Theriogenology 81 (2014) 1058–1066

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Dose-dependent effect of melatonin on postwarming development of vitrified ovine embryos

Sara Succu [a], Valeria Pasciu [a], Maria E. Manca [a], Sara Chelucci [a], Laura Torres-Rovira [a], Giovanni G. Leoni [a], Angelo Zinellu [b], Ciriaco Carru [b], Salvatore Naitana [a], Fiammetta Berlinguer [a,*]

[a] Department of Veterinary Medicine, University of Sassari, Sassari, Italy
[b] Department of Biomedical Science, University of Sassari, Sassari, Italy

ARTICLE INFO

Article history:
Received 6 November 2013
Received in revised form 14 January 2014
Accepted 20 January 2014

Keywords:
In vitro embryo production
Oxidative stress
Apoptosis
ATP
Total antioxidant capacity

ABSTRACT

After cryopreservation, embryos become sensitive to the oxidative stress, resulting in lipid peroxidation, membrane injury, and structural destruction. The present study aimed to assess the effect of increasing concentration of melatonin during postwarming culture on embryo's ability to restore its functions after cryopreservation. *In vitro*–produced blastocysts were vitrified, warmed, and cultured *in vitro* in TCM 199 with 5 different supplementations: control (CTR): 10% fetal calf serum; bovine serum albumin (BSA): 0.04% (wt/vol) BSA; and MEL^{-3}, MEL^{-6}, MEL^{-9}: BSA plus melatonin 10^{-3}, 10^{-6}, and 10^{-9} M. The medium with the highest melatonin concentration had the highest trolox equivalent antioxidant capacity, whose values were comparable with those determined in plasma sampled from adult ewes (8.7 ± 2.4 mM). The other media had lower trolox equivalent antioxidant capacity values ($P < 0.01$), below the range of the plasma. At the same time, embryos cultured with the highest melatonin concentration reported a lower *in vitro* viability, as evaluated by lower re-expansion and hatching rates, and lower total cell number compared with the other groups ($P < 0.05$). Their metabolic status was also affected, as evidenced by higher oxidative and apoptotic index and lower ATP concentration. The beneficial effects of melatonin on embryo development during postwarming culture were observed only at low concentration (10^{-9} M). These results suggest that melatonin at high concentration may exert some degree of toxic activity on preimplantation embryos. Thus, the dose at which the embryos are exposed is pivotal to obtain the desiderate effect.

1. Introduction

The ability to successfully cryopreserve embryos with limited loss in viability is essential for the success of assisted reproductive technologies. Currently, however, cryopreservation leads to a 30%–40% reduction in the implantation potential [1]. In addition, the survival of cryopreserved *in vitro*–produced (IVP) embryos, as measured either by postwarming survival in culture or by established pregnancies after embryo transfer, has lagged behind that of *in vivo*–derived embryos [2,3]. Although the background of this difference is not completely understood, it seems that differences in buoyant density between *in vivo*– and *in vitro*–produced embryos because of the different ratio of lipids and proteins may at least partly account for the lower cryo-withstand of IVP embryos [3]. In addition, it has been reported that although *in vivo* the oviduct provides the optimal red/ox environment for the

* Corresponding author. Tel.: +39 0 79 229904; fax: +39 0 79 229429.
E-mail address: berling@uniss.it (F. Berlinguer).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.01.032

embryo [4], *in vitro* culture conditions affect oxidative homeostasis [5,6] by increasing reactive oxygen species (ROS) exposure or mitigating antioxidant protection, thereby resulting in lower embryo quality.

The consequent oxidative stress (OS) is implicated in many different types of cell injuries, including membrane lipid peroxidation, oxidation of amino acids and nucleic acids, apoptosis, and necrosis, which may subsequently decrease the viability of IVP embryos [7,8]. Considering that the process of cryopreservation promotes ROS accumulation [7,9] and that cryopreserved embryos become even more amenable to the deleterious effects of ROS [10,11], the importance of ROS detoxification seems to play a major role in IVP embryo ability to restore its metabolic functions after warming.

OS occurs if disequilibrium takes place between ROS production and antioxidative capacity of the cell [12]. Antioxidants can provide beneficial effects for this balance because antioxidant administration decreases OS and cellular apoptosis and improves the *in vitro* development of mammalian embryos [11].

Melatonin is a potent free radical scavenger and antioxidant, and in contrast to most other known radical scavengers, this indolamine is multifunctional and universal. In addition, the metabolites of melatonin also participate in the interactions with ROS. This phenomenon is referred as a scavenging cascade reaction [13]. In addition to its direct interaction with ROS, melatonin also stimulates the activity of some antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and catalase [14–16]. Because of its multiple antioxidant capacities, melatonin prevents lipid peroxidation, protein, and DNA damage [17]. In addition, melatonin has been found to preserve optimal mitochondrial function and homeostasis by reducing and preventing mitochondrial OS [18], thereby curtailing subsequent apoptotic events and cell death [19].

Previous studies have reported the beneficial effects of melatonin supplementation to IVP embryos in mouse [20–23], ovine [24,25], bovine [26–28], and porcine [29,30], the potential mechanisms being related to its antioxidant and antiapoptotic capacities. The addition of melatonin (10^{-9} M) in the culture medium of *in vivo*–produced vitrified/warmed mouse two-cell embryos was accompanied by elevated intracellular levels of glutathione and reduced ROS production. These changes led to the reduction in apoptosis of blastocysts and the average apoptotic cell numbers/blastocyst in vitrified two-cell embryos [22]. Thus, melatonin may facilitate embryo ROS detoxification during postwarming culture, when embryos require high metabolic activity to complete the resumption of DNA and protein synthesis [31]. This action may be particularly effective for cryopreserved IVP embryos, considering their low cryotolerance.

In the current investigation, melatonin was added at different concentrations to the postwarming culture media of vitrified ovine blastocysts produced *in vitro*, thus exposing the embryos to culture conditions with different total antioxidant capacity. Thereafter, embryo vitality, total cell number, DNA fragmentation, intracellular ATP content, and oxidative status were evaluated. Plasma total antioxidant capacity was also determined in adult ewes to assess the *in vivo* conditions the embryo is likely to be exposed to.

2. Materials and methods

2.1. Chemicals

All chemicals used in the present study were purchased from Sigma Chemical (St Louis, MO, USA), unless stated otherwise.

2.2. Source of blastocysts: in vitro embryo production

Blastocysts used in this study were obtained by applying a standard protocol for *in vitro* embryo production. Ovaries from Sarda ewes of similar age and weight (4–5 years old, body weight 35–40 kg) were obtained from a commercial slaughterhouse and transported to the laboratory within 1 hour in Dulbecco's phosphate-buffered saline (PBS) at a temperature between 25 °C and 35 °C. After washing in fresh medium, ovaries were sliced using a microblade, and the follicle content was released in medium TCM 199 (with Earle's salts and bicarbonate) supplemented with 25 mM HEPES, penicillin and streptomycin, and 0.1% (wt/vol) of polyvinyl alcohol (PVA). The cumulus–oocyte complexes (COCs) comprised 4 to 10 layers of granulosa cells, and oocytes with a uniform cytoplasm, homogenous distribution of lipid droplets in the cytoplasm, and with an outer diameter of about 90 μm (mean) were selected for the experimental procedure. The selected COCs, after three washes in the same fresh medium, were *in vitro* matured in TCM 199 supplemented with 10% heat-treated estrous sheep serum, 1 IU/mL ovine follicle stimulating hormone (FSH), 1 IU/mL ovine luteinizing hormone (LH), and 100 μM of cysteamine. COCs were put in groups of 30 to 35, in 600 μL of the maturation medium in a four-well Petri dish (Nunclon; Nalgene Nunc International, Roskilde, Denmark), layered with 300-μL mineral oil and cultured for 24 hours in 5% CO_2 in air at 39 °C.

After maturation, the COCs were partially stripped of the granulosa cells and fertilized *in vitro*. The fertilization medium was composed of synthetic oviductal fluid (SOF) supplemented with 2% of heat-treated estrous sheep serum (vol/vol), 50 μg/mL streptomycin, and 50 IU/mL penicillin. Frozen/thawed spermatozoa from the same batch, obtained by pooling the ejaculates of three ram of proven fertility, were used across all the experimental procedures. The base medium used for semen cryopreservation was recommended ram extender (Tris 200 mM; citric acid 70 mM; fructose 55 mM; pH = 7.2, osmolality 300 mOsm/kg) supplemented with egg yolk 20% (vol/vol) and glycerol 4% (vol/vol). Freezing and thawing procedures were performed as previously described [32]. After thawing, motile spermatozoa were selected by using a swim-up procedure. Briefly, spermatozoa in the cryodiluent (0.25 mL) were placed gently in the bottom of a round-bottom glass tube that contained 1 mL of SOF and incubated in 5% CO_2, 5% O_2, and 90% N_2 at 39 °C. After 15 minutes, the upper layer of 0.3 mL was removed (aspirating the sperm suspension at a