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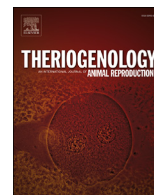

Effects of cytoplasts from Taiwan native yellow cattle on the cellular antioxidant ability of cloned Holstein cattle and their offspring

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ABSTRACT

We previously demonstrated that the cellular thermotolerance of cloned cattle produced by combination of ooplasm (o) derived from Taiwan native yellow cattle (Y) and the donor (d) nucleus derived from Holstein (H) cattle (Yo-Hd) transmits to their offspring (Yo-Hd-F1). In the present study, the responses of mitochondria in these cloned cattle and their offspring after heat shock were investigated to elucidate influence of cytoplasmic input (i.e., ooplasm) during the course of heat stress. After heat shock, oxidative phosphorylation proteins (Complex III and IV) of ear fibroblast cells with Y-originated cytoplasm (including Y, Yo-Hd, and Yo-Hd-F1 cattle) were significantly greater ($P < 0.05$) than those of ear fibroblast cells with H-originated cytoplasm (including H, Ho-Hd, and Ho-Hd-F1 cattle). However, the expressions of Complex I and Complex II protein in heat shocked cells derived from Yo-Hd-F1 cattle were significantly ($P < 0.05$) higher than those of cell derived from cattle with the H-cytoplasm. The catalase (CAT) expression in heat shocked ear fibroblast cells derived from Yo-Hd and Yo-Hd-F1 cattle were significantly ($P < 0.05$) higher than that of cells derived from Ho-Hd-F1 cattle. However, the level of glutathione peroxidase (GPx) expression was higher ($P < 0.05$) in ear fibroblast cells with Y-originated cytoplasm compared to cells with H-originated cytoplasm. In conclusion, the expression of proteins involved in mitochondrial oxidative phosphorylation and antioxidant enzymes after heat shock was increased in ear fibroblast cells from cattle with Y-originated cytoplasm, which can be transmitted to their offspring.

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1. Introduction

Environmental stress such as heat stress affects animal reproductive efficiency in farm animals [1,2]. Thermotolerant cattle breeds (*Bos Indicus*, e.g. Brahman) regulate body temperature better in response to heat stress than thermosensitive cattle breeds (*Bos Taurus*, e.g. Holstein). At the cellular level, deleterious effects of elevated temperature on cellular function are less for cells from *Bos Indicus* breeds (Brahman) than cells from *Bos Taurus* breeds (Angus and Holstein) [3,4]. The rate of blastocyst formation in embryos produced from oocytes derived from heat-tolerant breeds is greater after heat shock than in embryos produced from oocytes derived from heat-sensitive breeds [5]. Moreover, our previous studies demonstrate that thermotolerance is greater in cloned embryos [6] and ear fibroblast cells [7] from cattle reconstructed using Taiwan native yellow cattle (Y; *Bos Indicus*) ooplasm and Holstein (H, *Bos Taurus*) donor nuclei (Yo-Hd) compared to embyos or fibroblasts from cattle with cells reconstructed using Holstein ooplasm and Holstein nuclei (Ho-Hd). These results suggest that the ooplasm plays an important role in thermotolerance.

Mitochondria play a critical role in the generation of metabolic energy by mitochondrial oxidative phosphorylation. However,

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mitochondrial dysfunction may be affected by environmental stress such as heat stress. Heat stress causes mitochondrial damage/dysfunction, which is characterized by reduced energy production through oxidative phosphorylation [8]. Hyperthermia induces mitochondrial damage in rat cardiomyocytes [9], rat CNS neurons [8], and bovine mammary epithelial cells [10]. Hyperthermia was proposed to be responsible for stimulating reactive oxygen species (ROS) production to mediate cellular injury [11]. ROS are generated during mitochondrial oxidative metabolism as well as in cellular response to heat stress [12]. Antioxidant systems, including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), are mainly involved in cellular ROS degradation [13]. SOD converts the superoxide radical ($O_2{\bullet}^-$) into hydrogen peroxide, which is metabolized by GPx and CAT [13].

We have demonstrated greater thermotolerance in cloned embryos [6], cloned cattle ear fibroblast cells [7] and in cells from offspring [14] with Y-originated cytoplasm compared to embyos or cells with H-originated cytoplasm. However, there is no direct evidence regarding changes in mitochondria after exposure to heat shock in ear fibroblast cells with different cytoplasmic origins. In the present study, ear fibroblast cells of cloned cattle (Ho-Hd and Yo-Hd) were derived from our previous study [15], and ear fibroblast cells of their F1 offspring were produced by insemination with Holstein semen [14]. Heat-induced expression of oxidative phosphorylation-related proteins and antioxidant enzymes were examined in ear fibroblast cells derived from these cloned cattle and their offspring.

2. Materials and methods

2.1. Origin of ear fibroblast cells

The sample of ear fibroblast cells which harvested from different breeds in this study are consistent with the our previous research [14], included two cloned cows (1 Ho-Hd and 1 Yo-Hd), four cloned offspring (2 Ho-Hd-F1 and 2 Yo-Hd-F1), three purebred Taiwan native yellow cattle (Y, heat-tolerant breed), and three purebred Holstein cattle (H, heat-sensitive breed). The cloned cows were produced by using ooplasm derived from H (Ho-Hd) and Y (Yo-Hd) and donor nuclei derived from the same Holstein cow (i.e. donor nuclei with identical genetic backgrounds). The cloned offspring of Ho-Hd-F1 ($n = 2$) and Yo-Hd-F1 ($n = 2$) were produced by means of embryo transfer which artificial inseminated to Ho-Hd and Yo-Hd separately by the same semen of Holstein (heat-sensitive breed).

2.2. Chemicals and reagents

The chemicals and reagents employed in this study were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA), except for those specified items purchased from other companies.

2.3. Preparation of fibroblast cells and culture

Ear fibroblast cells were harvested as previously indicated [14,16]. Briefly, ear tissues (3 mm^2 per piece) were excised from each animal (<3-month of age) and cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 1% (v:v) penicillin/streptomycin (Gibco, 15140–122) and 10% (v:v) fetal bovine serum (FBS, Gibco, 10270–106). Culture was at 38.5 °C with 5% CO_2 in air. After intact pieces of ear tissue were discarded and adhered fibroblast cells were continuously cultured until passage 3, and then were stockpiled in FBS containing 10% (v:v) dimethyl sulfoxide (DMSO) in liquid nitrogen until utilization.

2.4. Heat shock treatment

Heat shock treatment and the temperature (42 °C) chosen for bovine ear fibroblast cells was as indicated by Lee et al. [7] and Kesorn et al. [14]. Briefly, cells (passage 3) in DMEM plus 10% FBS and 1% penicillin and streptomycin were incubated in 5% CO_2 in air at 38.5 °C for 24 h and then were trypsinized (passage 4) to release the cells from the plate. The cells were resuspended (1×10^6/mL) in DMEM supplemented with 10% FBS and incubated under saturated humidity at 38.5 °C with 5% CO_2 in air for the control group or at 42 °C with 7% CO_2 in air for the heat-shocked group. After 12 h of treatment, cells of the fifth passage were harvested for protein extraction.

2.5. Western blotting

Expression levels of oxidative phosphorylation related proteins representing Complex I, Complex II, Complex III and Complex IV in ear fibroblast cells, with or without heat shock treatment, were assessed by immunoblotting as previously delineated [14,17]. In short, ear fibroblast cells (with or without heat shock) were rinsed two times in DPBS and cells were then digested on ice for 15 min with 50 μL radioimmunoprecipitation assay (RIPA) lysis buffer (Millipore, Temecula, CA, USA). Proteinase inhibitor cocktail (Roche, Germany) was then added. Cell lysate was centrifuged at $20{,}000\times g$ for 15 min and the supernate was used for immunoblot analysis. Total protein concentration was determined by the Bio-Rad protein assay kit II (Bio-Rad, Hercules, CA, USA). SDS-PAGE (8–12% polyacrylamide gel) was loaded with 50 μg protein for each sample and electrophoresed (80 V) for 2 h. The gel proteins were transferred onto membranes of polyvinylidene difluoride (PVDF) (Amersham Biosciences, GE Healthcare Europe GmbH, Diegem, Belgium). Membranes of PVDF were blocked with 2% BSA at 37 °C for 1 h. Anti-Complex I (NADH-ubiquinone oxidoreductase α subunit 9) (1:2000; Mouse anti bovine, Thermo Fisher Scientific, Waltham, USA), anti-Complex II (succinate dehydrogenase, subunit 70 kDa; 1:2000; Rabbit anti human, Santa Cruz Biotechnology, Heidelberg, Germany), anti-Complex III (ubiquinol-cytochrome c reductase complex core protein 2; 1:2000; Mouse anti bovine, Novus Biologicals, Bangkok, Thailand), anti-Complex IV (cytochrome *c* oxidase subunit III; 1:2000; Rabbit anti human, Thermo Fisher Scientific) and anti-actin (1:5000; Millipore) were utilized as primary antibodies. After washing, horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Millipore) were added. The specificity of these antibodies to bovine proteins was confirmed by Western Blotting. The relative protein expression level was assessed via chemiluminescence assay (using an ECL kit, Millipore). The image was obtained by an illumination imaging system (UVP, Upland, CA, USA) and the amount of proteins were analyzed by densitometric assessment using a Bio-imaging camera system. The quantity of each protein was calculated based on the amount of actin detected in the same sample. The relative expression of each protein in the heat shocked groups was determined by dividing its arbitrary unit over that of the arbitrary unit of the control (i.e. cells that did not undergo heat shock).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Expression of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in ear fibroblast cells, with or without heat shock treatment, was investigated using a sandwich ELISA assay [14,18]. In brief, unlabeled capturing antibodies against GPx (1:500, Rabbit anti human, Santa Cruz Biotechnology), SOD (1:500, Mouse anti human, Santa Cruz Biotechnology) and CAT (1:500, Goat anti human, Santa Cruz Biotechnology) were coated onto 96-

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