Theriogenology 120 (2018) 147–156

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Embryotropic effects of vascular endothelial growth factor on porcine embryos produced by *in vitro* fertilization

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ARTICLE INFO

Article history:
Received 28 March 2018
Received in revised form
20 July 2018
Accepted 23 July 2018

Keywords:
Vascular endothelial growth factor (VEGF)
Apoptosis
Nrf-2
Viable embryos

ABSTRACT

Current research suggests that supplementing *in vitro* culture (IVC) media with vascular endothelial growth factor (VEGF) may have beneficial effects on the development of porcine embryos *in vitro*. However, the molecular signaling mechanisms underlying this effect are unclear. Therefore, we aimed to investigate the effects of VEGF on molecular signaling events during *in vitro* embryonic development of porcine embryos. Porcine oocytes matured *in vitro* were fertilized, and the resultant zygotes were cultured with 5 ng/mL of VEGF supplemented with or without fetal bovine serum from day 4 till day 7. Without VEGF and/or FBS served as the control group. Real-time quantitative PCR was used to detect expression patterns of apoptosis- and oxidative stress-related genes in day 7 blastocysts (BLs). Early-stage apoptosis was detected by annexin-V assays in day 2 and day 7 embryos. We found that the addition of VEGF throughout the culture period with or without FBS supplementation significantly improved embryo survival and development. Supplementation with VEGF in the IVC medium significantly increased early BL formation ($p < 0.05$), although addition of FBS on day 4 significantly increased hatched BL formation ($p < 0.05$) regardless of VEGF supplementation. However, supplementation of media with both VEGF and FBS increased the formation of expanded BLs synergistically. The average total cell numbers per BL were significantly ($p < 0.05$) higher in embryos supplemented with VEGF and FBS than in those supplemented with either VEGF or FBS alone. We also found that accumulation of reactive oxygen species in VEGF-treated embryos was significantly lower ($p < 0.05$) than that in untreated embryos. The mRNA levels of caspase-3 were significantly lower ($p < 0.05$), and those of Bcl-2 and Nrf-2 were significantly higher ($p < 0.05$) in embryos grown in VEGF-supplemented media than in embryos grown in non-supplemented media. Furthermore, on day 2, the numbers of viable embryos ($44.06 \pm 3.94\%$) and blastomeres ($67.18 \pm 3.60\%$) were significantly higher ($p < 0.05$), and the numbers of early apoptotic embryos (55.94 ± 3.94) and blastomeres (23.23 ± 4.22) were significantly lower ($p < 0.05$) in VEGF-treated BLs than in controls. Furthermore, the numbers of early apoptotic cells in BLs on day 7 were also significantly lower ($p < 0.05$) in VEGF-treated BLs than in controls. Overall, our results indicate that supplementing IVC media with VEGF during *in vitro* culture of porcine embryos increases their developmental potential.

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https://doi.org/10.1016/j.theriogenology.2018.07.024
0093-691X/

1. Introduction

Although much research has been focused on developing *in vitro* porcine oocyte maturation and embryo production systems, there has been little progress in improving the survival and development of embryos produced *in vitro*. At present, embryos produced *in vitro* are less robust than their *in vivo* counterparts, especially with regard to implantation [1–5]. There are several factors that contribute to this low viability of *in vitro* embryos, many of which are due to suboptimal culture conditions [6]. As preimplantation embryos are very sensitive to environmental factors such as lack of appropriate growth factors or nutrients in culture media and gas concentrations in incubators, ensuring successful *in vitro* embryo development is not only dependent on the quality and viability of gametes, but also on culture conditions.

Embryos are graded on the basis of their morphological features under a stereomicroscope. However, they can also be graded using non-invasive techniques, such as cryotolerance [7] and metabolic test [8], and invasive techniques, such as gene analysis [9] and apoptosis analysis [10]. During *in vitro* embryo development, apoptosis is induced, at least partially, by suboptimal culture conditions and may also be an indicator of embryo quality [6,11]. Apoptotic blastomeres are common in blastocysts (BLs) produced *in vitro*, whereas few or no apoptotic blastomeres are present in BLs that develop *in vivo* [12–14]. Suboptimal *in vitro* culture conditions also reduce BL development rates, increase apoptosis incidence, and decrease blastomere numbers, all of which subsequently contribute to higher incidences of implantation failure and fetal resorption of embryos produced and cultured *in vitro* [15–17]. Apoptosis or programmed cell death plays an important role in normal embryo development by eliminating blastomeres with chromosomal abnormalities or other defects [18–20]. If the ratio of apoptotic blastomeres in a BL exceeds the threshold level, the embryo becomes unviable and eventually dies. Suboptimal culture conditions can induce changes that may trigger unscheduled apoptosis in blastomeres, thereby delaying cleavage and ultimately affecting the quality, quantity, and viability of the BLs produced [16,17]. Key regulators of apoptosis in embryos are proteins of the Bcl-2 family, which affect both antiapoptotic and proapoptotic pathways [21,22]. The ratios of these proteins, along with those of the caspase family of proteins are critical determinants of cell fate [23]. Accordingly, a strong correlation has been detected between the incidence of apoptosis and the levels of caspase activation in cryopreserved cord-blood hematopoietic cells [24] and hepatocytes [25]. Besides, DNA fragmentation, which is an important step in and a typical indicator of apoptotic cell death, is linked to early embryonic loss, with ~90% of embryos produced *in vitro* and 56–71% of embryos produced *in vivo* exhibiting DNA fragmentation [26]. To prevent apoptosis and improve embryonic development, several strategies involving the addition of growth factors have been used.

Supplementation of culture media with epidermal growth factor (EGF) and insulin-like growth factor (IGF) has been found to increase BL production by reducing apoptosis in bovine embryos [27–29]. However, another study found that IGF-1 alone could reduce apoptosis in porcine embryos during *in vitro* culture [30] and that IGF-1 and IGF-1 receptors were widely expressed in embryos throughout the preimplantation stages [31]. As of now, several growth factors have been used to optimize *in vitro* developmental conditions for porcine oocytes including EGF, IGF, fibroblast growth factor (FGF), amphiregulin [32], VEGF [33], porcine granulocyte-macrophage colony-stimulating factor (GM-CSF) [34], growth differentiation factor 8 (GDF8) [35], and others. Although these growth factors had some beneficial effects such as inhibition of apoptosis, promotion of metabolic activities, and induction of required kinases, on embryo development, they failed to replicate the effects of the complex mixture of growth factors and cytokines that play a major role in embryo survival, growth, and development *in vivo* [36]. Because of these setbacks, it is crucial to not only determine an optimal mixture of growth factors and nutrients for *in vitro* embryo survival and growth but also investigate the mechanisms underlying the effects of these growth factors on the developing embryos.

VEGF is a potent mitogen used for the propagation of micro- and macrovascular endothelial cells and is known to influence gene expression patterns of antiapoptotic genes in these cells during serum-starvation culture. In the female reproductive tract, VEGF is involved in many processes, including ovulation, early embryonic implantation, and subsequent development of the embryo. VEGF is already known to reduce apoptosis in porcine parthenotes [37] and may improve the maturation of ovine gametes by activating the mitogen-activated protein kinase (MAPK) pathway [38]. However, little is known about the molecular signaling events involved in the effects exerted by VEGF in gamete and embryo development during *in vitro* culture. On the contrary, usually, 10% FBS is added during *in vitro* culture of porcine zygotes on day 4 during compaction. Addition of FBS on day 4 to porcine parthenotes deteriorates embryo production by influencing apoptosis-related gene expression during *in vitro* culture [39], whereas VEGF has an antiapoptotic effect on other tissues. This result suggests that the components of FBS either counteract the antiapoptotic effects of VEGF or interact with supplemented VEGF or both. Therefore, we designed this study to investigate the expression patterns of embryotropic genes in embryos produced by *in vitro* fertilization (IVF) and grown in VEGF-supplemented media in the presence and/or absence of fetal bovine serum (FBS). In this study, we show that supplementation of *in vitro* culture (IVC) media with VEGF can improve the quality of porcine embryos produced *in vitro*.

2. Material and methods

2.1. Chemicals

All the chemicals used in this experiment were bought from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated.

2.2. Ovary collection, aspiration, and in vitro maturation of porcine immature oocytes

Ovaries of prepubertal gilts were collected from a commercial local abattoir just after slaughter and the ovaries were transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution supplemented with penicillin-G (100 U/mL) and streptomycin sulfate (100 mg/mL) at 30–35 °C. The follicular fluid with cumulus-oocytes complexes (COCs) was aspirated from 3 to 6 mm antral follicles with a 10-mL disposable syringe and 20-gauge needle and sampled in a 15-mL conical tube at 37 °C for 5 min. The supernatant was discarded, and the precipitate was resuspended in Tyrode's Lactate HEPES-buffered medium (TLH) containing 0.05% (wt/vol) polyvinyl alcohol (TLH-PVA). COCs were recovered under a stereoscopic microscope; those with at least three layers of compact cumulus cells and homogeneous cytoplasm were selected for *in vitro* maturation. The selected COCs were transferred and cultured in 500 μL of tissue culture medium 199 (TCM 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. pFF was aspirated from 3 to 7 mm follicles of prepubertal gilt ovaries.