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Supplementation with nanomolar concentrations of verbascoside during in vitro maturation improves embryo development by protecting the oocyte against oxidative stress: a large animal model study

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ABSTRACT

The effects of verbascoside (VB), added at nanomolar concentrations during in vitro maturation (IVM) of juvenile sheep oocytes, on in vitro embryo development and its mechanisms of action at the oocyte level were analyzed. Developmental rates, after IVM in the presence/absence of VB (1 nM for 24 h; 1 nM for 2 h; 10 nM for 2 h), were evaluated. The bioenergetic/oxidative status of oocytes matured after IVM in the presence/absence of 1 nM VB for 24 h was assessed by confocal analysis of mitochondria and reactive oxygen species (ROS), lipid peroxidation (LPO) assay, and quantitative PCR of bioenergy/redox-related genes. The addition of 1 nM VB during 24 h IVM significantly increased blastocyst formation and quality. Verbascoside reduced oocyte ROS and LPO and increased mitochondria/ROS colocalization while keeping mitochondria activity and gene expression unchanged. In conclusion, supplementation with nanomolar concentrations of VB during IVM, in the juvenile sheep model, promotes embryo development by protecting the oocyte against oxidative stress.

1. Introduction

Recent studies have demonstrated that some polyphenols, phytochemical compounds present in fruit and vegetables, show disease-preventing properties, such as anticarcinogenic, antimutagenic, anti-inflammatory and antioxidant activities, as assayed by in vivo and in vitro models [1,2]. Verbascoside (VB) or acteoside is a polyphenol belonging to the family of phenyl propanoids. It is present in plants widely cultivated in the Mediterranean area [1,3], especially *Aloysia citrodora* and *Olea europea*, whose fruits and derived-products are widespread in the so-called "Mediterranean diet". Verbascoside is structurally characterized by a caffeic acid linked by a β-(D)-glucopyranoside to 4,5-hydroxyphenylethanol (hydroxytyrosol) bound through ester and glycosidic links, with a rhamnose in sequence [1–3] to the glucose molecule [3] and has been reported as showing antioxidant effects in animal experiments as well as in human clinical studies [1,2].

The antioxidant effects of VB observed in several cell systems have been related to different mechanisms of action: (1) short-term reactive oxygen species (ROS) scavenging effects, due to prevention of ROS-related damage in different ways, such as by interfering with

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initial ROS-generating reactions, or by scavenging the free oxygen molecules required to begin ROS production, or by chelating metals that speed up oxidative processes [2–5]; or (2) long-term genomic effects for up-regulation of endogenous detoxifying systems [2,4–6] or down-regulation of genes coding for pro-oxidant enzymes [2,7]. As reported in current literature, the biological mechanisms activated by VB seem to be mediated by linking to different receptor types, such as membrane, cytosolic and nuclear receptors, depending on cell type and functional status [2,7,8–10].

In Assisted Reproductive Technologies (ARTs), there is a need to establish reliable and affordable in vitro treatments with antioxidants for gametes and embryos from women with oxidative stress-based reproductive pathologies or environmental exposure or lifestyle-related fertility decline [11–13]. Additionally, antioxidants may improve the outcome of oocyte in vitro maturation (IVM), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), in vitro embryo culture (IVEC), oocyte and embryo cryopreservation. Indeed, it is well known that oocytes and embryos are vulnerable to oxidative stress conditions occurring in vivo [13] or induced by in vitro culture systems [13]. Various synthetic and natural antioxidants have been added to in vitro culture systems to improve the maturation of oocytes and the developmental competence of preimplantation embryos [14–25]. In recent years, naturally occurring antioxidants are receiving renewed interest because they occur in nature, in many cases being derived from plant sources and presumed to be safe [18]. For instance, beneficial effects on embryo development have been reported after addition of anthocyanin [19], melatonin [20,21] resveratrol [22–24] and sericin [25] during IVM.

Ruminant in vitro models are increasingly being considered as very relevant for human preimplantation reproductive research [26–28]. The ovine, a monovular species like human, could potentially represent an optimal animal model, being closer to human reproductive physiology than other species [29]. Because these models are not hampered by restrictive ethical constraints, they provide great support to research into fertility preservation in women of reproductive age and in prepubertal girls [30–32].

In a previous study performed in the juvenile sheep model, we reported that VB, added at micromolar concentrations using a continuative 24 h IVM exposure protocol, acted as a prooxidant molecule, by impairing oocyte bioenergetic potential and oxidative status and embryo developmental competence. This prooxidant activity was hypothesized to be due to an excessively high tested concentration, as suggested by uptake data and/or by prolonged exposure time in culture media (24 h) which probably induced H_2O_2 production [33]. Thus, it highlighted the need to evaluate whether lower VB concentrations may exert antioxidant effects.

The first aim of the present study was to test, in the same juvenile sheep model, the effects of supplementation with low (nanomolar) concentrations of VB during IVM on oocyte meiotic and developmental competence. Because significant improvements to embryo yield and quality were observed, we thereafter investigated whether these effects could be determined by the antioxidant activity of VB on in vitro matured oocytes.

2. Materials and methods

2.1. Chemicals

All chemicals for in vitro cultures and analyses were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise indicated. Verbascoside was extracted, purified and quantified following a previously described protocol [34]. Verbascoside was added at the concentrations of 1 nM and 10 nM. These concentrations were selected on the basis of previous studies reporting that following ingestion, plasmatic levels of polyphenolic compounds rarely exceed nanomolar concentrations [2]. A stock solution of 1 μM VB was prepared by dissolving lyophilized VB in TCM-199 and stored at −20 °C. Working solutions of 1 nM and 10 nM VB were prepared on the day of use.

2.2. Oocyte collection

Ovaries from sheep under 6 months of age were recovered at local slaughterhouses and processed by the slicing procedure as previously described [35,36]. Only cumulus-oocyte complexes (COCs) with intact cumulus cell layers and homogeneous cytoplasm were selected.

2.3. In vitro maturation (IVM)

In vitro maturation was performed following previously reported procedures [35,37]. Briefly, selected COCs were matured in vitro in TCM 199 supplemented with 10% heat-treated oestrus sheep serum (OSS), 0.1 IU/mL FSH, 0.1 IU/mL LH and 100 mM cysteamine for 24 h at 38.5 °C under 5% CO_2 in air. Verbascoside was added at the concentrations and exposure times as reported in the experimental design.

2.4. In vitro fertilization (IVF) and in vitro embryo culture (IVEC)

As described by Bogliolo et al. [35] in vitro matured oocytes were fertilized in Synthetic Oviductal Fluid (SOF; 38) with 2% oestrous sheep serum (OSS), 1 μg/mL heparin, 1 μg/mL hypotaurine for 22 h at 38.5 °C and under a 5% CO_2, 5% O_2, 90% N_2 atmosphere in four-well Petri dishes with frozen-thawed spermatozoa selected by the swim-up technique (1×10^6 spermatozoa/mL^{-1}). Presumptive zygotes were cultured for 8 days in four-well Petri dishes in SOF with essential and nonessential amino acids at oviductal concentration [39] and 0.4% Bovine Serum Albumin (BSA) under mineral oil, in maximum humidified atmosphere with 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C. The cleavage rate was recorded at 30 h after IVF.

2.5. Nuclear chromatin evaluation of oocytes

To evaluate nuclear chromatin, oocytes underwent cumulus cell removal by incubation in TCM-199 with 20% FCS and 80 IU hyaluronidase/mL and aspiration in and out of finely drawn glass pipettes. Oocytes were stained with 2.5 μg/mL Hoechst 33258 in 3:1 (v/v) glycerol/phosphate buffered saline (PBS) solution and mounted on microscope slides covered with cover slips, sealed with nail polish, and kept at 4 °C in the dark until observation. Oocytes were evaluated in relation to their meiotic stage under an epifluorescence microscope (Nikon Eclipse 600, 400× magnification) equipped with the B-2A (346 nm excitation/460 nm emission) filter, as germinal vesicle (GV), metaphase to telophase I (MI to TI), MII with 1 st polar body (PB) extruded, or degenerated [36].

2.6. Blastocyst evaluation and cell count

Blastocyst formation was assessed at day 8 and blastocysts were classified according to degree of expansion and hatching status [40]: blastocyst (normal blastocyst with a blastocoel equal or up to half of the embryo volume), expanded blastocyst (a large blastocyst with a blastocoel greater than half of the embryo volume or blastocyst with a blastocoel completely filling the embryo), and hatching blastocyst (hatching or already hatched blastocyst). Analysis of blastocyst cell number was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cell compartments [35]. To differentially stain ICM and TE nuclei, blastocysts derived from treated and control groups were exposed to 1% Triton X-100