REPRODUCTION
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Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study

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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 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. 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 between the 1- and 4-cell stage displayed biochemical characteristics of apoptosis. Consequently, a significant correlation (0.57) between developmental arrest and apoptosis could only be established for embryos arrested after embryonic genome activation.

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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 1994, 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.* 1998*a*, Alikani *et al.* 1999). Similar data in pig embryos are presently lacking.

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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* 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 equine chorionic gonadotrophin (eCG) (Folligon 1500 IU i.m. Intervet, Boxmeer, The Netherlands) three days after weaning, followed by human CG (hCG) (Chorulon 1500 IU i.m.) 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 15 ml of pre-warmed Hepes-buffered North Carolina State University-23 (NCSU-23) medium to collect the embryos. After ten 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 (Carl Zeiss NV-SA, Zaventem, Belgium). The chamber was regularly flushed with a humidified and warmed gas mixture consisting of 5% CO_2, 5% O_2, and 90% N_2. 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 (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 h post insemination (hpi) (3 h 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 min 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) (Fig. 1). Embryos exhibiting minimal fragments, usually in association with a single blastomere were designated as fragmentation pattern 1 (FP1). FP2 was used to characterize highly localized

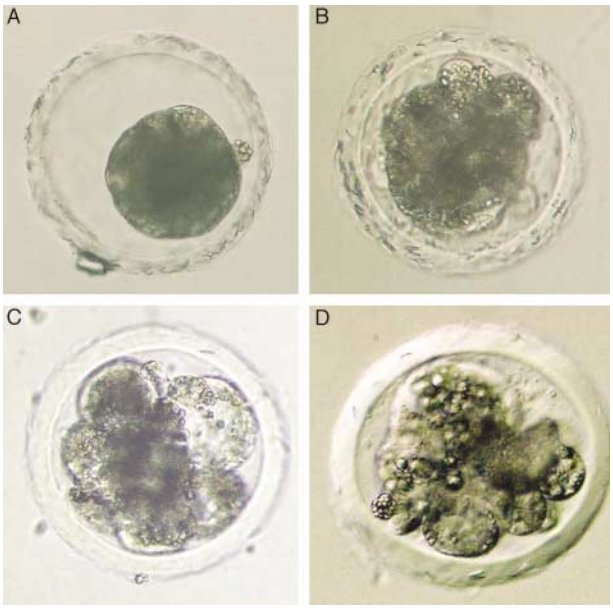

Figure 1 Four distinct patterns of fragmentation in porcine morulae: (A) fragmentation pattern 1 (FP1) is minimal in volume and fragments are associated with only one blastomere, (B) FP2 is characterized by highly localized fragments which often appear in a cluster or a column, (C) FP3 with many small fragments seen throughout the cleavage cavity and perivitelline space, (D) FP4 embryos have many large fragments which are randomly distributed and always associated with uneven cells.

fragments which often appear in a cluster or a column. In FP3, many small fragments could be seen throughout the cleavage cavity and perivitelline space. FP4 also had many large fragments which were randomly distributed and always associated with uneven cells.

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, OR, 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 1 μM staurosporine to induce apoptosis. Day 7 embryos were first washed for 5 min in annexin binding buffer at 37 °C and incubated for 15 min in the presence of FITC conjugate of annexin V (25 μl/ml) and PI solution (3 μg/ml) according to the manufacturer's recommendations for the assay kit. Then embryos were washed for 5 min in PBS and transferred to a drop of prewarmed 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 h in polyvinyl pyrrolidone (PVP) solution (1 mg PVP/ml PBS). After washing, they were permeabilized with 0.5% Triton X-100 in PBS for 20 min 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 three more washings in PVP solution, controls and samples were incubated in RNase A (50 μg/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 washed three times with PVP solution and embryos were mounted in glycerol with 1,4-diazabicyclo (2.2.2) octane (25 mg/ml). Samples were examined by laser scanning confocal microscopy. TUNEL-positive nuclei appeared bright yellowish-green, and 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 3 μm 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 50 μl NCSU23 under oil at 39 °C in 5% CO_2, 5% O_2, and 90% N_2. 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-, 4-, 5-, 6-, 7- and 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 hpi were cultured in groups of 25 in 500 μl NCSU23 at 39 °C in 5% CO_2 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 Olympus Belgium NV, Aartselaar, Belgium) 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 performed then 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 four categories: no fragmentation, 0–5% fragmentation, 5–15% and >15%. Additionally, the fragmentation pattern was investigated as a categorical variable with the categories: no fragmentation, and fragmentation pattern 1 (FP1), FP2, FP3 and FP4. Time points at which embryos attained a specified cell stage with the highest significant odds of becoming a 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 (Collette 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. 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

Experiment 1: cinematographic analysis of porcine embryonic morphology and development

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 three 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.

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 ± 5.4 h ($n = 25$), whereas the blastomeres of 4-cell stage embryos cleaved asynchronously with an average interval between the first and the last blastomere cleavage of 9.2 ± 10.8 h ($n = 55$). 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 Fig. 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 unlike embryos that reached the blastocyst stage

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

Cell stage	Mean time (hours ± S.E.M.) post insemination to reach cell stage
3-cell	55.61 ± 2.42
4-cell	57.41 ± 2.36
5-cell	85.64 ± 1.73
6-cell	87.83 ± 1.71
7-cell	89.81 ± 1.96
8-cell	92.78 ± 1.54
Early morula	103.43 ± 1.43
Compacted morula	112.30 ± 2.41
Early blastocyst	135.77 ± 2.94
Expanded blastocyst	148.34 ± 3.10
Hatching blastocyst	161.50 ± 9.57
Hatched blastocyst	176.04 ± 24.57

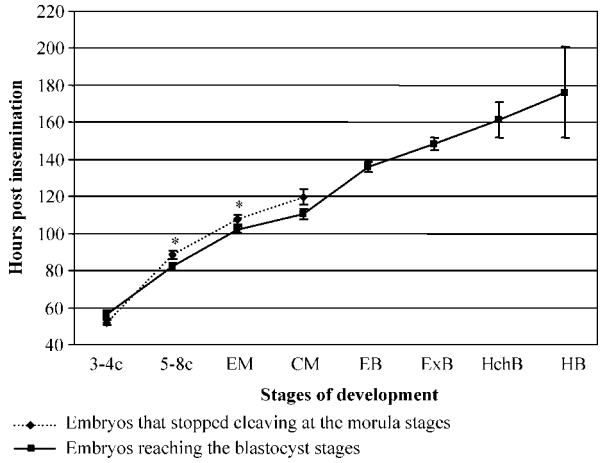

Figure 2 The developmental kinetics of embryos that arrested at the morula stage in comparison with the development of embryos that reached the blastocyst stage. *Significant difference within a stage of development ($P < 0.05$). Stages of development: 3–4c, 3- to 4-cell stage; 5–8c, 5- to 8-cell stage; EM, early morula; CM, compacted morula; EB, early blastocyst; ExB, expanded blastocyst; HchB, hatching blastocyst; HB, hatched blastocyst. ◆, embryos that stopped cleaving at the morula stage; ■, embryos reaching 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 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) ($P = 0.019$).

Time-lapse recordings showed two 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 with 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. FP1 ($n = 45$) and FP2 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 ($P = 0.007$).

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

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 7 days 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 with embryos that reached the blastocyst stage at 7 dpi ($P < 0.05$). Also, the average fragmentation percentage was higher for arrested embryos compared with 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 with 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 with 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 ($P < 0.01$).

As shown in Tables 3 and 4, the percentage 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

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.

State of development	% (ratio) of embryos with fragmentation	Average fragmentation % ± S.E.M.	% (ratio) of embryos with apoptosis	Average ACR (%) ± S.E.M. of embryos
Arrested 1-to 4-cell	50.0^{a} (4/8)	10.0^{a} ± 4.2	25.0^{a} (2/8)	8.3^{ab} ± 5.5
Arrested 5-cell to morula	77.4^{a} (41/53)	10.8^{a} ± 1.2	71.7^{b} (38/53)	16.1^{b} ± 2.2
Blastocyst	14.1^{b} (10/71)	2.5^{b} ± 1.0	14.1^{a} (10/71)	3.4^{a} ± 1.2

^{a,b}Within a column, values with a different superscript differ significantly ($P < 0.05$. ACR, apoptotic cell ratio.

Table 3 The relationship between fragmentation % and average apoptotic cell ratio (ACR) in day 7 cultured pig embryos ($n = 132$)

Fragmentation %	No. of embryos	% (ratio) of embryos with apoptosis	Average ACR (%) ± S.E. of embryos with apoptosis
0	77	0.0^{a} (0/77)	$0.0^{a} \pm 0$
0–5	11	90.9^{b} (10/11)	$12.7^{b} \pm 2.0$
5–15	27	94.7^{b} (26/27)	$28.8^{c} \pm 3.5$
>15	17	82.3^{b} (14/17)	$23.2^{c} \pm 3.4$

[a,b,c]Within a column, values with a different superscript differ significantly ($P < 0.05$).

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

Fragmentation	No. of embryos	% (ratio) of embryos with apoptosis	Average ACR (%) ± S.E. of embryos with apoptosis
No fragmentation	77	0.0^{a} (0/77)	$0.0^{a} \pm 0$
FP1	31	96.8^{b} (30/31)	$22.3^{b} \pm 2.9$
FP2	10	80.0^{b} (8/10)	$17.8^{b} \pm 4.2$
FP3	13	84.6^{b} (11/13)	$19.6^{b} \pm 4.3$
FP4	1	100.0^{b} (1/1)	$37.9^{b} \pm 0$

[a,b]Within a column, values with a different superscript differ significantly ($P < 0.05$).

fragmentation percentage of >5% ($P < 0.05$) (Table 3). The correlation detected between fragmentation and apoptosis was 0.87 ($P < 0.05$). Occurrences of biochemical cell changes indicative for apoptosis of fragmented embryos are presented in Fig. 3.

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 2002). This is not surprising, since 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 8-cell stage. For bovine embryos, a comparable time interval of 9.2 h was detected between the 9- and 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 with 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

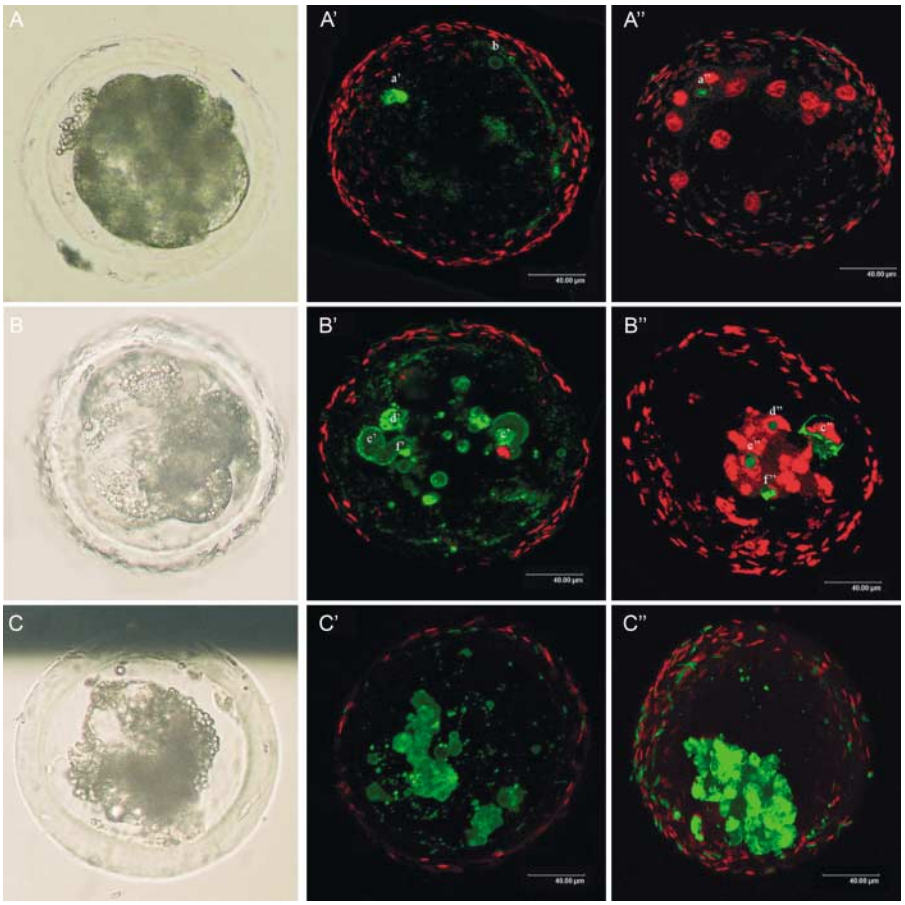

Figure 3 Differential interference contrast microscopic and confocal laser scanning images of fragmented *in vivo* fertilised and *in vitro* cultured porcine embryos stained with propidium iodide, annexin V and TUNEL (bar = 40.00 μm). (A, B and C) Embryos under differential interference contrast microscopy. (A′, B′ and C′) Confocal images of live embryos stained with propidium iodide and annexin V. (A″, B″ and 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 in A′ is condensed and displays TUNEL labelling (a″). (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″) Notice in blastomere c″ that TUNEL labelling is not only confined to the nucleus, but diffusely stains the blastomere, indicating 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) Image of a highly fragmented embryo (>15%) with a blastocoele. (C′ and C″) Many of the blastomeres show simultaneous annexin V and TUNEL labelling.

et al. 1995). 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 with 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.* 1998*b*) 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 & Keefe 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 (FP2) or small, scattered fragments distributed all over the embryonic cell mass (FP3) 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 & 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 key morphological 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 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 between the 5-cell and morula stage, but not for embryos arrested before or at the 4-cell stage. Only two out of eight embryos arrested at the 1- to 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 significantly 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 the *in vitro* 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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