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The evolution of porcine embryo *in vitro* production

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ABSTRACT

The *in vitro* production of porcine embryos has presented numerous challenges to researchers over the past four decades. Some of the problems encountered were specific to porcine gametes and embryos and needed the concerted efforts of many to overcome. Gradually, porcine embryo *in vitro* production systems became more reliable and acceptable rates of blastocyst formation were achieved. Despite the significant improvements, the problem of polyspermic fertilization has still not been adequately resolved and the embryo *in vitro* culture conditions are still considered to be suboptimal. Whereas early studies focused on increasing our understanding of the reproductive processes involved, the technology evolved to the point where *in vitro*-matured oocytes and *in vitro*-produced embryos could be used as research material for developing associated reproductive technologies, such as SCNT and embryo cryopreservation. Today, the *in vitro* procedures used to mature oocytes and culture embryos are integral to the production of transgenic pigs by SCNT. This review discusses the major achievements, advances, and knowledge gained from porcine embryo *in vitro* production studies and highlights the future research perspectives of this important technology.

1. Introduction

Forty years ago, procedures fundamental to swine artificial breeding, such as semen collection, estrous synchronization, and artificial insemination, had only recently been described and were still being developed. In the very first issue of volume one of *Theriogenology*, a study on the induction of parturition in the sow was reported [1], reflecting the applied animal production focus of reproductive biology research at that time. Research into the production of embryos by IVF predominantly utilized the gametes of laboratory animal species, and was largely the domain of clinical researchers. It was not until the 1970s that the gametes of livestock species were commonly used for *in vitro* studies of oocyte and embryo biology, and the development of advanced reproductive technologies. Whereas the *in vitro* production (IVP) of cattle and sheep embryos became well established in the 1980s, the IVP of porcine embryos was not achieved to an acceptable level until the early 1990s.

After the initial successes in producing live offspring of livestock species using IVM and IVF procedures, it was clear that this technology could be harnessed to increase the yield of embryos obtainable from genetically superior females. In addition to utilizing more of their ovarian pool of immature oocytes, thereby reducing reproductive wastage, oocytes could potentially be recovered post-mortem from valuable females and subjected to IVM and IVF for the purpose of genetic rescue. An increase in the rate of genetic gain could also be achieved by applying this technology to immature oocytes recovered from juvenile females, which would significantly reduce the generation interval. However, it was the need for large numbers of embryos for research purposes and the production of transgenic animals in the late 1980s and 1990s that drove the continued development of porcine embryo IVP systems.

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Table 1
Milestones of porcine embryo manipulations and *in vitro* production procedures that have resulted in the birth of live piglets.

Year	Details of manipulation/*in vitro* production procedure	Reference
1985	*In vivo* zygotes/transgene insertion by PN microinjection	Brem et al. [196]
		Hammer et al. [197]
1986	*In vivo* oocytes/IVF with fresh ejaculated sperm	Cheng et al. [2]
1988	*In vivo* oocytes/IVF with FT epididymal sperm	Nagai et al. [6]
1989	IVM oocytes/IVF with extended ejaculated sperm	Mattioli et al. [7]
	In vivo oocytes/NT using 4-cell stage blastomeres	Prather et al. [130]
	In vivo embryos/FT at the peri-hatching blastocyst stage	Hayashi et al. [116]
1993	IVM oocytes/IVF with fresh ejaculated sperm	Yoshida et al. [198]
1995	*In vivo* embryos/FT at the 4-cell stage[c]	Nagashima et al. [120]
1997	*In vivo* oocytes/IVF with sex-sorted sperm	Rath et al. [199]
1998	IVM oocytes/IVF with sex-sorted sperm	Abeydeera et al. [200]
2000	*In vivo* oocytes/SCNT embryos	Onishi et al. [127]
		Polejaeva et al. [128]
	IVM oocytes/SCNT embryos	Betthauser et al. [129]
	In vivo oocytes/NT using 4-cell stage blastomeres	Li et al. [145]
2001	IVP embryos/IVC to the 2- to 4-cell and blastocyst stages	Marchal et al. [25]
	SCNT embryos[a]/GM donor cells	Park et al. [136]
2002	IVP embryos/IVC to the blastocyst stage[b]	Kikuchi et al. [96]
	In vivo zygotes/IVC medium chemically defined[b]	Yoshioka et al. [105]
	SCNT embryos[a]/targeted GM donor cells	Dai et al. [131]
2003	IVM oocytes/IVF and IVC media chemically defined[b]	Yoshioka et al. [108]
2006	SCNT embryos[a]/IVC to the blastocyst stage[b]	Lagutina et al. [134]
	SCNT embryos[a]/FT at the blastocyst stage[bc]	Li et al. [117]
2007	IVP embryos/FT at the 4- to 8-cell stage[c]	Nagashima et al. [119]
	SCNT embryos[a]/handmade cloning[b]	Du et al. [132]
2009	IVP embryos/IVM, IVF and IVC media chemically defined[b]	Akaki et al. [88]
	IVP zygotes/FT at the PN stage	Somfai et al. [122]
	SCNT embryos[a]/handmade cloning/GM donor cells[b]	Kragh et al. [133]
2011	SCNT embryos[a]/FT at the morula stage[bc]	Nakano et al. [121]
	SCNT embryos[a]/handmade cloning/targeted GM donor cells[b]	Luo et al. [135]
2012	IVP embryos/non-surgical embryo transfer[b]	Yoshioka et al. [107]
	IVP embryos/FT at the morula stage[b]	Maehara et al. [118]

Abbreviations: FT, frozen-thawed or vitrified-warmed; GM, genetically modified; IVC, *in vitro* culture; IVF, *in vitro* fertilized/fertilization; IVM, *in vitro* matured/maturation; IVP, *in vitro* produced/production (IVM, IVF and IVC); NT, nuclear transfer; PN, pronuclear.

[a] IVM oocytes were used to generate the recipient cytoplasts.

[b] Embryos were cultured *in vitro* for 5 or 6 days before transfer to recipient females.

[c] Embryos were delipated, which involved the physical removal of cytoplasmic lipid droplets by centrifugation [121] and micromanipulation [117,119,120].

2. Early porcine embryo IVP

Early efforts to produce porcine embryos *in vitro* focused on developing effective IVF procedures and usually utilized ovulated oocytes collected from donor animals. The first birth of live piglets derived from IVP embryos [2] (Table 1) was reported in *Theriogenology* in an abstract presented at the 12th annual conference of the International Embryo Transfer Society. After a 4-hour pre-incubation of ejaculated boar sperm, Cheng et al. [2] successfully fertilized ovulated oocytes *in vitro* and cultured the resulting embryos to the two- to four-cell stage. A total of 19 piglets were born after transfer of these embryos to recipient gilts [2]. The IVF system they described transformed research in this area, which had been plagued by a lack of success up until this time. Important features of this system included a pre-incubation medium adjusted to pH 7.8 that was supplemented with fetal calf serum, sodium pyruvate, and calcium lactate, and an IVF medium that contained a relatively high level of calcium (4.7 mmol/L) [2,3]. Of course, it would be remiss not to acknowledge the contributions that numerous researchers made before this milestone (reviewed in [4]), which paved the way to achieving successful sperm capacitation *in vitro* in the pig.

Although the initial successes in producing piglets from IVP embryos were achieved using ejaculated sperm [2,3], the difficulties associated with capacitating ejaculated sperm prompted others to use sperm recovered from the epididymides of slaughtered boars. Nagai et al. [5] observed striking differences in the ability of ejaculated sperm and epididymal sperm to penetrate oocytes after co-incubation for 20 hours, and concluded that even a brief exposure of sperm to seminal plasma caused their pre-incubation conditions to be ineffective for inducing capacitation. Nagai et al. [6] subsequently used pre-incubation conditions similar to those described by Cheng et al. [2], and IVF medium supplemented with 10 mg/mL BSA and 2 mmol/L caffeine to fertilize ovulated oocytes with frozen-thawed epididymal sperm. Piglets were produced after transfer of the resulting embryos to the oviducts of recipient gilts [6] (Table 1). The use of BSA and caffeine as capacitating agents, an important breakthrough first achieved in other species, proved to be instrumental for successful fertilization in the pig (Fig. 1).

Because of their ready availability and low cost, ovaries collected at local abattoirs were widely used as an abundant source of immature oocytes in the majority of studies. In 1989, Mattioli et al. [7] reported that abattoir-sourced