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High mobility group box 1 (HMGB1) enhances porcine parthenotes developing in vitro in the absence of BSA

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Abstract

High mobility group box 1 (HMGB1) is considered a component of chromatin and membranes with a role in a variety of biologically important processes. The aim of this study was to determine the effects of HMGB1 on the viability and development of porcine diploid parthenotes cultured in vitro. In vitro derived 4-cell parthenotes were cultured to blastocysts, with or without recombinant HMGB1, in the presence or absence of BSA. The addition of 1, 10, 100 or 1000 ng/mL HMGB1 into NCSU 23 medium containing 0.4% BSA did not enhance the development of 4-cell parthenotes to the blastocyst stage and did not change the total number of nuclei in the blastocysts. However, addition of 10 or 100 ng/mL HMGB1 into NCSU 23 medium in the absence of BSA increased ($P < 0.05$) both the development rate of parthenotes to the blastocyst stage and total cell numbers. When cultured in NCSU23 medium supplemented with 10 or 100 ng/mL HMGB1 and without BSA, apoptosis in parthenotes at the blastocyst stage was decreased ($P < 0.05$). Based on real time RT-PCR, the addition of HMGB1 to the culture medium in the absence of BSA decreased mRNA expression of pro-apoptotic genes *Bak* ($P < 0.005$) or Caspase3 (*Casp3*, $P < 0.01$), but not Bcl-xL (*Bcl2l2*). In conclusion, we inferred that recombinant HMGB1 in the culture medium in the absence of BSA prevented apoptosis of porcine parthenotes and enhanced porcine embryo viability.

Keywords: HMGB1; Apoptosis; Parthenotes; In vitro culture; Pig

1. Introduction

High mobility group box 1 (HMGB1, previously termed HMG1 or amphoterin [1]) was first isolated from calf thymus as an abundant nuclear protein [2]. It has been implicated in a variety of biologically important processes including transcription, DNA repair [3,4], differentiation, development and extracellular signaling [5,6], cellular migration and tumor invasion [7]. More recent work has shown that HMGB1 inhibits cell death in yeast and mammalian cells, possibly by regulating apoptosis related genes such as *Bcl-2* family or Caspase 8 [8].

Programmed cell death or apoptosis is crucially involved in development and differentiation of embryos. Environmental stresses, such as those imposed by in vitro culturing can induce unscheduled apoptosis in cultured embryos, which may lead to arrest or abnormal development and lower viability of embryos. There are at least two major protein families involved in the regulation of apoptosis, namely, *Bcl-2* and *Bax*. The *Bcl-2* family members, including *Bcl-2*, *Bcl-xL*, *Bcl-x* and *Bcl-w*, protect cells from apoptosis, whereas the *Bax*

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family members *Bad, Bax* and *Bak* induce apoptosis in somatic cells. These patterns also appear to hold for embryonic development, as poor quality bovine embryos undergoing more apoptosis express more *Bax* and less *Bcl* as compared to good quality embryos [9]. Nutt et al. [10] reported that apoptosis results from changes in mitochondrial integrity caused by various effectors, such as Ca^{2+}, reactive oxygen species (ROS), or production of *Bax*, that lead to the release of cytochrome *c* and activation of the Caspase cascade.

There is a high incidence of polyspermy with in vitro fertilization (IVF) of porcine embryos; development of these embryos to the blastocyst stage is compromised. However, development of porcine parthenogenetic diploids to the blastocyst stage resembles normal development of embryos [11–14]. Perhaps parthenogenetic diploids could be used as model embryos for studies of early development and establishment of in vitro culture systems in the pig.

In the present study, we determined for the first time the effect of HMGB1 on development and apoptosis in porcine presumptive diploid parthenotes cultured in vitro. In addition, we used real time RT-PCR techniques to quantify the expression of apoptosis related genes, such as Bcl-xL (*Bcl2l2*), *Bak* and Caspase3 (*Casp3*).

2. Materials and methods

2.1. In vitro porcine oocyte maturation and parthenogenic activation

Prepubertal porcine ovaries were collected from a local abbatoir and transported to the laboratory at 25 °C in Dulbecco's phosphate-buffered saline (PBS) supplemented with 75 mg/mL penicillin G and 50 mg/mL streptomycin sulfate. Cumulus-oocyte complexes (COC) were aspirated from follicles of 3–6 mm in diameter with an 18-gauge needle and disposable 10 mL syringe. The COC were washed three times with Hepes-buffered Tyrode's medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). Each group of 50 COC was matured in 500 mL tissue culture medium (TCM)-199 (with Earle's salts; Gibco; Grand Island, NY, USA) supplemented with 0.57 mM cysteine (Sigma, St. Louis, MO), 10 ng/mL EGF, (Sigma), 10 IU/mL PMSG (Sigma) and 10 IU/mL hCG (Sigma) under paraffin oil at 39 °C for 44 h. Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/mL hyaluronidase for 2–3 min. Oocytes were activated for parthenogenesis with 50 uM Ca^{2+} ionophore A23187 for 5 min. After 3 h of culture in North Carolina State University (NCSU) 23 medium containing 7.5 mg/mL cytochalasinB (CB, Sigma), embryos were washed three times in NCSU 23 medium with 0.4% (w/v) BSA and cultured in the same medium for 48 h at 39 °C in an atmosphere of 5% CO_2 and 95% air.

2.2. Embryo culture

After 48 h of culture in NCSU23 medium containing 0.4% BSA, presumptive diploid 4-cell parthenotes embryos were collected and washed three times in NCSU23 medium with 0.4% (w/v) PVA and then randomly cultured in the same medium containing HMGB1 according to experimental design. The embryos were cultured for 5 days (Day 7 from parthenogenic activation) at 39 °C and 5% CO_2 in air; embryos that developed to the blastocyst stage were analyzed.

2.3. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

Blastocysts were washed three times in PBS (pH 7.4) containing polyvinylpyrolidone (PVP, 1 mg/mL), followed by fixation in 3.7% paraformaldehyde in PBS for 1 h at room temperature (RT). After fixation, embryos were washed in PBS/PVP and permeabilized by incubation in 0.5% Triton X-100 for 1 h at room temperature. Embryos were then washed twice in PBS/PVP and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (in situ Cell Death Detection Kit, Roche; Mannheim, Germany) in the dark for 1 h at 37 °C. After counterstaining with 50 mg/mL RNase A in 40 mg/mL propidium iodide (PI) for 1 h at 37 °C to label all nuclei, embryos were washed in PBS/PVP, mounted with slight coverslip compression and examined under a confocal microscope.

For the other experiments, embryos were fixed in 3.7% paraformaldehyde in PBS for 1 h RT, stained with 40 μg/mL PI for 1 h at 37 °C to label all nuclei and total cell numbers were counted under a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Real time reverse transcription polymerase chain reaction (real time RT-PCR)

Ten blastocysts were washed in Ca^{2+}- and Mg^{2+}-free PBS, snap frozen in liquid nitrogen and stored at −70 °C. Messenger RNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. Synethesis of cDNA was achieved by reverse transcription of the RNA by