Quality of porcine blastocysts produced in vitro in the presence or absence of GH

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Abstract

GH receptor (GHR) mRNA is expressed in bovine in vitro produced embryos up to the blastocyst stage and GH improves the quality of bovine embryos by increasing blastocyst cell numbers and reducing the incidence of apoptosis as evaluated by DNA strand-break labelling. Porcine in vitro produced blastocysts have lower cell numbers than in vivo blastocysts and exhibit higher incidences of apoptosis. Therefore we investigated the effects of 100 ng GH/ml NCSU23 medium during in vitro culture of presumptive in vitro fertilized sow zygotes on embryo development and blastocyst quality (defined by diameter, cell number, apoptosis and survival after non-surgical transfer). In vivo produced blastocysts were analysed concurrently as a reference value. GHR was expressed in embryos from the 2-cell to blastocyst stages. GH had no effect on blastocyst development or cell numbers, but increased the mean blastocyst diameter. The incidence of apoptosis, detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), was decreased by GH, but when non-TUNEL labelled apoptotic fragmented nuclei were included, no difference was seen. GH appeared to slow down the progression of apoptosis though. In vivo produced blastocysts presented no apoptotic nuclei, and contained higher cell numbers and larger diameters. Pregnancy rates on day 11 were similar for all groups, but survival was poorer for in vitro than in vivo produced blastocysts. In this study GH appeared to be beneficial only from the blastocyst stage, but the presence of GHR from early cleavage stages nevertheless indicates a role for GH throughout porcine embryo development and deserves further investigation.


Introduction

Growth hormone (GH) is a polypeptide hormone, mainly produced by the adenohypophysis (Meij et al. 1997, Costoya et al. 1999). Its regulatory effect on cell proliferation (Raff 1996, Costoya et al. 1999), follicular growth (Webb et al. 1992, Gong et al. 1993) and metabolism (Gluckman et al. 1987) is widely recognized. The exact role of GH during embryogenesis is unknown, but GH receptor (GHR) mRNA has been identified in the mouse, bovine and human from the fertilized oocyte up to the blastocyst stage (Pantaleon et al. 1997b, Kölle et al. 1998, Izadyar et al. 2000). This suggests a functional role of GH in preimplantation embryo development. Most recently the positive influence of GH on oocyte maturation in the bovine (Izadyar et al. 1996) and embryo development in both the mouse and bovine (Fukaya et al. 1998, Izadyar et al. 2000, Kölle et al. 2001, Mtango et al. 2003) has been identified. Addition of GH to in vitro culture (IVC) media stimulates growth and proliferation of in vitro produced bovine and mouse embryos by exerting distinct effects on the metabolic processes of the embryo (Pantaleon et al. 1997a, Kölle et al. 2001). GH also increases blastocyst yield and cell numbers in the mouse and bovine (Pantaleon et al. 1997b, Kölle et al. 2001), and enhances post-thaw survival of bovine blastocysts cultured with GH (Mtango et al. 2003). Furthermore, culture of bovine embryos in the presence of GH greatly reduces the incidence of double-strand DNA breaks in resultant blastocysts (Kölle et al. 2002). These promoting effects of GH on the developmental competence of preimplantation embryos in the mouse and bovine indicate a rudimentary role of GH during the early stages of in vitro embryo...
development, which could also be pertinent to embryo development in other species, such as the pig.

Many porcine embryo in vitro production laboratories are now as standard obtaining averages of 30% or more blastocysts from in vitro matured oocytes (Marchal et al. 2001, Wei et al. 2001, Wang & Day 2002), but despite these promising yields the quality of the resultant blastocysts remains questionable. Embryo quality is difficult to define, but chronologically and morphologically normal cleavage of the embryo are regarded as significant indicators of in vitro produced blastocyst quality and viability (Benkhalfa & Menezo 1993). The use of gross morphological criteria is subjective and not devoid of error due to possible bias of the evaluator (Farin et al. 1995, Van Soom et al. 2001), and moreover in the porcine no guidelines have been set by which in vitro produced blastocysts can be evaluated. Current parameters by which embryo quality can be measured include the nuclear and chromosomal status of the blastomeres contained in an embryo, indicative of apoptosis or programmed cell death. Late-stage apoptosis markers, such as nuclear condensation and fragmentation, are evaluated by methods such as 4,6-diamino-2-phenylindole (DAPI) staining for nuclear morphology, whereas the final stages of apoptosis are assessed by terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL), which enables in situ biochemical detection of single- and double-strand DNA breaks. Apoptosis is regarded as a side effect of in vitro embryo culture in the bovine (Kölle et al. 2002) but also in the pig (Hale et al. 1996, Long et al. 1998); in the latter little or no apoptotic DNA fragmentation is found in blastocysts produced in vivo. The degree of apoptosis in blastocysts, prevalent in any given in vitro embryo production system, can therefore be used as a tool for indicating the effectiveness and suitability of the system for the needs and developmental potential of the embryos involved. Ultimately, the definitive test of embryo viability remains the survival of embryos after transfer. Up to now the transfer of in vitro produced pig blastocysts has mainly been performed surgically (Hazeleger & Kemp 1999, Marchal et al. 2001, Kikuchi et al. 2002), but also survival rates remain unsatisfactory. Pregnancy rates comparable with those of surgical embryo transfer have been achieved after non-surgical transfer of in vivo produced blastocysts (Li et al. 1996, Yonemura et al. 1998, Hazeleger & Kemp 2001), but to the best of our knowledge non-surgical transfer of in vitro produced blastocysts has not been reported. Furthermore, data to correlate embryo morphological selection criteria and post-transfer survival rates are not available to date, and still need to be established in the actively developing field of in vitro pig embryo production.

Improvements in porcine in vitro embryo production regimens to enhance blastocyst viability, as well as the estimation thereof, are an all-important concern. Therefore, we investigated the effect of GH on in vitro preimplantation embryo development in comparison with in vivo produced embryos. The quality of the embryos was assessed by determining the diameter, blastocoel volume and cell number, the type of apoptosis, and survival after non-surgical transfer. In addition, the expression of mRNA for GHR was determined in early-stage embryos as an indication of the presence of the necessary molecular machinery.

Materials and Methods

Experimental design

RT-PCR was used firstly to ascertain the presence of the necessary molecular apparatus for potential utilization of GH, i.e. GHR mRNA. For this, embryos were produced in vitro and those at the 2-, 4- and 8-cell stages and Day 6 blastocysts were analysed. Upon confirmation of the presence of GHR, the effect of GH on in vitro embryo development was assessed. A total of 1263 (Control) and 1347 (GH) oocytes divided over seven replicates were evaluated for stage of development at 48 h (Day 2), 5 days (Day 5) and 6 days (Day 6) after the addition of sperm for in vitro fertilization (IVF). Of the seven in vitro embryo production replicates, three replicates were destined for blastocyst quality evaluation. These three replicates consisted of 268 oocytes in the Control and 278 oocytes in the GH group. From these oocytes 91 Control and 89 GH blastocysts were produced, of which 68 Control and 83 GH displayed the morphological characteristics required for non-surgical embryo transfer, and were analysed for quality parameters. In vivo derived blastocysts (n = 54) were randomly selected for embryo quality analyses from pooled blastocysts (n = 144) recovered from 26 donors. The remainder of the in vitro produced blastocysts were used for other studies.

The remaining four of the seven in vitro replicates were destined solely for non-surgical embryo transfer purposes. A total of 995 Control and 1069 GH oocytes subsequently led to 313 Control and 357 GH blastocysts. Of these blastocysts, a total of 200 per treatment group were transferred to 16 recipients (n = 8 Control and n = 8 GH). In addition, 80 extra embryos per group (20 per transfer replicate) were also transported to the embryo transfer site as a backup for eventual embryo losses during removal from the transport Eppendorf tubes. Of the remaining non-transferred blastocysts, 27 Control and 20 GH were set aside for ethidium homodimer (EthD-I) staining. For the transfers of in vivo derived blastocysts a total of 953 embryos (morula to expanded blastocyst stages) were recovered from 57 donors. Of these, 575 early to expanded blastocysts were allocated for transfer to 23 recipients. The number of blastocysts surviving after transfer was evaluated on Day 11 of the pregnancy, i.e. 6 days after transfer.

Animals, donors and recipients

All animal experiments were approved by the Ethical Committee for animal experiments of Wageningen University. Synchronization and embryo recovery was performed according to Hazeleger et al. (2000). Briefly, a total of 85
crossbred gilts, 7–11 months old (TOPIGS; Vught, The Netherlands), were used as donors and 39 multiparous crossbred sows (TOPIGS) as recipients (n = 8 for Control; n = 8 for GH; n = 23 for In vivo). Ovulation was synchronized using PG600 (Intervet International BV, Boxmeer, The Netherlands) for first oestrus induction and 2 × 500 μg Estramate (Schering-Plough, Maarsen, The Netherlands) 8 h apart around Day 13 of the first luteal phase. The next day follicular development was induced with 700 IU equine chorionic gonadotrophin (eCG) (Folligonan; Intervet International), followed by 600 IU human chorionic gonadotrophin (hCG) (Chorulon; Intervet International) after 72 h to induce ovulation. The gilts were artificially inseminated 24 and 36 h after hCG administration. Donors were killed 7 days (168 h) after hCG (120 h after estimated ovulation) for collection of Day 5 embryos. Immediately after stunning, bleeding and removal of the genital tract, the embryos were flushed from the uterus horns using Dulbecco’s PBS (DPBS) (BioWhittaker, Verviers, Belgium) supplemented with 1% heat-inactivated lamb serum (GIBCO, Paisley, Strathclyde, UK) and 1% PenStrep (penicillin-G 100 IU/ml and streptomycin sulphate 100 μg/ml; BioWhittaker) at 37°C. The flushing medium was then filtered, and the embryos retrieved by rinsing the filter with Dulbecco’s PBS (DPBS). An average of 16.3 ± 4.5 (mean ± S.D.) blastocysts was recovered per donor (19.5 ± 4.0 corpora lutea). The embryos were then directly transported to the laboratory in a temperature-controlled insulated container kept at 25°C. All non-fertilized oocytes and degenerated zygotes, as well as morulae and hatching or hatched blastocysts were discarded upon stereomicroscopic evaluation. The remaining unexpanded and expanded blastocysts were prepared for non-surgical transfer.

**IVC media**

For oocyte and embryo searching and selection, 25 mM Hepes-buffered Tyrode’s medium containing 0.1% polyvinyl alcohol (TL-Hepes-PVA) was used. The *in vitro* maturation (IVM) medium used for the first 22–24 h of oocyte IVM (IVM-I) was BSA-free North Carolina State University 23 medium (NCSU23) (Petters & Wells 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.8 mM cysteine, 25 μM β-mercaptoethanol, 101 IU/ml eCG and hCG (Folligonan and Chorulon). The second IVM (IVM-II) culture period (18–20 h) occurred without eCG and hCG added to the medium. pFF was collected from follicles 2-6 mm in diameter, centrifuged at 1900 g for 30 min (4°C), filtered through 0.8 μm syringe filter (Millipore SA, Molsheim, France) and stored at −20°C until use. Cumulus-oocyte complexes (COCs) were washed in IVM-wash medium (IVM-II medium with 20 mM Hepes). Modified Tris–buffered medium (mTBM) was used as fertilization medium. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2·2H2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, Caffeine (1 mM) and 0.1% BSA (w/v) (A-6003; Sigma, St Louis, MO, USA) were supplemented to mTBM for use as IVF medium. The *in vitro* embryo culture medium was NCSU23 containing 0.4% BSA (w/v) with 100 ng/ml bovine GH (NIH-B18; National Institute of Diabetes, Digestive and Kidney Diseases National Hormone and Pituitary Programme, NIH, Bethesda, MD, USA). Culture without GH served as a control. Before being placed in the IVC medium, presumptive zygotes were washed in IVC-wash medium, which is similar to the IVC medium, but containing 20 mM Hepes. All culture took place under oil (Heavy Mineral Oil for IVF; Reproline GmbH, Rheinbach, Germany).

**Recovery of oocytes and IVM**

Oocyte recovery, IVM, IVF and embryo culture proceeded as previously described (Kidson et al. 2003). Briefly, ovaries were collected from sows of unknown reproductive status at a local slaughterhouse and transported to the laboratory in insulated containers. Excess connective tissue and oviducts were cut from the ovaries, and they were then washed once at 30°C under running tap water. The ovaries were then placed in a beaker of pre-warmed sterile saline, supplemented with penicillin and streptomycin, and held at 30°C until aspiration. COCs were aspirated from 2–6 mm follicles with an 18-gauge needle fixed to a vacuum pump via a 50 ml conical tube. Contents were collected into the tube and allowed to settle for 10 min at room temperature. The supernatant was removed and sediment was resuspended in TL-Hepes-PVA at room temperature and allowed to settle. This treatment was repeated once more and the content of the tube was observed under a stereomicroscope on a heated stage (38.5°C). COCs surrounded by two or more layers of compact cumulus investment and containing oocytes of equal size were selected, washed twice in IVM-wash medium which had been pre-warmed to 38.5°C prior to use, and transferred in groups of 40–50 to a four-well dish humidified air. After 22–24 h all the oocytes were washed twice in IVM-wash medium and placed in 500 μl IVM-II medium for an additional 16–18 h of culture.

**IVF and embryo culture**

After maturation the oocytes were placed in a wash dish containing pre-warmed equilibrated IVF medium. Using a micropipette, the contents of the dish were vigorously pipetted for 30 s to remove the expanded cumulus cells. The denuded oocytes were washed once more in IVF medium before being placed in 50 μl droplets of equilibrated IVF medium, in groups of 40–50, and incubated at 38.5°C in 5% CO2 in humidified air. After 22–24 h all the oocytes were washed twice in IVM-wash medium and placed in 500 μl IVM-II medium for an additional 16–18 h of culture.
Fresh room temperature Beltsville thawing solution-extended (Johnson et al. 1988) semen was diluted 1:2 in IVF medium (also at room temperature). Sperm were centrifuged in a conical tube for 4 min at 700 g. The supernatant was removed and sperm resuspended in IVF medium and centrifuged again. Following resuspension the sperm concentration was determined and adjusted to achieve a final concentration ± 1000 motile cells/oocyte. After warming to 38.5 °C for 30 min, 50 μl of the sperm suspension were added to the 50 μl IVF droplets containing the oocytes and co-incubated with the oocytes for 20–24 h at 38.5 °C at 5% CO2 in air. Twenty-four hours after insemination, the oocytes were removed from the IVF droplets and washed twice in IVC-wash medium. They were then gently pipetted to remove excess sperm attached to the zona pellucida and transferred in groups of 40–50 into 500 μl IVC medium under oil in a four-well dish. At 48 h after the addition of sperm for IVF, cleavage rate was determined (structures judged to be degenerated or uncleaved were not removed), and on Days 5 and 6 of embryo culture, blastocyst formation was evaluated.

**Determination of cleavage, blastocyst size, volume and fixation**

To determine the efficiency of the IVC systems, embryos were scored morphologically on Days 2, 5 and 6 after the addition of sperm for IVF (Day 0). On Day 2 the percentage of cleaved embryos displaying two to eight evenly sized blastomeres was recorded; embryos with fragmented or uneven-sized blastomeres were categorized as degenerated. The percentage blastocysts, expressed on the basis of the number of oocytes placed into maturation, was evaluated on Day 5 and Day 6. At the termination of the culture period on Day 6, all blastocysts displaying a clear inner cell mass were selected for further evaluation. Both *in vivo* and *in vitro* derived blastocysts were measured for diameter using a graduated eyepiece, fixed individually in 2% paraformaldehyde and stored at 4 °C until further processing. Day 5 *in vivo* produced embryos were regarded to be the equivalent of Day 6 *in vitro* blastocysts due to the retardation of growth experienced in *in vitro* produced embryos (Machaty et al. 1998, Han et al. 1999). Blastocysts were categorized as more developed large expanded blastocysts (Large) when no perivitelline space was visible which corresponded to a diameter ≥180 μm. Blastocysts of lesser development and diameter were categorized as small blastocysts (Small). Blastocyst volume was determined using the formula: 4/3 × π × (diameter/2)³.

**TUNEL and cell counting**

Biochemical detection of DNA strand breaks was performed on each individual blastocyst by using TUNEL (fluorescein isothiocyanate (FITC)-conjugated dUTP and TdT; Roche, Mannheim, Germany) according to the manufacturer’s instructions, with 0.05 μg/ml DAPI (Sigma) as a counterstain. After 3 or more days of fixation the zona pellucida becomes elastic, subsequently allowing complete flattening under the coverslip upon mounting. Fixed blastocysts were washed twice in TL-Hepes-PVA and then permeabilized for 15 min on ice in 0.1% Triton X-100 (0.1% sodium citrate in PBS). Following two more rinses in TL-Heps-PVA, blastocysts were incubated in microdrops (25 μl per 1–12 embryos) of the TUNEL reaction mixture, for 1 h under oil in a humidified atmosphere in the dark. After TUNEL culture the embryos were washed twice in TL-Heps-PVA and then stained with DAPI (5 μl/ml) for 5 min at room temperature in the dark. The embryos were then mounted in a minimal amount of DAPI fluid, flattened completely by applying firm pressure to the cover slip, and examined (× 200 magnification) using a fluorescence microscope (BH2-RFCA; Olympus, Tokyo, Japan) to assess the total number of nuclei and the proportion showing DNA fragmentation. Overlap of nuclei was negligible in all groups. Nuclear morphology (i.e. fragmentation) was assessed whilst viewing through the microscope, but for determining cell numbers two digital images (Nikon Coolpix 990; Nikon Corporation, Tokyo, Japan) of each blastocyst were recorded: (i) using the UV filter for the DAPI image, and (ii) using the FITC filter for the TUNEL image. The number of nuclei was counted after printing of the images.

Five embryos from each treatment group were treated with DNase before TUNEL staining as a positive control for TUNEL labelling; for a negative control the terminal transferase enzyme was omitted during TUNEL labelling. To rule out necrosis, a number of embryos from each group (*in vitro* Control n = 27; *in vitro* GH n = 20) were stained with 4 μM EthD-I (Molecular Probes Europe BV, Leiden, The Netherlands) before TUNEL and DAPI staining. In the case of the *in vivo* derived blastocysts all were triple stained with EthD-I, TUNEL and DAPI.

**Nuclear morphology assessment for apoptosis**

Nuclei were classified according to three nuclear morphologies: (i) ‘healthy’ interphase nuclei with uniform DAPI staining and a clear outline but without TUNEL staining, also including mitotic nuclei; (ii) fragmented nuclei with no TUNEL labelling (FT −); (iii) TUNEL-labelled nuclei which were condensed (T +) and/or fragmented (FT +). Embryos containing only fragmented non-TUNEL stained nuclei, and no other apoptotic morphologies, were designated under the ‘fragmented’ classification. The total nuclei count consisted of all nuclei, whether they displayed apoptosis or not. TUNEL, fragmented and total apoptotic indices were calculated for each embryo as follows: TUNEL index = (no. of TUNEL-positive nuclei, either fragmented or condensed/(total no. of nuclei) × 100); fragmented index = (no. of TUNEL-negative fragmented nuclei)/(total no. of nuclei) × 100; total apoptotic index = (TUNEL-positive nuclei, either fragmented or condensed) + (no. of TUNEL-negative fragmented nuclei)/(total no. of nuclei) × 100.
Non-surgical embryo transfer

Transfer of in vivo and in vitro blastocysts took place as previously described (Hazeleger & Kemp 1999), except that in addition to expanded blastocysts, non-expanded blastocysts were transferred as well. Recipients were prepared similarly and synchronously to the donor animals described above. On the day of transfer in vitro produced blastocysts displaying a clear inner cell mass, and with less than 25% of the blastocyst volume containing extrudeds cells, were placed in Eppendorf tubes, containing transfer medium (DOPBS PBS with 10% lamb serum) at 38.5°C, in batches of 25. They were then allowed to cool to 25°C for transport to the transfer location. In vivo derived embryos were collected as previously described (see Animals), and transported to the transfer station similarly to the in vitro produced embryos. Upon arrival at the transfer station (<2 h after initial collection) the embryos were removed from the Eppendorf tubes, placed in a small Petri dish and photographed using a Polaroid camera (Polaroid Microcam; Polaroid Europe BV, Enschede, The Netherlands) to document the blastocyst diameters prior to transfer. Each batch of 25 embryos was then aspirated into the tip of the transfer catheter (Swinlet; Institute for Pig Genetics, Beuningen, The Netherlands). The transfer procedure consisted of careful passage of the instrument through the cervical folds into the uterine body. The catheter, containing the embryos, was then passed through the instrument and the embryos were deposited in the uterine body with <0.1 ml transfer medium. Recipients were not sedated during the transfer procedure.

On Day 11 after ovulation (6 days after transfer) the recipients were killed to evaluate the survival of transferred embryos. Blastocyst numbers were recorded following recovery from the uterus. Recipients were regarded as having been pregnant following the recovery of one or more blastocysts from the uterus.

RT-PCR

RT-PCR was used to assess the presence of the GHR mRNA in early in vitro produced embryos at the 2-, 4- and 8-cell stage, and the blastocyst stage on Day 6 of embryo culture. The embryos were washed four times in TL-Hepes-PVA, placed in Eppendorf tubes in groups of ten and frozen at -80°C until use. For each developmental stage, 30 embryos, divided over three replicates, were analysed.

Poly(A)⁺ RNA was isolated, following the manufacturer's instructions, from groups of ten 2-, 4- and 8-cell embryos and Day 6 blastocysts using a Dynabeads mRNA Direct Micro kit (Dynal, AS, Oslo, Norway). Briefly, 100 μl lysis/binding buffer (100 mM Tris–HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% (w/v) lithium dodecysulphate (LiDS), 5 mM dithiothreitol (DTT)) were added to the frozen samples and pipetting was repeated to obtain complete lysis. Twenty microlitres of prewashed Dynabeads Oligo (dT)25 were added to each tube and mixed thoroughly. After 5 min incubation at room temperature the beads were separated using a Dynal MPC-E-1 magnetic separator. The beads were washed in 100 μl washing buffer (10 mM Tris–HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% (w/v) LiDS) and twice in 100 μl washing buffer 2 (10 mM Tris–HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA). The mRNA was then eluted from the beads by incubating in 20 μl RNAse-free water at 65°C for 2 min. Reverse transcription was done in a total volume of 20 μl containing 10 μl sample RNA, 4 μl 5 × reverse transcriptase buffer ( Gibco BRL, Breda, The Netherlands), 8 U RNAseIn (Promega Benelux BV, Leiden, The Netherlands), 150 U SuperScript II reverse transcriptase (GIBCO BRL), 0.036 U random primers (Life Technologies BV, Leiden, The Netherlands) and final concentrations of 10 μM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C, for 5 min at 80°C, and stored at -20°C. Minus RT blanks were prepared under the same conditions but without reverse transcriptase.

Oligonucleotide primers used for amplification of the GHR mRNA were based on the porcine GHR cDNA sequence as described in the Genbank database (Genbank accession number X544291995). Amplification of the cDNA was performed in two stages with GHRrL1 (5'-TGTCCAGAGCCTCAGAGTC-3', sense, position 105–124) and GHRrL1 (5'-GTCTCTAGTTCAGGTGAACG-3', antisense, position 187–206). The second round of PCR was performed to increase the recovery of the final product. Reactions were carried out in 200 μl tubes (Eurogentec, Seraing, Belgium) using 1 μl cDNA as template in 25 μl PCR mixture containing final concentrations of 2 mM MgCl₂, 200 μM of each dNTP and 0.5 μM each of primers and 0.625 U Taq DNA polymerase (HotStarTaq; Qiagen, Valencia, CA, USA) in 1 × PCR buffer. The thermal cycling profile for the first round was: initial denaturation and activation of the polymerase for 15 min at 94°C, followed by 40 cycles of 15 s at 94°C, 30 s at 55°C and 45 s at 72°C. Final extension was for 7 min at 72°C. For the second round of amplification, 1 μl of the first-round product was transferred to another 200 μl tube containing 24 μl PCR buffer as above, and amplified for 30 cycles according to the same profile. All PCRs were performed in a 24-well thermocycler (Perkin-Elmer, Gouda, The Netherlands). Ten microlitres of the second-round product were resolved by 1% agarose gel containing 0.4 μg/ml ethidium bromide. A 100 bp ladder (GIBCO BRL) was included as a reference for fragment size. An image of the gel was taken using a digital camera (Olympus C-4040; Olympus, New York, NY, USA) and stored in digital form. A standard sequencing procedure (ABI PRISM 310 Genetic analyser; Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) was used to verify the analytical specificity of the PCR product, and compared with the Genbank database as described by Cioffi et al. (1990).
Statistical analyses

All experiments consisted of a minimum of three replicates. Statistical analysis of embryo development data was carried out using Fisher's exact test for the cleavage and blastocyst-formation rates, as well as for the incidence of apoptosis. After testing for normality (Kolmogorov–Smirnov test with Lilliefors correction) and testing for equal variances (F-test for two groups and Bartlett's test for multiple groups) the means of blastocyst diameters, nuclei count and nuclear damage were compared by unpaired t-test, or ANOVA (followed by Bonferroni multiple pair wise comparison) where appropriate. Data are presented as means ± S.E.M. Differences at *P* ≤ 0.05 were considered significant. All analyses were done using the statistical analysis program GraphPad Prism (Graphpad Software, San Diego, CA, USA).

Results

Expression of GHR mRNA

Amplification of cDNA from different stages of porcine preimplantation embryo development, primed with GHR-specific primers, resulted in one abundant PCR product after two rounds of amplification in all the samples (Fig. 1). Sequence analysis on the amplified GHR band showed 100% homology with the sequence described in the Genbank database.

Embryo development

There were no differences in the percentage of embryos displaying 2, 4 or 8 evenly sized blastomeres on Day 2, or blastocysts developing on Day 5 and Day 6 of IVC after IVF, in the two treatment groups (Fig. 2). The proportions of fragmented/degenerated embryos and uncleaved oocytes also were not different between the treatment groups (data not shown). Overall, 34% of oocytes placed in IVF had undergone cleavage up to the 8-cell stage on Day 2 of IVC, and almost completely developed to the blastocyst stage on Day 6, constituting a 97 and 103% conversion of embryos to the blastocyst stage in the Control and GH treatment groups respectively.

Diameter, volume and nuclei count of in vitro Day 6 and in vivo Day 5 blastocysts

In general, treatment of embryos with GH significantly increased the blastocyst diameter, but *in vitro* derived blastocysts irrespective of treatment were smaller in diameter than their *in vivo* counterparts (Table 1). The proportion of Large:Small blastocysts was similar in all groups. Blastocysts classified as Small did not differ in diameter according to either treatment or source of embryo. Large blastocysts in the Control group were on average smaller than those treated with GH, which again were smaller than *in vivo* produced Large blastocysts. The blastocoel volume did not differ for treatment within the Small blastocysts.
group. Large in vitro derived blastocysts had a significantly smaller volume than in vivo blastocysts, and this was similar when all blastocysts from each treatment group were taken into account. Similarly, blastocysts cultured in the presence of GH had an equal mean number of nuclei to embryos cultured in the absence of GH (41 ± 1 and 40 ± 2, for GH and Control respectively), which was significantly lower than that of in vivo produced blastocysts (51 ± 3). GH treatment also did not benefit the number of nuclei contained in Large blastocysts, but in vivo derived Large blastocysts had a greater number of nuclei than both in vitro derived groups (P < 0.001; ANOVA). The total number of nuclei ranged from 18 to 73 and 18 to 71 in the Control and the GH-treated groups respectively, and from 22 to 102 in the in vivo group.

### Apoptosis in Day 6 in vitro and Day 5 in vivo produced blastocysts

No indications of apoptosis (either morphological or biochemical) were found in blastocysts that had been produced in vivo. Large blastocysts in the in vitro produced Control group were more predisposed to biochemical apoptosis labelling by TUNEL than Small blastocysts, whereas GH-cultured blastocysts, irrespective of size (Table 2), contained fewer TUNEL-labelled nuclei overall. No necrosis was found in any embryos in any of the three groups. In the Control group, the majority of blastocysts containing nuclei presenting apoptotic morphology (fragmentation) in the absence of TUNEL labelling were found in the Small group, with only a small percentage of Large Control blastocysts presenting this characteristic. An equal proportion of the GH-treated Small and Large blastocysts contained TUNEL-positive apoptotic nuclei. Differences in appearance were apparent for fragmented TUNEL-positive nuclei (FT +) and fragmented TUNEL-negative nuclei (FT -) as presented in Fig. 3: FT + consisted of either two larger nuclear fragments, or one large and one small fragment; whereas FT - consisted of mostly a larger number of highly condensed very small fragments. The total incidence of apoptosis (TUNEL-positive

### Table 1 The effect of GH treatment during porcine in vitro embryo culture on different embryo quality parameters in Day 6 in vitro produced blastocysts, as compared with in vivo produced Day 5 blastocysts.

<table>
<thead>
<tr>
<th></th>
<th>Small blastocysts (&lt;180 µm)</th>
<th>Large blastocysts (&gt;180 µm)</th>
<th>All blastocysts</th>
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<tr>
<td></td>
<td>Control</td>
<td>GH</td>
<td>In vivo</td>
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<tr>
<td>No. of blastocysts</td>
<td>40</td>
<td>48</td>
<td>32</td>
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<tr>
<td>Per cent of all blast</td>
<td>58.8</td>
<td>57.8</td>
<td>59.3</td>
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<td>Diameter (µm)</td>
<td>159.4 ± 1.4b 161.2 ± 1.1a</td>
<td>159.1 ± 2.0d 195.1 ± 3.5f</td>
<td>201.5 ± 4.4b 226.9 ± 5.1c</td>
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<tr>
<td>Volume (µm³ × 10⁶ ± S.E.M.)</td>
<td>2.1 ± 0.3a 2.2 ± 0.3a</td>
<td>4.0 ± 0.3d 4.5 ± 0.3a</td>
<td>6.8 ± 0.3b 2.9 ± 0.2a 3.2 ± 0.2a 4.6 ± 0.2b</td>
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<tr>
<td>Number of nuclei (mean ± S.E.M.)</td>
<td>38 ± 2a 40 ± 2a</td>
<td>34 ± 1a 42 ± 2a</td>
<td>65 ± 3b 40 ± 2a 41 ± 1a 51 ± 3b</td>
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</tbody>
</table>

Diameter: a, b, c: Within rows for each stage of development, values with different superscripts are significantly different; P < 0.001 (ANOVA). Number of nuclei: a, b, c, d: Within rows for each stage of development, values with different superscripts are significantly different; P < 0.05 (ANOVA).

### Table 2 Apoptosis in Day 6 porcine Small or Large blastocysts cultured in the presence or absence of GH following IVM and IVF of sow oocytes.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small blastocysts (&lt;180 µm)</td>
<td>Large blastocysts (&gt;180 µm)</td>
</tr>
<tr>
<td>No. of blastocysts (N)</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>TUNEL (DNA damaged) (%)</td>
<td>35.0**</td>
<td>60.7**</td>
</tr>
<tr>
<td>Fragmented only (%)</td>
<td>20.0*</td>
<td>3.5*</td>
</tr>
<tr>
<td>Total apoptosis (%)</td>
<td>55.0</td>
<td>64.3</td>
</tr>
<tr>
<td>TUNEL index</td>
<td>5.5 ± 0.8 (n = 496)</td>
<td>6.2 ± 1.1 (n = 684)</td>
</tr>
<tr>
<td>Fragmented index</td>
<td>5.0 ± 1.1 (n = 242)</td>
<td>3.7 ± 0.0 (n = 53)</td>
</tr>
<tr>
<td>Total apoptotic index</td>
<td>6.2 ± 0.7</td>
<td>7.9 ± 1.3b</td>
</tr>
</tbody>
</table>

|                  | Small blastocysts (<180 µm) | Large blastocysts (>180 µm) |
| GH               | 48                 | 35                     |
| TUNEL (DNA damaged) (%) | 14.6b        | 20.0b      |
| Fragmented only (%) | 33.3           | 28.6b       |
| Total apoptosis (%) | 47.9             | 48.6         |
| TUNEL index | 4.2 ± 0.9 (n = 214) | 5.5 ± 1.0 (n = 265) |
| Fragmented index | 4.9 ± 1.0 (n = 677) | 4.0 ± 0.5 (n = 383) |
| Total apoptotic index | 5.0 ± 0.8       | 4.8 ± 0.6e |

Within rows and between treatment groups: a, b, c: P < 0.05; a, b, c, d: P < 0.01; d: P < 0.001; within rows and within treatment groups: *P < 0.05.

N = total number of blastocysts per category containing ≥1 apoptotic nuclei.

n = total number of nuclei in blastocysts containing ≥1 apoptotic nuclei.

†Total = combination of TUNEL and fragmented only.
and fragmented only blastocysts) did not differ between the treatment groups nor did the size of the blastocysts (Table 2). GH treatment significantly reduced the percentage of apoptotic nuclei (apoptotic index) in Large blastocysts containing ≥1 apoptotic nuclei, but not in Small apoptotic blastocysts. Also, the percentage of either TUNEL-labelled or fragmented nuclei per blastocyst did not differ for treatment group or blastocyst diameter. When taking into account both Small and Large in vitro produced blastocysts, Fig. 4 shows the differences in apoptosis morphology between the Control and GH group blastocysts cultured with or without GH. The manner in which apoptosis was manifested differed greatly between the Control and the GH-treated blastocysts, of which the latter contained fewer TUNEL-labelled apoptotic nuclei.

**Embryo transfer**

As shown in Table 3, the non-surgical transfer of in vitro produced blastocysts resulted in pregnancy rates comparable with those achieved when in vivo produced blastocysts were transferred. Although GH-treated blastocysts had a higher pregnancy rate than Control blastocysts, the difference was not significant. Furthermore, the number of blastocysts surviving transfer was greatly reduced in the in vitro blastocyst litters. Albeit that care was taken to randomly distribute blastocysts according to size, retrospective analysis of blastocyst diameters (images were recorded before transfer as described in the section on Non-surgical embryo transfer) revealed that both the mean diameter ($P < 0.01$) and volume ($P < 0.001$) of the batches of transferred embryos were significantly greater for those that led to the establishment of pregnancies, irrespective of in vivo

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**Figure 3** Phenotypic appearance of normal or apoptotic nuclear fragmentation in Day 6 in vitro produced porcine blastocysts. (Ia) DAPI-filter image of a blastocyst containing A = condensed nucleus, B = fragmented nucleus, S = sperm on zona pellucida; (Ib) corresponding FITC-filter image of the blastocyst in (Ia) showing A = condensed TUNEL-positive nucleus and B = fragmented TUNEL-positive nucleus; (II) DAPI-filter image of a blastocyst containing F = fragmented TUNEL-negative nuclei and M = mitotic nucleus; (III) DAPI-filter image of a healthy blastocyst containing Int = normal interphase nuclei (Bar = 35 μm).
or in vitro treatment group. All non-pregnant batches consisted of ‘Small’ blastocysts on average.

**Discussion**

In the present study we have examined the effect of GH on in vitro embryo development in the pig. In addition, to gain more insight into the functional aspects of blastocyst quality in the pig we compared in vitro with in vivo derived blastocysts with regard to blastocyst diameter, volume, cell number, apoptosis and survival following non-surgical embryo transfer. In general, in vitro produced porcine embryos are known to be retarded in their development when compared with their in vivo counterparts and also contain fewer nuclei (Papaioannou & Ebert 1988, Han et al. 1999). Following the positive effects that GH has on bovine in vitro embryo development and quality (Kölle et al. 2001, 2002, Mtango et al. 2003) it presented a promising tool for improving the quality of in vitro produced embryos in the porcine. The present data, though, show for the first time that GH addition during in vitro porcine embryo culture does not improve blastocyst formation or cell numbers, nor reduce apoptosis, but alters the pattern of apoptosis in Day 6 blastocysts. Another novel finding is related to the diameter-expanding effect of GH, which points to modulation of the metabolic activity of GH-treated blastocysts.

First, despite the presence of GHR during early in vitro embryo development in the pig, no effect of GH on rate of blastocyst formation, or blastocyst cell numbers, was found. Although both the maternal and the embryonic genome express GHR (porcine embryonic genome is activated at the 4-cell stage (Prather & Day 1998, Anderson et al. 1999, 2001)), the question now arises whether the GHR is functional in pre-blastocyst stage pig embryos. In the bovine (Izadyar et al. 2000, Kölle et al. 2001) and mouse (Drakakis et al. 1995, Fukaya et al. 1998) the functionality of the GHR from early cleavage stages has been clearly illustrated where GH significantly improved cleavage and blastocyst development rates. The absence of any GH-mediated effect on porcine embryo development could be due to the specific metabolic needs of the porcine embryo. In the mouse it has been reported that GH significantly stimulates glucose uptake in blastocysts in a dose-dependent manner (Pantaleon et al. 1997a,b) by direct recruitment of glucose transporters. In preimplantation prepertual gill embryos, glucose has recently been shown to inhibit early development in vitro while a significant increase in glucose uptake was found at the blastocyst stage (Swain et al. 2002). This effect is similar in the mouse (Chatot et al. 1989), hamster (Seshagiri & Bavister 1989) and rat (Miyoshi et al. 1994) where glucose is known to suppress embryo development before compaction or before the blastocyst stage. As porcine embryos do not utilize glucose until the blastocyst stage (Swain et al. 2002), pre-blastocyst stage embryos could not take advantage of the glucose-uptake-promoting effect of GH, and hence no cell proliferation effect of GH was seen.

![Figure 4](image-url) The distribution of different apoptotic morphologies among the cohort of in vitro produced porcine blastocysts containing ≥1 apoptotic nuclei and cultured in vitro with (n = 83) or without (n = 68) GH (FT–: TUNEL-negative fragmented nuclei; FT+: TUNEL-positive condensed nuclei; FT+T+: TUNEL-positive fragmented nuclei).

**Table 3** Pregnancy rates and embryo characteristics after non-surgical transfer of in vitro produced (IVP) and in vivo produced porcine blastocysts.

<table>
<thead>
<tr>
<th></th>
<th>In vivo blastocysts</th>
<th>Control IVP blastocysts</th>
<th>GH IVP blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of transfers</td>
<td>23</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>No. of embryos transfered in total</td>
<td>575</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Pregnant</td>
<td>192.30 ± 2.1*</td>
<td>186.42 ± 2.87*</td>
<td>187.41 ± 2.59*</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>173.10 ± 1.1</td>
<td>175.77 ± 2.44</td>
<td>177.14 ± 2.21</td>
</tr>
<tr>
<td>Mean blastocyst diameter (μm ± S.E.M.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean blastocyst volume (μm³ ± S.E.M.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>3.7 ± 0.2**</td>
<td>3.4 ± 0.2**</td>
<td>3.5 ± 0.2*</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>2.7 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>No. of embryos recovered in total</td>
<td>28</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Per cent of embryos pregnant on Day 10</td>
<td>30 (7/23)</td>
<td>25 (2/8)</td>
<td>38 (3/8)</td>
</tr>
</tbody>
</table>

Within columns pregnant differs significantly from non-pregnant: *P < 0.01; **P < 0.001.
The main effect of GH was seen in the modulation of apoptosis, which has been prominent in the literature during the past 2 years. In the human (Hardy 1999), mouse (Brison & Schultz 1997) and bovine (Watson et al. 2000) the occurrence of apoptosis in preimplantation embryos has been identified both in vivo and in vitro. It is likely that blastocyst stage apoptosis acts to eliminate damaged cells no longer required (i.e. undifferentiated trophoblast cells inappropriately present in the inner cell mass), or developmentally incompetent, and is thought to be a part of the cellular ‘quality control’ within the developing embryo (Byrne et al. 1999). In this study and other studies from our laboratory (Rubio-Pomar et al. 2004) in vivo produced sow embryos presented none of the classical features of apoptosis such as nuclear condensation and blebbing/fragmentation (karyorhexis), or DNA fragmentation (karyolysis). This is in contrast to the findings of Long et al. (1998), who found apoptotic nuclei in the majority of expanded in vivo (70.8%) and in vitro (90.3%) produced blastocysts. The differences in results may be due to donor age, as all in vivo derived blastocysts in this study were collected from older gilts than those of Long et al. (1998) and oocytes for in vitro embryo production were harvested from slaughterhouse sows, and not prepubertal gilts. Sow and prepubertal gilt oocytes differ in their ability to support normal fertilization, and subsequent blastocyst quality is superior in sow embryos produced in vitro (O’Brien et al. 2000, Marchal et al. 2001). Although no comparisons have been made between sow and gilt blastocysts regarding the incidence and degree of apoptosis it is highly likely that they would differ in this aspect as well. This study, in concurrence with previous studies from our laboratory (Rubio-Pomar et al. 2004), thus strongly indicates that under ideal in vitro circumstances porcine blastocysts should not contain any apoptotic cells.

In this current experimental design, in agreement with bovine studies (Kölle et al. 2002) GH significantly reduced the incidence of karyolysis as detected by TUNEL, as well as the apoptotic index (percentage apoptotic nuclei in each blastocyst presenting ≥1 apoptotic nuclei). However, the combined levels of karyolysis and karyorhexis (total incidence of apoptosis) were not decreased by GH addition to the culture medium. In the majority of papers investigating apoptosis in embryos only karyolysis is taken into account when determining the incidence of apoptosis. The fact that some nuclei display apoptotic morphology (karyorhexis) in the absence of TUNEL staining may have different reasons and deserves careful consideration. Evaluation of both morphological and biochemical characteristics supports the comprehensive assessment of apoptosis, first to distinguish the stage of apoptosis progression and secondly because different apoptotic pathways have specific substrate targets during the execution phase of apoptosis (Guthrie & Garrett 2001). During the final stages of apoptosis, karyolysis is thought to precede karyorhexis (Collins et al. 1997, Hardy 1999, Gjørret et al. 2003), which means that a larger proportion of non-GH-treated blastocysts displayed earlier stages of apoptosis than the GH-treated blastocysts. In the light of the recently published glucose requirements of pig embryos (Swain et al. 2002) it may thus be hypothesized that GH exerts its effect only from the early blastocyst stage onwards where it then prevents or reduces the onset of ‘new’ apoptosis as blastocyst development progresses. On Day 6 of embryo development, GH-treated blastocysts are thus ‘healthier’ as they are not suffering from the cumulative effects of IVC induced apoptosis.

Alternatively, it is a known fact that dissociation between apoptosis and DNA fragmentation can occur in blastocysts as well as other cell types (Sakahira et al. 1999, Cavaliere et al. 2001, Taylor et al. 2001, Hinck et al. 2003). It is thought to be linked to the pathway or specific trigger of apoptosis. In studies investigating apoptosis in the bovine (Gjørret et al. 2003) and apoptosis and the metabolism of human (Spanos et al. 2000), mouse (Kamjoo et al. 2002) and rat embryos (Hinck et al. 2003) nuclear fragmentation in the absence of TUNEL labelling has been reported. A mitochondrial protein, bcl-2, involved in the modulation of caspase activity, has been shown to protect the rat blastocyst from chromatin degradation due to hyperglycaemic culture conditions, but not from nuclear fragmentation (Pampfer et al. 2001). In both the mouse and bovine blastocysts (Jurisicova et al. 1998, Kölle et al. 2002) GH increases the expression of bcl-2 which is known to suppress caspase-3 cleavage, and inhibits TUNEL-detected apoptosis in this way (Lamothe & Aggarwal 2002, Liang et al. 2002). Caspase-3, via caspase-activated DNase, leads to DNA fragmentation, whereas caspase-6, via lamin/nuclear-mitotic apparatus protein, brings about chromatin condensation and fragmentation (Guthrie & Garrett 2001, Hinck et al. 2003). It is thus conceivable that, in porcine embryos, GH reduces caspase-3-dependent apoptosis but does not influence caspase-6-dependent apoptotic pathways. The potential interaction of GH and glucose on early in vitro produced porcine embryos should be ascertained to establish the relationship with specific apoptotic pathways in subsequent blastocysts.

Embryo diameter is as yet an unexplored gauge for evaluating blastocyst quality, as only one report exists in which larger in vivo produced pig blastocysts led to a higher percentage of pregnancies and litter size after nonsurgical transfer (Hazeleger et al. 2000). In this respect GH had indeed slightly improved blastocyst quality by increasing the mean diameter of the blastocysts, albeit that the cell numbers had not been improved. In contrast to the in vivo produced blastocysts, the diameter of both the groups of in vitro blastocysts had increased without a concurrent increase in cell number. This finding could point to anomalies in either the cell proliferation activity of the in vitro produced blastocysts or the osmotic/secretory activity of the blastomeres responsible for blastocoel formation. When taking into account the blastocyst volume, although not statistically significant, the increase in diameter induced by GH constitutes a
10% increase in blastocyst volume. This effect could indicate superior metabolic activity of the larger, as opposed to the smaller, in vitro derived blastocysts. The trophoderm plasma membrane sodium pump, Na\(^{+}/K^{+}\) ATPase, plays a critical role in the formation and maintenance of the blastocoel, which in turn is essential for preparing the embryo for implantation (reviewed by Watson 1992). A direct relationship exists between glucose consumption of the embryo for implantation (reviewed by Watson 1992). A the blastocoel, which in turn is essential for preparing the embryo for implantation (reviewed by Watson 1992). A.


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Acknowledgements

The authors thank: the EU Commission (grant number QLK3-CT 1999-00104); the National Institute of Diabetes, Digestive and Kidney Diseases National Hormone and Pituitary Programme, NIH, Bethesda, MD, USA for kindly supplying bovine GH (NIH-B18); Varkens Aktiviteiten Centrum, Bunnik, The Netherlands, for the supply of boar semen; Ramon Cabrera for logistical support; and Hiemke Knijn for critical comments on the manuscript.


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Received 28 May 2003
First decision 5 August 2003
Revised manuscript received 22 September 2003
Accepted 20 October 2003