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Leptin and nonessential amino acids enhance porcine preimplantation embryo development *in vitro* by intracytoplasmic sperm injection

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ABSTRACT

Intracytoplasmic sperm injection (ICSI) has been considered one of the strong assisted reproductive technologies for producing transgenic animals as well as treating infertility in animals and humans. However, in porcine ICSI, embryos produced by *in vitro* methods show low pregnancy rates with high abnormal offspring and blastocyst formation rate as well as quality are poor compared with those in other species. For these reasons, developing a protocol for porcine ICSI is essential to efficiently generate transgenic pigs. Since amino acids were introduced to embryo development because of their beneficial effects, many embryologists have been using nonessential amino acid (NEAA) in culture medium for embryonic development in pig and other species. Leptin also has been shown to be beneficial in embryonic development for increasing rate of cleavage and blastocyst development. However, the effects of NEAA and leptin were not fully understood in the development of porcine ICSI-derived embryos. Here we investigated the optimization of NEAA and leptin supplementation in culture medium to improve developmental competence and quality of preimplantation embryos after ICSI in pig. The proportion of embryos that developed to the blastocyst stage was significantly greater when 1% vol/vol NEAA (24.6%) or 100 ng/mL leptin (27.1%) was supplemented in the culture medium compared with other concentrations or no supplement. When NEAA and leptin (24.8%) were supplemented together, blastocyst formation was significantly higher than other single supplementation groups. We also evaluated the effects of different supplementation periods of NEAA or leptin on the preimplantation embryonic development after ICSI. Both NEAA and leptin showed that supplementation for the entire 7 days significantly increased the blastocyst formation rate compared with the other groups of supplementation for the first 4 days and for the subsequent 3 days. A second goal of our research was to evaluate the quality of developed blastocysts after ICSI. The supplementation of 100 ng/mL leptin in culture medium made blastocysts express less of the proapoptosis genes *BAX* and *BAK* and more of the antiapoptosis genes *BCL-XL* and *BCL-2* after the ICSI procedure. Furthermore, terminal deoxynucleotidyl transferase dUTP nick end labeling index, fragmentation, and total apoptosis were significantly decreased and the total cell number was significantly increased when the ICSI-derived embryos were cultured to blastocyst stage in the presence of the combination of NEAA and leptin. These results suggest that NEAA and leptin could improve not only the quantity but also quality of ICSI-derived porcine embryos during *in vitro* culture with the optimal concentration of each reagent.

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1. Introduction

For more than two decades, intracytoplasmic sperm injection (ICSI) has been known as a valuable technique providing opportunities for studying fertilization, treating human infertility, and producing transgenic animals [1]. Not only in facilitating fertilization but also in propagating mammalian species, ICSI has been used and has enhanced the potential of assisted reproductive technologies [2,3]. In the porcine system, polyspermy has been known to occur very frequently, influencing the embryonic development to the blastocyst stage during *in vitro* fertilization [4–6]. Thus, ICSI is considered to be very useful in assisted reproductive technologies [7], and is especially well-known for generating porcine transgenesis [8]. Even though many species have been successfully generated as live ICSI-derived offspring, live ICSI-derived piglets have only been recently reported with the use of *in vivo*- and *in vitro*-matured oocytes [9–10]. However, using ICSI, the porcine blastocyst formation rate and quality is poor compared with outcomes in other species. Porcine embryos produced by *in vitro* methods generally show reduced pregnancy rates and increased incidence of abnormal offspring based on low total cell numbers, altered inner cell mass ratios, high nuclear apoptosis, and fragmentation [11–14]. Even though the reasons for the low success rate in porcine research are not clear, culture conditions have been considered an important factor affecting the quality and developmental capacity of porcine embryos produced *in vitro* [15–17].

Encoded by the obese (*ob*) gene, leptin is a 16-kDa adipokine protein secreted mainly from adipose tissue and plays a crucial role for the regulation of food intake and energy expenditure [18–20]. Leptin deficient mice (*ob*/*ob* mice) show hyperphagia, obesity, and insulin resistance; the administration of leptin to these mice reverses these effects [21]. Leptin is present in human [22] and mouse [23,24] oocytes, in human follicular fluid [22,25], and in human granulosa and cumulus cells [22]. The importance of leptin has also become clear in reproductive function. The human endometrium and placenta are sites for local production as well as a target tissue for circulating leptin, and might be implicated in the human implantation process and maternal–fetal interactions [26,27]. Craig et al. showed the influence of leptin on porcine preimplantation embryo development [28]. Inclusion of leptin in culture medium has significantly increased not only embryo cleavage but also blastocyst development after IVF compared with embryos cultured without leptin [28].

The *in vitro* culture environment is an essential factor in the development of *in vitro*-produced embryos. Amino acids are important components that have shown beneficial effects on early embryo development [29]. Van Winkle summarized how amino acids play multiple roles such as protein biosynthesis, activation of the embryonic genome, energy production, and cell homeostasis during early development [29]. Koo et al. reported the beneficial effect of nonessential amino acids (NEAA) on porcine embryos produced by *in vivo* or *in vitro* fertilization [12]. Furthermore, some reports have demonstrated that the development of porcine embryos produced by *in vitro* fertilization, parthenogenesis, and somatic cell nuclear transfer, was improved by the addition of NEAA [30–32]. However, it is not fully understood whether NEAA influences the development of porcine embryos produced by ICSI.

This study was therefore designed to investigate the effects of leptin and exogenous NEAA on the development capacity and quality of porcine embryos produced by ICSI. Furthermore, we evaluated the expression of pro- and antiapoptosis-related genes by reverse transcription polymerase chain reaction (RT-PCR) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to assess the quality of the ICSI-derived embryos.

2. Materials and methods

Unless otherwise mentioned, chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.1. Porcine oocyte collection and IVM

Porcine ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory in saline supplemented with 75 mg/mL penicillin G and 50 mg/mL streptomycin sulfate within 2 h at 30 °C to 35 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 3 to 7 mm diameter with an 18-ga needle into a 10 mL disposable syringe. COCs with two or three layers of cumulus cells and uniform cytoplasm were selected and rinsed three times in saline supplemented with 5% fetal bovine serum, and washed three times in tissue culture medium (TCM)-199 supplemented with 10% porcine follicular fluid, 10 ng/mL EGF, 10 IU/mL pregnant mare serum gonadotropin, and 10 IU/mL human chorionic gonadotropin. Fifty COCs were matured in a four-well dish with 0.5 mL of the aforesaid medium for 44 h at 38.5 °C with 5% CO_2 in air. During the first 22 h of culture, the COCs were cultured with 10 IU/mL pregnant mare serum gonadotropin and 10 IU/mL human chorionic gonadotropin, and then washed three times before *in vitro* culturing without these hormones during the last half of the culture period.

2.2. IVF

After maturation, cumulus cells were removed from the oocytes by repeated pipetting in NCSU-23 containing 0.1% hyaluronidase. The cumulus-free oocytes with first polar body release and uniform cytoplasm were washed three times with the fertilization medium. The fertilization medium was a modified tris-buffered medium (mTBM) consisting of 113 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \bullet 2H_2O$, 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 1 mM caffeine, 0.57 mM L-cysteine, and 0.1% wt/vol BSA. For IVF, one straw of the frozen semen was rapidly thawed in a 37.5 °C water bath for 1 min, transferred into a 1.5 mL tube, and then washed once with 1 mL mTCM-199 medium consisting of TCM-199 medium supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM Na-pyruvate, 2.92 mM, Ca-lactate$\bullet 5H_2O$, 75 mg/L kanamycin, and 10% vol/vol fetal bovine serum, washed twice in 1 mL mTBM medium by centrifugation at $\times 700g$ for 3 min. After the last