In vitro embryo development in the pig

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Assisted reproductive technologies (ART), such as in vitro embryo production, cryopreservation and transfer, have become an integral part of commercially applied breeding techniques in a variety of animal species [1]. Whereas progress of genetics through ART has played a core role in food-producing domestic species, such as the cow and sheep, during the past two decades it has not shown the same progress in the pig. Its importance as a food-producing animal, as well as a biological model in human biomedical applications, such as transgenesis directed at the pig as donor of cells, tissues and organs (xenotransplantation) [2], has prompted increased efforts to improve the efficiency of reproductive technologies in the pig. Despite the establishment of several systems to generate embryos in vitro, the efficiency is still poor and the quality of resultant blastocysts inferior compared with their in vivo counterparts. This limits the improvement of other reproductive techniques, such as embryo transfer and embryonic stem cell technology, which are dependent on the blastocyst as source of material. Advanced reproductive technologies rely more on the basic techniques of oocyte maturation and fertilization, and the development and identification of baseline requirements for these processes are essential and still much in need of further refinement in the pig.

In vitro embryo production (IVP) places major demands on oocytes and embryos with regard to developmental transitions, i.e. oocyte maturation, fertilization, compaction, blastocyst formation, all of which need to be traversed successfully in order for the embryo to develop to a new individual. These transitions are defined by exquisite remodelling of the gamete, zygote and embryo at intra- and intercellular levels, which sustains the continuation of its developmental program. Oocytes and embryos need to summon many signal transduction mechanisms, cytoskeletal constituents and genes in order to successfully negotiate and complete all the necessary developmental processes. Support of all these functions and processes is of cardinal importance when applying in vitro embryo production techniques to produce embryos capable of generating functional cell lines or live healthy offspring. Therefore, IVP systems are generally comprised of three stage-specific culture environments, specially designed to supply in the needs of each stage of oocyte, zygote and embryo development: 1) in vitro oocyte maturation (IVM), 2) in vitro fertilization (IVF), and 3) in vitro embryo culture (IVC). Although culture media have been adapted to suit the specific individual needs of the pig oocyte and embryo (i.e. North Carolina State University-23 (NCSU-23) medium [3]), not much progress in the improvement of embryo viability has been made since the adoption of its use a decade ago. Chronic and cumulative stresses caused by sub-optimal culture conditions or insufficiencies in the constituents of the culture medium, can lead to oocyte or embryo anomalies at a genetic or metabolic level and severely jeopardise the viability of the resultant embryo [4]. Markers for the best developmental potential of pig oocytes and embryos are scarce, thus confronting us with a dilemma, as we cannot estimate the developmental outcome for the in vitro period or the subsequent remaining in vivo period. As we become more sophisticated in the in vitro production of pig embryos, so our battery of markers will increase and eventually improve our understanding and design of each separate phase of the IVP system for the pig.

In vitro maturation of oocytes

Oocyte maturation consists of two aspects, i.e. nuclear and cytoplasmic maturation (Figure 1).

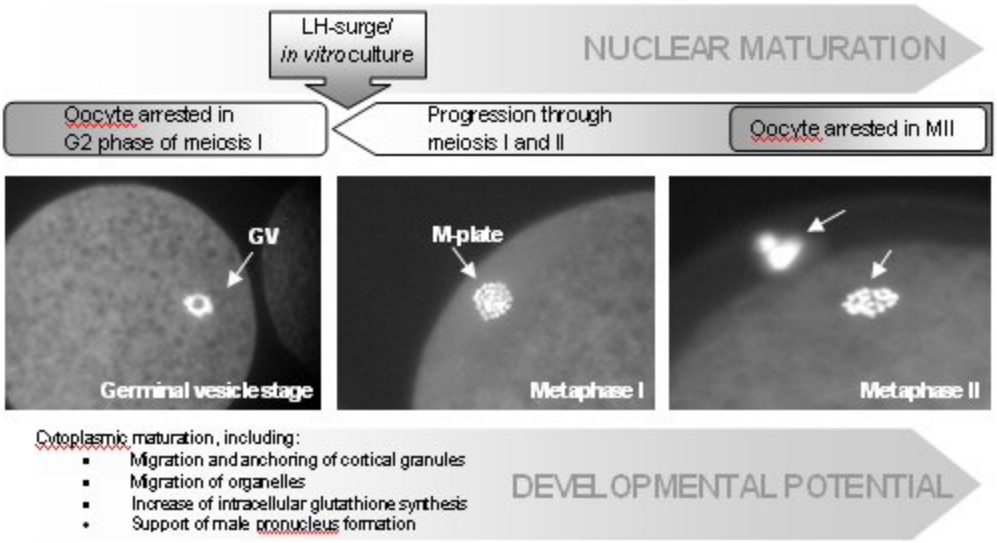

Figure 1. Follicular oocytes are arrested in the G2 phase (germinal vesicle stage) of the first meiotic division. The immature oocyte will resume meiosis and complete its final maturation upon the LH surge in vivo, or after being liberated from its follicular environment in vitro. Resumption of meiosis is characterized by condensation of the chromatin, breakdown of the germinal vesicle and organization of the chromosomes into the metaphase I (MI) plate and spindle. At this stage the oocyte enters into meiosis II and upon extrusion of the first polar body remains arrested in the metaphase II (MII) stage until activation of the oocyte takes place at fertilization. Parallel with nuclear maturation, cytoplasmic maturation/capacitation of the oocyte progresses and is characterized by migration of the cortical granules to the periphery of the ooplasm, migration of the mitochondria to the inner cytoplasm, and increased synthesis of glutathione (GV: germinal vesicle; M-plate: metaphase plate).

Nuclear maturation refers to the resumption of meiosis and progression to the metaphase II (MII) stage, whereas cytoplasmic maturation encompasses other, poorly understood, maturational events related to the cytoplasmic capacitation of the oocyte. These processes are believed to progress in parallel to one another, and synchronization of nuclear and cytoplasmic maturation are essential for establishing optimal oocyte developmental potential. Cumulus-oocyte-complexes (COCs) are typically harvested from the ovaries of slaughterhouse sows and prepubertal gilt, and specifically from follicles 3 to 6 mm in diameter. Selection of follicle size ensures the harvest of fully grown oocytes and hence the ability of the oocytes to resume and complete meiosis [5] [6] [7]. Hormonal supplements, such FSH, eCG or hCG, are added to the IVM medium in order to mimic the in vivo situation and stimulate nuclear maturation of the oocyte. Since its beneficial effects became known a decade ago [8] [9], it is now common practice in the pig to add these supplements only during the first half of maturation. Follicular fluid supplementation of the IVM medium has been proven to provide a beneficial microenvironment for the further development of the immature pig oocyte [8] [9]. Under these circumstances pig oocytes develop to MII without much difficulty, with a reasonable degree of cytoplasmic maturation. Nevertheless, the cytoplasmic component of oocyte maturation is not completely satisfactory and believed to be incomplete or delayed. Efficiency of cytoplasmic maturation includes the ability of the oocyte to block the penetration of more than one sperm [10], and also to support the decondensation of the sperm head within the ooplasm of the fertilized oocyte. Whereas nuclear maturation can be evaluated by simple nuclear staining methods, such as aceto-orcein or DAPI, cytoplasmic maturation can only be determined by indirect means such as the blastocyst yield and cell number, glutathione content of the oocyte and the percentage male pronucleus formation. Cytoplasmic maturation of pig oocytes can be improved by reducing oxidative stress caused by the COCs production of reactive oxygen species (ROS) due to the in vitro culture environment [11] [12]. Metabolic pathways, mediated by enzymes such as glutathione, control ROS cellular levels and protect the oocyte against the damaging effects of oxidative stress. Glutathione content of the oocyte can be increased by adding thiol compounds such as cysteine, cysteamine, glutamine, ß-mercaptoethanol and/or follicular fluid to the maturation medium [13] [14]. Apart from its protective action, glutathione also increases amino acid transport and stimulates DNA and protein synthesis [15] [16].

An important element for both nuclear and cytoplasmic maturation of the oocyte, are the layers of cumulus cells surrounding the oocyte. These cells act as a 'go-between' between the oocyte and the follicular or culture environment. Intracellular communication between the oocyte and the cumulus cells takes place via gap junctions which are facilitated by means of microfilamentar transzonal projections of the cumulus cells directly in contact with the zona pellucida, i.e. corona radiata cells [17]. These processes transverse the zona pellucida and terminate upon the oocyte plasma membrane (oolemma), thereby establishing a route of communication by which direct transfer of substances important for oocyte growth and maintenance of meiotic arrest can take place [18]. Cumulus cells also play a protective and metabolic role in oocyte cytoplasmic maturation, by reducing cystine to cysteine and promoting the uptake of cysteine in the oocyte [12], thereby protecting the oocyte against oxidative stress caused by ROS. By evaluating the degree of expansion in response to gonadotrophin stimulation [19] [20] [21] [22], cumulus cell function and viability can be estimated and serves as a gauge to approximate the COCs developmental potential. Hormonal stimulation also regulates remodelling of the actin cytoskeleton at the level of the cumulus cell transzonal processes, but also within the oocyte cortex. The actin cytoskeletal changes, mediated by the cumulus cells, are believed to stabilize the distribution of cortical granules in the cortex of the oocyte and aid in the migration of organelles such as mitochondria and the endoplasmic reticulum which also influences the ability of the oocyte to support normal fertilization [23] [24]. Other factors known to enhance nuclear and cytoplasmic maturation of the oocyte include somatic cell co-culture, or addition of growth factors to the IVM medium, which can have beneficial effects on embryo development and blastocyst cell number [25].

In vitro fertilization

The main feature, widely perceived to be a distinctive trait in porcine IVF, is the high prevalence of polyspermic fertilization. Polyspermy is an abnormality of the fertilization process (Figure 2), during which more than one sperm enters the oocyte due to failure of initiation or completion of the zona reaction to block the entry of multiple sperm (Figure 3).

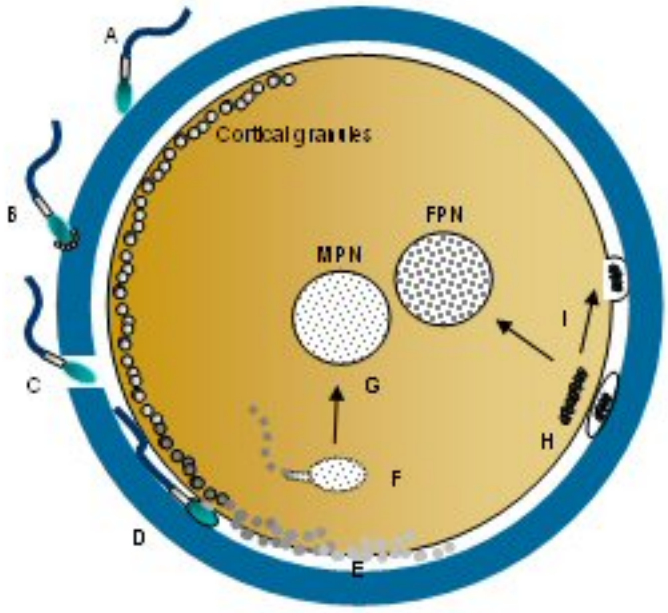

Figure 2. A schematic overview of the event sequence occurring during fertilization. (A) Acrosome intact, capacitated sperm bind to the zona pellucida, thereby (B) triggering the acrosome reaction. The enzymatic acrosomal contents lyse the zona pellucida (C), thus enabling the now hypermotile sperm cell to enter the perivitelline space, fuse with the oolemma (D) and activate the oocyte. Upon activation, release of cortical granule content takes place (E) which prevents the penetration of supplementary sperm. The sperm head, now incorporated within the ooplasm, begins to swell and decondense (F) eventually forming the male pronucleus (G). The oocyte, until now arrested in metaphase II (H),

commences progression through meiosis II to extrude the second polar body (I), and form the female pronucleus (I). The male and female pronuclei are now in apposition, as a final prelude to syngamy.

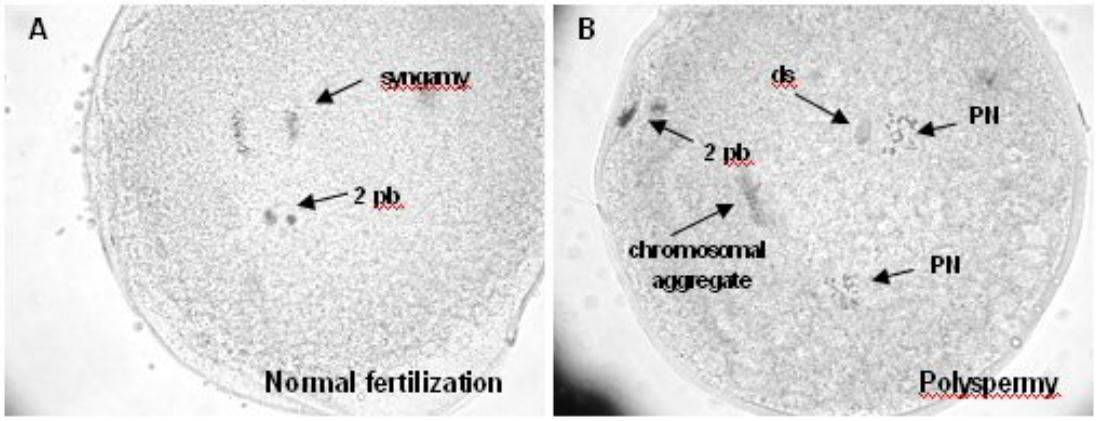

Figure 3. Micro-images of orcein stained porcine oocytes 20 h after the addition of sperm to the oocytes for IVF. In A) a normally fertilized oocyte is seen, with two polar bodies and the male and female chromosomal complement involved in the process of syngamy. In B), polyspermic fertilization has taken place and although two pronuclei can be seen, they are accompanied by a decondensed sperm head, and an extra set of aggregated chromosomal material. (2 pb: 2 polar bodies; ds: decondensed sperm head; PN: pronucleus)

It is a potentially lethal condition, which in the majority of cases leads to the early demise of the conceptus. The exact cause of this phenomenon, which has an exceptionally high prevalence in the pig, has not yet been determined. Only recently has it been linked to the age of the oocyte donor. In the greater majority of IVP studies in the pig, oocytes are harvested from the ovaries of prepubertal gilts out of necessity, due to the relative unavailability of adult sow ovaries. In other species, such as the cow, sheep and goat [26] [27] [28], differences in oocyte developmental potential between prepubertal and adult donors have been reported and the prepubertal oocyte used as a negative model for studying oocyte developmental competence [26]. Similar studies in the pig have shown that age of the donor appeared to be the main cause of the polyspermy phenomenon and subsequent poor developmental of the porcine oocyte [29] [30]. Conversely, penetration rates exceeding 80% are typically achieved in prepubertal gilt oocytes, but polyspermy rates rarely measure less than 40%. When using sow oocytes, polyspermy rates in the range of 10% are routinely achieved, but penetration rates remain low (40-50%) [29] [30]. An ideal IVF system should support a high sperm penetration rate (>80%) and a low polyspermy rate (<10%).

In neither donor age group has the optimal fertilization result been achieved yet. Various strategies for the reduction of polyspermy have been developed, focussing on enhancement of either oocyte cytoplasmic maturation or modulation and adaption of sperm and IVF treatments. This has included co-culture of the male and/or female gamete with oviductal secretions or proteins, supplementation of fertilization media with glycosaminoglycan or thiol compounds, and IVF in the presence of cumulus cells [31]. Although these approaches have led to some degree of improvement, the level of polyspermy in prepubertal gilt oocytes remains far from satisfactory or comparison with that achieved in sow oocytes.

Akin to the calf and lamb, prepubertal gilt oocytes show intrinsic differences in both cytochemical and ultrastructural aspects [28] [29] [32]. Redistribution of organelles (cortical granules and mitochondria), also linked to cytoplasmic maturation, does not occur to the same extent in prepubertal gilt as in ovulated oocytes [33], although cortical granule (CG) exocytosis appears to be normal [34]. In sheep, the size of the mitochondria and CGs are smaller, and the glutamine metabolism lower in prepubertal than in adult oocytes. The size of the perivitelline space (PVS) has also been linked to the ability of the oocyte to prevent polyspermy, as ovulated oocytes have a much wider PVS than those matured in vitro [34]. Culture of oocytes in low NaCl medium results in a wider PVS and a also a reduction in polyspermy [9]. As pre-ovulatory oocytes have a narrower PVS and suffer a similar fate as in vitro maturated oocytes [35], post-ovulatory contact with the oviductal environment appears to supply the oocyte with the cytoplasmic machinery

required to prevent polyspermy. Furthermore, after ovulation cumulus cells surround the oocyte at the time of sperm contact and thus also play an important role in promoting fertilization [18]. In the pig, though, due to the initial high polyspermy rates following IVF of cumulus-intact oocytes in early studies [36] [37] [38] [39], it is now common practise to denude COCs prior to fertilization, which may further contribute to the poor fertilization results commonly experienced.

Baseline values and characteristics, for sow vs. prepubertal gilt oocytes, yet need to be established, and will help to elucidate the intrinsic age-related differences between prepubertal and adult oocytes in order to identify specific means by which the developmental potential of prepubertal oocytes can be enhanced.

Embryo culture and development

Advanced technologies, such as embryo transfer or embryonic stem cell studies, rely on the blastocyst as its source of material. The production of large numbers of viable blastocysts is therefore one of the main goals in the majority of porcine IVP laboratories [4]. In the pig, the initially experienced developmental block at the 4-cell stage was overcome by adapting the then available culture media to suit the development and specific needs of the pig embryo. Current embryo culture systems are now able to support in vitro-derived zygotes up to the blastocyst stage. In fact, a number of porcine IVP groups routinely obtain a blastocyst formation rate of 30% or more, from in vitro matured oocytes [30] [40] [41] which is on par with that achieved in other farm animal species [42] [43]. The percentage of zygotes cleaved to the four to eight-cell stage 48 h after IVF, can be used as a gauge to estimate the potential of cleaved zygotes to develop to the blastocyst stage. Those embryos that consist only of evenly sized blastomeres are believed to have a better chance of further development than zygotes with cytoplasmic fragments, which frequently contain no nuclei. To which extent fragmentation is linked to polyspermy is not yet fully established, but cytoplasmic fragmentation has also been associated with oocyte aging and early developmental arrest [44].

Using the blastocyst yield as the ultimate parameter for evaluating the success of a given porcine IVP system is also not without its pitfalls. The resultant blastocyst should also be of optimal quality and viability. The term embryo quality is a fuzzy concept in any species, but even more so in the pig where non-invasive means or criteria for scoring blastocyst quality have not yet been developed. When taking into account the abovementioned problems with cytoplasmic maturation, and subsequent polyspermy, appearances can be deceptive as far as blastocyst quality are concerned in the pig. Polyspermic zygotes readily develop to blastocysts [45], but their viability after transfer is severely restricted: of blastocysts developing from polyspermic zygotes, 78% have been reported to suffer from abnormal ploidy [45] and also lag behind in development compared with their normospermic counterparts [46] [47]. In vitro produced blastocyst differ distinctly from their in vivo counters parts, with the former containing much lower numbers of nuclei. This is thought to be due to deficiencies in actin filament content and distribution within the cytoplasm [44], most likely related to poor cytoplasmic maturation of the original oocyte. Parameters used in other species to define embryo quality include blastocyst morphology, total and inner cell mass cell number, kinetics of development, post-cryopreservation survival, chromosomal abnormalities, metabolism, gene expression and apoptosis [48]. Of these parameters only blastocyst morphology and kinetics of development are non-invasive techniques, and neither has been developed for use in pig embryos yet. The remaining factors have indeed proven differences between in vivo and in vitro derived porcine blastocysts, but none have been utilised to distinguish viable from non-viable embryos.

One parameter of particular interest in the pig is apoptosis, as in vivo-produced blastocysts present few or no apoptotic cells, whereas in vitro produced embryos show a much higher incidence. Apoptosis, or programmed cell death, serves a variety of purposes such as elimination of inappropriately differentiated inner cell mass cells [49], removal of cells containing chromosomal abnormalities such as haploidy [50], or of cells failing to activate the embryonic genome [51] [52]. It is therefore a naturally occurring cellular process in which the cell literally commits suicide without the neighbouring cell noticing. Apoptosis differs from necrosis in that coordinated gene-directed energy-dependent processes are required for its execution [53]. A cell

undergoing apoptosis presents characteristic morphological features in which the cell shrinks, accompanied by organelle and cytoplasmic condensation, chromatic condensation, nuclear and DNA fragmentation, blebbing of the plasma membrane, cell fragmentation and finally phagocytosis by neighbouring cells [54]. The cascade of apoptotic events (Figure 4) is initiated by two pathways: an intrinsic and an extrinsic pathway [55] [56].

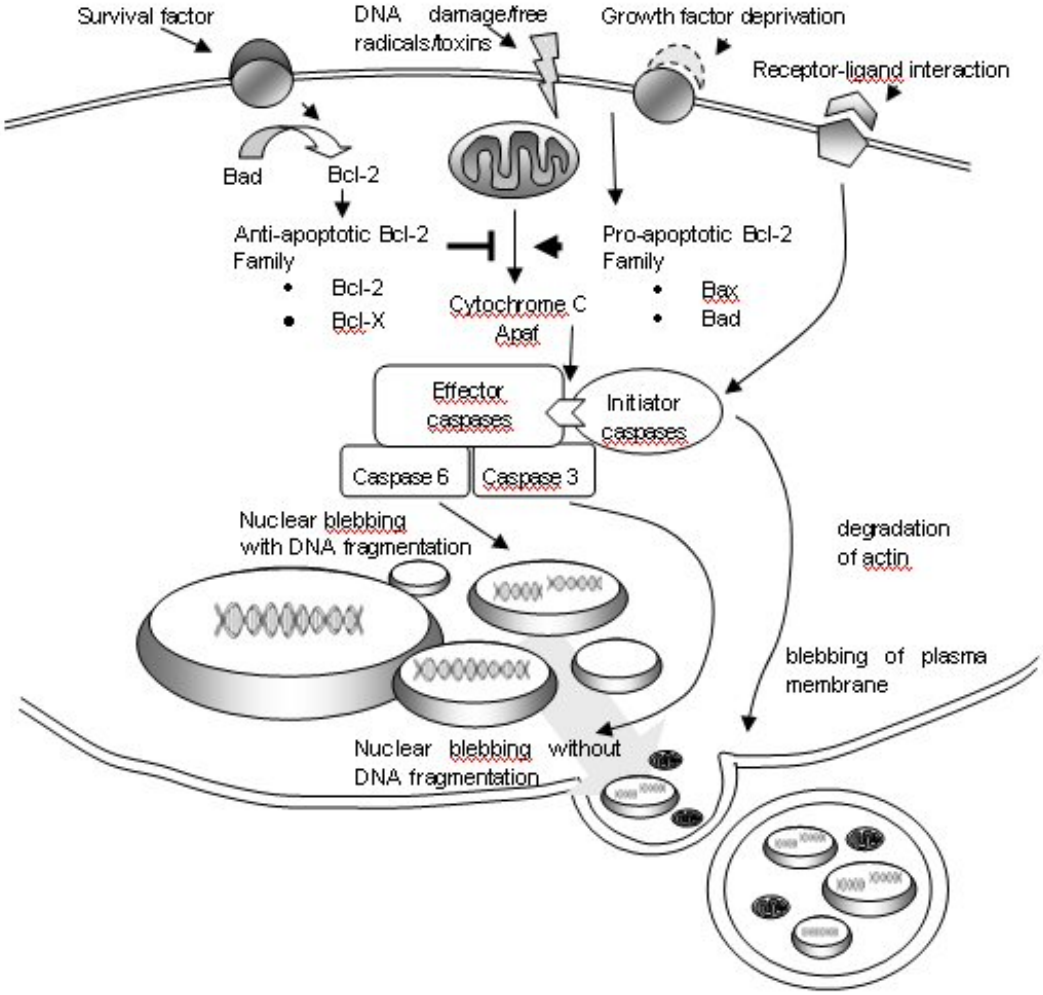

Figure 4. Schematic overview of the proposed major apoptotic pathways relevant in the embryo. The mitochondria-mediated 'intrinsic' pathway can be triggered by a wide range of stimuli, including deprivation of growth factors, presence of toxic substances or excess free radicals or metabolic stress. In contrast, apoptosis can be inhibited by presence of survival factors, which include growth factors and hormones. The system is regulated by the interplay of proapoptotic and antiapoptotic proteins of the Bcl-2 family. The proapoptotic proteins, such as Bad, contain a domain which forms a heterodimer with Bcl-2, blocking its survival promoting activity. The abundance of these pro- and antiapoptotic proteins determines the susceptibility of the cell to apoptosis. The proapoptotic proteins act on the mitochondrial membrane by decreasing the transmembrane potential and promoting leakage of cytochrome c. In the presence of dATP, cytochrome c activates Apaf, which in turn activates downstream caspases which results in apoptosis. The "extrinsic" receptor-dependent pathway, is initiated by ligand-receptor binding which acts directly on the initiator caspases. This family of caspases are responsible for proteolytic events e.g. cleavage of cytoskeletal components such as actin which are thought to be responsible for plasma membrane blebbing.

The intrinsic pathway, mediated via the mitochondria, may be activated by culture stress conditions including deprivation of growth factors, presence of toxic substances, excess free radicals or metabolic stress. These stimuli act directly on the pro-apoptotic Bcl-2 family, by phosphorylating Bad/Bax which in turn acts on the mitochondria causing the leakage of cytochrome c, which activates the apoptosis protease activating factor (apaf) [55] [56]. The initiator caspases are then cleaved leading to activation of downstream caspases which in turn cause nuclear and/or DNA fragmentation. The extrinsic, or receptor dependent pathway, is

initiated by ligand-receptor binding which acts directly on the initiator caspases. This family of caspases are responsible for proteolytic events e.g. cleavage of cytoskeletal components such as lamin/actin which are thought to be responsible for plasma membrane blebbing. The nuclear apoptotic features are telltale signs of apoptosis which can be visualized using relatively simple fluorescent DNA-labelling techniques [52].

Embryo transfer

The acid test for determining the developmental potential of in vitro produced embryos is their survival after transfer to the uterus of a recipient female. In the pig, embryo transfer is still in its development stages which further complicate the progression of the associated ART-forms. Determining blastocyst viability is only one of many potential applications of ET, and the combination of IVP and ET holds much promise for both the agricultural and biomedical application of porcine embryo-related technologies. At the top of the pig breeding pyramid, the combination of ET and IVP could introduce complete genomes of a superior combination of animals (instead of 50% through sperm of a superior boar), minimize the risk of pathogen transfer associated with the introduction of breeding animals into a herd, and also reduce the costs and welfare issues of live animal transport. The immediate impetus of ET/IVP, though, lies in the potential progression of developing technologies such as transgenesis and nuclear transfer [57] [58], which of course relies on IVP for the production of blastocysts suitable for transfer.

Transfer of in vivo produced pig blastocysts has mainly been performed surgically, with reasonable success rates [59]. At present, 60% of transfers lead to pregnancies, with 60% of transferred embryos surviving to farrowing. Recently, it was reported that the transfer of IVP blastocysts could lead to pregnancies and live births [30] [60], albeit at a low degree of efficiency. In these surgical transfer studies, vast numbers (50 to 100) of randomly chosen IVP blastocysts were transferred to uterus of recipient animals resulting in less than 20 % survival to farrowing. Explanations for these poor survival rates are most likely found in the inherent poor quality of in vitro produced blastocysts, worsened by the lack of selection criteria. It is therefore not yet clear whether an accurate picture of the survivability of IVP blastocysts is created by the recent transfer results. Data to correlate embryo morphological selection criteria and post-transfer survival rates therefore still need development to serve the needs of the actively developing field of in vitro pig embryo production.

Surgical transfer of porcine embryos is not allowed in the Netherlands due to the invasive nature of the procedure, which has necessitated the development of non-surgical ET (nsET). Being a less invasive procedure, requiring neither surgery nor sedation [61], nsET promises to be a valuable tool for both research and commercial purposes, due to its on-farm applicability and reduced need for special facilities. The design of a pig-specific transfer instrument allowed the transcervical deposition of embryos into the uterine body of the recipient sow [59] [61] [62]. Pregnancy rates comparable to that of surgical ET have been achieved after the non-surgical transfer of in vivo produced blastocysts [61] [63], but embryo survival rates are slightly lower necessitating the transfer of larger numbers of embryos per recipient. One of the problems affecting embryonic survival is the synchronicity of the donor embryos and the recipient uterus.

The ultimate culture environment for pig embryos is the pig reproductive tract itself, but due to the complex nature of the female reproductive tract with its convoluted cervical canal, and long coiled nature of the uterine horns, the non-surgical retrieval of oocytes and embryos cannot be achieved. Surgical embryo collection is also not an option, due to animal welfare considerations and the labor intensive nature of the procedure. Ovaries collected from female pigs, slaughtered at abattoirs, provide a favorable alternative for the supply of large numbers of oocytes. Such oocytes, though, are still immature and require further maturation before they can be utilized for any of the purposes described above.

Our studies briefly reviewed here have explored various modifications of our core porcine in vitro embryo production system, by addition of selected biological supplements during in vitro oocyte maturation and the following phases of in vitro embryo production. Fundamental features of oocyte maturation and fertilization were studied in order to improve our understanding of the mechanisms critical to the overall developmental proficiency of resultant blastocysts.

Due the relative experimental nature of the non-surgical embryo transfer procedure and the cost and labor involved, an alternative method was therefore necessary to estimate or predict the overall health and wellbeing of in vitro produced porcine embryos. We subsequently developed a novel method for testing blastocyst function, namely cytochalasin-B (CytB) challenge. When blastocysts were exposed to this actin-filament depolarizing agent for a short period of time, until collapse of the blastocoel, and placed back into culture a direct correlation between blastocyst quality and its ability to re-expand after CytB removal was evident. Similar to the use of sucrose which causes shrinkage of the viable blastomeres, blastocoel collapse by CytB also allowed for more precise assessment of cells extruded in the perivitelline space and thus an accurate evaluation of blastocyst morphology. As CytB does not affect the viability of oocytes or embryos [64] [65], it is believed to be a non-invasive technique which could allow for the accurate selection of quality and function-proven blastocysts. Such embryos could then be applied for transfer or other purposes such as its use as embryonic stem cell donor.

Final remarks - ex ovo omnia -

The in vitro production of embryos is a complex assembly of procedures, based on our very limited knowledge and understanding of the intracellular processes fundamental to oocyte development. It is therefore quite remarkable that oocytes matured and embryos produced in vitro, still maintain a reasonable degree of function and developmental competence. In our studies we investigated a number of culture modifications, based on the conditions present in the female reproductive tract, to improve the developmental outcome of our IVP procedures. In vitro conditions cannot truly replicate in vivo conditions, and features of oocyte and embryo morphology, metabolic and biochemical properties can be altered by the in vitro environment. These alterations can become evident as errors that may be compatible with early embryonic development, but deleterious for the viability of the embryo. We have learned that treating the cause instead of the symptoms produces the most encouraging results. That is to say, the early development of the embryo appears to depend on the maturation microenvironment of the oocyte. By simply augmenting the oocyte culture environment with elements from the naturally occurring milieu in the mature sow follicle, significant improvements in the overall outcome of the IVP procedure can be obtained. As we become more sophisticated in our experience with in vitro embryo production in the pig, so will our knowledge increase and eventually improve our understanding and design of each separate phase of the IVP system for the pig.

> "Keep sowing your seeds, for you never know which will grow, perhaps they all will."
>
> Ecclesiastes 11:6

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