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Vitamin K2 improves developmental competency and cryo-tolerance of *in vitro* derived ovine blastocyst

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ABSTRACT

Vitamin K2 (VK2), acts as an electron carrier in mitochondria and thereby effects reactive oxygen species (ROS) and ATP production. This study evaluates role of VK2 on *in vitro* developmental competency and cryo-survival of pre-implantation ovine embryos. Initially the optimal and beneficial concentration of VK2 on compaction and blastocyst formation rates was defined (0.1 μM). Subsequently, it was shown that 0.1 μM VK2, at blastocyst stage, reduces H2O2 production, increase the expression of mitochondrial related gene and improved embryos quality. We further assessed presence VK2 supplementation before and/or after vitrification of *in vitro* derived blastocysts. Our results reveal that presence of VK2 before and after vitrification improves rates of blastocysts re-expansion (88.19± 3.37% vs 73.68± 1.86%, $P < 0.05$) and hatching (49.55± 4.37% vs 32.7± 3.32%) compared to control group. These observation were consistent with reduction in H2O2 production and improved in expression of mitochondrial related genes. However, VK2 before or after vitrification, not only had no positive effect on these two parameters, but also significantly reduced these parameters. Therefore, in concordance with pervious report in bovine, we show that VK2 supplementation post genomic activation (Day 3–7) improved developmental competency of ovine *in vitro* derived embryos. We also showed that presence of VK2 after vitrification improves the cryo-survival of ovine embryos.

1. Introduction

Rate of *in vitro* embryos fabrication is severely effect by composition of culture media. Despite extensive back ground studies in the last two decades to improve composition of culture media, blastocyst formation and hatching rates are limited and appear to be more room for further improvement. One of the cellular component which plays a key role in embryo development is mitochondria. Mitochondria are unique organelles, involved in different cellular processes including ATP synthesis, calcium homeostasis, cell survival and cell death [1,5,30]. In addition, mitochondria contains its own DNA which has important role in its biogenesis [14]. Inappropriate composition of culture media can have unfavorable effects on structure and functions mitochondria and thereby can influence quality of embryos [6]. Moreover, culture medium can have significant impact on expression of genes related to embryo viability and quality [26,28]. Oocyte of mammals contain 100000 mitochondria [25]. These organelles are the main source of cellular energy and have indispensable role in biosynthesis of lipids, nucleic acids and as well as amino acids [5,30].

Even though mitochondria are considered as the main source for production of reactive oxygen species (ROS) they are also susceptible to damage by ROS [9]. Indeed, one of the main reasons associated with early developmental block or retarded embryo development is pathological production of ROS, possibly related to inappropriate composition of culture medium and conditions [3,13,26,36]. This dearth has been marginal improved by supplementation of culture medium with different types of antioxidant [8,10,23]. Baldoceda et al. [2] believe that antioxidant therapy can lead to limited improvement, due to fact that ROS are not the main source of the problem and it is the multifaceted functions of mitochondria which is compromised even when ROS levels are reduced by antioxidants.

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Vitamin K2 (VK2) beside its other functions, like ubiquinone, plays an electron carrier role in the mitochondrial electron transport chain complex resulting in more efficient oxygen use and production of ATP [38]. This property of VK2 was first demonstrated by Vos et al. [38] in Drosophila. They showed the deleterious effect of mitochondrial function induced by mutation of genes involved in Parkinson's disease is counteract by VK2. Based on these findings Baldoceda et al. [2] improve rate of blastocysts formation by supplementation of culture media with VK2. Ploured et al. also showed that immature oocytes aspirated post-mortem appeared to carry a mitochondrial dysfunction and VK2 supplementation can increase ATP production needed for *in vitro* embryo development and overexpression of genes involved in mitochondrial functions [26].

Vitrification of embryo is a useful tool in both human and animal assisted reproductive technology. In human, it allows preservation of supernumerary embryos and for livestock it provides opportunity for long preservation of genetically golden embryos, livestock embryos and embryos of species in danger of extinction. Despite the aforementioned benefits, vitrification can lead to biochemical and structural changes which can jeopardize future developmental competency of vitrified embryos [15,16,40] In this regard Martino et al. [19] showed that vitrification has developmentally-related and mild effects on morphology, nuclear and bioenergy/oxidative parameters of mouse embryos. Somoskoi et al. [31] also showed that vitrification effect mitochondrial distribution pattern and Romão et al. [29] believe that reduce cry-tolerance of ovine *in vitro* in comparison to *in vivo* derived embryos is likely due to alteration in lipid content and metabolism related to mitochondrial dysfunction. Despite extensive research on vitrification, there are limited studies regarding how composition of culture media can improve aforementioned side effects of vitrification. Therefore, the current study aims to investigate the effect of supplementation of culture medium with VK2 administration before, and after vitrification of ovine embryos. To our knowledge, this is the first report on effect of VK2 on embryo cryo-preservation.

2. Material and methods

2.1. In vitro production of ovine embryos

Ovine embryo production method was conducted according to previous studies [21]. Ovaries were collected from local slaughterhouses at 2–4 pm, transferred to the laboratory by 6 p.m. and stored at 15 °C until time for harvesting the oocytes based on previously set protocols [39]. Briefly, follicles with 2–6 mm diameters were sucked by needles connected to 15 ml conical tubes containing Hepes-supplemented tissue culture medium- 199 (HTCM199) + 10% FBS (fetal bovine serum) solution. Next, clear and vivid Cumulus-oocyte Complex (COC) with uniform cytoplasm were isolated and washed in H-TCM199 + 10%FCS.Then, they were cultured in 50 μL droplets of maturation medium [MM: tissue culture medium- 199 (TCM199) +10% FBS supplemented with 10 μg/ml FSH (Follicle-stimulating hormone-sigma F8174), 10 μg/ml LH (Luteinizing hormone-sigma L5269), 100 mM 17-beta estradiol 0.1 mM cysteamine, 10 ng/ml EGF (epidermal growth factor) and 100 ng/ml IGF1(insulin-like growth factor 1)] under mineral oil for 22–24 h at 38.5 °C, 5% CO2, in maximum humidified air.

Fresh ram ejaculated were collected from trained rams by artificial vageninto 15 mL tubes and promptly moved to laboratory. Capacitation was induced by washing semen and maintaining sperm in Tyrode's albumin lactate pyruvate medium at 38.5 °C, 5% CO2, in maximum humidity for 30–40 min.

For *in-vitro* fertilization (IVF), 10 COCs were transferred to 50 μL drops of fertilization medium comprised of (NaCl 114 mM, KCl 3.15 mM, NaH2PO4 0.39 mM, Na-lactate 13.3 mM, CaCl2 2 mM, MgCl2 0.5 mM, Na-pyruvate 0.2 mM, Penicillin 50 IU/ml, Streptomycin 50 μg/ml, NaHCO3 25 mM, Heparin 10 μg/ml). Next, COCs were inseminated by capacitated sperm at a ratio of approximately 5000 sperm/matured oocyte. Inseminated dishes were incubated at 38.5 °C, 5% CO2, and maximum humidity for 18 h. Finally, six presumptive zygotes were transferred into 20 μL drops of culture medium comprising synthetic oviduct fluid (SOF) supplemented with 8 mg/ml bovine serum albumin (BSA) at 38.5 °C, 5% O2, 5% CO2 under mineral oil for 3 days. To allow embryos to reach blastocyst stage, in day 3, cleaved embryos were cultured in modified SOF {mSOF: SOF + charcoal stripped serum (10%) and glucose (1.5 mM)}for another 4–5 days at 38.5 °C, 5% O2, 5% CO2 in maximum humidity under mineral oil.

2.2. Embryo vitrification

Vitrification procedure was based on studies of Martinez et al. [18]. Initially, blastocyst were transferred to basic medium (BM: phosphate buffer saline (PBS) and 20% FBS) in room temperature for1 minutes. Next, blastocysts were moved to equilibrium solution (ES: BM+ 7.5% EG+7.5% DMSO) for 5 min and subsequently to vitrification solution (VS: BM+15% EG+ 15% DMSO + 0.5M sucrose) for 35–45 s. Finally, blastocysts were placed on cryotop (Cryologic; CVM™, Fibreplug & Sleeve, Australia), and quickly transferred into liquid nitrogen (LN2). For warming, blastocysts on the cryotop were immediately exposed to warming solutions 1 (WS1: BM+ 1M sucrose) at 37 °C for 1 min. Subsequently, blastocysts were washed in WS2 and WS3 containing 0.5 and 0.0 M sucrose for 3 and 5 min, respectively. Finally, blastocysts were transferred into mSOF for 22–24 h for re-expansion and hatching.

2.3. Experimental design

First, in order to choose the best concentration of VK2, embryos were exposed to various concentration of VK2 (0.0, 0.1, 0.5 and 10 μM) from day 3 to day 7 of *in vitro* culture (IVC) and the number of developed embryo to morula and blastocysts stage were documented on days 5 and 7 respectively. 0.1 μM was considered as optimal concentration for VK2 and used for the rest of experiments. Cultured embryos in media without VK2 were considered as control group. In all experiments, hatched blastocysts were collected on days 7 and 8 of IVC and used for assessment of ROS or quality of blastocysts.

This study consisted of four experimental groups including: 1) IVC^{+}/PW^{+}: embryos which were treated with VK2 (0.1 μM) from day 3–7, vitrified/warmed and again treated with VK2, 2) IVC^{+}/PW^{-}: embryos which were treated with VK2 from day 3–7, vitrified/warmed but were not treated with VK2 after warming, 3) IVC^{-}/PW^{+}: embryos which were not treated with VK2 during IVC, vitrified/warmed and treated with VK2 after warming and 4) IVC^{-}/PW^{-}: embryos not treated with VK2 before and/or after vitrified/warmed. Finally, blastocysts in different groups were used to determine Total Cell Number (TCN), ROS measurement and gene expression.

2.4. Differential staining

The quality of blastocysts were assessed by differential staining according to Moulavi et al. [22]. Hatched blastocysts were washed in H-TCM supplemented with 5 mg/mL BSA and fixed for 30 s in the same solution containing 0.5% Triton X-100. Subsequently, fixed blastocysts were exposed to 30 μg/mL PI (propidium iodide) in H-TCM + 5 mg/mL BSA for 1 min and next, they were washed and stained with 10 μg/mL Hoechst in ethanol for 15 min at 4 °C. Finally,