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In Vitro Production of Porcine Embryos

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ABSTRACT

There have been intensive attempts to establish reliable methods for in vitro production (IVP) methods forof porcine embryos. Although a great deal of progress has been made, our current IVP systems still need to be improved. In this review, we focused on studies about in vitro maturation and fertilization (IVM-IVF) of porcine oocytes and their in vitro culture (IVC), especially on an excellent piglets production system using modified IVP system producing porcine blastocysts with high quality.

INTRODUCTION

Pigs have been playing an important role in meat production. They produced over 25% of the energy and 5% of the protein that human obtained from animal sources in early 1990s (1), and the percentages may be getting higher now because of BSE in cattle. Currently, new applications of pigs outside animal industry are drawing attention, especially in the field of human medicine. It is hoped that pig organs can be used as human replacement organs in the face of the serious shortage of human organs for xenotransplantation (2). The use of pigs as experimental animals is also gaining ground; for example, they are useful models for studying arteriosclerosis (3). Thus, their application in fields other than traditional animal industry is expanding. The new technologies to produce transgenic and cloned pigs also provide us with the potential of very rapid genetic manipulation and dissemination. All such technologies involve manipulating oocytes

and/or embryos in vitro. Thus the concept of in-vitro maturation (IVM) and in vitro fertilization (IVF) of oocytes in pigs has a particular importance now. Since Mukherjee (4) reported that mouse oocytes can be matured and fertilized in vitro and developed to term in 1972, there have been intensive attempts in pigs, and Mattioli et al. (5) succeeded in getting piglets from IVM-IVF oocytes in 1989. Although a great deal of progress has been made, our current IVM-IVF systems still suffer from two major problems: a high rate of polyspermy and a low rate of development of IVM-IVF embryos to the blastocyst stage and their low quality compared with in vivo produced embryos in pigs (6). Recently Kikuchi et al. (7), however, reported an excellent IVC system afterforfor IVM-IVF oocytes using a low oxygen tension during IVM and modified IVC system producing high quality of blastocysts and succeeded in getting piglets. In this review, we would like to focus on studies about in vitro production (IVP) of porcine oocytes embryos and then try to suggest practical ways to enhance the efficiency of this reproductive technology.

IVM OF OOCYTES AT REDOX STATE: RELEVANT TO THE IMPROVEMENT OF OOCYTE QUALITY

Control of Glutathione content in IVM oocytes

Several lines of evidence suggest that glutathione (GSH) is a crucial intra-oocyte factor for events after maturation in both cattle and pigs. Yoshida et al. (8) reported that the concentration of GSH in porcine oocytes increased according to the concentration of cysteine added to IVM medium, and at the same time the rate of male pronucleus formation became higher. Cysteine is a critical component amino acid of GSH, because GSH is a thiol tripeptide synthesized by the γglutamyl cycle where availability of cysteine is crucial (9). The GSH has an important role in providing cells with a reducing environment and in acting to protect against the toxic effect of oxidative damage (9). Therefore the promoting effect of GSH on male pronucleus formation is speculated to act synergistically in the following ways: glutathione promotes breaking of disulfide bond (s-s) of protamine in penetrated sperm head by making oocyte cytoplasm in redox state; and/or the GSH reacts as a substrate of glutathione peroxidase and acts as a scavenger of free radicals in oocytes, enhancing their competence as a whole (1).

The impact of high GSH content during porcine oocyte maturation was proven by the fact that Yoshida et al. (10) produced piglets derived from IVM-IVF oocytes by using a culture medium (TALP) of relatively simple components supplemented with cysteine for maturation. Yamauchi and Nagai (11) reported that cysteamine, a thiol with reducing function, increased the content of GSH and promoted male pronucleus formation even in cumulus denuded porcine oocytes (DOs). Probably in this case, cysteamine converted (reduced) cystine to cysteine in TCM 199, a cystine-rich medium. When DOs and cumulus-oocyte complexes (COCs) were cultured in the medium supplemented with cysteamine, the GSH content of COCs was significantly higher than that of COCs (11). Furthermore, most recently Mori et al. (12) demonstrated elegantly that gap junctional communication between the porcine oocyte and cumulus cells might play an important role in regulating GSH inflow from cumulus cells, by using a gap junction inhibitor, heptanol. The importance of gap junctional communication, coupling between oocytes and follicular cells (corona cells), for normal maturation of porcine oocytes was reported (13,14). Thus the GSH is probably one of the most crucial molecules to be transported through the junction.

Considering these facts, the efficiency of GSH synthesis in COCs should be best when the environment is rich in cysteine, because both oocytes and attached cumulus cells can uptake cysteine resulting in high GSH content and developmental competence in oocytes.

IVM of Oocytes Under a Low Oxygen Tension

In general, in vitro culture is maintained at a higher tension of O2 (20%) than that of the in vivo environment and, therefore, results in an increased production of reactive oxygen species (ROS) (15). ROS can cause lipid peroxidation and enzyme inactivation, resulting in cell damage by promoting hydroxyl radical formation (16). To decrease the production of ROS, reduction of the oxygen tension used for the culture of embryos from 20% to 5% has been found to have a beneficial effect on embryo development in pigs (17).

However, there have been only two reports investigating the effects of oxygen tension during maturation culture on the nuclear maturation and their subsequent developmental ability. Kikuchi et al. (7) cultured porcine COCs in vitro under 5% O2 and 20% O2 conditions, and IVM oocytes were fertilized in vitro and subsequently cultured for 6 days in vitro. They found that nuclear maturation and blastocyst formation rates were not different, however, the quality of blastocysts measured by the total cell number was higher (mean cell number = 43.5) after IVM under 5% O2 than that under 20% O2 (37.8).

Bing et al. (18) cultured porcine DOs and COCs in the medium supplemented with or without cysteamine under 5% O2 and 20% O2 conditions to measure GSH content of oocytes, and IVM oocytes were fertilized in vitro to assess their ability to form male pronucleus. Although the GSH content of DOs cultured in the absence of cysteamine was not affected by the oxygen tensions, DOs cultured under the low oxygen tension had a significantly higher rate of male pronucleus formation than that of DOs cultured under the high oxygen tension. In this instance, the small (0.5 pmol/oocyte) non-significant increase in the GSH content of DOs cultured without cysteamine under 5% vs 20% oxygen may have been sufficient to result in the significant increase in the rate of male pronucleus formation. Alternatively, culture under 5% O2 may have changed the metabolism of oocytes so that they showed a different response to sperm penetration, promoting male pronucleus formation. Under 5% O2, despite the male pronucleus formation rates of DOs cultured in medium with and without cysteamine being the same, DOs cultured with cysteamine showed a significantly higher GSH content (6.63 pmol/oocyte) than that of DOs cultured in medium without cysteamine (1.83 pmol/oocyte) ($P<0.05$). These results indicate that even in the absence of cysteamine the metabolism of DOs may be enhanced by culture under 5% O2, resulting in improved promotion of male pronucleus formation. To verify this proposal a further study is needed.

Anyway, it is recommended in practical in vitro producing (IVP) systems to use IVM systems in which COCs are cultured under 5% O2 (7).

IMPORTANCE OF CUMULUS CELLS DURING IVM OF PORCINE OOCYTES

It is generally accepted that cumulus cells during maturation period support IVM of oocytes to the metaphase-II stage and are involved in the cytoplasmic maturation needed for developmental competence of post-fertilization such as male pronucleus formation in porcine oocytes (19). Without cumulus cells, porcine oocytes could not develop beyond the 4 cell stage even after maturing in the medium containing cysteamine and having a high content of GSH and a high rate of male pronucleus formation after IVF (11).

Furthermore, Bing et al. (18) cultured porcine DOs and COCs in the medium supplemented with or without cysteamine under 5% O2 and 20% O2 conditions to compare GSH content of oocytes, and IVM oocytes were fertilized in vitro to assess their ability to form male pronucleus. The GSH content of DOs cultured in the presence of cysteamine was significantly higher than that of COCs cultured in the absence of cysteamine under both oxygen tensions (5% O2: 6.63 vs 2.67 and 20% O2: 5.85 vs 2.57 pmol/oocyte). However, in contrast with this result, cysteamine-treated DOs showed a significantly lower rate of male pronucleus formation than COCs under 5% O2 (54.1% vs 76.7%), and the same rate as that of COCs without cysteamine treatment under 20% (67.0% vs 79.3%). In addition, when cultured in the absence of cysteamine, despite the GSH contents of COCs and DOs being similar under both oxygen tensions (5% O2: 2.67 vs 1.83 and 20% O2: 2.57 vs 1.32 pmol/oocyte), a significantly higher rate of male pronucleus formation was obtained for COCs than for DOs (5% O2: 76.7% vs 41.0% and 20% O2: 79.3% vs 24.4%). Although it should be noted that both DOs and COCs cultured under 20% O2 condition showed significantly higher GSH contents and rates of male pronucleus formation when cultured with cysteamine than when cultured without cysteamine (18), these results suggest that the absolute amount of GSH is not the sole determinant of male pronucleus formation. Therefore it can be concluded that the existence of cumulus cells during IVM may be involved in enhancing male pronucleus formation in oocytes after IVF.

It is recommended in practical IVP systems to use IVM systems in which the healthy communication between cumulus cells and oocytes are properly maintained (20).

POLYSPERMIC FERTILIZATION

A weak and delayed cortical granule release, which is important for zona reaction to prevent polyspermic fertilization, was reported with porcine IVM-IVF oocytes (21) and has been thought to be the main reason for a high rate of polyspermic fertilization (22). However, the use of oviduct fluid from sows on the 20th or 21st day of estrus (added to maturation medium in 10, 30, or 100% concentration) to culture oocytes for 1.5 h before IVF reduced the polyspermic fertilization rate without lowering the IVF rate (23). The observations of IVM-IVF oocytes by laser microscope revealed that IVM oocytes exposed to oviduct fluid and ovulated oocytes have a similar cortical granule reaction (23). Furthermore, it was suggested that the ability of porcine oocytes to undergo the zona reaction to inhibit polyspermic fertilization is not fully developed until their exposure to the oviduct (24). Therefore the biggest problem, a high rate of polyspermic fertilization, in IVM-IVF of porcine oocytes is not only related to the cytoplasmic maturation but also to the modification of zona pellucida and/or cytoplasm of oocytes in the oviduct after ovulation. Considering the difficulty of obtaining a large amount of oviduct fluid from pigs, in vitro synthesis of active ingredients that can be obtained by isolation and purification from oviduct fluid and their application to the IVM-IVF system are expected in the future.

Furthermore, Funahashi and Nagai (25) invented a new in vitro fertilization system designated as a climbing-over-a-wall (COW) IVF method in which highly motile spermatozoa can be selected physically, and demonstrated that COW-IVF method can increase the normal penetration of frozen-thawed boar spermatozoa into IVM oocytes without any reduction in the sperm penetration rate.

IVC OF PORCINE IVM-IVF OOCYTES

Our previous reports (26,27) suggest that IVP blastocyst were in low quality emphasizing that the IVC system is not optimal because the only one or two days-IVC of IVM/IVF embryos resulted in low developmental competence to term after transfer to the recipients. To overcome incompleteness of IVP system, many laboratories are trying to improve the system, especially aiming successful term after transfer of blastocysts to recipients, however, the challenges resulted in failure except only two reports (7,28).

According to Kikuchi et al. (7), when porcine IVM-IVF oocytes were cultured in IVC medium supplemented with pyruvate and lactate for the first 2 days and then in the medium containing glucose for subsequent 4 days under 5% O2, the rate of blastocyst formation (25.3%) has a higher tendency compared with that of glucose supplement for the first 2 days (14.5%), and the cell number is significantly higher in that IVC medium (48.7) than that of glucose supplemented (35.4). This result clearly indicates that glucose in IVC medium for the first 2 days of culture is detrimental for the development of embryos. Furthermore, conditioned medium, which was obtained from supernatant of the medium co-cultured with epithelial cells for 2 days and supplemented with pyruvate and lactate, during the first two days has a significant effect on the cell number of the blastocyst (58.3) compared with the control (48.4). When expanding blastocysts after 5 days of culture (Cell number: 49.7) were transferred to an estrus-synchronized recipient (50 blastocyst per a recipient), the recipient was pregnant and farrowed 8 normal piglets. On the other hand, expanded blastocysts after 6 days of culture (80.2) were transferred to 2 estrus-synchronized recipients, all of them were pregnant and farrowed total 11 piglets. These results suggest that excellent piglets production system is established using modified IVP system producing high quality of blastocysts. This system will give advantages for generating of cloned and transgenic pigs.

OUTLOOK

Considering a fact that the advantage of using IVM oocytes as recipients for nuclear transfer, it will be a great advantage to collect immature oocytes of small size from a vast number of preantral follicles in the ovaries of a pig and grow (IVG) and mature them in vitro (3929). If a reliable IVG method is established and combined with IVM-IVF method, the efficiency of IVP of embryos will be greatly improved.

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