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Sequential treatment with resveratrol-trolox improves development of porcine embryos derived from parthenogenetic activation and somatic cell nuclear transfer

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ABSTRACT

We investigated the effect of resveratrol supplementation during IVM and/or trolox during IVC on the development of porcine embryos derived from parthenogenetic activation (PA) and SCNT. In this study, we evaluated intracellular glutathione (GSH) and reactive oxygen species (ROS) levels, gene expression in blastocysts, and embryonic development after PA and SCNT. To determine the combined effects of resveratrol during IVM and trolox during IVC on PA embryos, we selected optimal concentrations (2 μM of resveratrol and 200 μM of trolox) and designed four groups: (1) control, (2) resveratrol, (3) trolox, and (4) combined. All treatment groups showed significantly increased intracellular GSH levels and decreased ROS levels. Resveratrol supported significantly higher cleavage and blastocyst formation rates than the control (80.3% and 38.0% vs. 71.1% and 22.4%, respectively) by downregulating *Bax*/*Bcl-2*, *Caspase-3*, and *Bak*. Trolox showed significantly increased blastocyst formation rates (36.7%) compared with the control (22.4%) by downregulating only *Caspase-3*. The combined group had significantly higher cleavage and blastocyst formation rates and greater total cell numbers than the control (81.7%, 36.3%, and 67.1 vs. 71.1%, 22.4%, and 47.8, respectively) by downregulating *Bax*/*Bcl-2*, *Caspase-3*, and *Bak*. On the basis of these results, we applied sequential treatments with resveratrol and trolox to SCNT, and blastocyst formation rates and total cell numbers were significantly increased compared with the control (17.2% and 52.1 vs. 11.8% and 36.6, respectively), with increased GSH, decreased ROS levels, upregulated proliferating cell nuclear antigen, and downregulated *Bax*/*Bcl-2* and *Caspase-3*. These results indicate that sequential treatment with resveratrol during IVM and trolox during IVC improved the development of PA and SCNT porcine embryos by regulating intracellular GSH, ROS levels, and gene expression.

1. Introduction

Pigs have been widely used as models for studying disease and disease resistance and to provide genetically defined models for surgery and xenotransplantation because of the similarity of their anatomy and physiology to those of humans [1,2]. Several techniques for IVP of preimplantation porcine embryos, including IVF, intracytoplasmic sperm injection, and SCNT, have made it possible to produce animals for specific purposes [3–5].

However, the developmental ability of porcine embryos produced *in vitro* is still inferior to that of embryos generated *in vivo*. One reason for this is thought to be reactive oxygen species (ROS) generated during aerobic metabolism, even

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under basal conditions. In both oocytes and embryos, endogenous ROS can be produced by various metabolic pathways and enzymes [6–8]. In particular, ROS such as hydrogen peroxide (H_2O_2), superoxide anions ($^{\bullet}O_2^-$), and hydroxyl radicals ($^{\bullet}OH$) are produced during intermediate steps of oxygen reduction [9]. Overproduction of ROS during oocyte maturation and early embryonic development causes lipid peroxidation of the cell membrane [9,10], DNA fragmentation [11], and alterations in RNA transcription [12,13] and protein synthesis [14]. These damaging changes consequently lead to mitochondrial alterations, embryo cell development blocks, adenosine triphosphatase depletion, and apoptosis (programmed cell death) [15–17].

Cells generate endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase [18] to protect themselves from ROS by scavenging free radicals. However, imbalance between ROS generation and antioxidant activity, particularly that of glutathione (GSH), causes an oxidative stress condition and apoptosis. Glutathione, a ubiquitous intracellular free thiol compound, protects cells from ROS [19] and regulates the intracellular redox mechanism [20].

Therefore, to increase GSH, reduce ROS, and subsequently improve the developmental ability of *in vitro*–matured oocytes and *in vitro*–cultured embryos, various antioxidants such as L-ascorbic acid, α-tocopherol [21–23], β-mercaptoethanol [24], cysteine [25], L-carnitine [26], resveratrol [27,28], and trolox [24,29] have been added to IVM and/or IVC systems.

Resveratrol (3,4′,5-trihydroxystilbene) is a phytoalexin, a secondary plant metabolite, generated by the interaction between plants and a microorganism found in grapes, plums, and peanuts [30]. It plays an important role in the plant's natural defense system against fungal and bacterial infections [23]. Resveratrol has a wide variety of pharmacologic activities including anti-inflammatory, antioxidant, antiproliferative, immunomodulative, and cardioprotective effects [31–34]. It can also affect the expression of a wide range of genes related to DNA synthesis, the cell cycle [35], proliferation [36], and apoptosis [37].

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analog of vitamin E reported to scavenge peroxyl radicals in a manner similar to vitamin E (α-tocopherol), and therefore, it could provide antioxidant protection for embryos. Vitamin E and Trolox prevent the chain reaction of ROS formation by trapping peroxyl radicals as the longer-lived tocopheryl radical [38], thus reducing the generation of ROS. Trolox also appears to be a promising candidate for improving cryopreservation of spermatozoa and oocytes, acting as an antioxidant, because it stabilizes cell membranes by reducing oxidation in membrane lipids [39,40].

Supplementation with resveratrol during IVM was beneficial for *in vitro*–fertilized and parthenogenetically activated porcine embryos [27], but information is lacking about its effects in SCNT porcine embryos. Although some studies have suggested beneficial effects of trolox treatment during IVC in mouse [41], bovine [24,29], and ovine embryos, little attention has been focused on effects of trolox on porcine embryos. Therefore, we investigated the effects of resveratrol during IVM of oocytes and/or trolox during IVC of porcine embryos derived from parthenogenetic activation (PA) and SCNT.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in this study were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2.2. Oocyte collection and in vitro maturation

Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory within 3 hours in physiological saline at 32 °C to 35 °C. Cumulus–oocyte complexes (COCs) were aspirated from 3- to 6-mm-diameter superficial ovarian follicles using an 18-ga needle attached to a 10-mL disposable syringe and allowed to settle down as sediment in 50-mL conical tubes at 37 °C for 5 minutes. The supernatant was discarded, and the sediment was washed three times in washing medium containing 9.5 g/L of tissue culture medium-199 (Invitrogen, Carlsbad, CA, USA), 5-mM sodium hydroxide, 2-mM sodium bicarbonate, 10-mM HEPES, 0.3% polyvinyl alcohol, and 1% penicillin–streptomycin (Invitrogen). Subsequently, the COCs were recovered while observing under a stereomicroscope. Only COCs having 3 or greater uniform layers of compact cumulus cells and a homogeneous cytoplasm were selected and washed three times in washing medium. Approximately 60 COCs were transferred to IVM medium comprising tissue culture medium-199 supplemented with 10 ng/mL of EGF, 0.57-mM cysteine, 0.91-mM sodium pyruvate, 5 μL/mL of insulin–transferrin–selenium solution (ITS-A) 100X (Invitrogen), 10% porcine follicular fluid, 10 IU/mL of eCG, and 10 IU/mL of hCG. The selected COCs were incubated at 39 °C with 5% CO_2 in 95% humidified air for IVM. After 21 to 22 hours of maturation with hormones, the COCs were washed twice in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 21 to 22 hours.

2.3. Parthenogenetic activation of oocytes

After 42 to 44 hours of IVM, the COCs were denuded by gently pipetting with 0.1% hyaluronidase, washed three times in Tyrode's albumin lactate pyruvate (TALP) medium, and then gradually equilibrated in activation medium consisting of 0.28-M mannitol, 0.5-mM HEPES, 0.1-mM $CaCl_2$, and 0.1-mM $MgSO_4$. For activation, denuded oocytes with homogeneous cytoplasm were placed between electrodes covered with activation medium in a chamber connected to a BTX Electro-Cell Manipulator 2001 (BTX Inc., San Diego, CA, USA). Oocytes were activated with a single direct current (DC) pulse of 1.5 kV/cm for 60 μs. Then, electrically activated oocytes were washed three times in fresh porcine zygote medium-5 (PZM-5; Funakoshi Corporation, Tokyo, Japan), placed into 20-μL PZM-5 droplets (five gametes per drop) covered with prewarmed mineral oil, and then cultured at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days.