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Supplementation with vascular endothelial growth factor during *in vitro* maturation of porcine cumulus oocyte complexes and subsequent developmental competence after *in vitro* fertilization

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Abstract

The aim of the present study was to investigate whether the effects of vascular endothelial growth factor (VEGF) on porcine cumulus oocyte complexes (COCs) and subsequent blastocyst formation following *in vitro* fertilization are attributable to improved fertilization and cytoplasmic maturation. Porcine COCs were cultured for 42 h in TCM199 medium with 5 ng/mL human recombinant VEGF, and the resultant metaphase II oocytes were fertilized *in vitro*. COCs without VEGF supplementation served controls. Supplementation with VEGF during *in vitro* maturation (IVM) significantly ($P < 0.05$) improved the blastocyst formation rate and total cell number (46.7 $\pm$ 3.1% and 82.8 $\pm$ 6.7, respectively) compared with controls (32.5 $\pm$ 3.4% and 64.1 $\pm$ 5.6, respectively). On day 2, the percentage of four-cell stage embryos was significantly higher in the VEGF-matured group (49.1 $\pm$ 2.7%) than in the control (33.1 $\pm$ 5.8%), and the percentage of two-cell stage embryos was significantly higher in the control group (10.4 $\pm$ 1.4%) than in the VEGF-matured group (6.6 $\pm$ 0.9%). At 10 h after the onset of *in vitro* fertilization (IVF), oocytes with two pronuclei were considered as monospermically or normally fertilized, and oocytes with more than two pronuclei were considered as polyspermically fertilized. Monospermy was significantly higher in VEGF-matured oocytes (47.2 $\pm$ 4.3%) than in the control (20.0 $\pm$ 2.4%), and polyspermy and sperm penetration per oocyte were significantly higher in the control group (54.4 $\pm$ 3.8% and 2.3 $\pm$ 0.1, respectively) than in the VEGF-matured oocytes (43.9 $\pm$ 3.6% and 1.8 $\pm$ 0.1, respectively). Supplementation with VEGF during IVM significantly ($P < 0.05$) improved male pronuclear formation as compared with the control (91.1 $\pm$ 1.9 vs 74.4 $\pm$ 3.8%). Type III cortical granule distribution in oocytes was more common in VEGF-matured oocytes (78.0%) than in the control (52.1%). These results suggest that VEGF supplementation during IVM enhanced the developmental potential of porcine IVF embryos through higher male pronuclear formation and higher monospermic fertilization rates as a consequence of improved cytoplasmic maturation.
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Keywords: VEGF; Polyspermy; MPN; Cortical granule

1. Introduction

Polyspermic penetration is a major problem encountered with the in vitro fertilization (IVF) of porcine oocytes which reduces the efficiency of in vitro embryo production (IVP) and still it is an unresolved problems in the porcine IVF programme [1,2]. For in vitro systems, the rate often exceeds 50% [3,4]. It can be induced in vivo when abnormal conditions are imposed experimentally upon oocytes, including the postovulatory ageing of oocytes [5], injection of progesterone beneath the serosal layer of the fallopian tubes or sys-

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temically, and introduction of an excessive number of spermatozoa into the fallopian tubes shortly before ovulation. The incidence of polyspermic penetration observed for *in vivo* porcine eggs us substantially higher (30-40%) than the other species [6]. In mice, hardening of the zona pellucida (ZP), which is associated with the exocitosis of cortical granules (CG) in the cortical reaction and cleavage of ZP2 protein, is thought to be a major component of the protective mechanisms against polyspermy [7].

Polyspermy causes aberrant development and the death of early embryos [6]. It is associated with insufficient cytoplasmic and nuclear maturation, which leads to low fertilization and blastocyst rates. The success of nuclear and cytoplasmic maturation of oocytes is crucial for efficient IVM/IVF embryo production. During cytoplasmic maturation, the cell becomes prepared for fertilization, activation, and embryo development. In various model organisms, this includes the ability of competent female germ cells to fuse with sperm, decondensation of sperm chromatin, formation of pronuclei, and prevention of polyspermy.

Vascular endothelial growth factor (VEGF) is a homodimer with a subunit molecular mass of 23 kDa [8]. It is a potent mitogen for micro- and macrovascular endothelial cells [9,10]. In the female reproductive system, VEGF is involved in many processes such as ovulation, periodic changes of the endometrium, embryo implantation, and development [11]. There is evidence that adding exogenous VEGF during *in vitro* maturation of bovine oocytes significantly improves the rates of cleavage and zygote development [12]. This effect occurs synergistically with follicle-stimulating hormone (FSH) [13] or cumulus cell expansion [14]. Whether improved cleavage rates and embryonic development of porcine oocytes during IVM/IVF are attributable to enhanced fertilization is not clear. However, we previously have shown that VEGF supplementation of porcine IVM medium influences the developmental potential of parthenogenetic and somatic cell nuclear transfer embryos by increasing intracellular glutathione (GSH) levels [15]. Optimal cytoplasmic maturation may be involved in the prevention of polyspermy during *in vitro* embryo production and for increased male pronuclear formation after fertilization. The present study was designed to investigate the effects of VEGF on *in vitro* maturation of porcine oocytes and subsequent developmental competence after *in vitro* fertilization.

2. Material and methods

2.1. Ovary collection, recovery, and in vitro oocyte maturation

Ovaries of prepubertal gilts were collected from a commercial abattoir and transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution at 30 to 35 °C. The follicular fluid with oocytes was aspirated from 3–7 mm antral follicles with a 10-mL disposable syringe and 20 gauge needle and collected in a 15 mL conical tube. COCs with at least three layers of compact cumulus cells and with homogenous cytoplasm were selected for IVM. The selected 40–45 COCs were transferred and cultured in 500 μL of tissue culture medium 199 (Life Technologies, Rockville, MD, USA) supplemented with 26 mM sodium bicarbonate, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 0.5 IU/mL porcine luteinizing hormone, 0.5 IU/mL porcine follicle stimulating hormone, 10% (v/v) porcine follicular fluid (pFF), 75 μg/mL penicillin-G, and 50 μg/mL streptomycin. The pFF was aspirated from 3–7 mm follicles of prepubertal gilt ovaries and were prepared according to Hyun *et al.* [16] and stored at –20 °C until use. The COCs were then statically cultured at 39 °C in a humidified atmosphere containing 5% CO_2 with 10IU/mL eCG (Intervet International, BV). After 20–22 h of maturation with hormones, the oocytes were washed twice in a fresh maturation medium before being cultured in hormone-free medium for additional 20 h.

2.2. In vitro fertilization

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in TL-HEPES-PVA and washed three times with mTBM containing 1 mM caffeine and 0.1% BSA (Sigma). After washing, 10–15 oocytes were placed in 40 μL mTBM drops that had been covered with warm mineral oil. For IVF, 0.5 mL liquid semen was washed two times by centrifugation at 1900 $\times$ g for 2 min in Dulbecco's PBS (Gibco, Grand Island, NY) supplemented with 0.1% BSA. Thereafter, the sperm pellet was re-suspended with IVF medium and appropriate sperm concentration was made and subsequently 5 μL of sperm suspension was added in IVF drop that contained MII oocytes (final concentration 2.5 $\times$ 10^5 sperm/mL). Oocytes were co-incubated with spermatozoa at 39 °C in an atmosphere of 5% CO_2 in air for 20 min. After that, the oocytes were gently washed with TL-HEPES-PVA and transferred to a fresh 50 μL droplet of the same medium without spermatozoa and the

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