

## Mitochondrial dysfunction in cumulus-oocyte complexes increases cell-free mitochondrial DNA

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**Abstract.** This study examined the concentration of cell-free mitochondrial DNA (cf-mtDNA) in porcine follicular fluid (FF) and explored whether the cfDNA level in the culture medium could reflect mitochondrial dysfunction in cumulus cell-oocyte complexes (COCs). cfDNA concentration was higher in the fluid of small-sized follicles, compared to that in larger follicles. The length of cfDNA ranged from short (152 bp) to long (1,914 bp) mtDNA in FF, detected by polymerase chain reaction (PCR). cfDNA concentration in FF significantly correlated with the mtDNA copy number in FF but not with the number of one-copy gene (nuclear DNA) in FF. When the COCs were treated with the mitochondrial uncoupler, namely carbonyl cyanide m-chlorophenyl hydrazone (CCCP), for 2 h and incubated for 42 h, subsequent real-time PCR detected significantly higher amount of cf-mtDNA, compared to nuclear cfDNA, in the spent culture medium. The mtDNA number and viability of cumulus cells and oocytes remained unchanged. When the oocytes were denuded from the cumulus cells following CCCP treatment, PCR detected very low levels of cfDNA in the spent culture medium of the denuded oocytes. In contrast, CCCP treatment of granulosa cells significantly increased the amount of cf-mtDNA in the spent culture medium, without any effect on other markers, including survival rate, apoptosis of cumulus cells, and lactate dehydrogenase levels. Thus, cf-mtDNA was present in FF in a wide range of length, and mitochondrial dysfunction in COCs increased the active secretion of cf-mtDNA in the cultural milieu.

**Key words:** Cell-free DNA, Granulosa cell, Mitochondria, Oocyte

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**C**ell-free DNA (cfDNA) in circulation and body fluids is a non-invasive marker of the physiological condition of the body, and its concentration is associated with several diseases [1]. cfDNA consists of nuclear as well as mitochondrial genome, and the concentration of mitochondrial cfDNA (cf-mtDNA) in circulation is particularly associated with certain specific diseases [2–5]. cfDNA is generated from the extracellular nucleic acid of dead cells or from active secretion of living cells [6–8]; however, the precise mechanism underlying the release of cfDNA from cells is still unclear.

Follicular fluid (FF) contains cfDNA, and its concentration reflects the quality of oocytes. Scalici *et al.* [9] have shown that the amount of cfDNA in FF is related to the rate of fragmentation of the embryos, derived from corresponding oocytes. The amount of cfDNA in FF has also been shown to positively correlate with the apoptosis of granulosa cells and to negatively correlate with the ability of oocytes to reach the blastocyst stage [10]. Furthermore, the embryo itself secretes cfDNA into the cultural milieu. Stigliani *et al.* [11] have detected both genomic and mtDNA in the spent culture medium of human embryos and suggested that the amount of cf-mtDNA in the cultural

milieu reflected mitochondrial selection, *i.e.*, bottle-neck purification of embryos during development. Furthermore, Dantham *et al.* [12] have shown that mutations in the mitochondrial protein-coding genes decreased the ratio of cf-mtDNA to nuclear cfDNA and that nutritional intervention using omega-3 fatty acids helped restore the ratio. From these reports, it may be hypothesized that mitochondrial dynamics in oocytes and embryos, affect the level of cf-mtDNA in circulation as well as in the cultural milieu. Mitochondria are strictly regulated by *de novo* synthesis and degradation, and treatment of oocytes with the mitochondrial membrane uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) has been reported to enhance mitochondrial biogenesis and degradation [13]. However, no study has discussed the effect of mitochondrial dysfunction on the amount of cfDNA in cultural milieu.

In this study, we investigated the amount of cf-mtDNA in FF and examined the effect of CCCP treatment of cumulus cell-oocyte complexes (COCs) during oocyte maturation on the amount of cf-mtDNA and nuclear cfDNA in the spent culture medium, and investigated the origin of cfDNA.

### Materials and Methods

#### *Chemicals, media, and culture conditions*

All chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated. Porcine oocyte medium (POM) [14], containing 3 mg/mL polyvinyl alcohol (PVA) was used for incubating COCs and oocytes (hereafter referred to as IVM medium). Medium

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199 supplemented with 5% (v/v) fetal calf serum (FCS) (5703H; ICN Pharmaceuticals, Costa Mesa, CA) was used for granulosa cell culture. Incubation was performed at 38.5°C in an atmosphere of 5% CO<sub>2</sub> and saturated humidity. CCCP (Sigma-Aldrich) was diluted in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Osaka, Japan). The final concentration of CCCP used in this experiment was 10 μM.

#### *COC and granulosa cell culture*

Ovaries were collected from the gilts obtained at a local slaughterhouse and transported to the laboratory within 30 min. COCs were aspirated from 3–5 mm antral follicles by using a syringe connected to an 18-gauge needle, and they were picked up from the follicular contents under a stereomicroscope by using a Pasteur pipette. Only the COCs surrounded by thick and compact cumulus cells were selected for subsequent culture. *In vitro* maturation of oocytes was performed by incubation of COCs in 100 μl of IVM medium (10 COCs/drop) under paraffin oil (tissue culture grade; Nacalai Tesque) for 44 h. To obtain granulosa cells, the cellular suspension was passed through a 40-μm nylon mesh (BD Falcon, Bedford, MA) to remove cellular debris, and centrifuged (200 × g) for 1 min to obtain a pellet. The GCs were dispersed using an enzyme cocktail (Accumax; Innovative Cell Technologies, San Diego, CA) as per the manufacturer's instructions. These cells were re-suspended in the culture medium (TCM-199: 1 mM taurine, 100 IU/ml penicillin, 0.1 μg/ml streptomycin, 50 μg/ml gentamicin, and 5% FCS) and cultured in 500 μL of medium in a 4-well dish (Nunc; Thermo Fisher Scientific, Waltham, MA) at a density of 2.0 × 10<sup>5</sup> cells/well. Twenty-four hours after the initiation of incubation, un-attached cells were removed by gentle pipetting, and the remaining cellular sheet was subjected to further experiments.

#### *DNA extraction from FF*

FF was collected from the antral follicles from each donor gilt and transferred to 1.5-ml micro tubes, followed by centrifugation (5000 × g for 1 min), to remove cellular content. The DNA present in 240 μl of FF was extracted using a DNA purification kit (NucleoSpin® Plasma XS; MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Extracted DNA was diluted in 10 μl of water. The cfDNA concentration (ng/μl) in the suspension and that in 1 μl of FF was measured using an analyzer (e-spect; BM Equipment, Tokyo, Japan). Two microliters of cfDNA was used for electrophoresis in 2% agarose gel for 30 min, followed by ethidium bromide staining. The image was captured using Alpha Imager® mini (Alpha Innotech, San Leandro, CA).

#### *DNA extraction from oocytes, granulosa cells, and spent culture medium*

The DNA in each oocyte and granulosa/cumulus cell was extracted in lysis buffer (20 mM Tris, 0.4 mg/ml proteinase K, 0.9% (v/v) Nonidet P-40, and 0.9% (v/v) Tween 20) by incubation at 55°C for 30 min, followed by 95°C for 5 min. To extract the DNA present in the spent culture medium, the medium was transferred to a micro tube and centrifuged at 4,000 × g for 1 min to remove cells, and the spent medium was mixed with the same volume of lysis buffer (2 ×: 40 mM Tris, 0.8 mg/ml proteinase K, 0.9% (v/v) Nonidet P-40, and 1.8% (v/v) Tween 20) and treated as described above.

#### *Real-time polymerase chain reaction and mtDNA copy number in cfDNA, cumulus cells, and oocytes*

mtDNA copy number was determined by real-time PCR using a Corbett Rotor Gene 6000 Real-Time Rotary Analyzer (Corbett Research, Sydney, Australia). The primers targeting porcine mitochondrial genome (LOCUS NC\_000845.1: 16,613 bp) and one-copy gene (154 bp, GCG glucagon, Gene ID: 397595, LOCUS NC\_010457.4,) were designed using Primer-BLAST. The primer set used to determine mtDNA copy number was 5'-cgagagagcacttc-caagg-3' and 5'-ctaattcgggtgtgtgct-3' targeting short mitochondrial sequences (152 bp: 9,985–10,136). The primer set used to detect the length of mitochondrial genome was 5'-tcccaacacccaaacgactc-3' and 5'-aacctcgcaagggcgatcg-3', which targeted the long sequence of the mitochondrial genome (1,914 bp: 9,211–11,124). The primer set used for one-copy gene was 5'-agcagaatcaacaccatcggt-3' and 5'-tggtccaccatagaatgc-3'. To amplify the mitochondrial short sequence and one-copy gene, the PCR was performed as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles at 98°C for 5 sec and 59°C for 11 sec. To amplify the mitochondrial long sequence, the PCR program was as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 sec, 62°C for 60 sec, and 72°C for 130 sec. SYBR green fluorescence was measured at the end of each extension step. To determine the mitochondrial copy number in each cell, the mtDNA number obtained by real-time PCR targeting the mitochondrial short sequence was divided by the cell number in the sample, which was determined by real-time PCR targeting one-copy gene. mtDNA copy number in the oocytes was determined for each oocyte. A standard curve was generated for each run by using 10-fold serial dilutions of the representative copies of the external standard. The external standard was the PCR product of the corresponding gene sequence, cloned into a vector by using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The product was sequenced for confirmation prior to use. All analyses were performed in duplicate. Amplification efficiency at all instances was > 1.96.

#### *CCCP treatment of COCs and granulosa cells*

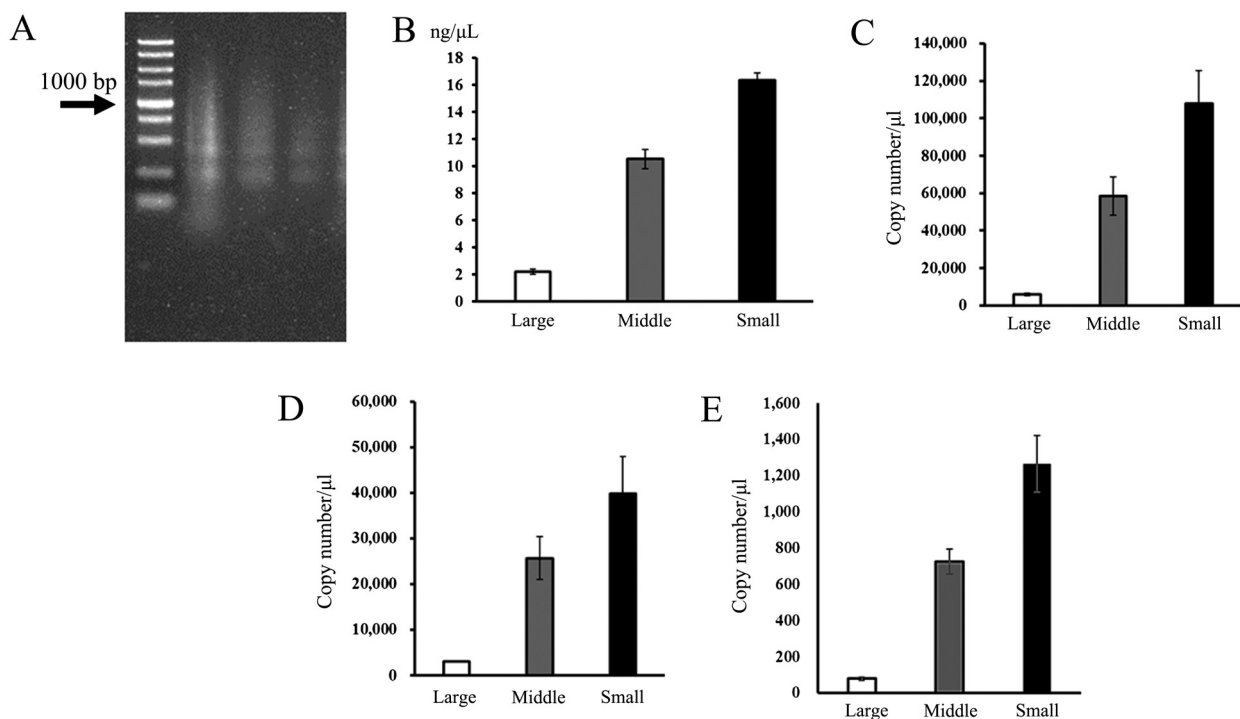
COCs and granulosa cells were incubated in the culture medium (POM and medium 199, respectively) containing 10 μM CCCP or vehicle (DMSO) for 2 h, followed by vigorous washing. After CCCP treatment, the COCs were incubated for 44 h, while the granulosa cells were cultured for 24 h. At the end of the culture period, the spent culture medium was collected.

#### *Cumulus and granulosa cell number and survival rate determination*

Cumulus cells were collected from the COCs adding hyaluronidase (0.1%, v/v), to the culture medium and vortexing. Granulosa cells attached to the culture plate were collected using an enzyme cocktail (Accumax). These cells were stained with propidium iodide and Hoechst 33342, and observed under the fluorescence microscope (Olympus, Tokyo, Japan). The survival rate was determined by counting 200 cells. The cellular number was determined by real-time PCR targeting one-copy gene, as described above.

#### *Mitochondrial membrane potential of COCs*

The COCs were incubated in medium containing CCCP for 3 h,



**Fig. 1.** Comparison of cell-free DNA (cfDNA) in follicles of different sizes. A: Representative image of electrophoresis of cfDNA extracted from small-, mid-, and large-sized follicles; B: Amount of cfDNA (y-axis, ng/μl); C–D: mitochondrial (mtDNA) copy number predicted by real-time polymerase chain reaction (PCR) using primer sets targeting short (152 bp) and long (1,916 bp) mitochondrial genome (y-axis, mtDNA copy number); E: nucleic DNA copy number predicted by real-time PCR using primer set targeting one-copy gene (154 bp, GCG glucagon).

followed by JC-1 staining for 20 min. The cells were mounted on a glass slide and observed under a fluorescence microscope (Leica). Images were captured, and the ratio of fluorescence intensity of JC-1-positive red cells (active mitochondria) to that of green cells (whole mitochondria) was determined.

#### Measurement of lactate dehydrogenase (LDH) activity in spent culture medium

The spent culture medium was collected and centrifuged ( $10,000 \times g$  for 5 min) to remove cells, and the supernatant was used for LDH assay using the Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions.

#### Apoptosis of granulosa cells

Apoptotic cells were detected using Annexin V-FITC kit (Miltenyi Biotec, Tokyo, Japan). Granulosa cells attached to the culture plate were stained using Hoechst 33342 and Annexin V, according to the manufacturer's instructions. The cells were observed under a fluorescence microscope (Leica DMI 6000 B; Leica, Wetzlar, Germany), and the apoptotic rate (number of apoptotic cells/total cell number) was determined. A total of 200 cells were counted, and the experiment was repeated eight times.

#### Statistical analysis

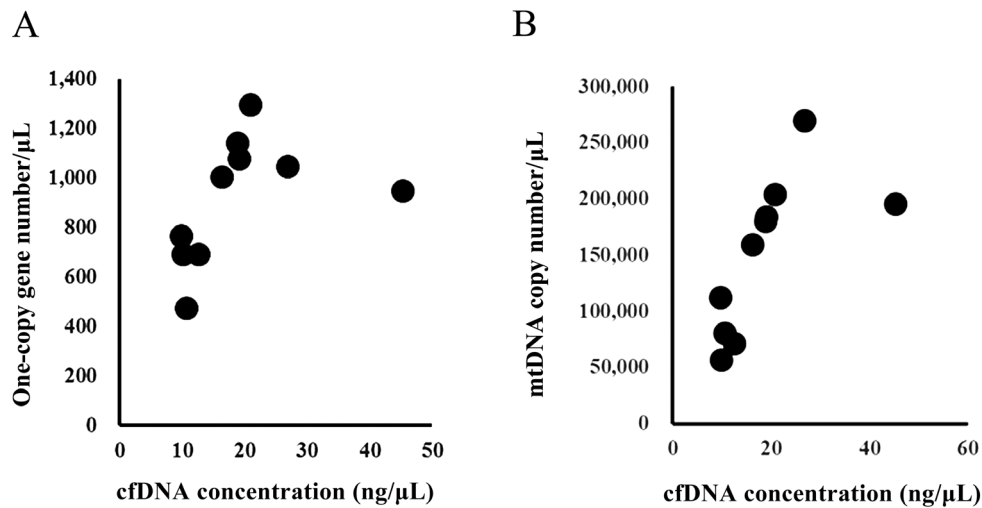
Student's *t*-test was used for analyzing data of two groups, while

data from three or more groups were analyzed using analysis of variance (ANOVA), followed by Tukey's post-hoc test. Percentages were arcsine-transformed prior to analyses.  $P < 0.05$  was considered significant.

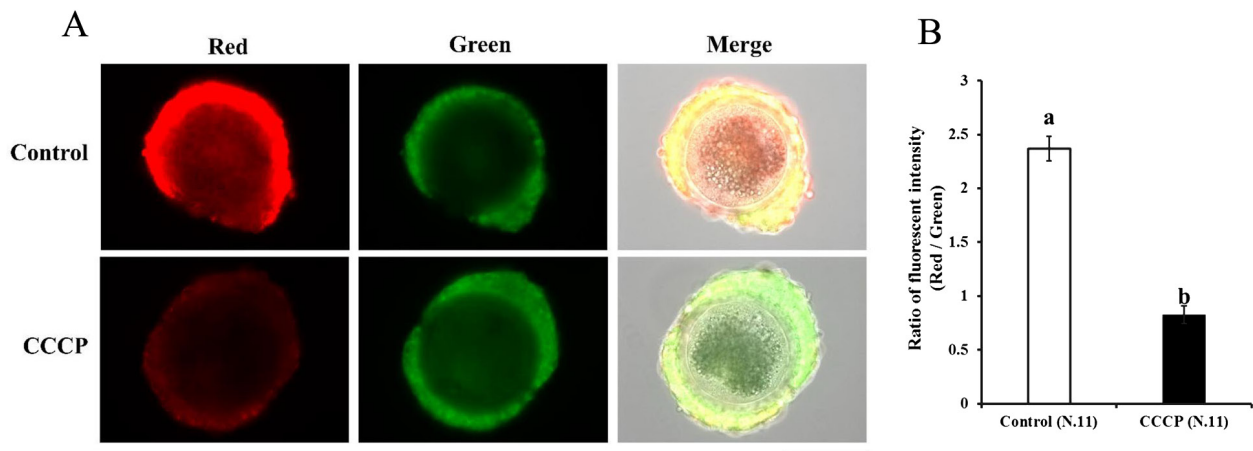
## Results

#### *In FF, the intrinsic oocyte cumulus complex milieu contained cf-mtDNA*

We examined the cf-mtDNA content in FF. DNA was extracted from FF collected from small (1–2 mm, S-FF), middle (3–5 mm, M-FF), and large (> 6 mm in diameter, L-FF) antral follicles of at least 15 gilts, and three batches of each of S-, M-, and L-FFs were created. High cfDNA content was observed in FF of small antral follicles while the length of cfDNA was found to vary (Fig. 1A). cfDNA content in FF decreased as the follicle developed, the average concentration in FF derived from S-, M-, and L-FF was  $16.4 \pm 0.5$ ,  $10.5 \pm 0.7$ , and  $2.2 \pm 0.2$  ng/μl, respectively (Fig. 1B), and the real-time PCR targeting short (152 bp) and long (1,989 bp) mitochondrial sequences detected corresponding mitochondrial genome in the cfDNA. mtDNA copy number and nucleic gene copy number (number of one-copy gene) also decreased as the follicle grew (Fig. 1C, D and E). Next, we examined how mt-cfDNA could reflect the cfDNA content of FFs. FFs were collected from mid-sized antral follicles of 10 gilts and DNA was extracted. The concentration of cfDNA (ng/μl) in the FF did not correlate with the number of one-copy gene (Fig. 2A, r



**Fig. 2.** Comparison of cell-free DNA (cfDNA), mitochondrial DNA (mtDNA), and nuclear DNA copy number predicted by real-time polymerase chain reaction (PCR). A: Relationship between the number of one-copy gene (y-axis) and the amount of cfDNA (ng/μL) (x-axis); B: Relationship between mtDNA copy number (y-axis) and amount of cfDNA (ng/μL) (x-axis). mtDNA copy number was determined by real-time PCR targeting short mitochondrial sequences (152 bp; 9,985–10,136).



**Fig. 3.** Effect of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) treatment on oocyte membrane potential. Cumulus cell-oocyte complexes (COCs) were treated with CCCP for 2 h, followed by mitochondrial membrane potential determination using JC-1 staining. A: Representative images of COCs subjected to JC-1 staining; red color indicates active mitochondria, and green color indicates whole mitochondria; B: Ratio of active mitochondria, that is, ratio of fluorescence intensity of red to that of green. Bar = 100 μm.

= 0.45,  $P > 0.05$ ) but was significantly related to the mtDNA copy number in the FF (Fig. 2B,  $r = 0.67$ ,  $P < 0.05$ ).

*CCCP treatment increased cf-mtDNA content in the spent culture medium, without any change in viability and mtDNA copy number in the oocytes and cumulus cells*

After 120 min of CCCP treatment, JC-1 staining showed that the treatment decreased the ratio of active mitochondria to whole mitochondria in COCs (Fig. 3 A and B). At the initial point of *in vitro* maturation, CCCP treatment of COCs did not affect either the final maturation rate of the oocytes or the survival rate of the

cumulus cells (Table 1). We examined how CCCP treatment affected mitochondrial DNA copy number in oocytes, cumulus cells, and spent culture medium. One hundred and ten COCs were treated with CCCP and cultured *in vitro* (10 COCs/100 μL droplet). Comparable mitochondrial DNA copy number was observed in CCCP- and vehicle-treated oocytes and granulosa cells (Table 2). Although CCCP treatment did not affect the nuclear cfDNA (one-copy gene) content of the spent culture medium, that of CCCP-treated COCs contained significantly greater amount of cf-mtDNA compared to that of vehicle-treated COCs.

**Table 1.** Effect of CCCP treatment on the nuclear maturation of porcine oocytes and viability of cumulus cells

Groups	No. of trials	Oocytes		Cumulus cells
		No. examined	MII rate	Survival rate (%)
Control	5	86	91.7 ± 3.1	81.3 ± 3.5
CCCP	5	85	90.6 ± 1.4	81.2 ± 3.1

Data are represented as mean ± SEM values.

*CCCP treatment did not affect the amount of cf-mtDNA in the spent culture medium of denuded oocytes but increased cf-mtDNA content in the spent culture medium of granulosa cells, without any change in the cellular viable markers*

To confirm whether the cfDNA in spent culture medium came from oocytes or cumulus cells, oocytes were denuded from surrounding cumulus cells after CCCP treatment, and a group of 10 oocytes were cultured in the medium (100 µl) for 24 h. cf-mtDNA in the spent culture medium of denuded cells was 5.54 ± 4.69 and 6.51 ± 3.77/100 µl for the vehicle- and CCCP-treated groups, respectively (Table 3), which implied that almost all cf-mtDNA in spent culture medium derived from cumulus cells. To explore whether the granulosa cells secreted CCCP-unresponsive cfDNA, granulosa cells were cultured in 4-well plates at a concentration of 2 × 10<sup>5</sup> cells/ml and the cells were treated with CCCP for 2 h, followed by a 24-h incubation. CCCP treatment did not affect cellular viability, including survival rate and ratio of apoptotic cells determined by Annexin staining and necrosis marker, LDH. Moreover, CCCP treatment did not affect mitochondrial copy number in granulosa cells, although the cf-mtDNA content significantly increased.

## Discussion

The present study showed that FF contained cf-mtDNA of a wide range of size, and the amount of cf-mtDNA reflected the content of

total cfDNA in FF. Furthermore, mitochondrial dysfunction induced by CCCP increased the secretion of cf-mtDNA (but not of nuclear cfDNA) from cumulus cells in the spent culture medium, without any change in the conventional cellular markers such as survival rate, apoptosis, and LDH activity.

cfDNA concentration in human FF is known to decrease with the development of the follicle; its concentration ranges from 1 to 3 ng/µl in the antral follicles (8–12 mm in diameter) [9, 15, 16]. Consistent with this, the present study showed that the cfDNA content in FF decreased with follicle development, and the concentration in FF derived from S-, M-, and L-FF was 16.4 ± 0.5, 10.5 ± 0.7, and 2.2 ± 0.2 ng/µl, respectively (Fig. 1B). The presence of cf-mtDNA in circulation has been reported to be associated with several diseases [4, 17]. Although in the present study, no physical information about the donor gilts was available, the cfDNA content in FF of antral follicles (3–6 mm in diameter) collected from 10 gilts ranged between 9.9 and 45.4 ng/µl. The results indicated that cfDNA concentration depended on the condition of the ovaries and the physiological condition of the donors.

Electrophoresis showed that cfDNA comprised various lengths of DNA ranging from small (< 100 bp) to large (> 2,000 bp). Furthermore, real-time PCR targeting both 152- and 1,914-bp fragments detected mtDNA in the cfDNA (present in FF). Small-sized DNA, especially ~166 bp, are believed to indicate apoptosis-derived DNA [18, 19]. Therefore, our results suggest that at least a part of the cfDNA present in FF does not originate from apoptotic cells. To the best of our knowledge, the present study is the first to report the presence of long stretches of mtDNA in FF. The mtDNA copy number determined by real-time PCR targeting the short sequence closely correlates with the amount of cfDNA (ng/µl), but not much correlation was found between the number of one-copy gene and amount of cfDNA (ng/µl). This finding may be attributed to the fact that mitochondrial number per cell is much higher than that in nuclear genome. Moreover, the mtDNA copy number determined by real-time PCR is considered as a useful indicator of the cfDNA content in FF. A pioneering study has

**Table 2.** Effect of CCCP treatment of COCs on mitochondrial DNA copy number and nucleic DNA copy number in cumulus cells, oocytes, and spent culture medium

Groups	Cumulus cells		Oocyte		Spent culture media (100 µl)			
	No. of examined	mtDNA	No. of examined	mtDNA	No. of examined	mtDNA	Nucleic DNA	Ratio of mt/nucleic
Control	11	514.7 ± 22.2	58	170,334 ± 8,642.7	11	1482.9 ± 142.9 <sup>a</sup>	90.3 ± 11.5	17.7 ± 1.4 <sup>a</sup>
CCCP	11	497.3 ± 12.1	58	152,143 ± 9,791.0	11	2190.5 ± 185.6 <sup>b</sup>	84.9 ± 11.2	29.1 ± 3.5 <sup>b</sup>

Data are represented as mean ± SEM value. a–b: P < 0.05. Mitochondrial DNA copy number (mtDNA) was determined by real-time PCR targeting mitochondrial short sequences.

**Table 3.** Effect of CCCP treatment on the survival of granulosa cells and mtDNA copy number in granulosa cells and spent culture medium

Group	No. of trials	Cell number in well	Survival rate (%)	Annexin positive (%)	Relative value of LDH	mtDNA in granulosa cell	mtDNA in medium (/100 µl)
Vehicle	8	143125 ± 6186	91.6 ± 0.1	5.4 ± 0.2	1.0 ± 0.02	590.7 ± 25.3	12,494.6 ± 668.8 <sup>a</sup>
CCCP	8	137000 ± 5463	88.3 ± 2.5	5.1 ± 0.2	0.96 ± 0.00	545.9 ± 31.6	15,005.1 ± 905.7 <sup>b</sup>

Data are represented as mean ± SEM value. Average LDH values of control are defined as 1.0. a–b: P < 0.05. Mitochondrial DNA copy number (mtDNA) was determined by real-time PCR targeting mitochondrial short sequences.



shown that the amount of cfDNA in FF was related to the apoptosis of granulosa cells and that addition of cfDNA to the culture medium induced the latter [10]. On the other hand, accumulating evidence suggests that the amount of cfDNA reflects the ovarian reserve and possible outcome of *in vitro* fertilization [15, 16]. In the present study, however, the phenomenon by which cfDNA (present in FF or culture medium) reflects oocyte ability and follicle condition has not been elucidated. Nevertheless, we are the first to report that cf-mtDNA content in the spent medium reflects mitochondrial condition, without any detectable cellular damage. CCCP is a potent inducer of mitochondrial uncoupling, and in the present study, the effect of CCCP on mitochondrial membrane potential is confirmed by JC-1 staining. In addition, this CCCP-induced mitochondrial dysfunction was not accompanied by any change in the mtDNA copy number, as well as viability of oocytes and cumulus cells. CCCP treatment has been used to enhance mitochondrial quality control in cells [20]. We showed that CCCP treatment of bovine and porcine COCs did not reduce developmental ability but enhanced mitochondrial biogenesis and degradation in oocytes [13, 21]. From these facts, it is conceivable that short duration of CCCP treatment may reversibly affect mitochondrial conditions in oocytes and cells, and enhance mitochondrial kinetics in both cells. In the present study, CCCP treatment increased mtDNA copy number, but not one-copy gene in the spent culture medium. It is possible to speculate that the mitochondrial dysfunction induced by CCCP enhanced the secretion of cf-mtDNA from the COCs, without any detectable deterioration of cells and oocytes. We further addressed the following questions: 1) Which oocytes or granulosa cells secreted cfDNA in the spent culture medium? 2) Did cfDNA come from dead or living cells? When denuded oocytes were cultured in the medium, following CCCP treatment, we detected a very low amount of cf-mtDNA in the spent culture medium of oocytes, indicating that almost all cf-mtDNA came from the surrounding cells. Furthermore, CCCP treatment did not affect either the number of granulosa cells in the culture well, survival rate, apoptosis of cells, or the LDH concentration in the spent culture medium. Bronkhorst *et al.* [22] have shown that 143B cells secreted cfDNA without detectable apoptosis and necrosis. Thus, we deduced that once CCCP induced mitochondrial dysfunction in the granulosa cells, these cells actively secreted cf-mtDNA into the culture medium, without causing any serious damage.

To conclude, the cfDNA content and its length substantially vary across donors. In addition, the amount of cfDNA predicted from real-time PCR closely correlates with the actual amount of cfDNA. We propose that mitochondrial dysfunction likely induces the secretion of cf-mtDNA from live cumulus cells.

Conflict of interest: The authors declare that there is no conflict of interest that can be perceived as prejudicing the impartial nature of the research reported.

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