonic survival rate of a sow was defined as the number of viable embryos collected at D21 divided by the number of transferred embryos at D7. Serum samples from recipient sows were taken the day of the embryo transfer and 14 days later and tested for antibodies against PCV2 using an immunoperoxidase monolayer assay (IPMA) as described by Labarque et al. (2000).

**Detection of viral antigen expression in day 21 embryos**

Embryos were fixed in 4% paraformaldehyde, embedded in paraffin and sections were made with intervals of 15 µm. Immunoperoxidase staining was performed to detect PCV2-positive cells. Slides were suspended in a sodium citrate buffer solution before being placed in a
microwave of 800 Watt for antigen retrieval. After being rehydrated in ethanol solutions (100%, 95%, 70% and 50%) slides were washed with a TRIS buffer solution. Subsequently, slides were incubated in a TRIS buffer with sodium azide (10%) and hydrogen peroxide (0.5%) to block the endogenous peroxidase activity. Following three 5 min washes in a TRIS buffer, slides were incubated for 1 h at 39 °C with a 1:100 dilution of PCV2 monoclonal antibody (F190) (McNeilly et al., 2001). Monoclonal antibody 13D12, directed against pseudorabies virus protein gD (Nauwynck and Pensaert, 1992) was used as a control. Afterwards, slides were washed with TRIS, incubated for 1 h with biotinylated goat-anti-mouse secondary antibodies (Dako, Denmark) and washed again with TRIS. Next, the slides were incubated for half an hour with streptavidin-biotin HRP solution (Dako, Denmark). The reaction was visualized with 3,3'-diaminobenzidine (Dako Liquid DAB plus, Dako, Denmark). A haematoxylin-eosin staining was performed to identify organs and degenerative processes. After mounting the embryos in glycerol with 1,4-diazabicyclo (2.2.2) octane (25mg/ml), embryonic structures and organs were evaluated for antigen-positive signals using a Leica DM/RBE fluorescence microscope at a magnification of 400 x and 600 x.

Statistical analysis

Antibody titres against PCV2 of the recipient sows were compared between the two groups of sows at the day of ET (D7) and 14 days after ET (D21) using a Kruskal-Wallis test. Differences in pregnancy results and the ratio of viable embryos between the two groups of sows was analyzed using Chi-square analysis. Fisher’s exact tests were applied when small numbers were involved. Variables were considered to be significant at a 0.05 level (two-sided). The statistical analyses were performed using SPSS (SPSS 14, SPSS Inc. Illinois 60611, USA, 2006).
Chapter 5: Porcine circovirus type 2 infection during early pregnancy

RESULTS

Clinical monitoring and antibody titers against PCV2 of recipient sows

None of the recipient sows developed clinical signs in the two-week period following the embryo transfer. One sow belonging to the control group was injured the day of embryo transfer and became lame. For that reason, she was taken out of the experiment. The antibody titres against PCV2 the day of ET and 14 days later were similar for negative control sows and sows that had received PCV2-exposed embryos. Also, none of the sows showed any rise in antibody titres against PCV2, 14 days after ET.

Pregnancy results after embryo transfer

The two sows that received negative control embryos were pregnant 14 days after ET (A and B). They contained viable embryos and had active corpora lutea. In two out of the 5 sows that received infected embryos, embryos and corpora lutea were detected (C and D). However, the embryos of sow D were not viable and therefore the sow was designated as not pregnant. The remaining 3 sows that had received PCV2-exposed embryos were not pregnant (E, F and G). Embryos were not found and the sows had follicles of 6-10 mm in diameter without any corpora lutea on the ovaries. These sows also displayed early oestrous symptoms 14 days after ET.
Macroscopic scoring of embryos 14 days after ET (Table 1)

A significantly lower embryonic survival rate was detected in sows that had received PCV2-exposed embryos (6.4%, 7/110) compared to the survival rate in the negative control sows (65.4%, 34/52) (P<0.05).

Table 1. Results of embryo transfers using negative control or PCV2-exposed embryos.

<table>
<thead>
<tr>
<th>Sow</th>
<th>Transferred embryos</th>
<th>Number of transferred embryos at D7</th>
<th>Number (%) of recovered embryos at D21</th>
<th>Number (%) of viable embryos at D21</th>
<th>Number (%) of non-viable embryos at D21</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>Negative control</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>Negative control</td>
<td>26</td>
<td>19 (73)</td>
<td>19 (73)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C</td>
<td>Negative control</td>
<td>26</td>
<td>17 (65)</td>
<td>15 (58)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>D</td>
<td>PCV2-exposed</td>
<td>20</td>
<td>13 (65)</td>
<td>7 (35)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>E</td>
<td>PCV2-exposed</td>
<td>23</td>
<td>3 (13)</td>
<td>0 (0)</td>
<td>3 (13)</td>
</tr>
<tr>
<td>F</td>
<td>PCV2-exposed</td>
<td>20</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>G</td>
<td>PCV2-exposed</td>
<td>18</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>H</td>
<td>PCV2-exposed</td>
<td>29</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Sow A* was taken out of the experiment because she showed severe lameness the day after ET.

Histological and immunohistochemical assessment of embryos

All 30 positive control embryos that were examined by IIF 48 h after PCV2 incubation had PCV2-positive cells (on average 5.3% per embryo). None of the embryos collected from negative control sows showed PCV2-positive signals. It was impossible to distinguish different organs in the non-viable embryos since they were highly degenerated, but PCV2 antigens were detected in tissues of all non-viable embryos collected from sows that had received PCV2-exposed embryos. In 4 out of the 7 viable embryos recovered from sows that received PCV2-positive embryos, individual or small clusters of PCV2-positive cells were detected in the placenta (2/4), the mesonephros (2/4, Fig. 1), the neural tube and the liver (2/4). In the remaining 3 viable embryos, PCV2-positive signals were not detected.
Figure 1. Detection of porcine circovirus type 2 (PCV2) viral antigens by immunoperoxidase staining (brown) in a mesonephros cell of a D21 porcine embryo. Magnification = 600x; bar = 25µm.
DISCUSSION

A number of studies have already used the porcine ET model to gain more insight in the effect of viruses on embryos and recipient sows (Wrathall and Mengeling, 1979a; Bolin et al., 1982). In the present study, the virological and clinical outcome of transferring PCV2-exposed embryos was investigated on embryo and sow level. Results showed that a PCV2 infection of embryos can lead to embryonic death and subsequent pregnancy loss.

The embryonic survival rate of PCV2-exposed embryos (6.4%) was significantly lower than that of the negative control embryos (65.4%) which was comparable to common survival rates (60%) after porcine surgical ET (Youngs, 2001). In 3 out of the 5 sows receiving PCV2-exposed embryos, major embryonic losses must have occurred before the 12th day of gestation because these 3 sows experienced a regular return to oestrus. Sows having less than 4 viable embryos in the uterus at the 12th day of the cycle will not maintain pregnancy and can return to oestrus around D21 (Polge et al., 1966). Since preimplantation embryos before 14 days of age are totally resorbed within 5 days after embryonic death (Christianson, 1992), it was impossible to detect or analyse any embryonic remnants of embryos that died before the 12th day of the cycle at the end of the experiment (D21). Although hard conclusions concerning the cause of these early embryonic deaths cannot be drawn, it is expected that PCV2 infection was involved.

In the 2 remaining sows that received PCV2-exposed embryos and did not show a regular return to oestrus, a total of 9 non-viable, degenerated embryos were found. In one of these sows, also 7 viable embryos were collected. In all of the non-viable embryonic remnants, clusters of PCV2-positive cells were detected in degenerated tissues. In 4 out of the 7 viable embryos, individual or small foci of PCV2-positive cells without any gross histological lesions were detected in the placenta, the mesonephros, the neural tube and the liver. We
could not detect any PCV2-positive signal in the 3 remaining viable embryos. There seems to be an individual difference in susceptibility of embryos for a PCV2 infection since in a small proportion of the embryos only minimal amounts or no antigen-positive cells were detected 14 days after virus inoculation. The embryos that were transferred to a recipient originated from different donor sows as they were randomly allocated to the recipients. This individual different susceptibility for PCV2 infection has already been described in foetuses and piglets by several authors (Sanchez et al., 2003; Ladekjær-Mikkelsen et al., 2002). Another possible explanation for the fact that 3 embryos became negative for viral antigens 14 days after transfer may be related to apoptosis, the process that embryos use during mammalian preimplantation development to eliminate unwanted or damaged cells (Hardy and Stark, 2002). Since 48 hours after the virus inoculation only a small proportion of the blastomeres (on average 6%) of the hatched blastocysts were infected (Mateusen et al., 2004), it is possible that through a confined apoptotical process this limited number of infected cells were expelled without any effect on further embryonic development (Mateusen et al., 2005). Further research will focus on possible apoptotic effects PCV2 will have on embryos. The finding that 4 embryos had PCV2-positive cells in specific organs without producing excessive damage raises the question whether a persistent PCV2-infection of embryos can occur. In the present study, embryos were analyzed only 14 days after inoculation which means that the time period was too short to really define the embryos as being persistently infected. A persistent viral infection may explain how PCV2 can cover the time period between the moment of intra-uterine inoculation at insemination and the start of pathology in several foetal stages of development as described by Cariolet et al. (2002). In this case, the factor that would trigger the increase in replication of PCV2 still remains to be identified.

The organ distribution of PCV2-positive cells in the viable embryos was different from that in foetuses and neonates where the heart and lymphoid tissues, respectively, were the main
target organs (Sanchez et al., 2003). It is known that in cell cultures, PCV2 requires actively
dividing cells for replication (Allan et al., 1995). Developing embryonic stages are full of
mitotic activity, but only a few organs had PCV2-positive cells. This means that in embryos,
next to the mitotic factor also other factors play a role in the replication of PCV2.
In the present study, immune recipient sows were used. They had high virus-antibody titres at
the moment of embryo transfer. None of the recipient sows in the study showed clinical
symptoms or seroconverted against PCV2 in the 14 days after ET. This suggests that the virus
that was replicating in the embryos did not replicate in maternal tissues. This is in accordance
with serological and clinical results after intra-uterine or intra-fetal inoculation of PCV2
where a serological response was never detected until 4 weeks after inoculation and where no
clinical symptoms apart from fetal death and mummification were observed (Sanchez et al.,
2001; Cariolet et al., 2002). The pregnancy result after ET with PCV2-exposed embryos (1/5)
was considerably lower than the ET result using negative control embryos (2/2). The
difference was not statistically significant due to the small numbers of recipient sows
involved.
The results of the present study show that transferring PCV2-exposed embryos to PCV2-
erseropositive recipient sows affects pregnancy outcome. The replication of PCV2 in embryos
before the 21st day of pregnancy leads to embryonic death in most embryos. However, in
some embryos no or only a limited number of PCV2-positive cells were detected 14 days
after inoculation.

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CHAPTER 6

GENERAL DISCUSSION
CHAPTER 6: GENERAL DISCUSSION

Background

The swine industry of today is dominated by a limited number of international pig breeding organizations that are constantly trying to maximize and uniformize the genetic potential of their sows. Their high value breeding animals are produced worldwide in only a limited number of nucleus herds. In order to introduce new genetics in these nucleus herds, the use of ET is generally considered as most appropriate since the risk of disease transmission is lower compared to purchase of animals or transfer of semen (Thibier, 2006). Because of this increased demand of the swine industry for ET and the recent major progress in non-surgical ET and embryo cryopreservation techniques, porcine ET will likely be used more frequently in the future. Two key elements for the success of future porcine ET were investigated in this thesis. Firstly, parameters for assessing the quality of porcine embryos were defined based on invasive and non-invasive markers of embryo viability. Secondly, more knowledge concerning the risk of pathogen transmission through porcine embryos was obtained by investigating the interaction between embryos and three porcine viral pathogens with a tropism for the reproductive tract (PCV2, PRV and PRRSV).

The experiments in this thesis were conducted not only to facilitate the efficiency and safety of porcine ET, but they were also useful for basic scientific reasons. With the increased use of pigs as a model for research such as in vitro fertilisation (IVF), cloning and transgenesis, a comparative ‘gold’ standard for porcine embryo development needed to be established. We therefore decided to culture in vivo-derived porcine embryos in vitro in order to describe the “physiological” timing of pig development. This approach is best suited since evaluation of embryo development in vivo is impossible within an individual sow with the currently
available methods. When *in vivo* derived porcine zygotes or early cleavage stage embryos (which have been matured and fertilized under ideal *in vivo* circumstances) are subsequently being cultured in identical conditions as those used for embryos produced *in vitro* or “treated” in some way (e.g. pig embryos derived from IVF, nucleus transfer or virus inoculated embryos), we assume that any differences in timing of embryo development or in embryo morphology have been caused by embryonic origin or by the treatment imposed on the embryo. By establishing a timetable of the first week of porcine preimplantation embryo development, comparisons between studies of *in vitro* embryo development can be made more easily. Moreover, since commercial porcine embryo transfers are being performed with *in vivo*-derived embryos, establishment of reliable non-invasive embryo quality parameters is essential. In general, mammalian embryo quality assessment is mainly based on the evaluation of cleavage rate and embryo morphology (Alikani et al., 2002). Published information on morphological porcine embryo quality assessment was rather limited in early 2003 when I started my investigations.
Assessing the quality of porcine embryos

In the present thesis, a time-lapse cinematography has been applied to predict developmental competence of embryos based on early cleavage of porcine *in vivo*-derived embryos cultured *in vitro*. Although a variety of environmental and genetic factors can affect embryo cleavage rate, it has been shown for several mammalian species that timing of particular events during preimplantation development is important for sustained development (Van Soom et al., 1992; Grisart et al., 1994; Holm et al., 1998; Lonergan et al., 1999; Racowsky et al., 2000). Appropriate time-windows for these events can be used as non-invasive indicators of the developmental potential of embryos. For the porcine embryo culture model used, an important reference time point for developmental capacity was the attainment of at least the 5-cell stage before 77 hours post insemination. Embryos that completed this event in a timely manner almost had a tenfold greater chance of attaining the early blastocyst stage compared to slower developing embryos. The exact reason why faster developing embryos are more capable of developing is not known, but they are probably better equipped with transcripts necessary for early embryo development. In cattle, higher levels of histone H2A (Dode et al., 2006) and histone H3A (Fair et al., 2004) have been detected in fast cleaving embryos, which is logical because histones are needed to replace the protamines of the sperm nucleus after fertilization and to assemble embryonic DNA into chromatin for the first cleavages. Other transcripts, which were differentially expressed in fast cleavers, were stem-loop binding protein (SLBP), which is involved in histone stabilisation (Donnison and Pfeffer, 2004), and isocitrate dehydrogenase (IDH), which modulates oxidative damage (Dode et al., 2006).

Under *in vivo* circumstances, it is critical that the rate of the embryo development is synchronized with the changes in the uterus in order to sustain pregnancy (Wilmut and Sales,
1981; Brownell and Warner, 1988). In our study, the formation of a blastocoel cavity was considered as the main indicator of developmental competence of a given embryo. Although blastocoel formation is generally accepted as the best non-invasive morphological embryo quality end-point for *in vitro* development (Watson, 1992), embryonic viability can *strictu sensu* only be assessed after ET (Bavister, 1995). However, ET is laborious, expensive and it is difficult to perform on a large scale for experimental purposes.

The time-lapse cinematography used in our study also enabled us to capture all morphological characteristics of embryos during cleavage such as asynchrony between blastomere cleavage, extrusion of blastomeres, and embryonic fragmentation. We could not detect any negative effect of asynchronous cleavage or blastomere extrusion on *in vitro* developmental potential although in human embryology, it clearly affects pregnancy and implantation rate (Hardarson et al., 2001). In order to investigate such effects on the viability of porcine embryos, ET experiments are necessary.

Next to setting reference points for the timing of development of porcine embryos, porcine embryo fragmentation was investigated in relation to embryo development and apoptosis. Embryonic fragmentation is a defect that is frequently observed in *in vitro*- and *in vivo*-derived mammalian embryos. Although it is generally considered as a negative embryo quality characteristic (Lindner and Wright, 1983; Puissant et al., 1987), in human embryology the degree (Ebner et al., 2001) and specific spatial patterns of fragmentation rather than the occurrence of fragmentation *per se* seem to be more closely related to developmental competence (Antczak and Van Blerkom, 1999; Alikani et al., 1999). In line with these results, our study showed that the degree (as a percentage of the embryonic volume) and the pattern of fragmentation (as described by Alikani et al., 1999) was related with subsequent embryo development. Fragmentation exceeding 15% of the embryonic volume had a significantly adverse effect on subsequent porcine embryo development, whereas minor fragmentation (0-
5%) did not affect development. Also fragmentation patterns characterized by a more severe fragmentation that either could be localized in a cluster or column (fragmentation pattern 2), or scattered throughout the cleavage cavity and the perivitelline space (fragmentation pattern 3) were typical for embryos with an impaired developmental potential. Recently, Booth et al. (2007) showed that for in vitro fertilized porcine embryos, fragmentation occurs in earlier stages of development compared to in vivo embryos, and further development is compromised if more than 10% of the embryonic volume is fragmented.

The underlying mechanisms, which determine the relationship between embryo fragmentation and embryo viability, remain to be clarified. Independent from its cause, fragmentation in itself can exert a negative effect on the embryo since fragment removal can improve development (Alikani et al., 1999) and viability of affected embryos (Keltz et al., 2006). The negative effect might be caused by an interference with normal cell-to-cell contact between blastomeres, or by an induction of degenerative processes in adjacent blastomeres (Alikani et al., 1999). The origin of fragmentation also plays a determinant role in the viability of embryos. There are several hypotheses concerning the origin of embryo fragmentation that have been investigated predominantly in human embryology. Embryonic fragmentation in the stages before embryonic genome activation (EGA) is probably not related to apoptosis, since spontaneous apoptotic processes are only seldom described at these stages. The morphological characteristics of fragmentation following developmental arrest in the stages before EGA are consistent with an autophagic mode of cell death as described by Levine and Klionsky (2004). The blastomeres of these early arrested embryos shrink and cytoplasmic vesicles are formed. In an attempt to survive, these cells seem to catabolize their own cytoplasm to produce energy. Members of the Bcl-family also contribute to this process by destroying mitochondria, ultimately leading to embryo demise (Tolkovsky et al., 2002).
The embryonic fragmentation that occurs in the period between EGA and the compaction stages of development may arise from apoptotical processes or may also originate from spatial specific cytoplasmic blebbing (Van Blerkom et al., 2001). Severe, mostly nucleated fragmentation at these cleavage stages is induced by apoptosis and is often accompanied by cleavage arrest (Jurisicova et al., 1996) or by complete destruction of all blastomeres (Yang et al., 1998). More subtle and discrete fragmentation at these stages of development might be caused by a subset of the cell death machinery that removes specific areas of cytoplasm with pro-apoptotic gene products without leading to subsequent cell death (Antczak and Van Blerkom, 1999). This survival mechanism of the embryonic blastomere could be mediated by caspases, since caspase-positive fragments have been observed to detach from blastomeres retaining their cleavage potential (Martinez et al., 2002).

The embryonic fragmentation that occurs in the postcompaction stages is mostly characteristic of the classical apoptotic mode of cell death (Jurisicova and Acton, 2004). Starting from compaction, cell differentiation occurs with a division in inner cell mass and trophodermal cells and there is a shift from totipotent (one cell can give rise to one individual) to pluripotent (one cell can give rise to all tissues of the body) cells. Apoptosis within the inner cell mass is probably caused by an attempt of the embryo to remove cells that have retained the trophodermal potential (Pierce, 1991).

In our experiments described in Chapter 3 where in vivo-derived porcine embryos were collected at day 2 and cultured for up to 5 days in vitro and then fixed at the 7th day after insemination, a highly significant correlation (r=0.87) between fragmentation and apoptosis was found. However, this does not imply there is a causal relationship between these two variables. Eighty percent (40/50) of the embryos with apoptosis also showed arrested development and since it has been described that a prolonged culture of arrested embryos can trigger the apoptotic machinery (Hardy, 1999), the correlation between fragmentation and
apoptosis for normal developing porcine embryos might be lower. A better experimental set-up to specifically investigate a possible causative relation between apoptosis and fragmentation would be to induce apoptosis in embryos in a controlled way, and to assess the subsequent effects on embryo fragmentation. This controlled apoptosis could be performed by activating the intrinsic pathway of apoptosis using microinjection of cytochrome c in the cytoplasm of blastomeres. This model is well-defined for several cell types (Li et al., 1997; Zhivotovsky et al., 1998) and has been successfully used to induce apoptosis in the microinjected cell and gap-junction-related bystander cells (Cusato et al, 2003; Frank et al., 2005). Cytochrome c, released in the cytoplasm, will bind with cytoplasmic apoptosis protease-activating factor-1 (Apaf-1). In the presence of 2'-deoxyadenosine 5'-triphosphate (dATP), this complex will recruit and activate pro-caspase-9 to form an apoptosome. Subsequently, the activated caspase-9 is released from the complex and effector caspase-3, -6 and -7 will be activated (Li et al., 1997; Slee et al., 1999; Zou et al., 1999). These effector caspases are the real executioners of apoptosis since they cleave a broad range of cellular substrates in the apoptotic pathway. In this way, apoptosis can be induced in a controlled number of blastomeres and the effects on fragmentation and embryo development could be assessed. Also, threshold apoptotic values for normal development in different embryonic stages could be determined.

In embryology, apoptosis is a natural process during mammalian preimplantation development, providing a defense mechanism against unwanted, damaged or potentially harmful cells. In this latter category, also virus-infected cells could be included. If embryos that are infected with viruses develop apoptosis in a part of their cells and become fragmented, the chance of being selected for ET will be very low, since only morphological excellent or good embryos are utilized for ET (Stringfellow, 1998). The three viruses investigated in this thesis have been shown to induce apoptosis both in in vivo and in in vitro experiments. For PCV2, an open
reading frame (ORF) 3 has been characterized as a non-structural protein that plays a major role in the virus-induced apoptosis in cell cultures by activating initiator caspase-8 and effector caspase-3 pathways (Liu et al., 2005). Regarding PRV, it has been demonstrated that it induces apoptosis in inflammatory cells of the trigeminal ganglia during an acute infection (Aleman et al., 2001). Also for PRRSV, it has been shown that infection induces apoptosis, mainly in macrophages and mononuclear cells (Sirinarumitr et al., 1998; Sur et al., 1998; Choi and Chae, 2002; Labarque et al., 2001; Labarque et al., 2003; Miller and Fox, 2004). To investigate if these viruses can also induce apoptosis in embryonic cells it first had to be elucidated whether they were able to reach and infect porcine embryonic cells.
Studies of the effects of viruses on porcine embryos

An embryonic viral infection can theoretically occur after vertical transmission via the oocyte or the fertilizing spermatozoon. At present, no paper on infected offspring of domestic livestock due to vertical viral transmission via the oocyte has been published, not even on retroviruses. The possibility that some viruses can be carried into the oocyte by spermatozoa has been described in in vitro models for Simian virus 40 and rabbit spermatozoa (Brackett et al., 1971), and for Sendai virus, influenza virus and Semliki Forest virus exposed to bull spermatozoa (Nussbaum et al., 1993). Under in vitro and in vivo circumstances, this phenomenon has been described for human immunodeficiency virus type 1 (Bacetti et al., 1994). Experimental data on other human viruses that have the capability of entering spermatozoa such as human papilloma virus also suggest that these viruses can be carried into the oocyte at fertilization (Chan, 1992). In swine, PRRSV has been detected by IHC and ISH in abnormal cells of spermiogenic origin and in multinucleated giant cells (Sur et al., 1997). Although PRRSV was never detected in mature spermatozoa, the fact that PRRSV was able to enter progenitor cells of spermatozoa raises concerns for a possible carry-over of virus into the oocyte through the male gamete. Since the virus was only detected in abnormal cells of spermiogenic origin and not in motile fertile spermatozoa, the risk of infecting the oocyte however seems to be low.

In the first stages after fertilization, embryonic cells can only be reached by viruses if the virus can pass the zona pellucida (ZP). Data in this thesis have shown convincingly that ZP intact porcine embryos remain negative for viral antigens after incubation with PCV2, PRV or PRRSV. These results confirm the hypothesis that an intact ZP in pigs forms an efficient barrier against most viral pathogens. The ZP is an extracellular matrix shell that surrounds the oocyte and early embryo until the process of hatching, which takes place 6 to 7
days after conception in pigs (Hunter, 1974). It plays an important role as sperm recipient at fertilization and it also forms a physical barrier controlling molecular transport between the embryonic cells and their environment (Turner and Horobin; 1997). The ZP is a network of filaments with numerous pores and holes that physically prevent cell-to-cell contact of the embryo with immunocompetent cells and potential pathogens. The thickness of the ZP is species dependent and this may play a role in species differences for trespassing the ZP. For mouse embryos that have a narrow ZP (5 µm), some of the smallest viruses seem to able to reach the blastomeres (Gwatkin and Auerbach, 1966; Heggie and Gadis, 1979). Pig embryos have a ZP of approximately 13-18 µm in thickness (Wang et al., 1999). Once, transzonal infection of ZP-intact porcine embryos with porcine parvovirus was reported by Bane et al. (1990).

The ultrastructure of the ZP consists of a loose network of filaments with numerous holes and meshes. This specific architecture may be the basis for its barrier function. In experiments using different sized inert microspheres, we found that the smaller the microspheres the deeper they entered the ZP. Microspheres sized 20 and 26 nm in diameter were even able to cross the ZP. This diameter is only slightly larger than that of PCV2 (diameter of 17 nm - Tischer et al., 1982), one of the smallest viruses, which indicates that PCV2 might be able to cross the ZP. In a study by Turner and Horobin (1997), it was found that not only the size but also the hydrophilic-lipophilic character of molecules influences the ease of penetration through a ZP. Their model showed that lipids and most lipid containing molecules will penetrate the ZP with relative ease. Since the envelope of viruses is a pleomorphic lipid bilayer, one might assume that the penetration dynamics of enveloped viruses such as PRV and PRRSV is only limited by their size. Whether proteins and nucleic acids will cross the ZP of the pig could not be answered in our study as this would require investigations of the kind used by Turner and Horobin (1997). However, the fact that the
blastomeres of ZP-intact embryos remained resistant to PCV2 infection suggests that penetration across the ZP by non-enveloped viruses is limited not only by their size but also by other factors. PCV2 consists of a genome packaged in a viral capsid. The PCV2 capsid is constituted by capsomeres, these are proteins with a high affinity for glycosilated proteins (Misinzo et al., 2006). Since the porcine ZP is composed of glycoproteins (pZPA, pZPB, pZPC) (Harris et al., 1994), an interaction between the viral capsid and these glycoproteins might be responsible for the sticky behaviour of the porcine ZP towards PCV2. Also other virus-binding sites can be present in the porcine ZP. This was shown in chapter 4.2. as the ZP showed foci of intense poliovirus receptor-related 1 (PVRL1; formerly known as nectin 1) staining. This species specific characteristic of the ZP is most likely responsible for the fact that washing procedures according to the IETS manual (Stringfellow et al., 1998) are not always capable of removing all viral materials from embryos (Bureau et al., 2005; Hebia et al., 2007).

Results of this thesis also show that porcine embryonic cells up to the hatched blastocyst stage can be infected with PCV2 and PRV but, that they are resistant to a PRRSV infection. Differences in viral susceptibility of these cells at different stages of development can, at least in part, be related to onset of expression of specific virus receptors by those stages. For example, embryos become susceptible to PRV infection from the 5-cell stage onwards. This coincides with the cellular expression of a known PRV entry receptor, PVRL1. The role of virus receptors in the susceptibility of embryos was explicitly shown by the differences between the results of the microinjection experiments compared to those obtained by PRV incubation of ZP-free embryos after protease treatment. Embryos were refractory to infection when the ZP was digested by pronase. The latter finding was most probably caused by the destruction of the PRV receptors during proteolysis. Protease treatment caused a disruption of the 3-dimensional structure of nectin-1, since the conformation-dependent MAb
CK41 (Krummenacher et al., 2000) was not able to bind to the V-like ectodomain of PVRL1. The finding that enzymes such as proteases have an effect on viral receptors throws doubt on the results of previous studies (e.g. Bolin et al., 1981) regarding virus susceptibility of embryonic cells. The present results of the subzonal microinjection experiments indicate that this technique is the method of choice for investigating the interaction between pathogens and embryos prior to hatching. All pre-implantation stages up to the hatched blastocyst remain resistant to PRRSV. The absence of sialoadhesin, the internalization receptor for PRRSV, is likely to be of major importance for this refractory state of early embryos.

The effect of an embryonic PCV2 infection during the first 3 weeks of pregnancy was investigated using a porcine ET model. The 3-week endpoint was chosen because domesticated sows have oestrus intervals of 3 weeks. Sows that show a regular return to oestrus had less than 4 viable embryos in their uterus the 12th day of the cycle (Polge et al., 1966). The results showed that an embryonic infection with PCV2 leads to embryonic death in most cases. Only a small subset of the PCV2-exposed embryos (6.4%) remained viable in the 2 weeks after ET. In some of these embryos there were no detectable PCV2-positive cells whereas others had just small numbers of such cells in the placenta, mesonephros, neural tube and liver without any gross pathology. These data indicate an individual variation among later stage embryos for their susceptibility to a PCV2 infection. The phenomenon of individual variation in susceptibility to viral infection has already been described for PCV2 in foetuses and piglets (Ladekjaer-Mikkelsen et al., 2002; Sanchez et al., 2003).

The finding that 4 embryos had PCV2-positive cells in specific organs without excessive damage may be an indication of a persistent PCV2-infection of embryos. However, to really define it as a persistent infection, the time between exposure of the embryos to virus and the stage at which they are assayed for the virus must be longer than the two week period
used in our study. Further research is needed to relate our findings with the PCV2-pathology observed in the foetal stages of pregnancy.

In conclusion, in vivo-derived porcine embryos with good developmental potential can be selected for transfer based on a) their developmental stage in relation to the time since fertilisation, and b) an absence of excessive fragmentation. As long as the ZP of the selected embryo is intact and washing procedures are performed according to the IETS manual (Stringfellow, 1998) the risks of transmitting PCV2, PRV or PRRSV through an ET seem minimal. However, when the barrier function of the ZP is impaired by manipulations or treatments, or by hatching, embryos can get infected with PCV2 or PRV and disease transmission through ET may occur. Porcine embryos do not appear to be susceptible to PRRSV at these early stages, even when the ZP is removed.

Based on the results of this thesis it still would be interesting to focus future work on establishing threshold apoptotic and fragmentation values for porcine embryo viability in different embryonic stages. This could be done by inducing a controlled apoptotic process using microinjection of cytochrome c. Also, although I have shown that specific stages of development are susceptible to viral infections, further research on the way the virus can reach an embryo is necessary in order to really assess the risk of getting an infected embryo. Another question that remains unanswered in the present thesis is when porcine embryos become susceptible to a PRRSV infection. Based on the results of this thesis we can conclude that porcine embryos up to the hatched blastocyst stage (day 7) are refractory to a PRRSV infection. To investigate whether and when further embryonic stages become susceptible, intra-uterine inoculation experiments or PRRSV receptor expression experiments could be performed.
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Chapter 6: General discussion


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CHAPTER 7

SUMMARY – SAMENVATTING
CHAPTER 7: SUMMARY

Interest in embryo transfer (ET) as a practical method for exchanging porcine germplasm has increased greatly in recent years due to progress with techniques such as embryo cryopreservation and non-surgical collection and transfer. However, key aspects which need further investigation include criteria for selecting viable embryos, and how best to ensure that potential pathogens are not transmitted via ET.

Chapter 1 begins with a review of current methods for assessing the quality and/or viability of in vivo derived porcine embryos. Inferior embryos should not be selected for transfer, so the determination of non-invasive criteria that predict continued embryo development and viability are needed. A better understanding of biosecurity risks which may be associated with international trade of pig embryos is another important research objective. Specifically the dangers associated with viruses which have a preference for the reproductive tract, such as porcine circovirus type 2 (PCV2), pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) have been covered. In particular, attention has been paid to what has been observed in the field and what has been done in experimental studies. Possible pathways of contamination of pig embryos by means of semen, oocytes/follicular fluid or uterine environment are listed. Finally, the four definitions which describe the different in vivo and in vitro approaches to test the risk for viral infection by means of embryo transfer are explained in detail.

In Chapter 2, the major aims of this thesis are outlined: the first aim was to define parameters for evaluating porcine, in vivo-derived embryos and the second aim was to investigate the interaction between preimplantation pig embryos and porcine viruses with a predilection for the genital tract, namely PCV2, PRV and PRRSV.
In Chapter 3 we established parameters for the quality of in vivo-derived pig embryos based on timing of development in vitro, embryo morphology and the presence of apoptosis. The kinetics of development and morphological parameters were investigated in a time-lapse cinematographic experiment. Possible links between embryo morphology and apoptosis were examined via a confocal laser scanning experiment, analysing nuclear changes, annexin V and terminal dUTP nick-end labelling. The timing of early cleavages was firmly linked to embryo developmental competence in vitro. Attainment of at least the 5-cell stage before 77 h post insemination and attainment of the morula stage before 102 h post insemination significantly increased the odds for reaching the early blastocyst stage. Overall, a negative effect of fragmentation percentage and fragmentation pattern on subsequent embryonic development was observed, but the developmental potential of embryos experiencing slight fragmentation (0-5%) was not different from embryos without fragmentation. Significant correlations were detected between developmental arrest and fragmentation and apoptosis. By using the developmental criteria described above, we now can either select the best embryos for transfer or we can use the settings as a gold standard when evaluating the developmental potential of in vitro produced pig embryos.

Since we believed that porcine embryos flushed from infected donor sows could represent a possible hazard for international trade in pig embryos, we next focused on the interaction of viruses with preimplantation pig embryos. In chapter 4.1, the initial aim was to determine if porcine circovirus type 2 (PCV2) was able to infect embryonic cells of in vivo-derived porcine embryos with and without zona pellucida (ZP). ZP-intact and ZP-free morulae, early blastocysts and hatched blastocysts were exposed to 105.0 TCID50 PCV2 per ml. At 48 h post-incubation, significantly different percentages of infected embryos were detected: 15% for ZP-free morulae, 50% for ZP-free early blastocysts and 100% for hatched
blastocysts. The percentage of cells that expressed viral antigens was similar for the three stages of development. PCV2 exposure did not affect the in vitro development of the embryos. All ZP-intact embryos remained negative for viral antigens. In an additional experiment, the diameter of the channels in the porcine ZP was determined. After incubation of early blastocysts with fluorescent microspheres of three different sizes, microspheres with a diameter of 20 nm and 26 nm both crossed the zona whereas microspheres with a diameter of 200 nm did not. The study showed that PCV2 was able to replicate in in vivo produced ZP-free morulae and blastocysts and that the susceptibility increased during development. The ZP forms a barrier to PCV2 infection, but based on the size of the channels in the ZP, the possibility that PCV2 particles cross the ZP cannot be excluded.

In a following study (Chapter 4.2), we looked deeper into the temporary refractoriness of porcine embryonic cells against a specific type of viral infection by evaluating the expression of virus receptors on the embryo, in particular poliovirus receptor-related 1 (PVRL1; formerly known as nectin 1) which is a known receptor for PRV and sialoadhesin, which is used to internalize PRRSV. Embryonic cells of ZP intact embryos incubated with PRV remained negative for viral antigens. Interestingly, no antigen-positive cells could be detected after PRV incubation of protease-treated embryos, since the protease disrupted the expression of PRVL1. However, starting from the five-cell-stage onwards, viral antigen-positive cells were detected after subzonal microinjection of PRV. At this stage, the first foci of PVRL1, also a known cell adhesion molecule, were expressed. At the expanded blastocyst stage, a lining pattern of PVRL1 in the apicolateral border of trophectoderm cells was present, whereas the expression in the inner cell mass was low. Furthermore, PVRL1-specific monoclonal antibody CK41 significantly blocked PRV infection of trophectoderm cells of hatched blastocysts, while the infection of the inner cell mass was only partly inhibited. Viral antigen-positive cells were never detected after PRRSV exposure of preimplantation embryos
up to the hatched blastocyst stage. Also, expression of sialoadhesin in these embryonic stages was not detected. The study showed that the use of protease to investigate the virus embryo interaction can lead to misinterpretation of results. It was also shown that blastomeres of five-cell embryos up to the hatched blastocysts can become infected with PRV, but there is no risk of a PRRSV infection.

In Chapter 5 we performed ET in sows with PCV2 infected embryos and evaluated the effects of PCV2 on porcine embryos and their receptor sows during the first 21 days of pregnancy. All of the non-viable PCV2-exposed embryos (n=9) displayed immunohistochemical positive signals for PCV2-antigen in degenerated tissues. In the PCV2-exposed embryos that were categorized as viable at D21, small clusters (n=4) or no PCV2-positive cells (n=3) were detected. The pregnancy results of the receptor sows that received PCV2-exposed embryos (1/5) were considerably different from the negative control receptors (2/2), with 3 out of 5 sows displaying a regular return to oestrus. The study documented that PCV2 can replicate in embryos and might lead to embryonic death. In a small proportion of embryos, PCV2 exposure did not have a detrimental effect on embryo development before D21.

In the General Discussion (Chapter 6), two key elements for the success of future porcine ET that were investigated in this thesis, were summarized and discussed. Firstly, morphological embryo quality parameters for porcine embryos were defined based on invasive and non-invasive markers of embryo viability. We developed a gold standard for the timing of development for in vivo derived pig embryos and investigated porcine embryo fragmentation in relation to embryo development and apoptosis. Secondly, the interaction between porcine embryos and the three viral pathogens was investigated. Our data have shown very convincingly that ZP intact porcine embryos remain negative for viral antigens after incubation with PCV2, PRV or PRRSV, which reinforces the belief that an intact ZP in
pigs forms an efficient barrier against most viral pathogens. We also showed that porcine embryonic cells up to the hatched blastocyst stage can be infected with PCV2 or PRV but that they are resistant to a PRRSV infection. There is a difference in viral susceptibility of these cells at different stages of development that can at least in part be related to the expression of specific virus receptors. And in our ET study, we showed that there was an individual variation among embryos for their susceptibility to a PCV2 infection. We can conclude that we have established criteria for selection of in vivo derived pig embryos with a high developmental potential, and we have confirmed that the ZP represents a barrier against PCV2, PRV and PRRSV. ET therefore represents a biosecure technique, which can be applied in practice to transfer pig genetics all over the world.
SAMENVATTING

De laatste jaren is de interesse voor embryotransplantatie (ET) als een praktisch middel om genetisch materiaal van varkens uit te wisselen sterk toegenomen. Met de recente vooruitgang in technieken zoals cryopreservatie van embryo’s en niet-chirurgische ET zijn de meeste obstakels verdwenen. Er is echter dringend bijkomend onderzoek nodig om te na gaan welke criteria gebruikt kunnen worden om goede embryo’s te selecteren en hoe de verspreiding van pathogene agentia d.m.v. ET kan voorkomen worden.

In Hoofdstuk 1 wordt een overzicht gegeven van de verschillende methoden die momenteel kunnen gebruikt worden om de kwaliteit en de vitaliteit van in vivo verkregen varkensembryo’s te beoordelen. Embryo’s van mindere kwaliteit dienen niet geselecteerd te worden voor transplantatie omdat er een reële kans bestaat dat ze zich niet zullen ontwikkelen tot foeti of levensvatbare biggen. Zowel invasieve als niet-invasieve methoden voor de bepaling van de embryokwaliteit worden besproken.

Het onderzoek naar niet-invasieve criteria die gebruikt kunnen worden voor de beoordeling van varkensembryo’s en die ook een voorspellende waarde hebben ten aanzien van de verdere embryonale ontwikkeling of voor het geboren worden van vitale biggen is van groot belang. Daarnaast blijkt onderzoek naar de bioveiligheidsaspecten die geassocieerd zijn met de internationale handel van varkensembryo’s dringend noodzakelijk te zijn. Vooral virussen die een tropisme hebben voor de geslachtstractus, zoals het porcien cirvovirus type 2 (PCV2), het Aujeszky virus (AV) en het porcien reproductief en respiratoir syndroom virus (PRRSV), vormen een belangrijk potentieel gevaar. In het bijzonder wordt aandacht besteed aan het onderscheid tussen de praktijksituatie en de situatie onder experimentele omstandigheden. Mogelijke contaminatiebronnen via het sperma, oöcyten, folliculair vocht en de uteriene omgeving worden besproken. Tenslotte worden vier benaderingen beschreven die kunnen gebruikt worden om het risico op een virale infectie na ET te bestuderen.
De doelstellingen van de thesis worden in Hoofdstuk 2 vermeld. De eerste doelstelling was om parameters voor de evaluatie van porciene, *in vivo* verkregen embryo’s te definiëren. De tweede doelstelling was om de interactie te bestuderen tussen pre-implantatie embryo’s en porciene virussen die de genitaaltractus kunnen aantasten namelijk PCV2, AV en PRRSV.

De beoordeling van de kwaliteit van *in vivo* verkregen varkensembryo’s wordt beschreven in Hoofdstuk 3. De parameters die gebruikt werden voor de beoordeling van de kwaliteit van *in vivo* verkregen varkensembryo’s zijn gebaseerd op de ontwikkelingscapaciteit *in vitro*, de embryo morfologie en de aanwezigheid van apoptose. De ontwikkelingskinetiek en de morfologische parameters werden onderzocht via een time-lapse cinematografische studie. Mogelijke associaties tussen embryomorfologie en apoptose werden nagegaan m.b.v. confocaal laser scanning onderzoek waarbij eventuele veranderingen in de kern, in de membraan en in de fragmentatie van het DNA werden nagegaan.

Het optreden van vroege delingen was sterk geassocieerd met de ontwikkeling van het embryo *in vitro*. Het bereiken van het 5-cellig stadium in minder dan 77 uur na inseminatie en het bereiken van het morula stadium in minder dan 102 uur na inseminatie waren beiden significant geassocieerd met een hogere kans om het vroege blastocyst stadium te bereiken. Globaal genomen werd een negatief verband vastgesteld tussen het percentage fragmentatie en het fragmentatiepatroon enerzijds en de ontwikkelingscapaciteit van de embryo’s anderzijds, maar de ontwikkelingscapaciteit van embryo’s met een geringe fragmentatie (0-5%) was niet verschillend van embryo’s zonder fragmentatie. Er werden significante correlaties aangetoond tussen het stopzetten van de ontwikkeling, fragmentatie en apoptose. Slechts een minderheid van de embryo’s die zich niet verder ontwikkelden tussen het 1- en 4-cellig stadium vertoonden biochemische eigenschappen die op apoptose wezen. De ontwikkelingseigenschappen kunnen aangewend worden om de beste embryo’s te selecteren.
voor transplantatie, en kunnen tevens als gouden standaard gebruikt worden voor het beoordelen van de ontwikkelingscapaciteit van *in vitro* geproduceerde varkenseembryo’s. Omdat embryo’s die verkregen worden uit met bepaalde virussen geïnfecteerde donorzeugen niet in aanmerking kunnen komen voor internationaal transport, werd in een volgend deel van het onderzoek uitvoerig aandacht besteed aan de interactie van virussen met pre-implantatie varkenseembryo’s. In **Hoofdstuk 4.1** wordt beschreven in welke mate PCV2 in staat was om embryonale cellen van *in vivo* verkregen porciene embryo’s met en zonder zona pellucida (ZP) te infecteren. ZP-intacte en ZP-vrije morulae, jonge blastocysten en uitgekipte blastocysten werden hiertoe blootgesteld aan $10^{5.0}$ TCID$_{50}$ PCV2 per ml. Na 48 uur incubatie waren de percentages geïnfecteerde embryo’s significant verschillend: 15% voor de ZP-vrije morulae, 50% voor de ZP-vrije jonge blastocysten en 100% voor de uitgekipte blastocysten. Het percentage cellen dat expressie vertoonde van virale antigenen was voor de 3 ontwikkelingsstadia hetzelfde. De blootstelling aan PCV2 had geen invloed op de *in vitro* ontwikkeling van de embryo’s. Alle ZP-intacte embryo’s bleven negatief voor virale antigenen. In een bijkomend experiment werd de diameter van de poriën in de ZP van de embryo’s bepaald. Na incubatie van jonge blastocysten met fluorescente microsferen van drie verschillende groottes, bleek dat de microsferen met een diameter van 20 nm en 26 nm beiden de ZP konden passeren, terwijl de microsferen met een diameter van 200 nm dit niet konden. Met deze studie werd aangetoond dat PCV2 in staat is om te vermenigvuldigen in *in vivo* verkregen ZP-vrije morulae en blastocysten en dat de gevoeligheid van de cellen toeneemt tijdens de ontwikkeling van het embryo. De ZP vormt een barrière tegen PCV2 infectie, maar op basis van de diameter van de poriën in de ZP blijkt dat de mogelijkheid dat PCV2 partikels de ZP passeren, niet kan uitgesloten worden. In een volgende studie (**Hoofdstuk 4.2**) werd onderzocht in welke mate porciene embryonale cellen tijdelijk refractair zijn tegen een specifiek type virale infectie door de expressie na te
gaan van virusreceptoren op het embryo, in het bijzonder van poliovirus receptor-gerelateerd 1 (PVRL1; voorheen nectine 1 genoemd) dat een bekende receptor is voor het AV, en van sialoadhesine, dat gebruikt wordt door het PRRSV om cellen binnen te dringen. Embryonale cellen van ZP intacte embryo’s die geïncubeerd werden met het AV, bleven negatief voor virale antigenen. Interessant was ook dat er geen antigen-positieve cellen opgespoord konden worden na incubatie van met protease-behandelde embryo’s met het AV. Dit is wellicht te danken aan het feit dat protease de expressie van PVRL1 verhindert. Echter, vanaf het vijf-cellig stadium werden virus antigen-positieve cellen vastgesteld na subzonale micro-injectie van het AV. In dit stadium werden de eerste foci van PVRL1, ook een bekende celadhesiemolecule, tot expressie gebracht. Vanaf het stadium van uitgekipte blastocyst was er een lijnpatroon van PVRL1 in het apicolateraal deel van de trofectodermcellen, terwijl de expressie in de kiemschijf laag was. Bovendien was het mogelijk om d.m.v. PVRL1-specifieke monoclonale antistoffen CK41 een AV-infectie van de trofectodermcellen van uitgekipte blastocysten te blokkeren, terwijl infectie van de kiemschijf slechts gedeeltelijk werd geïnhibeerd. Virus antigen-positieve cellen werden nooit vastgesteld na infectie met PRRSV van pre-implantatie embryo’s tot het stadium van uitgekipte blastocyst. Evenmin werd er expressie vastgesteld van sialoadhesine in deze embryonale stadia. De studie toonde aan dat het gebruik van protease om virus-embryo interacties te bestuderen, tot verkeerde interpretaties van de resultaten kan leiden. Er werd tevens aangetoond dat blastomeren van vijf-cellige embryo’s tot het stadium van uitgekipte blastocyst vatbaar zijn voor infectie met het AV, maar dat er geen risico is voor PRRSV infectie.

In Hoofdstuk 5 werd ET toegepast bij zeugen met PCV2-geïnfecteerde embryo’s en werden de effecten van PCV2 op de porciene embryo’s en de receptorzeugen onderzocht gedurende de eerste 21 dagen van de dracht. Alle afgestorven embryo’s die aan PCV2 werden blootgesteld (n= 9), waren op basis van immunohistochemisch onderzoek positief voor PCV2
in de gedegenereerde weefsels. In de embryo’s die aan PCV2 werden blootgesteld en die levend waren op dag 21 van de dracht, werden ofwel kleine clusters (n=4) ofwel geen PCV2-positieve cellen (n=3) vastgesteld. De drachtigheidsresultaten van de receptorzeugen die embryo’s hadden gekregen die aan PCV2 waren blootgesteld, waren significant lager (1/5) dan die van de negatieve controlezeugen (2/2), en drie van de vijf zeugen werden opnieuw berig op dag 21. De studie toonde aan dat PCV2 zich kan vermengvuldigen in varkensembryo’s en dat dergelijke infectie kan leiden tot embryonale sterfte. Bij een klein percentage van de embryo’s had PCV2-blootstelling geen nadelig effect op de embryonale ontwikkeling tijdens de eerste drie weken van de dracht.

In de algemene discussie (Hoofdstuk 6) werden de twee belangrijke voorwaarden voor een succesvolle ET, die in de thesis werden onderzocht, samengevat en bediscussieerd. Eerst werden morfologische criteria voor embryokwaliteit gedefinieerd gebaseerd op invasieve en niet-invasieve markers van embryovitaliteit. Er werd m.b.t. de ontwikkelingssnelheid van de embryo’s een gouden standaard ontwikkeld voor in vivo verkregen porciene embryo’s, en de fragmentatie van porciene embryo’s werd onderzocht in relatie tot embryo-ontwikkeling en apoptose. Als tweede luik van het onderzoek werd de interactie tussen varkensembryo’s en deze drie pathogene virussen onderzocht. Tijdens de eerste fases na de bevruchting kunnen embryonale cellen enkel bereikt worden door virussen indien deze laatste de ZP kunnen passeren. De resultaten van het onderzoek tonen duidelijk aan dat ZP-intacte varkensembryo’s negatief kunnen blijven voor virale antigenen na incubatie met PCV2, AV en PRRSV. Ze bevestigen de hypothese dat een intacte ZP een efficiënte barrière vormt tegen de meeste virale pathogenen. Er werd ook aangetoond dat porciene embryonale cellen tot het stadium van uitgekipte blastocyst geïnfecteerd kunnen worden met PCV2 of AV, maar dat ze resistent zijn tegen een PRRSV infectie. Er is een verschil in gevoeligheid van deze cellen in verschillende stadia van ontwikkeling, en dit kan althans gedeeltelijk verklaard worden door
de expressie van specifieke virusreceptoren. In de ET studie bleek dat er een individuele variatie was tussen embryo’s betreffende de gevoeligheid voor een PCV2 infectie. Als conclusie kan gesteld worden dat dit doctoraatsonderzoek criteria heeft vastgelegd voor de selectie van *in vivo* verkregen varkensembryo’s met een hoog ontwikkelingspotentieel, en dat de functie van de ZP als effectieve barrière tegen een PCV2, PRV en PRRSV bevestigd is. ET kan daarom vanuit bioveiligheidsstandpunt als een veilige techniek beschouwd worden die onder praktijkomstandigheden kan gebruikt worden om genetisch materiaal van varkens wereldwijd te verspreiden.
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Bart
Curriculum Vitae

Sinds juli 1999 was hij verbonden aan de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde van deze faculteit diergeneeskunde. Vanaf 2001 droeg hij als assistent bij aan het onderwijs van de laatstejaarsstudenten diergeneeskunde optie varken, pluimvee en konijn en nam hij deel aan praktijkgericht onderwijs voor de studenten optie herkauwers. In 2001 behaalde hij het getuigschrift Bedrijfskunde aan de EHSAL managament school.


Hij behaalde in 2006 met grote onderscheiding het diploma vakdierenarts varken aan de faculteit diergeneeskunde. In datzelfde jaar slaagde hij voor het examen aan het European College of Animal Reproduction (ECAR) en werd hij tevens diplomate van het European College of Porcine Health Management (ECPHM).

Bart Mateusen is auteur of mede-auteur van 19 wetenschappelijke publicaties in internationale en nationale tijdschriften.

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