Journal of Reproduction and Development, Vol. 67, No 2, 2021

—Original Article—

Mitochondrial reactive oxygen species regulate mitochondrial biogenesis in porcine embryos

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Abstract. The number of mitochondria in blastocysts is a potential marker of embryo quality. However, the molecular mechanisms governing the mitochondrial number in embryos are unclear. This study was conducted to investigate the effect of reduced mitochondrial reactive oxygen species (ROS) levels on mitochondrial biogenesis in porcine embryos. Oocytes were collected from gilt ovaries and activated to generate over 4 cell-stage embryos at day 2 after activation. These embryos were cultured in media containing either 0.1 μM MitoTEMPOL (MitoT), 0.5 μM Mitoquinol (MitoQ), or vehicle (ethanol) for 5 days to determine the rate of development to the blastocyst stage. The mitochondrial number in blastocysts was evaluated by real-time polymerase chain reaction (PCR). Five days after activation, the embryos (early morula stage) were subjected to immunostaining to determine the expression levels of NRF2 in the nucleus. In addition, the expression levels of *PGC1α* and *TFAM* in the embryos were examined by reverse transcription PCR. One day of incubation with the antioxidants reduced the ROS content in the embryos but did not affect the rate of development to the blastocyst stage. Blastocysts developed in medium containing MitoT had lower mitochondrial DNA copy numbers and ATP content, whereas MitoQ showed similar but insignificantly trends. Treatment of embryos with either MitoT or MitoQ decreased the expression levels of NRF2 in the nucleus and levels of *PGC1α* and *TFAM*. These findings indicate that reductions in mitochondrial ROS levels are associated with low mitochondrial biogenesis in embryos.

Key words: Blastocyst, Embryo development, Mitochondrial biogenesis, Mitochondrial number, Reactive oxygen species

(**J. Reprod. Dev. 67:** 141–147, 2021)

Technology for *in vitro* embryo production has been developed for humans and cows, and blastocysts developed *in vitro* are used for embryo transfer worldwide. Through these numerous efforts, the quality of *in vitro*-developed embryos has been improved but remains lower than that of *in vivo*-developed embryos. Mitochondria are important organelles for oocytes and embryos, and their number is thought to be an important marker of oocyte quality. The mitochondrial number in oocytes is notably greater than that observed in cells, and increases in mitochondrial number have been observed between early antral follicles (approximately 50,000) and antral follicles (200,000–350,000) in pigs [1–4]. A larger mitochondrial number in oocytes is closely associated with high oocyte maturation, fertilization, and developmental competence [5–7]. After fertilization, the mitochondrial number decreases and is maintained at a low level until the blastocyst stage because of the low mitochondrial biosynthesis activity [8]. In blastocysts, the mitochondrial number increases and mitochondrial morphology changes to meet the active proliferation requirement of trophoblasts [9, 10]. Therefore, a greater mitochondrial number is considered as an important factor underlying proper post-implantation development [11]. However, the relationship between mitochondrial number and reproductive potential in blastocysts is not completely clear. Accumulating evidence supports the quiet embryo hypothesis [12], which states that blastocysts with higher mitochondrial DNA copy numbers have low quality or low reproductive ability [13–16].

Mitochondrial biogenesis is regulated at the transcriptional, translational, and post-translational levels [17], and peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α) is a key regulator of mitochondrial biogenesis [18]. In addition, NRF2, NRF1, and downstream TFAM are the main regulators of mitochondrial biosynthesis [19, 20].

However, the factors that stimulate mitochondrial biogenesis during implantation development are unclear. In an exercise model, it was suggested that reactive oxygen stress acts as a signal to increase mitochondrial biogenesis through the NRF2-PGC1a-TFAM pathway [20]. Accumulating evidence has shown that the expression levels of NRF-1, NRF-2, PGC1α, and PGC1β are upregulated by oxidative stress [21–23], indicating a close link between reactive oxygen species (ROS) and mitochondrial biogenesis [24]. Furthermore, ROS levels are considered as signaling molecules that regulate the redox regulation of cellular processes including proliferation, differentiation, and apoptosis [25, 26]. 2,2,6,6-Tetramethyl-4-[[5-(triphenylphosphonio) pentyl]oxy]-1-piperidinyloxy bromide (MitoTEMPOL: MitoT) and 10-(2,5-dihydroxy-3,4-dimethoxy-6-methylphenyl)decyl] triphenyl-phosphonium, monomethanesulfonate (Mitoquinol: MitoQ) are specific inhibitors of mitochondrial reactive oxygen that can

Received: September 5, 2020
Accepted: February 2, 2021
Advanced Epub: February 20, 2021

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reduce the generation of mitochondria-specific ROS or superoxide [27]. Therefore, we hypothesized that modulation of ROS levels in mitochondria affects mitochondrial biogenesis in embryos. To evaluate this hypothesis, we added mitochondrial antioxidants to the culture medium of porcine parthenogenetic-activated embryos and evaluated embryo development and mitochondrial DNA copy number.

Materials and Methods

Ethics statement

The animal ethics committee of the Tokyo University of Agriculture approved the use of oocytes from gilt ovaries collected from a slaughterhouse where ovaries are otherwise discarded.

Chemicals and media

All drugs used in this study were purchased from Nacalai Tesque (Kyoto, Japan) unless stated otherwise. The medium for *in vitro* maturation (IVM) of oocytes was TCM199 (Gibco, Grand Island, NY, USA) supplemented with 10% v/v porcine follicular fluid, 0.5 mM L-cysteine, 1 mM L-glutamine, 0.9 mM sodium pyruvate, 10 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 5% fetal calf serum, 10 IU/ml equine chorionic gonadotropin (ASKA Pharma Co., Ltd., Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (Fuji Pharma Co., Ltd., Tokyo, Japan). Porcine follicular fluid was aspirated from the antrum follicles (3–5 mm in diameter) of gilts, centrifuged (10,000 × *g* for 5 min), and stored at –20°C until use. The medium for *in vitro* culture (IVC) of embryos was porcine zygote medium 3 (PZM3) [19, 28]. IVM was performed under atmospheric conditions of 5% CO_2 and 95% air at 38.5°C, and IVC was performed under atmospheric conditions of 5% O_2, 5% CO_2, and 90% N_2 at 38.5°C. MitoTEMPOL (MitoT; 18796, Cayman Chemical, Ann Arbor, MI, USA) and Mitoquinol (MitoQ; 89950, Cayman Chemical) were diluted in water and ethanol by1000-fold to obtain the final concentrations (0.1 and 0.5 μM for MitoT and MitoQ, respectively). The concentrations of the antioxidants were determined in a previous study [29, 30]. In addition, in our preliminary experiments, we found that 0.1 μM MitoT and 0.5 μM MitoQ were the most effective among 0, 0.1, and 1 μM of MitoT and 0, 0.5, and 5 μM MitoQ, respectively. Control groups were prepared in the same volume of vehicle (1/1000 ethanol). Supplementation of 1/1000 ethanol to the culture medium did not affect developmental rate to blastocyst stage (Control 16.7 ± 3.6%, Ethanol 19.0 ± 2.4, P = 0.59).

Oocyte collection and in vitro maturation

Cumulus-oocyte complexes (COCs) were collected and used for IVM according to our previous method [31]. Briefly, ovaries were collected from gilts at the slaughter house, stored at 37°C in phosphate-buffered saline containing antibiotics, and transported to the laboratory within 1 h. The COCs were aspirated from antral follicles (3–5 mm in diameter) using a syringe with a 21-G needle (Terumo, Tokyo, Japan). The COCs were cultured in IVM medium for 44 h (10 COCs/100 μl drops) and then subjected to activation.

Parthenogenetic activation (PA) of oocytes and in vitro culture of embryos

After IVM, all oocytes were subjected to parthenogenetic activation. Polyspermy occurs frequently in porcine oocytes fertilized *in vitro*, and the rate of polyspermic fertilization varies to a great extent among ovary batches. In addition, the ploidy of the embryos affects mitochondrial numbers in the resultant blastocysts [31]. To avoid these differences, we conducted parthenogenetic activation rather than *in vitro* fertilization. The oocytes were denuded from surrounding cumulus cells and activated in a solution containing 280 mM mannitol, 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$ using a single electrical pulse of 100 V for 0.1 ms and CUY500P1 electrode (NEPA21 (NepaGene Ltd., Chiba, Japan) followed by incubation in PZM3 containing 10 μM cytochalasin B and 10 μM cycloheximide for 4.5 h. The embryos were then cultured in IVC media (10 embryos/50 μl drops). After 2 days of PA, cleaved embryos with normal morphology (> 4-cell stage) were individually cultured in 6 μl of IVC media supplemented with mitochondrial antioxidants (0.1 μM of MitoT and 0.5 μM of MitoQ, or 1/1000 ethanol vehicle) for 5 days. Embryos at the > 4-cell stage after 2 days of PA were predicted to have good subsequent developmental ability. We used greater than 4-cell stage embryos for the experiments to obtain a uniform developmental background.

Evaluation of developmental competence of embryos

After 5 days of incubation (day 7 post-activation), the blastocyst rate was examined, and the total cell number of randomly selected blastocysts was counted after Hoechst 33342 staining under a fluorescence microscope (Olympus, Tokyo, Japan). Approximately 20–25 embryos (> 4-cell stage embryos) were cultured for each replicate and repeated 25 times, and the rate of embryos (day 2) reaching the blastocyst stage at day 7 was calculated. All blastocysts derived from each of the 6 trials were used for ATP (29 embryos were used for each experimental group, respectively) and mitochondrial DNA copy number measurement; 31, 24, and 32 embryos were used for control, MitoT, and MitoQ groups), and blastocysts derived from others (13 trials) were used to measure the total cell number of the blastocysts. To obtain cleaved embryos, 3,350 oocytes were used, and the total cleaved rate (over 4-cell stage embryos/oocytes at 48 h post-activation) was 60.0%. To measure the total cell number of the blastocysts, embryos were fixed in 4% paraformaldehyde overnight, followed by treatment with Triton-X 100 (0.25%) for 30 min and mounted on a slide glass with antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). The number of nuclei was examined under a fluorescence microscope (DMI 6000B, Leica, Wetzlar, Germany).

Evaluation of mitochondrial function and number

Blastocysts obtained by IVC with or without antioxidants were used for the experiment. The ATP content of individual blastocysts was measured based on the luminescence generated in an ATP-dependent luciferin-luciferase reaction (ATP Assay Kit; TOYO B-Net., Ltd., Tokyo, Japan). Luminescence was measured immediately after the reaction using a luminometer (Spark 10M; Tecan, Männedorf, Switzerland). Blastocysts were individually transferred into 50 μl of distilled water and stored at −20°C until measurement. DNA from individual blastocysts was extracted by incubation in 30 μl of lysis buffer (final concentration: 20 mM Tris, 0.4 mg/ml proteinase K, 0.9% Nonidet P-40, and 0.9% Tween-20) at 55°C for 30 min, followed by incubation at 98°C for 10 min. The mitochondrial number was determined by PCR using a real-time system (Bio-Rad Laboratories

Hercules, CA, USA) and KAPA SYBR Fast (Kapa Biosystem, Wilmington, MA, USA) as described previously [32–35]. Real-time PCR was performed at 95°C for 3 min, followed by 40 cycles at 98°C for 5 sec and 60°C for 11 sec. The primer set was designed using Primer-BLAST (NC_000845.1: forward, 5-atccaagcactatccatcacca-3 and reverse, 5-ccgatgattacgtgcaaccc-3; 10277-10431, 155 bp). A standard curve was generated for each assay using 10-fold serial dilutions, representing copies of the external standard, which was the PCR product of the corresponding gene cloned into a vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). To obtain the copy number of the standard DNA, the concentration of the standard DNA, molecular weight of the vector, and Avogadro's number were used. Prior to its use, this product was sequenced for confirmation.

Measurement of mitochondrial ROS

We examined whether mitochondrial antioxidants reduced mitochondrial ROS. Embryos (> 4-cell stage embryos at day 2 post-activation) were cultured in medium containing vehicle (ethanol), MitoT (0.1 μM), or MitoQ (0.5 μM) for 24 h, and ROS in mitochondria were measured using MitoSOX (mitochondrial superoxide indicator, Invitrogen). Embryos were incubated with 5 μM MitoSox for 10 min, and then observed under a fluorescence microscope (Leica) to obtain images. Fluorescence intensity was calculated using ImageJ software (NIH https://imagej.nih.gov/ij/).

Reverse transcription PCR (RT-PCR)

Mitochondrial biogenesis starts from the morula stage, and when mitochondrial DNA copy number was compared between embryos, differential mitochondrial DNA copy number was detected from day 4 post-activation in pigs [31]. Therefore, embryos on day 5 (morula-stage 130 h after PA) were used for RT-PCR. Two days post-activation, cleaved embryos (> 4-cell stage) were transferred into medium containing MitoT, MitoQ, and vehicle (ethanol), and cultured for 3 days. Then, 30 embryos from each group were used for RNA extraction. RNA was extracted using an RNAqueous total RNA isolation kit (Invitrogen) and reverse-transcribed into cDNA using SuperScript IV Reverse Transcriptase and oligo(dT) primer. Next, cDNAs were diluted by 20-fold with DNAase and RNAase-free water. PCR was conducted with 8 μl of cDNA, 10 μl of KAPA SYBR® FAST qPCR (Kapa Biosystems), and 2 μl of primer set (final concentration: 0.2 μM). The primer sets are shown in Table 1. All PCRs were conducted at 95°C for 3 min, followed by 40 cycles of 98°C for 8 sec and 60°C for 10 sec. PCR products were examined by melting curve analysis and electrophoresis. Gene expression was determined by absolute quantification using a serially diluted standard plasmid into which each PCR product was cloned using the Zero Blunt™ TOPO™ PCR Cloning Kit. According to the minimal information for publication of Quantitative Real Time PCR Experiments guidelines [36], the expression levels of *PGC1α* and *TFAM* were normalized to that of *B2M*. The amplification efficiency was > 1.98. The experiment was repeated 4 times using differential embryo batches.

Expression levels of NRF2 in the nucleus

Embryos were immunoassayed against NRF2, and the levels of NRF2 in the nuclei of embryos were examined. Embryos (> 4-cell stage embryos at 2 days post-activation) were cultured in IVC medium containing MitoT, MitoQ, and vehicle (ethanol) for 3 days and subjected to immunostaining. Embryos were fixed in 4% paraformaldehyde overnight, followed by treatment with Triton-X 100 (0.25%) for 30 min, blocking in 5% bovine serum albumin-phosphate-buffered saline for 1 h, and immunostained as previously described [37]. The primary antibody was rabbit polyclonal anti-NRF2 (1:200, overnight, ab31163, Abcam), and the secondary antibody used was anti-rabbit IgG (H+L) F(ab')2 fragment (1:500, 1 h, Alexa Fluor® 555 Conjugate) (Cell Signaling Technology, Danvers, MA, USA), which were diluted in blocking solution, mounted onto glass slides using an anti-fade reagent containing DAPI, and observed under a Leica DMI 6000B microscope using LAS AF software (Leica). Nuclei in the blastomeres were visualized by DAPI staining. The fluorescence intensities of NRF2 in the nuclear region evidenced by DAPI and those of the whole embryo were captured to calculate the ratio of NRF2 nuclei expression level per cytoplasm for each embryo (Fig. 1). Next, 43, 40, and 43 embryos were examined for their NRF2 expression levels in the control (vehicle, ethanol), MitoT, and MitoQ groups, respectively. The fluorescence intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

All data were analyzed by one-way analysis of variance followed by Fisher's post hoc test. The percentage of embryo development was arcsine-transformed prior to analysis. P-values less than 0.05 were considered as statistically significant.

Results

Mitochondrial antioxidants decreased the levels of mitochondrial reactive oxygen.

When early cleavage-stage embryos (> 4 cells at day 2 after activation) were cultured in a medium containing mitochondrial

Table 1. Primer sequences used for reverse transcription PCR

Gene		Primer	Accession number	Amplicon size (bp)
PGC1α	F	ttccgtatcaccacccaaat	NM_213963.2	137
	R	atctactgcctggggacctt		
TFAM	F	ggcagactggcaggtgta	NM_001130211.1	165
	R	cgaggtctttttggttttcca		
B2M	F	gatcagtatagctgccgcgt	NM_213978.1	87
	R	ctgtgatgccggttagtggt		

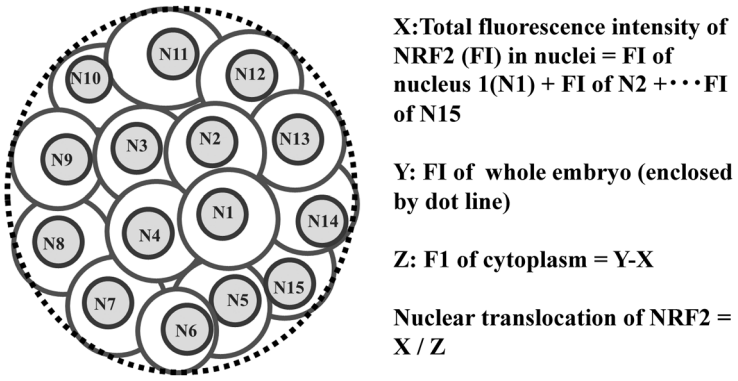

Fig. 1. Evaluation of nuclear translocation of NRF2. Representative image of an embryo immune-stained against NRF2, and formulas to obtain fluorescence intensity of NRF2 in nuclei per fluorescence intensity of cytoplasm.

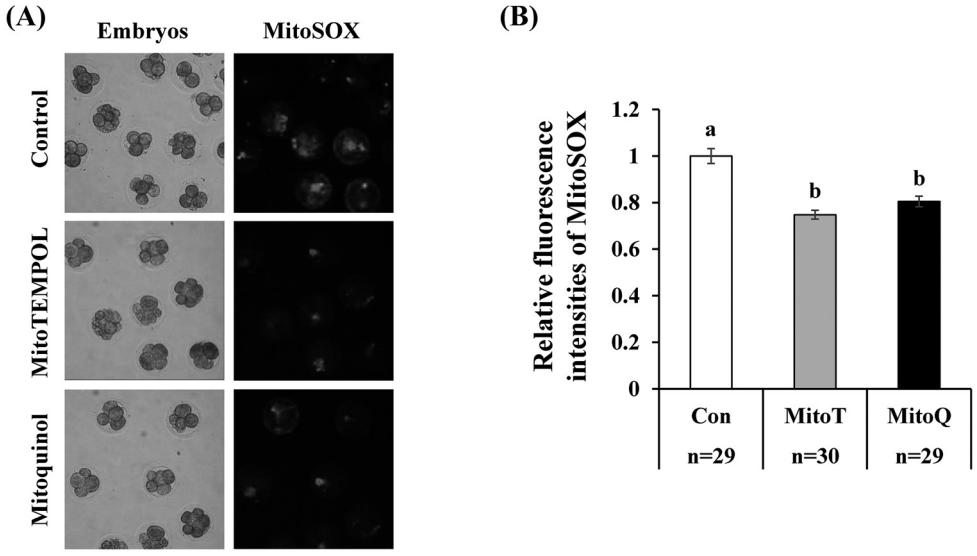

Fig. 2. Effect of mitochondrial antioxidants on mitochondrial ROS levels in embryos. Cleaved embryos (> 4-cell stage at 2 days after parthenogenetic activation) were cultured in medium containing vehicle (ethanol 1:1000, Con) or mitochondrial antioxidants (0.1 μM MitoT or 0.5 μM MitoQ) for 24 h. Relative fluorescence intensities of embryos stained with MitoSOX were compared among the three groups. The fluorescence intensity of the control was normalized to 1.0. a–b, $P < 0.05$. Number of embryos (n) examined is stated at the bottom of the figure.

antioxidants (MitoT or MitoQ) for 24 h, the amount of mitochondrial ROS determined by MitoSOX significantly decreased compared to that in cleavage-stage embryos cultured with the vehicle (ethanol) (Fig. 2).

Developmental competence and mitochondrial function and quantity in blastocysts

After culturing the embryos (> 4-cell stage embryos) in medium containing mitochondrial antioxidants, the developmental rate to the blastocyst stage and total cell number of the blastocysts did not change among the experimental groups (MitoT, MitoQ, and vehicle (ethanol)) (Table 2). Supplementation with MitoT and MitoQ led to decreased ATP and mitochondrial DNA contents in the blastocysts compared to in those developed with the vehicle, but the difference was not significant for MitoQ (Figs. 3A, 3B).

Mitochondrial antioxidants decreased mitochondrial biogenesis

RT-PCR revealed that the expression levels of *PGC1a* and *TFAM* were significantly lower in embryos cultured with mitochondrial antioxidants than in their vehicle (ethanol)-treated counterparts (Fig. 4). Immunostaining revealed that the expression levels of NRF2 in the nucleus were significantly decreased in embryos cultured with mitochondrial antioxidants compared to in their vehicle-treated counterparts (Fig. 5).

Discussion

MitoT and MitoQ mitochondrial superoxide scavengers reduced mitochondrial ROS levels in early cleaved embryos and reduced mitochondrial biogenesis during embryonic development. In addition,

Table 2. Effect of mitochondrial antioxidants on developmental competence of porcine embryos

Group	No. of replicates	No. of embryos	No. of blastocysts	Rate of blastocyst (%)	n=	TCN
Control	25	522	147	28.5 ± 3.2	89	37.8 ± 1.1
MitoT	25	526	158	30.5 ± 3.2	105	37.5 ± 1.0
MitoQ	25	534	171	32.2 ± 3.2	113	35.0 ± 1.3

At day 2 post-activation, > 4-cell stage embryos were cultured in medium containing ethanol (Con, 1:1000), and antioxidants (0.1 μM MitoT or 0.5 μM MitoQ) for 5 days, and the rate of blastocyst per embryos were examined. Total cell number (TCN) of the blastocysts and number of blastocyst (n) examined are shown. Data are presented as the mean ± SEM.

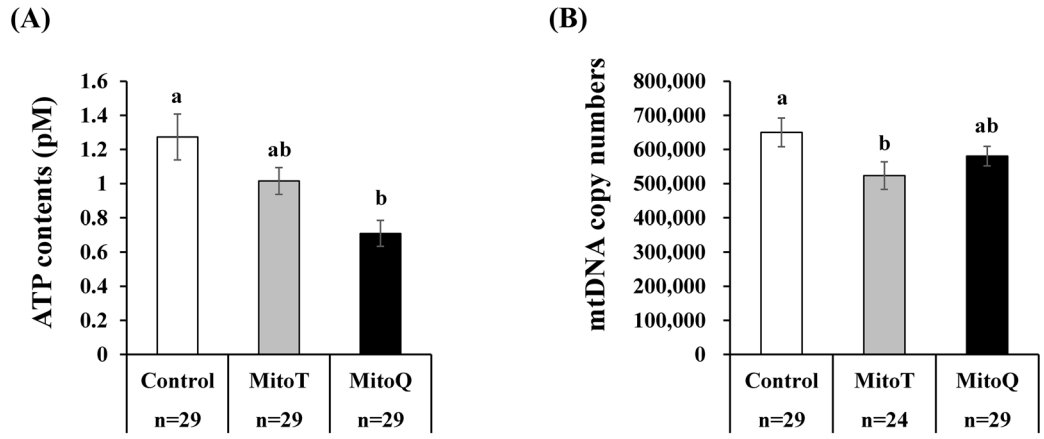

Fig. 3. Effect of mitochondrial antioxidants on mitochondrial function and quantity in blastocysts. Cleaved embryos (> 4-cell stage embryos at 2 days after parthenogenetic activation) were cultured in medium containing vehicle (ethanol 1:1000, Con) or mitochondrial antioxidants (0.1 μM MitoT or 0.5 μM MitoQ) for 5 days. The ATP content (A) and mitochondrial DNA copy number (B) in blastocysts were compared among the three groups. a–b, $P < 0.05$. Number of embryos (n) examined is stated at the bottom of the figure.

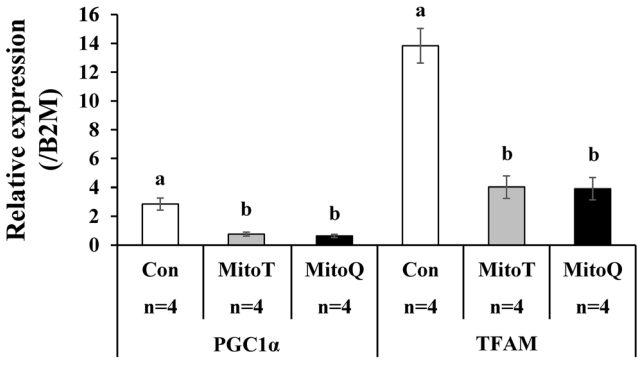

Fig. 4. Effect of mitochondrial antioxidants on expression levels of genes associated with mitochondrial biogenesis. Cleaved embryos (> 4-cell stage at 2 days after parthenogenetic activation) were cultured in medium containing vehicle (ethanol 1:1000, Con) or mitochondrial antioxidants (0.1 μM MitoT or 0.5 μM MitoQ) for 3 days. The expression levels of genes were normalized to that of beta-2-microglobulin (B2M) (A). a–b or c–d, $P < 0.05$. The experiment was repeated 4 times (n) using embryos derived from differential oocyte batches.

exposure to MitoT resulted in blastocysts with a low mitochondrial DNA copy number, while exposure to MitoQ resulted in blastocysts with low ATP content.

Mitochondria are crucial in the production of energy in cells. ROS are a common by-product of ATP generation in mitochondria. Although normal levels of ROS play a role in intercellular signaling, excessive ROS can cause mitochondrial dysfunction, damage cellular organelles, and induce apoptosis [38]. We confirmed that 24 h of incubation with MitoT and MitoQ reduced the levels of mitochondrial ROS in embryos. However, these antioxidants did not increase the rate of development to the blastocyst stage or total cell number of blastocysts compared with those treated with the vehicle. Yang *et al*. [29] reported that supplementation of the culture medium of porcine embryos with MitoT in an atmosphere of 5% CO_2 in air improved embryonic development and increased cellular numbers. It is widely accepted that low oxygen tension is beneficial for embryonic development [39], and that high oxygen tension changes the morphology of mitochondria and decreases the development of mouse embryos, resulting in lower cell numbers and higher ATP content [40]. This suggests that under low oxygen tension, the amount of ROS in embryos was not sufficient to improve embryo development.

Interestingly, supplementation of the culture medium with MitoT decreased the mitochondrial DNA content in the blastocysts. In

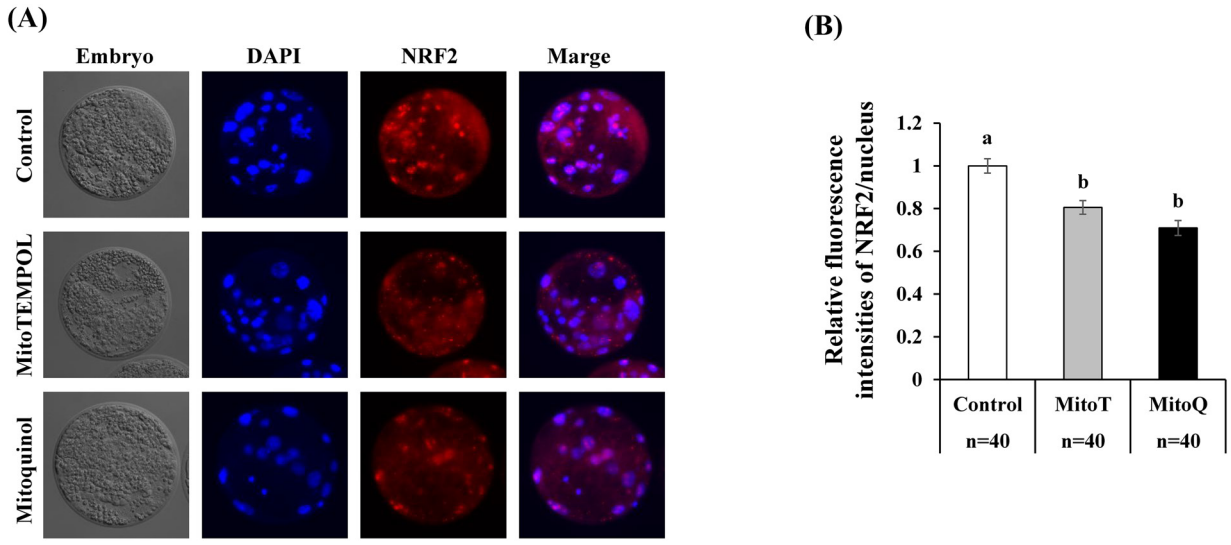

Fig. 5. Effect of mitochondrial antioxidants on expression levels of NRF2 in the nucleus. Cleaved embryos (> 4-cell stage embryos at 2 days after parthenogenetic activation) were cultured in medium containing (ethanol 1:1000, Con) or mitochondrial antioxidants (0.1 μM MitoT or 0.5 μM MitoQ) for 3 days. A: Representative images of embryos stained to visualize NRF2 and nuclei (DAPI) to examine the nuclear translocation of NRF2. B: Relative fluorescence intensities of NRF2 in the nucleus were compared among the three groups. The fluorescence intensity of control embryos was normalized to 1.0. a–b, $P < 0.05$. Number of embryos (n) examined is stated at the bottom of the figure.

addition, these antioxidants induced inactive mitochondrial biogenesis, as indicated by decreased expression levels of *PGC1α* and *TFAM* expression as well as by low translocation of NRF2 into the nucleus. Mitochondrial biogenesis is well-regulated by crosstalk between mitochondria and the nucleus. Mitochondrial ROS are involved in the crosstalk, although their precise role remains unclear [41]. It has been reported that key factors regulating mitochondrial biogenesis, such as NRF1, NRF2, PGC1-1α, and PGC1-β, are upregulated by oxidative stress [21–24], and nuclear translocation of NRF2 was induced by oxidative stress [42, 43]. Therefore, mitochondrial ROS regulate mitochondrial biogenesis in embryos. One question remains as to why MitoT was more effective than MitoQ in reducing ATP and mitochondrial DNA copy number in embryos, even though both drugs reduced mitochondrial biogenesis and mitochondrial ROS. MitoT is a superoxide dismutase mimetic that reduces mitochondrial O_2^- to H_2O_2. MitoT combines an antioxidant moiety with the lipophilic cation triphenylphosphonium, which allows it to pass through lipid bilayers and accumulate in mitochondria [44]. MitoQ is a mitochondria-targeted antioxidant and a reduced form of mitoquinone [45]. Both drugs target mitochondrial ROS but exert their effects under different molecular mechanisms. Unraveling the differential extent of their effects is a target for future research.

In the present study, blastocysts that developed in the presence of mitochondrial antioxidants had low mitochondrial DNA copy numbers and ATP content. It is unclear whether these embryos have high developmental competence. However, we found a higher mitochondrial copy number in *in vitro*-grown bovine blastocysts than in those grown *in vivo* [46]. It is widely accepted that *in vitro* culture conditions are closely related to ROS generation. In our previous report, granulosa cells cultured *in vitro* showed increased mitochondrial DNA content and ATP generation, whereas under low oxygen tension, both mitochondrial number and ATP were decreased [47]. In addition, it has been reported that low mitochondrial numbers in blastocyst-stage embryos are associated with the high implantation potential of the embryos [14]. In addition, embryos with abnormal ploidy have a large number of mitochondria in humans and pigs [35, 48]. In cells with abnormal ploidy, an imbalanced contribution of the mitochondrial genome and nuclear genomes induces an imbalance of mitochondrial proteins, which induces ER stress and generation of ROS [49]. In addition, dysfunction in the electron transport chain and increased misfolding of mitochondrial proteins induce increased ROS generation, which stimulates the biogenesis of mitochondrial DNA and thus an increased copy number [10]. Based on these reports and the results of the present study, ROS in embryos may be a key factor determining the mitochondrial number in blastocysts.

In conclusion, our results suggest that reducing the mitochondrial ROS level attenuates mitochondrial biogenesis during embryo development and results in blastocysts with low mitochondrial DNA copy number and function.

Conflicts of Interests: The authors declare that there are no conflicts of interest regarding this study.

Acknowledgments

We thank Kanagawa Meat Center for providing the ovaries.

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