Theriogenology 85 (2016) 125–134

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years

Raquel Romar [a], Hiroaki Funahashi [b], Pilar Coy [a,*]

[a] Department of Physiology, Faculty of Veterinary, University of Murcia, Campus Mare Nostrum, IMIB-Arrixaca, Murcia, Spain
[b] Department of Animal Science, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan

ARTICLE INFO

Article history:
Received 20 April 2015
Received in revised form 8 July 2015
Accepted 12 July 2015

Keywords:
IVF
Pig
Oviduct
Polyspermy

ABSTRACT

Assisted reproduction technology (ART) protocols are used in livestock for the improvement and preservation of their genetics and to enhance reproductive efficiency. In the case of pigs, the potential use of embryos for biomedicine is being followed with great interest by the scientific community. Owing to the physiological similarities with humans, embryos produced *in vitro* and many of those produced *in vivo* are used in research laboratories for the procurement of stem cells or the production of transgenic animals, sometimes with the purpose of using their organs for xenotransplantation. Several techniques are required for the production of an *in vitro*–derived embryo. These include *in vitro* oocyte maturation, sperm preparation, IVF, and further culture of the putative zygotes. Without doubt, among these technologies, IVF is still a critical limiting factor because of the well-known, but still unsolved, question of polyspermy. Despite the improvements made in the past decade, current IVF systems hardly reach 50% to 60% efficiency and any progression in porcine ARTs requires an unavoidable improvement in the monospermy rate. It is time, then, to learn from what happens under *in vivo* physiological conditions and to transfer this knowledge into ART. This review describes the latest advances in porcine IVF, from sperm preparation procedures to culture media supplements with special attention paid to molecules with a known or potential role in *in vivo* fertilization. Oviductal fluid is the natural medium in which fertilization takes place, and, in the near future, could become the definitive supplement for culture media, where it would help to solve many of the problems inherent in ARTs in swine and improve the quality of *in vitro*–derived porcine embryos.

1. Introduction

Owing to its relevance in the production of embryos for commercial purposes or for biomedical studies, pig IVF has been the focus of attention for research laboratories, and several review articles on this topic have been published over the years [1–5]. Citing just a few examples, experiments have been conducted into the role of different molecules included in the culture media [6,7], as well as the culture conditions themselves. Gamete coincubation times [8,9], sperm concentration [10,11], the source of spermatozoa [12–14], the source of oocytes [15,16], or the effect of co-culture with somatic cells [17–19] are all factors that influence embryo production. Overall, the main objective of these studies was to improve the frustratingly low success rates of pig IVF by reducing the consistently high levels of polyspermy.

More recently, specific studies have tried to recreate *in vitro* the ideal conditions for the concurrence of the physiological mechanisms that lead to fertilization. Molecular biology, microarray technologies [20], or, more recently, RNA sequencing [21] mean that it is now possible to determine the main genes that are upregulated or downregulated in the oviductal tissue at specific time points before and after the gametes encounter each other [22]. Similarly, liquid chromatography-tandem mass

* Corresponding author. Tel.: +34 868884789; fax: +34 868884147.
E-mail address: pcoy@um.es (P. Coy).

0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.07.017

spectrometry (LC-MS/MS) [23] and techniques for relative isotope-coded affinity tag [24], or, in the near future, absolute selected reaction monitoring in tandem MS quantitation will provide knowledge of protein profiles and concentrations in the oviductal epithelium and fluid at the time of fertilization; these data could potentially be transferable to guide the composition of culture media. However, the extensive information that these tools will generate (when a significant number of studies in pig become available) is difficult to translate into useful laboratory protocols, so that the molecules or procedures of interest will need to be carefully chosen. Similarly, video microscopy and other imaging technologies would enable the visualization of sperm and oocytes in oviductal explants [25], or even *in vivo*, leading to a reinterpretation of the cell ratios at fertilization, the patterns of sperm movement, or the time interval for the release of spermatozoa from their oviductal epithelial cell attachments in the isthmus reservoir. Such data will also be useful for designing new protocols for sperm treatment before IVF.

The present review aims to collate the most recent information about porcine IVF and porcine oviductal molecular and microscopic physiology to help researchers obtain the best rates of *in vitro* porcine embryos.

2. Sperm preparation methods: Can we move toward more physiological protocols?

In general, fresh epididymal or ejaculated boar spermatozoa, in some cases after liquid preservation or cryopreservation, have been prepared for the IVF of porcine oocytes. Seminal plasma and/or extender contain components that function as decapacitation factors that must be removed before coincubation with oocytes. The fertilization medium, besides, contains chemicals that induce capacitation at suitable concentrations. However, sperm preparation methods seem to affect sperm capacitation status and penetrability *in vitro* [13]. Here, we discuss current methods for preparing spermatozoa intended for IVF.

2.1. Boar semen in the female reproductive tract

Under *in vivo* physiological conditions, epididymal spermatozoa are mixed with seminal plasma in the male reproductive tract just before ejaculation, the components of which play an active role in the transportation and survival of viable spermatozoa in the female reproductive tract [26]. After ejaculation, boar spermatozoa are already coated with a large amount of spermadhesins (AQN-1, AQN-2, AQN-3, AWN-1, and AWN-2), which are multifunctional proteins involved in boar sperm capacitation and gamete recognition [27]. Because most of these spermadhesins are removed from the surface of ejaculated spermatozoa during capacitation, a large subpopulation of boar spermadhesins are believed to function as decapacitation factors, whereas the remaining ones, which are tightly bound to the spermatozoa, may play a role as positive capacitation factors and/or in gamete recognition [27]. Porcine seminal plasma proteins I and II (PSP-I/PSP-II) have been reported to exert a decapacitation effect on highly extended boar spermatozoa [28]. The seminal plasma PSP-I/PSP-II spermadhesin, when present *in vitro*, blocks sperm–ZP binding [29]. Cholesterol is also known to be the predominant inhibitor of capacitation [30].

After artificial insemination, spermatozoa, seminal plasma, and semen extenders in the female reproductive tract all play roles in the induction of postmating uterine inflammation characterized by increased levels of cytokines, polymorphonuclear leukocytes, and mononuclear cells [31–34]. Seminal plasma suppresses polymorphonuclear leukocytes migration into the uterus after mating and enhances the rate of disappearance of uterine inflammation [35]. Moreover, contact between seminal plasma and the epithelium of the uterotubal junction is essential for the transduction of the local signals involved in the advancement of ovulation [36]. This means that part of the seminal plasma somehow must reach the uterotubal junction after insemination. There is also evidence that seminal factors influence ovarian function [31,37], the timing of ovulation, CL development, and progesterone synthesis [38]. Seminal plasma also stimulates the active transport of spermatozoa through the female reproductive tract [39] and increases the number of fertilized oocytes attaining the viable blastocyst stage [38]. As spermatozoa pass through the female reproductive tract from the cervix to the uterotubal junction, the seminal plasma may be reduced in volume by diffusion and backflow, and, consequently, spermatozoa may be separated from seminal plasma. The major proteome of boar seminal plasma and the association between specific seminal plasma proteins and semen parameters have been recently published opening the basis for determination of molecular markers of sperm function in the swine species [40].

In some species, in which spermatozoa are ejaculated into the vagina, ovulatory cervical mucus is a candidate for the removal of cholesterol and glycerophospholipids from the sperm plasma membrane, acting as a “sperm membrane scrubber” [41]. However, in pigs, in which semen is ejaculated into the cervix entering the uterus, the detailed mechanism of how seminal plasma is eliminated probably differs from the way in which it occurs in other species. Seminal plasma is somehow separated from spermatozoa, and cholesterol may be partially removed from the sperm plasma membrane. This is probably as a result of high uterine sterol sulfatase activity, promoting an increase in membrane fluidity [41]. After reaching the uterotubal junction, carbohydrate-mediated binding with the epithelium traps the spermatozoa. Although carbohydrate-binding proteins (AQN-1) of uncapacitated spermatozoa can bind to the exposed high-mannose type N-glycans of oviductal membrane glycoproteins (LAMP-1/2 and others), the coating proteins dissociate from the surface, exposing proteins of the multimeric receptors (AWN, AQN-3, P47, and others) in capacitated cells, allowing binding to the ZP through the recognition of a set of neutral complex N-glycans [42]. Cholesterol appears to be further removed from the sperm plasma membrane to increase membrane fluidity, which is a prerequisite for subsequent membrane fusion, i.e., acrosome reaction by albumin and high-density lipoprotein in the oviduct fluid [43] after scramblase activation *via* a bicarbonate adenylate cyclase protein kinase A signaling pathway [44].