Changes in Distribution of Active Mitochondria during Oocyte Maturation and Fertilization in the Hamster

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Abstract: The localization and activity of mitochondria in hamster oocytes during maturation and fertilization were monitored by using three different fluorescent probes: rhodamine 123 (Rh123), MitoTracker Red (MT-Red) and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$). Oocytes aged in vivo were also examined. The germinal vesicle (GV) oocytes exhibited a range of mitochondrial organizations, from a uniform distribution to a more cortical restriction. Metaphase I (MI) and MII oocytes included numerous mitochondria, among which the fluorescence intensity increased two-fold over that of GV oocytes. About half (54.5%) of the MII oocytes exhibited a mitochondria free zone at the cortex. An approximately 10% decline in the density of mitochondria was observed in the oocytes aged 10 h post-ovulation, despite no significant changes. After fertilization, mitochondria became progressively aggregated and localized in the perinuclear region of all eggs examined. These comparative studies show that the signals for Rh123 and MT-Red were virtually coincident, but that for $DiOC_6(3)$ displayed wider areas of bright organizations regardless of the very low concentration used in this study (5 ng/ml). In addition, transition from a relatively homogeneous distribution of active mitochondria to perinuclear clustering may reflect energy production and utilization during oocyte maturation and fertilization in hamsters, as reported in other species.

Key words: Hamster, Oocyte Maturation, Mitochondria

Introduction

Nuclear changes during oocyte maturation and fertilization are coordinated with cytoplasmic maturation and organization, through the movements of organelles, including mitochondria. During these processes, mitochondria function to provide energy/metabolites to specific regions in the oocyte [1–3] and are associated with energy production to ensure subsequent embryo development [3–7]. Inappropriate distribution of mitochondria, namely alterations in the energy production could result in early embryonic failure. In mice, large numbers of mitochondria surround the nucleus during chromosome condensation and then accompany the spindle to the cortex [1, 2, 8]. Similar translocation of active mitochondria has been observed during oocyte maturation in cows [9], pigs [10], and humans [11–13]. In hamsters, however, limited data are available on changes in the mitochondrial distribution during oocyte maturation and fertilization. Only one study has reported that both follicular and oviductal unfertilized eggs showed a homogeneous distribution of mitochondria throughout the cytoplasm [4]. This description may be inconsistent with those reported in the other species mentioned above [9–13].

In the present study, three different fluorescent probes were used to compare and determine the distribution and activity of active mitochondria during maturation and fertilization of hamster oocytes. Aged oocytes after ovulation were also examined. Here, we used the mitochondria-specific fluorescent probes rhodamine 123 (Rh123) [14], MitoTracker Red (MT-Red) [15], and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) [16] to identify all active mitochondria. Rh123 localizes mitochondria in a charge-dependent manner [14]. MT-Red stains not only active mitochondria, but reacts with thiols on proteins and

Received: May 9, 2005
Accepted: June 12, 2005
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peptides in the mitochondrial matrix [23]. $DiOC_6(3)$ localizes mitochondria and other cellular membranes in response to the high membrane potential [16]. At low concentrations, $DiOC_6(3)$ accumulates in mitochondria due to their large negative membrane potential [16].

Materials and Methods

Collection of eggs

Golden hamsters (*Mesocricetus auratus*) were housed in a room controlled for temperature (21–23°C) and light on a 14-h light: 10-h dark photoperiod (light-on at 5:00 a.m.). They were given laboratory chow (MF, Oriental Yeast Co. Ltd., Tokyo) and water *ad libitum*. Cycling female hamsters 8–10 weeks old were super-stimulated with an intraperitoneal injection of weight-dependent dose [17] of pregnant mare serum gonadotropin (PMSG, Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan). According to a previous report [18], oocytes at the germinal vesicle (GV) stage were collected by puncture of antral follicles 51 h post-PMSG. To obtain oocytes at the metaphase I (MI) and metaphase II (MII) stages, PMSG-injected hamsters were given a further administration of human chorionic gonadotropin (hCG, Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) 58 h later in a weight-dependent dose [17]. The MI-stage oocytes were collected by puncturing antral follicles 3 h post-hCG, and the MII-stage oocytes were collected from the ampullary region of the excised oviducts 16 h post-hCG. In addition, the oocytes were recovered 20 and 24 h post-hCG, and assigned to aged oocytes (6 and 10 h after the estimated time of ovulation, respectively). Pronuclear zygotes were collected from PMSG-primed females at 2:00 p.m. on the day following mating [19]. Oocytes and zygotes were collected in HECM-3 supplemented with 1.0 mM hypotaurine (HECM-3ht) [20] equilibrated with 10% CO_2, 5% O_2, 85% N_2 at 37.5°C. Cumulus cells were dispersed with 0.05% hyaluronidase (Sigma, St. Louis, MO, USA) in Ca-free Dulbecco's phosphate buffer. The experimental design was approved by the Ethical Committee for Experimentation with Animals, Hirosaki University.

Staining of mitochondria

Our preliminary study confirmed that the staining pattern of Rh123 was completely different in fixed vs. fresh eggs as reported by Barnett *et al*. [4], who indicated that fixation affected the membrane potential of the mitochondria. Therefore, fresh material was used for all Rh123 staining. The eggs were stained with Rh123 (10 μg/ml, Sigma) for 15 min in HECM-3ht under 10% CO_2, 5% O_2, 85% N_2 at 37.5°C [4], mounted onto slide glasses and imaged immediately after labeling. MT-Red (chloromethyl-X-rosamine, Molecular Probes, Eugene, OR) was used at a concentration of 0.1 μg/ml in HECM-3ht for 15 min under culture conditions (10% CO_2, 5% O_2, 85% N_2 at 37.5°C), and then fixed with 1% formaldehyde in Dulbecco's PBS containing 0.1% polyvinyl alcohol (Sigma) at room temperature for 1 h. For staining with $DiOC_6(3)$ (Sigma), eggs were fixed for 2–3 min in 0.25% glutaraldehyde in a sucrose-cacodylate buffer (0.1 M sucrose, 0.1 M sodium cacodylate, pH 7.4) and then stained for 30–60 sec with 5 ng/ml $DiOC_6(3)$ in the cacodylate buffer [16]. Double staining with Hoechst 33342 (10 μg/ml, Sigma) and mitochondrial probes were occasionally used to determine the nuclear status of maturation in oocytes.

Fluorescence imaging

The samples were viewed under a fluorescence microscope (BX-FLA, Olympus, Tokyo, Japan) as previously reported [21]. A U-MNIBA filter set (Olympus) was used for Rh123 and $DiOC_6(3)$, a U-MWIB set (Olympus) was used for MT-Red, and a U-MWU set (Olympus) for Hoechst 33342. A cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ, USA) was used to obtain images on a computer and color adjustment was performed by IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA, USA).

Quantify of images

To compare fluorescence intensity among the specific stages, the digital images were obtained at a constant exposure of 0.1 sec. The images of oocytes stained with Rh123 (10–15 oocytes for each different stage) were analyzed by quantifying the average pixel intensities on the central line (equal to the diameter) of the ooplasm after correction as follows. Areas of the germinal vesicle and the pronucleus were free of mitochondria and were usually observed as a dark sphere in the fluorescent images. Therefore, the average pixel intensities including the GV and pronuclear areas in the GV oocytes and zygotes may be underestimated more than those of the MI and MII oocytes. To correct this effect, values for the ooplasm were calculated by subtracting the average intensity of GV and pronuclear areas, as a background value, from the intensity on the central line for each ovum at different stages. The mean pixel intensities at different stages were analyzed by ANOVA.

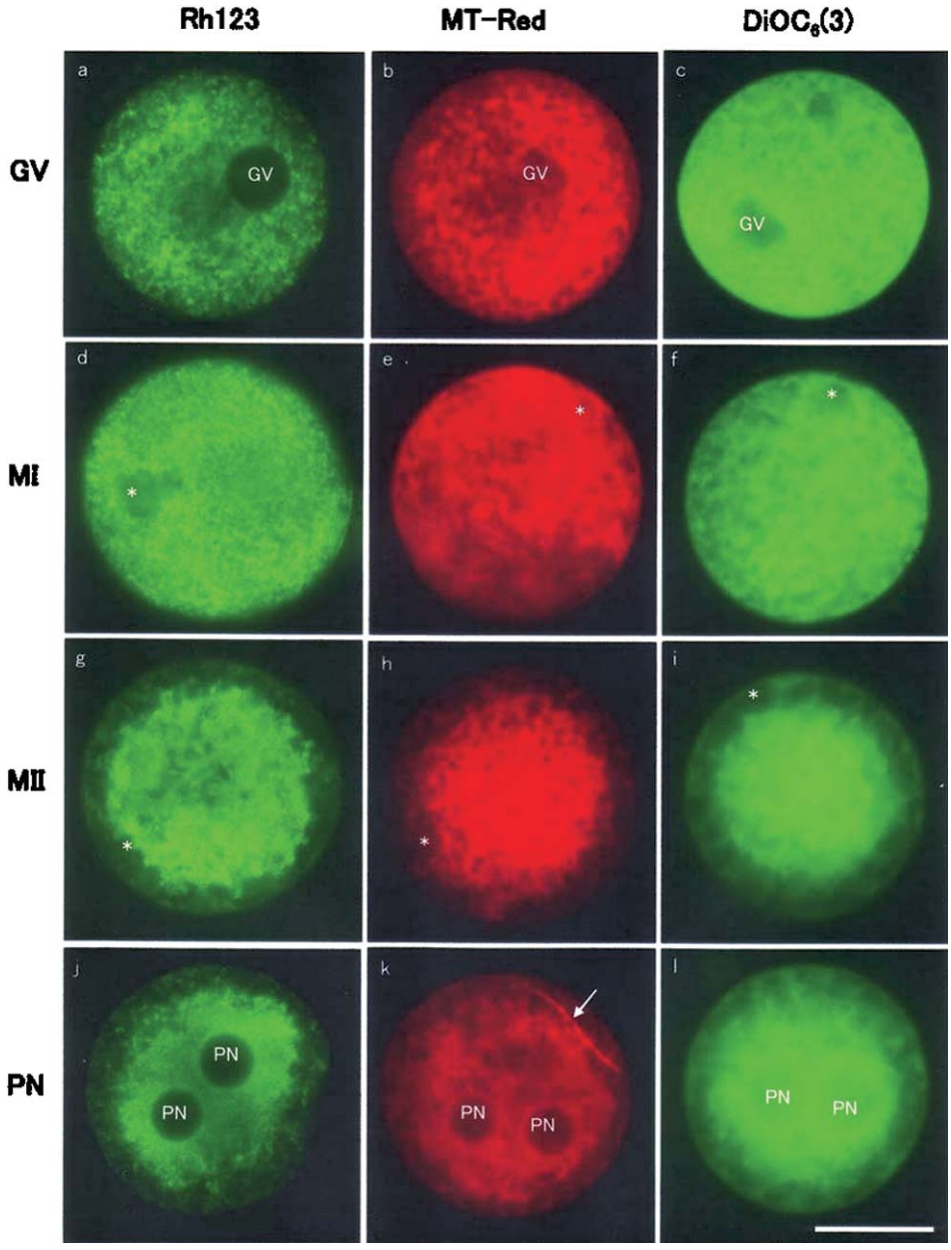

Fig. 1. Distribution of mitochondria in the hamster eggs stained with 3 different probes: Rh123 (left panels), MT-Red (middle panels) and $DiOC_6(3)$ (right panels). Rh123 reveals clearer and brighter clustering of mitochondria than MT-Red. $DiOC_6(3)$ shows uniform cytoplasmic fluorescence. The majority of GV oocytes (a–c) showed uniform cytoplasmic fluorescence, while some of them possess fewer mitochondria in the central ooplasm than in peripheral areas. The large germinal vesicle (GV) can be seen as a dark sphere. The MI oocytes (d–f) are characterized by increased density and intensity of mitochondrial fluorescence. In about half of the MII oocytes (g–i), mitochondria are usually absent from the cortical ooplasm. The asterisk indicates the location of the spindle in d–i. In zygotes (j–l), the mitochondria have become arranged in a dense cluster encircling the pronuclei with cortical cytoplasm showing little fluorescence. Arrow indicates the midpiece mitochondria of the penetrated sperm in k. The bar in l represents 50 μm for all photomicrographs.

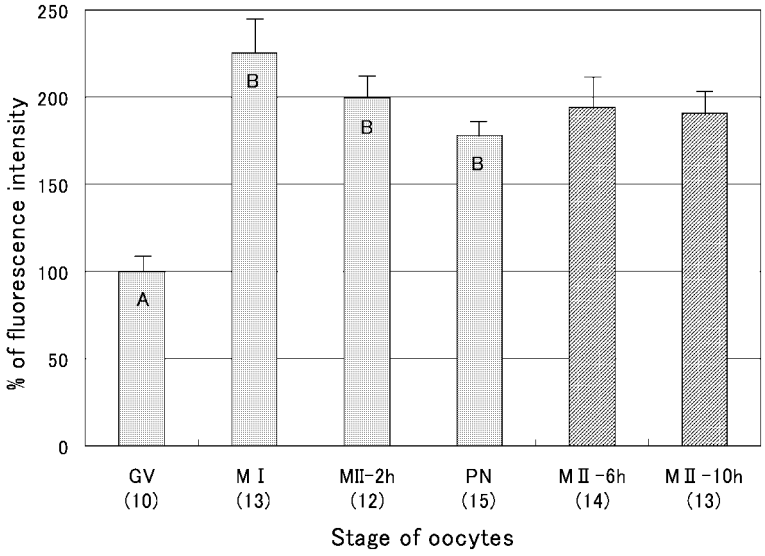

Fig. 2. Changes in fluorescence intensity of hamster oocytes during maturation. Values were calculated as percentages of the GV intensity, 100%. Data with different superscripts (gray bars) differ significantly ($P<0.01$). Aged MII oocytes (striped bars, 6 and 10 h post-ovulation, respectively) showed no significant difference in the intensity from the MII oocytes 2 h post-ovulation. Figures in parentheses are numbers of oocytes analyzed.

Results

Representative micrographs of mitochondrial localization in immature, matured and fertilized oocytes using three different fluorescent probes are presented in Fig. 1. The signals for Rh123 and MT-Red were virtually coincident, but Rh123 clearly revealed more distinct clusters of mitochondria than did MT-Red. On the other hand, $DiOC_6(3)$ provided much wider areas of bright signals than Rh123 and MT-Red. Despite a very low concentration used in this study, the membrane structures of not only mitochondria but also endoplasmic reticula may be stained with $DiOC_6(3)$ as previously reported [16]. Distribution patterns of active mitochondria in the eggs stained by the three different probes are summarized in Table 1 and the average fluorescence intensities are compared in Fig. 2. In all oocytes at the GV stage (n=174), mitochondria were spread throughout the ooplasm (Fig. 1a–c), although they seemed to be higher in the peripheral cytoplasm than in the central ooplasm. In some oocytes examined (21.4%, 38.1% and 10.4% for Rh123, MT Red and $DiOC_6(3)$, respectively), fewer mitochondria were evident in the center of the ooplasm than the cortex. At the MI stage, the fluorescence intensity of the ooplasm increased significantly compared to that of the GV ooplasm. Mitochondrial activity of the MI ooplasm, based on the fluorescence intensity, was more than doubled. The majority of oocytes (88.9%, n=158) at the MI stage showed a similar distribution pattern of mitochondria as seen at the GV stage, whereas the remaining 11.1% exhibited a mitochondria free zone at the cortex (Table 1). At the MII stage, the proportion of the oocytes with few mitochondria in the cortex was increased to 54.5% (n=196). In the present study, the accumulation of mitochondria around the MII spindle as reported in mice [1] was not noticed (Fig. 1g–i). The proportion of oocytes showing a mitochondria free zone at the cortex seemed to increase in the 10 h-aged group compared to the earlier groups (54.4%, 46.2% and 72.5% for 2 h, 6 h and 10 h post-ovulation groups, respectively) ($P=0.061$, by χ^2 analysis). In MII oocytes aged 6–10 h, the fluorescence intensity of the mitochondria seemed to decrease by approximately 10% compared to the MII oocyte 2 h post-ovulation, but no significant differences were found (Fig. 2).

After fertilization, mitochondria were aggregated around both male and female pronuclei, and the cortical region of the ooplasm had become devoid of active mitochondria in all zygotes examined (n=173, Fig. 1j–l).

Table 1. Changes in mitochondrial distribution during oocyte maturation and fertilization in the hamster

Stage	Probe*	No. of oocytes	Mitochondrial distribution:	
			Uniformly	Peripheral free**
GV	Rh123	84	100	0
	MT Red	42	100	0
	$DiOC_6(3)$	48	100	0
	Total/Mean	174	100	0
MI	Rh123	58	84.5	15.5
	MT Red	49	93.9	6.1
	$DiOC_6(3)$	51	88.3	11.7
	Total/Mean***	158	88.9 ± 2.7	11.1 ± 2.7
MII	Rh123	70	32.9	67.1
	MT Red	58	55.2	44.8
	$DiOC_6(3)$	68	45.6	54.5
	Total/Mean***	196	45.5 ± 6.5	54.5 ± 6.5
PN	Rh123	63	0	100
	MT Red	51	0	100
	$DiOC_6(3)$	59	0	100
	Total/Mean	173	0	100

GV, germinal vesicle; MI, the first meiotic metaphase; MII, the second meiotic metaphase; PN, pronuclear. *, See Materials and Methods. **, Showing mitochondria free zone at the cortex. ***, Mean ± sem.

Interestingly, the midpiece mitochondria of penetrated sperm were stained by MT-Red, but not by Rh123 around 12 h after the estimated time of sperm penetration [22] (Fig. 1k).

In 47.5% of the newly ovulated oocytes (n=70), strong fluorescence of Rh123 was detected in the first polar body, but the proportion of such oocytes decreased to 4.0% (n=39) and 8.8% (n=51) for the oocytes aged 6 and 10 h, respectively. In 11.1% of the zygotes (n=63), an intense signal was detected in the second polar body, but not in the first polar body.

Discussion

We examined the localization of active mitochondria in hamster oocytes by using 3 different staining methods, namely Rh123, MT-Red and $DiOC_6(3)$. Although the three different fluorescent dyes revealed a similar pattern of mitochondrial distribution during oocyte maturation in the present study, the high fluorescence output achieved with Rh123 may have been caused by a high mitochondrial membrane potential when unfixed oocytes were stained. Furthermore, the midpiece mitochondria of penetrated sperm were stained by Mt-Red, but not by Rh123 in this study, suggesting that the midpiece mitochondria may have lost their membrane potential by 12 h after the estimated time of sperm penetration [22]. Therefore, comparison of the areas stained with Rh123 and MT-Red in the same eggs may enable discrimination between active and inactive mitochondria.

The observations of the present study also demonstrate that active mitochondria are localized in the cortex rather than in the inner ooplasm at the GV stage, become homogeneously distributed at the MI stage, and at the MII stage decrease in density mainly at the cortex. After fertilization, mitochondria were obviously accumulated in the perinuclear region. The oocytes aged in the oviducts for 6–10 h showed decreases of 10% in density and flourescence intensity of mitochondria compared to those of the newly ovulated oocytes. Extended culture decreased the mitochondrial membrane potential in human oocytes [12], too.

The peripheral accumulation of mitochondria was also observed with the use of fluorescent probes in immature oocytes of mice [3], cows [9], pigs [10] and humans [12, 13]. Electron microscope observations also showed mitochondrial aggregation in the egg cortex in sheep [24], pigs [25] and cattle [26]. This pattern of distribution may be related to the high energy requirement of the cortex, as oocytes require cumulus cell support at this stage, and an intimate association between the oocyte and the cumulus cells is maintained

with the cumulus cell process ending on the surface of the oolemma [27–30]. In mice [1, 2, 8], mitochondria continue to aggregate around the perinuclear area between GVBD and MI. However, the present observations in the hamster showed that the mitochondria were homogeneously distributed throughout the ooplasm at the MI stage and that the perinuclear cluster did not become obvious until the pronuclear stage. The present study also demonstrated that the fluorescence intensity of mitochondria increased more than two-fold at the MI and MII stages as compared to the GV stage, suggesting the high energy requirement of these meiotic processes: e.g. spindle formation, chromosome condensation and movement, and polar body extrusion. Such strong staining of mitochondria in the ooplasm may represent an increase in mitochondrial activity and the aggregation of mitochondria for dynamic changes of the nucleus and organelle movement, as suggested by Van Blerkom and Runner [1] for mouse oocytes. The perinuclear clustering of the mitochondria in zygotes is consistent with the observation by Barnett *et al.* [4] in hamsters and the results obtained in pig [9], mouse [3] and human zygotes [7]. The pronuclear migration and apposition to form syngamy, probably being related to cytoskeletal reorganization, may require high concentrations of ATP [7, 9].

In summary, mitochondria in the ooplasm intensified their activities progressively from the GV stage to the MI stage and reorganized dramatically during oocyte maturation and fertilization in the hamster, as reported in other species. It has been reported that hamster 2-cell embryos exposed to blocking culture condition (addition of glucose and phosphate) suffer from the redistribution of mitochondria, showing metabolic disturbance [5]. A recent study revealed that mitochondrial dysfunction in mouse oocytes was directly responsible for the early arrest of preimplantation embryos *in vitro* [31]. In the hamster, the techniques for oocyte maturation and embryo culture *in vitro* have not been established yet. The present data on the distribution and activity of mitochondria in hamster oocytes may provide a basis for understanding the alterations in mitochondrial functions by compartmentalization of energy production and the necessary utilizations during oocyte maturation *in vitro*. The mitochondrial translocation may be associated with the potential of hamster eggs to develop *in vitro*.

Acknowledgements

The authors thank the staff of the Gene Research Center at Hirosaki University for the use of the image analyzing system.

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