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# Improving development of cloned goat embryos by supplementing α-lipoic acid to oocyte *in vitro* maturation medium

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ABSTRACT

α-Lipoic acid (LA) is a powerful antioxidant for clinical therapy of some metabolic diseases, but there are few reports about the effect of LA on animal occyte *in vitro* maturation (IVM). The objective of this study is to investigate the effect of supplementing LA to IVM medium on subsequently developmental competence of goat cloning embryos after somatic cell nucleus transfer (SCNT). Twenty-five micromolars LA significantly increased 12% oocyte maturation rate from control 57.8% to treated group 69.8% ($P < 0.05$). The reconstructed rate of cloning embryos in LA supplement group (67.3%) was significantly higher than control (56.5%, $P < 0.05$). Although the SCNT embryo cleavage rates did not have significant difference between the two groups (42.0% vs. 47.9%, $P > 0.05$), LA supplement group had significantly higher blastocyst formation rate and hatched rate than control (24.0% vs. 18.4% and 37.0% vs. 30.9%, respectively, $P < 0.05$). In addition, supplementing LA significantly reduced the cellular apoptosis rate of nucleus transfer blastocysts by inhibiting the expression of apoptotic activators, such as *Bax*, *Bad*, *Caspase-3*, and *CytC* genes and promoting cumulus-oocyte complexes to synthesize glutathione (GSH) and express antioxidant enzymes such as *GPX4* and *SOD* genes. In conclusion, supplement of LA to oocyte IVM medium could improve the maturation rate and antioxidant ability of oocytes and increase the developmental competence of oocytes after SCNT.



## 1. Introduction

The success of modern animal embryo engineering technologies, including animal cloning and transgenic animal production, mainly depends on *in vitro* embryo production. Oocyte maturation is the first and most critical step for successful *in vitro* embryo production. In most mammalian species, oocytes are formed during fetal life and arrested at the prophase stage of the first meiotic division until the ovulation. The oocyte maturation is defined as the reinitiation and completion of the first meiotic division from the germinal vesicle (GV) stage to metaphase II (MII) stage, accompanying cytoplasmic maturation necessary for fertilization and early embryonic development [1,2]. Cytoplasmic protein synthesis [3], maturation-promoting factor activity [4,5], and cytostatic factor [6] regulate the progress of GV to MII. The mechanisms for sperm head penetration, decondensation of chromatin, and the polyspermy block are all progressively acquired during the final days of maturation before ovulation. Optimally matured oocytes will have a great potential to develop into good quality embryos. Some studies have indicated that oocytes retrieved from larger follicles, with increased estrogen and inhibin A, show higher meiotic maturation, fertilization, and embryonic

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development rates [7]. The developmental competence of a fertilized oocyte is profoundly affected by the culture conditions. Therefore, an optimal culture condition is vital for a successful *in vitro* maturation (IVM) program.

Early research in oocyte IVM showed that the culture medium with protein and hormone supplements plays an important role in oocyte maturation and subsequent embryo development competence following *in vitro* fertilization (IVF) [8,9]. However, current IVM remains suboptimal. The main challenge resides in the relatively low reproductive outcomes [10]. Recent investigations implied that oxygen radicals, or reactive oxygen species (ROS) act as primary or secondary messengers to promote cell growth or death [11]. When an overabundance of ROS is produced and a cell cannot adapt, a phenomenon known as oxidative stress occurs. The oxidative stress can alter many important reactions that affect cell signaling pathways involved in proliferation, differentiation, and apoptosis. During IVM, oocyte is much more susceptible to oxidative stress due to lack the innate antioxidant defense. In order to reduce the cell damage caused by oxidative stress, adding antioxidants such as β-mercaptoethanol and vitamins C and E to culture media have been attempted in recent years [12].

α-Lipoic acid (LA), a naturally occurring disulfide molecule, is a powerful antioxidant that reportedly exerts beneficial effects in patients with advanced cancer by reducing the level of ROS and increasing glutathione peroxidase activity. LA acts as an important coenzyme for enzymes inside the mitochondria [13], such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase; therefore, it has been treated as a vitamin-like substance [14]. Recent study indicated that LA can decrease ROS concentration and increase follicular total antioxidant capacity during the mouse ovarian *in vitro* culture [15]. Currently, these studies have mainly been conducted on routine IVF or ICSI to produce *in vitro* embryos. However, in our animal cloning practice, it often needs a large amount of good quality matured oocytes for nucleus transfer. Thus, improving oocyte IVM technology plays an important role in the subsequent embryo cloning, nucleus transfer, and transgenic animal production. The objective of this study is to investigate the effect of supplementing LA to oocyte IVM medium on oocyte maturation rate and subsequent somatic cell nucleus transfer (SCNT) embryo development.

## 2. Materials and methods

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise stated.

### 2.1. Oocyte collection and IVM

Goat ovaries were obtained from a local slaughterhouse and transported to laboratory within 5 to 6 hours in a thermo-bottle in sterile physiological saline at 20 °C to 25 °C. Cumulus-oocyte complexes (COCs) were obtained by slicing the ovarian surface with a razor blade and collected under a stereo microscope. COCs surrounded by more than three cumulus layers and a finely granulated homogeneous ooplasm were selected for IVM. The basic maturation medium used for oocyte IVM was bicarbonate-buffered tissue culture medium-199 (TCM-199, Gibco, Grand Island, NY, USA) supplemented with 10% (vol/vol) FBS, 1 μg/mL 17β-estradiol, 0.075 IU/mL human menopausal gonadotropin, 10 ng/mL EGF, 1% (vol/vol) insulin-transferrin-selenium, 0.2 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin. COCs were cultured in 2-mL maturation medium supplemented with or without 25 μM LA according to the experimental design in 95% humidified air with 5% $CO_2$ at 38.5 °C. After 18 to 20 hours maturation, cumulus cells were completely dispersed from COCs by carefully pipetting in PBS containing 0.2% bovine testicular hyaluronidase. Oocytes with a first polar body and evenly granulated ooplasm were used for SCNT.

### 2.2. Production of SCNT embryos

Goat fetal fibroblast cell lines were isolated as described previously [16]. The cells were seeded on 48-well plate and grown to 100% confluence for 2 to 3 days before SCNT. The procedures of SCNT were carried out as described previously [17]. Briefly, the oocytes were incubated for 10 minutes in PBS containing 5 μg/mL Hoechst-33342 before enucleation. Then the oocytes were transferred to manipulation medium microdrops (TCM-199 containing 25 mM HEPES, 10% FBS, and 7.5 μg/mL cytochalasin B) overlaid with mineral oil. The first polar body and the small amount adjacent cytoplasm were removed with a 25-μm diameter pipette. Enucleated oocytes were checked for the absence of chromosomes under ultraviolet radiation. A single donor cell was injected into the perivitelline space of the enucleated oocytes. The karyoplast-cytoplast couplets were fused with a pair of platinum electrodes connected to the micromanipulator, and a double electrical pulse of 30 V for 10 μs was applied for fusion. Then the couplets were held in TCM-199 with 7.5 μg/mL cytochalasin B for 2 to 3 hours. Successfully fused embryos were activated by exposure to 5 μM ionomycin for 5 minutes and then incubated in mSOF medium containing 2 mM 6-dimethylaminopurine for 4 hours. The reconstructed embryos were cultured in 200 μL G1 (Vitrolife, Sweden) in a 25-mm concave glass dish covered with mineral oil in humidified atmosphere with 5% $CO_2$ in air at 38.5 °C. After 72 hours of culture, embryos were cultured in 200 μL G2 (Vitrolife) for another 96 hours. The developmental rates of 2-cell and blastocysts were monitored at 48 and 168 hours, respectively.

### 2.3. Apoptosis assays

Apoptosis assays were performed as described previously [18] using a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA). All steps were performed at room temperature, unless stated otherwise. Briefly, after washing the embryos with PBS containing 0.2% polyvinyl alcohol for three times, Day 8 blastocysts were fixed in immunol staining fix solution (Beyotime) for 1 hour. Embryos were then permeabilized with 0.2% Triton X-100 in PBS for 20 minutes. After three washes, the blastocysts were equilibrated with equilibration buffer for 8 minutes, and then incubated with terminal deoxynucleotidyl transferase (rTdT) incubation buffer (45 μL equilibration buffer, 5 μL nucleotide mix, 1 μL rTdT enzyme) in the dark at 37 °C