Theriogenology 91 (2017) 127–133

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Pyridoxine supplementation during oocyte maturation improves the development and quality of bovine preimplantation embryos

Mansour Aboelenain [a, b], Ahmed Zaky Balboula [a, b], Manabu Kawahara [a], Abd El-Monem Montaser [b], Samy Moawad Zaabel [b], Sung-Woo Kim [c], Masashi Nagano [d], Masashi Takahashi [a, *]

[a] Laboratory of Animal Breeding and Reproduction, Department of Animal Science, Graduate School of Agriculture, Hokkaido University, Hokkaido, 060-8589, Japan
[b] Department of Theriogenology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt
[c] National Institute of Animal Science, Animal Genetic Resources Research Center, Namwon, 55717, South Korea
[d] Laboratory of Theriogenology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido, 060-0818, Japan

ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form
21 December 2016
Accepted 21 December 2016
Available online 23 December 2016

Keywords:
Cathepsin B
Pyridoxine
In vitro maturation
Developmental competence
Bovine oocytes

ABSTRACT

Recently, inhibition of cathepsin B (CTSB) activity during *in vitro* maturation (IVM) and culture (IVC) improved the developmental competence and quality of bovine oocytes and embryos. E-64 is a widely used inhibitor to inhibit CTSB activity, however, E-64 inhibits not only CTSB activity but also the activities of other proteases including cathepsin L (CTSL), papain, calpain, and trypsin. Pyridoxine, the catalytically active form of vitamin B6, plays a crucial role in several cellular processes and has the ability to inhibit CTSB activity. However, whether pyridoxine has an improving effect during IVM of bovine oocytes is still unknown. In this study, we investigated the effect of pyridoxine supplementation during IVM on the developmental competence of bovine oocytes and the quality of the produced blastocysts. Supplementation of pyridoxine to the maturation medium significantly decreased the activity of CTSB in both bovine cumulus cells and oocytes. Moreover, pyridoxine improved both the blastocyst and hatched blastocyst rates. In addition, the presence of pyridoxine during IVM also significantly improved the quality of the produced embryos by increasing the total cell number as well as decreasing the CTSB mRNA expression and apoptotic rate. These results indicate that pyridoxine is a promising tool to improve the developmental competence of bovine oocytes and subsequent embryo quality.

1. Introduction

Many studies have been performed to improve the developmental competence of bovine oocytes and embryos [1–4]. However, the quality of *in vitro* produced (IVP) embryos remains incomparable to that of *in vivo* produced embryos [5–7]. Many stressors during *in vitro* maturation (IVM), either extrinsic such as medium composition or culture conditions, or intrinsic such as oocyte quality itself, affect the developmental competence and quality of IVP embryos [8,9]. As a result, it is necessary to find optimal conditions to overcome the negative factors affecting the developmental competence of bovine oocytes.

Cathepsin B (CTSB) is an abundant and ubiquitously expressed cysteine protease found in a wide variety of cells, including bovine oocytes and cumulus cells [10,11]. CTSB is involved in many physiological processes, including intracellular protein degradation in lysosomes, initiation of the apoptotic pathway [12,13], stress-induced response [14], autophagy [15], and differentiation of cancer cells [16]. We have shown that CTSB activity is inversely correlated with the quality and developmental competence of bovine oocytes [3,10]. Thus, inhibiting CTSB activity during *in vitro* maturation (IVM) has emerged as a new strategy to improve the developmental competence of bovine oocytes *in vitro* [10].

l-trans-Epoxysuccinyl-Leucylamido-(4-guanidino) Butane (E-64) and its derivative compounds (CA-030 and CA-074) are the most commonly used inhibitors to inhibit CTSB activity [17,18]. Unfortunately, the affinity of E-64 to bind an active thiol group in many cysteine proteases renders it a non-selective CTSB inhibitor

* Corresponding author.
E-mail address: mmasashi@anim.agr.hokudai.ac.jp (M. Takahashi).

http://dx.doi.org/10.1016/j.theriogenology.2016.12.022
0093-691X/

[19]. In fact, it can also inhibit many cysteine proteases including CTSL, papain, calpain, as well as trypsin [20]. In addition, application of oral administration of E-64 has been achieved for protection from bacterial and viral infections [21–23], that raises the possible improvement of oocyte quality by *in vivo* administration of CTSB inhibitor. However, large scale application of CTSB inhibitor such as *in vivo* administration is impractical because of the cost and the possible toxicity. Thus, it is necessary to find a natural alternative inhibitor that could be used to regulate CTSB activity in mammalian oocytes either *in vivo* or *in vitro*.

Vitamin B_6 coenzymes such as pyridoxine is very similar to vitamin B_6 enzyme in terms of amino acid metabolism and synthesis of nucleic acids [24]. Vitamin B_6 has an essential role in antioxidant activities [25]. Singlet oxygen resistance 1 (*SOR 1*) is involved in *de novo* vitamin B_6 biosynthesis. Pyridoxine quenches singlet oxygen at a rate comparable to that of vitamin C and E, two of the most highly efficient biological antioxidants [26]. Interestingly, vitamin B_6 coenzyme has an active aldehyde at position 4 of the pyridine ring, which has a binding affinity for the active SH-site of cysteine residues in CTSB. This unique structure can explain the inhibitory effect of pyridoxine on CTSB activity in helper T lymphocyte type-2 [27]. Using pyridoxine as a CTSB inhibitor outperforms others by its natural origin from whole-grain products (including cereals), starchy vegetables, fish, liver and organ meats [28,29]. In this study, we investigated the effect of pyridoxine on CTSB activity during oocyte maturation, and its effect on the subsequent development and quality of embryos.

2. Material and methods

2.1. Oocyte collection and IVM

According to the strict regulation of Sapporo slaughterhouse and after BSE screening test result, bovine ovaries were brought to our laboratory from a local abattoir within 12 h after slaughter. The ovaries were washed several times in a sterile saline. IVM was performed as described previously [30]. In brief, cumulus–oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) using an 18-gauge needle attached to a 10-ml syringe and washed three times in tissue culture medium (TCM)-199 medium (Invitrogen, Grand Island, NY, USA). Ten COCs were matured in a 50-μl drop of TCM-199 supplemented with 10% fetal calf serum (FCS; Invitrogen), follicle stimulating hormone (FSH; 0.02 units/ml; Kyoritsu Seiyaku Corp., Tokyo, Japan), estradiol-17β (1 μg/ml; Sigma-Aldrich, St. Louis, MO, USA), and gentamycin (50 μg/ml; Sigma-Aldrich) covered with mineral oil (Sigma-Aldrich) for 22–24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. In vitro fertilization (IVF)

IVF was conducted according to a procedure described previously [31]. Briefly, after thawing of frozen semen in warm water (37 °C) for 20 s, motile sperm were separated using percoll gradients (45 and 90%) (Sigma-Aldrich). COCs were co-incubated with motile sperm (5×10^6 cells/ml) in droplets (10 COCs/100 μl) of modified Brackett and Oliphant's isotonic medium containing 3 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich) and 2.5 mM theophylline for 18 h at 38.5 °C under a humidified atmosphere of 5% CO_2, and 90% N_2.

2.3. In vitro culture (IVC)

IVC of presumptive zygotes was performed as described previously [32]. Briefly, after fertilization, cumulus cells were removed by mechanical pipetting (internal diameter of the pipette, 150–180 mm) [33], and the presumptive zygotes were transferred to 50 μl drops (20–30 zygotes/drop) [34] of modified synthetic oviduct fluid (SOF) medium supplemented with amino acid solution (Sigma-Aldrich), 10 μl/ml insulin, 1 mM glucose, and 3 mg/ml fatty acid-free BSA at 38.5 °C under 5% CO_2, 5% O_2 and 90% N_2.

2.4. RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from twenty blastocysts per replication was extracted using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The extracted RNA was then immediately used for RT-PCR or stored at −80 °C until analysis. cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Conventional PCR was performed using the GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA). Primers specific for *CTSB* were designed and commercially synthesized (Eurofins Genomics, Co., Ltd., Tokyo, Japan). The primer information is presented in Table 1. The reactions were carried out in 96-well PCR plates, in a total volume of 10 μl containing 1 μl of 10 pmol/μl of each primer, 5 μl of the Thunderbird Sybr qPCR Mix (Toyobo), and 3 μl of cDNA. After centrifugation, the plates were placed in a Roche Light Cycler 480 II (Roche, Basel, Switzerland) and subjected to the following cycling conditions: a denaturation step at 95 °C for 30 s, an amplification step of 50 cycles at 95 °C for 10 s, 57 °C for 15 s, 72 °C for 30 s, a melting-curve step using a gradient of 55–95 °C with an increment of 2.2 °C/sec and continuous fluorescence acquisition, and a cooling step at 4 °C. Amplicons that consisted of PCR products were confirmed to have a single band for each target gene by electrophoresis and sequenced to verify their authenticity. The expression levels of the target genes were determined relative to that of the histone H2A family member Z (*H2AFZ*) [35].

2.5. Detection of CTSB activity

CTSB activity was measured by using Magic Red Detection Kit (MR-RR) 2 (Immunochemistry Technologies, LLC, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, IVM oocytes or COCs were stained in 250 μl of serum-free Dulbecco's modified Eagle medium (DMEM) containing 1 μl of reaction mix in a humidified atmosphere of 5% CO_2 at 38.5 °C for 30 min. For nuclei staining, Hoechst (H 33342; Sigma-Aldrich) was added and incubated in the same culture conditions for 5 min. After rinsing in phosphate-buffered saline (PBS), the stained oocytes or COCs were mounted onto a glass slide and observed under a fluorescence microscope BZ-9000 Biorevo (Keyence, Osaka, Japan). An excitation filter of 590 nm was used for CTSB detection (red), while an excitation filter of 365 nm was used for observing the cumulus cell nuclei (blue).

2.6. Differential staining

Differential staining for bovine embryos was carried out as described previously [36]. In brief, blastocysts were incubated at room temperature for 40–60 s in 0.2% (v/v) Triton-X100 with 0.1 mg/ml propidium iodide (P4864; Sigma-Aldrich). Blastocysts were then stained with 25 μg/ml of Hoechst reagent (Sigma-Aldrich) in 100% (w/v) EtOH at 4 °C for 3 h. The stained blastocysts were rinsed in glycerol, mounted onto a glass slide, and observed with a fluorescence microscope (Nikon, Tokyo, Japan). The nuclei of the inner cell mass (ICM) were stained in blue by Hoechst reagent and the nuclei of trophoectoderm (TE) cells were stained in pink by both Hoechst reagent and propidium iodide.