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Supplementation of lycopene in maturation media improves bovine embryo quality *in vitro*

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ABSTRACT

This study sought to modulate factors that reduce embryo quality in *in vitro* culture (IVC) systems. Over eight replicates, 3075 oocytes were cultured in *in vitro* maturation media containing various concentrations of lycopene, followed by *in vitro* fertilization and culture. The percentages of MII-stage oocytes, the presumptive zygotes that underwent cleavage and developed into blastocysts were significantly ($P < 0.05$) higher, the intracellular ROS concentrations reduced significantly ($P < 0.05$) in oocytes/blastocysts, TUNEL assay demonstrates reduced apoptosis and increased total cell number per blastocyst ($P < 0.05$), Immunocytochemistry confirmed that diminished protein expression of nuclear factor kappa B (NFκB), cyclooxygenase-2 (COX2), and 8-oxoguanine (indicated by ROS) and relative mRNA expression of the *Caspase-3, NFκB, COX2, iNOS* and *BCL2*-associated X (*BAX*) was significantly ($P < 0.05$) lower whereas the anti-apoptotic gene *BCL2* was significantly ($P < 0.05$) higher in the 0.2 μM lycopene-supplemented group than the control. In conclusion, lycopene improves blastocyst quality by overcoming unfavorable conditions in *in vitro* culture systems.

1. Introduction

The cattle industry must prepare to switch from artificial insemination, which has been routinely used over the past 75 years, to more advanced technologies that promote genetic progress. Super-ovulation, synchronization of estrus, ovum pick-up (OPU), and embryo transfer (ET) technology will become the primary means to obtain elite cattle embryos with rapid genetic progress. OPU and ET using *in vitro* fertilization (IVF) may also be routinely used to improve genetic progress within the cattle industry. OPU and ET are expected to be widely used within the next decade due to continued improvements in the fertility of cattle, and the rate of genetic progress is expected to increase to 15% and milk production will increase to 60% [1]. Oxidative stress is considered as important constraints that reduce the quality of embryos in *in vitro* culture (IVC) systems. Highly reactive oxygen species (ROS) are endogenously generated via the normal metabolic activity of embryos. ROS react with critical cellular biomolecules, such as lipids, proteins, and DNA, and thereby reduce the quality of embryos [2]. Highly reactive oxygen species (ROS) are short-lived molecules that can cause decreased embryonic development *in vitro*. Antioxidants block free radical formation or guard against their detrimental effects. When increased generation of ROS overcomes the antioxidants' capacity, leading to OS, prompt to oocytes injury. ROS may have a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis and luteolysis [3]. Antioxidants are molecules that block free radical formation or guard against their

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harmful effects, reduce apoptosis or cell death by inhibiting the peroxidation of lipids and lipoproteins and contribute to the defences against oxidative damage to embryo [4]. Supplementation of antioxidants to the culture media is effective in supporting bovine embryo development *in vitro* [5] as antioxidant defences against reactive oxygen species. During cellular metabolism, the large amounts of free radicals generated *in vivo,* which then mostly occupied by the built-in antioxidant defences of the ovarian follicle. But *in vitro*, it may then be the antioxidant compounds supplemented with IVM culture media, play the role of free radical scavengers, provide protection against the potential influences of oxidative stress (both intrinsic and extrinsic) [4].

Moreover, during IVM, oocytes are usually cultured under conditions with high concentration of oxygen, resulting in prematuration aging of oocyte by the increased production of ROS [4,6]. Premature aging of oocytes before MII stages are detrimental to oocyte nuclear and cytoplasmic maturation process causes poor fertilization and retarded embryo development. Antioxidants can help to protect against defective embryo development [7]. It is also very important that antioxidants are stored in the cultured oocytes (as mRNA transcripts or proteins) during its growth and maturation phases not only as kith and kin to the maturation process but also from the time when the initial stages of embryonic development over the activation of the zygotic genome [8]. Antioxidant supplementation to culture medium can increase the developmental potential of oocytes or embryos [9,10]. Addition of high concentrations of antioxidants to the IVM medium decreased the rate of blastocyst formation compared to treatment with low concentrations suggesting that the proper concentration of an antioxidant can contribute to the generation of high quality embryos [11]. Besides, improvements can be made by altering the culture conditions for oocyte maturation and embryo development, including the external oxygen concentration [8,12].

Lycopene is a bright-red carotenoid pigment and powerful *in vitro* antioxidant found in tomatoes and other red fruits and vegetables. Among naturally occurring carotenoids, lycopene has the strongest ability to scavenge free radicals [13] and chemically quench singlet oxygen ($^1O_2$) [2,14]. Lycopene quenches $^1O_2$ 2− and 10-fold more effectively than β-carotene and α-tocopherol, respectively [2]. Lycopene is one of the most potent antioxidants in the human body; its antioxidant potency is 100-fold higher than those of vitamins E and C [15]. Lycopene, which has a high free radical-scavenging capacity due to its unique structure (high number of conjugated double bonds) (Fig. 1), might also quench superoxide (O_2−) and other free radical anions, which are readily released in IVC systems.

Lycopene has a high $^1O_2$-quenching ability and thereby protects embryos against oxidative damage. Lycopene also efficiently scavenges other ROS [2]. During $^1O_2$ quenching, energy is moved from $^1O_2$ to lycopene, converting it into the energy-rich triplet state. Lycopene in the triplet state can be converted back to the basal state by disintegrating this energy as heat or via physical quenching, leaving the lycopene molecule intact and ready for further quenching activity. Lycopene is also an excellent $^1O_2$ quencher in biomolecules such as liposomes [16,17]. The capture and breakdown of other ROS, such as hydroxyl radicals (OH), NO_2, or peroxynitrite, by lycopene prevents oxidative damage [18]. In organic solutions, lycopene is the most rapidly destroyed carotenoid and reacts with peroxyl radicals [19,20], indicating that it functions in the first line of defense. ROS such as O_2−, hydrogen peroxide (H_2O_2), and OH− are produced during glycolysis and mitochondrial phosphorylation in oocytes [21]. Lycopene is a potent antioxidant not only *in vitro* but also *in vivo*, preventing oxidation of lipids, proteins, and DNA. OH− generated via the Fenton reaction is supposedly one of the greatest sources of DNA damage [21].

Lycopene is active at a concentration of 0.1 μM, whereas retinoic acid is only active at a higher concentration (1.0 μM) in comparable stimulations [22]. Lycopene is very common and readily available. This carotenoid not only potently scavenges free radicals but also helps to maintain the balance of the endogenous defense system in cells [23]. To our knowledge, the effect of lycopene in embryo IVC systems has not been studied. We investigated the effect of lycopene on the developmental potential of bovine embryos *in vitro*. The main objectives of this study were (a) to improve embryo developmental competence *in vitro* by modulating factors that negatively affect embryos, (b) to determine whether the excellent ROS-quenching ability of lycopene counteracts stressors in IVC medium, and (c) to determine the mechanisms by which lycopene reduces the effects of stressors in embryo IVC.

2. Materials and methods

2.1. Reagents

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

2.2. Animal ethics

All the experiments were approved and performed in accordance with the Gyeongsang National University guidelines for the care and use of laboratory animals (approval no. GAR-110502-X0017).

2.3. Preparation of lycopene

Lycopene was purified from tomato extracts (≥90%) (Cat. no. L9879). Tetrahydrofuran containing 250 mg/L butylated hydroxytoluene (≥99.0%) (Cat. no. 360589) was used as a vehicle for lycopene. Lycopene was solubilized in this vehicle to generate a stock solution of 100 μM and kept at −80 °C and the same concentration of THF was added in the control group during the eight replications. The treatment was given in the culture medium just immediately before the culture of oocytes. Moreover, during the study, we strictly follow the Safety Data Sheet (SDS) for lycopene from the Sigma Aldrich (Cat. no. L9879). All procedures were performed under dim light to prevent oxidation of the compounds.

2.4. Experimental design

The ROS-scavenging compound lycopene was added to IVM media to overcome the effects of ROS. The experiment was performed in two stages. Embryos were divided into six experimental groups (treated with 1.5, 1.0, 0.5, 0.3, 0.2, and 0.1 μM lycopene) and

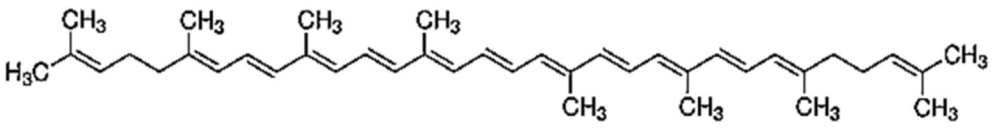

Fig. 1. Chemical structure of lycopene (synonym: ψ, ψ-Carotene, 2,6,10,14,19,23,27,31-Octamethyl-dotriaconta-2,6,8,10,12,14,16,18,20,22,24,26,30-tridecaene) [3].