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Histological Studies on the Onset of Meiosis and Changes in Mitochondria in Germ Cells in Fetal and Infant Rabbit Ovaries

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Abstract To estimate changes in cell activities of germ cells around the onset of meiosis, numbers of mitochondria in ovarian germ cells of rabbit fetuses and week-old does were counted histologically with Altmann-Kull stain and the ultrastructure of those mitochondria was observed.

The mean number of mitochondria per germ cell in each stage of mitotic (oogonium in mitotic and resting stage) and/or meiotic stages (oocyte in leptotene, zygotene and pachytene) were not dependent on stage of development. The distribution of germ cells by the number of mitochondria was roughly uniform in each stage of oogonia (mitotic and resting stage in mitotic division) and oocytes (leptotene, zygotene and pachytene in meiotic prophase I). The mean number of mitochondria per oogonium in mitotic and resting stage and per oocyte indicated approximately 40, 32 and 23, respectively.

With the transmitting electron microscope, the mitochondria in the oogonia in high mitotic activity were ultrastructually pleomorphic, suggesting cylindrical shape of the mitochondria. Such mitochondria usually contained many cristae traversing partially or completely the organella. On the other hand, most mitochondria in the germ cells transforming into oocytes showed a small circular shape, suggesting spherical mitochondria. Such mitochondria never contained many cristae, and the cristae traversed more incompletely through the matrix in comparison with those of mitochondria of the oogonia with high mitotic activities.

In general, the number of mitochondria in germ cells decreases and their function is declining duing the progress of cell division, from oogonia in mitotic stage via resting stage to meiotic prophase I. It is suggested that the decline of activities of division and of the function of the oocytes may be caused by the changes in the mitochondria.

INTRODUCTION

The total number of oocytes destined to ovulate, be fertilized, and develop is extremely smaller in the normal estrous cycle than the number of oocytes embedded in the ovarian tissue (BYSKOV, 1979; PETERS and MCNATTY, 1980). Recently, some attempts have been made to recover oocytes embedded in the ovarian tissue from not only normal estrous

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animals but also superovulated animals (Szybek, 1972; Bae and Foote, 1975; Nicosia *et al.*, 1975; Gould and Graham, 1976; Sorensen and Wassarman, 1976; Dunber and Raynor, 1980; Katska, 1984; Bar-Ami and Tsafriri, 1984; Fukunari *et al.*, 1989). Although many oocytes embedded in the ovarian tissue have the ability to develop in the future, they have not been explored sufficiently, since many of the attempts at recovery were carried out with ovaries form adult animals having many atretic follicles. It is also emphasized that only oocytes in large follicles located on the ovarian surface are usually recovered, in spite of growing follicles located on the ovarian surface are usually recovered, in spite of growing follicles located in deeper sites of the ovarian cortex (Peters and McNatty, 1980).

The number of germ cells in the ovarian tissue attains a maximum at the end of the proliferative phase of oogonia or at the time of onset of meiosis (Prepin *et al.*, 1985). The oocytes transformed from oogonia never increase in mumber and most of those degenerate with follicular atresia sooner or later. Since the oocytes embedded in the ovarian tissue have been never investigated sufficiently, changes in germ cells before and after the onset of meiosis have to be clarified for more effective utilization of oocytes. Although the transition of nuclei of germ cells during progress of meiotic prophase I and the histological changes of the ovaries in that period have been studied extensively, a biochemical approach has seldom been carried out, and even the energetic changes in cellular metabolism around the onset of meiosis have not been well studied.

The purpose of the present study was to estimate the changes in metabolic activities of germ cells around the onset of meiosis, judging from changes in the mitochondria as an index of the energy production within the cell.

MATERIALS and METHODS

Fifteen female fetuses of Japanese White rabbits at 22 days (4 does), 26 days (4 does), 28 days (4 does), and 30 days (3 does) of gestation and 4 does at 7 days of age were sacrificed by decapitation after weighing. To study the numerical changes in mitochondria in the cytoplasm of germ cells around the onset of meiosis, right ovaries from all fetuses and does were excised and fixed for 48 hours in Champy's solution immediately after removal. They were chromized with 3% potassium dichromate solution for eight days and then dehydrated. After embedding in paraffin wax for serial sections at 3 μm, each section was stained with Altmann-Kull stain. Germ cells with normal structure were observed microscopically in specimens in which the nuclei showed maximum size in serial sections, and the numbers of mitochondria in germ cells were counted ($\times$1000) in specimens for each day of gestation and age and each stage of mitotic (mitotic stage and resting stage) and meiotic (leptotene, zygotene and pachytene) division. The distribution pattern of mitochondria in germ cells in different stages of mitotic and meiotic division was checked, also. To study changes in ultrastructure of mitochondria after onset of meiosis, left ovaries from all rabbits were minced into approximately 3.5 mm^3 cubes with a pair of razors, and the cubes were fixed in 3% glutaraldehyde in 0.1 M Millonig's phosphate buffer for two hours at 4°C. They were rinsed several times with the phosphate buffer and postfixed in 1% osmium tetroxide in the phosphate buffer for one hour at 4°C, followed by graded alcohol dehydration and Quetol 812 infiltration. After polymerization overnight at 45°C and for 48

hours at 60°C, one μm-thick serial sections were made, stained with toluidine blue, to seek out the ovarian cortex involving germ cells in various stages of mitotic and meiotic diviion, then 90-100-thick sections were made with a glass knife using an ultramicrotome (SORVALL, model MT2-B). The ultra-thin sections were stained with uranyl acetate and lead citrate, and observed with a transmitting electron microscope (HITACHI HS-10, operated at 50 kV).

Statistical analysis

Data were examined by analysis of variance and differences among means of the number of mitochondria at each age and stage of mitotic and meiotic division were tested by Duncan's multiple range test.

RESULTS

1. *Distributions of numbers of mitochondria estimated by Altmann-Kull stain*

Table 1 shows the distribution of oogonia and oocytes in different stages of meiotic prophase I (leptotene, zygotene and pachytene), which appeared in ovaries with increasing ages from 22 days of gestation to seven days of age. On 22, 26 and 28 days of gestation, the ovaries were in the proliferative phase; about 9% of oogonia were in mitotic stage and all the other were in resting stage. At 30 days of gestation, oogonia in mitotic (about 4.5% of all germ cells) and resting (about 91%) stages and oocytes in leptotene (about 2%) and zygotene (about 1%) were recognized. At 7 days of age, oogonia in mitotic stage became very few (about 1.5%) and oocytes in pachytene appeared.

Table 1. The distribution of germ cells in different stages of mitotic and/or meiotic division on the various days of gestation and age.

Days of gestation and/or age[1)]	Rabbit no.	Body weight (g)	No. of germ cells counted[2)]	Oogonia (%)		Oocytes (%)		
				mitotic	resting	leptotene	zygotene	pachytene
P 22	P 22-1	7.04	1345	9.8	90.2	0	0	0
	P 22-2	6.22	1682	7.7	92.3	0	0	0
	P 22-3	6.86	732	11.2	88.8	0	0	0
P 26	P 26-1	28.57	1339	8.3	91.7	0	0	0
	P 26-2	28.64	635	11.3	88.4	0	0	0
	P 26-3	20.82	1093	9.5	90.5	0	0	0
P 28	P 28-1	32.74	1412	10.0	90.0	0	0	0
	P 28-2	41.05	1611	9.8	90.2	0	0	0
	P 28-3	41.27	1154	7.7	92.3	0	0	0
P 30	P 30-1	54.41	4452	3.9	95.0	1.0	0.2	0
	P 30-2	61.02	3130	5.3	91.6	2.2	0.9	0
	P 30-3	65.58	2225	2.9	90.5	3.6	3.0	0
D 7	D 7-1	125.0	2234	0	39.7	19.1	22.4	2.8
	D 7-2	94.0	7402	0.05	55.6	19.1	32.5	8.7
	D 7-3	140.0	5037	0.2	41.5	18.8	33.3	6.1

[1)] P22, P26, P28, P30 and D7 mean day 22, 26, 28, 30 days of gestation and seven days of age, respectively.

[2)] Total number of germ cells counted in every tenth serial section.

Comparison between mean numbers of mitochondria in oogonia in various stages (mitotic prophase, metaphase, anaphase, telophase and resting stage) in all fetal ovaries showed that the numbers of mitochondria of oogonia both in prophase and resting stage were significantly smaller than those in metaphase, anaphase and telophase ($p<0.01$). However, there were no significant differences in mitochondrial numbers between prophase and resting stage, or among metaphase, anaphase and telophase (Table 2).

When numbers of mitochondria per oogonium and/or oocyte are divided into the following groups, 0-9 (group 0), 10-19 (group 10), 20-29 (group 20), 30-39 (group 30), 40-49 (group 40), 50-59 (group 50) and 60-69 (group 60), distributions of incidences (%) of germ cells of each group for each age and stage of cell division were as shown in Text-fig. 1.

The distribution pattern of mitochondrial numbers in germ cells was basically uniform through each stage of oogonia (mitotic and resting stage in mitotic division) and oocytes (leptotene, zygotene and pachytene in meiotic prophase I).

In mitotic stage, oogonia had comparatively larger numbers of mitochondria, and the mode, which was shown by the ratio of the number of oogonia in each group to all oogonia in mitotic stage, was in group 30 or 40.

In resting stage, oogonia with large numbers of mitochondria were decreased in number in comparison with those in mitotic stage, and the mode was in group 20 or 30.

The distributions of oocytes in each meiotic prophase showed that the modes of oocytes were in group 10 or 20. Oocytes with larger number of mitochondria were fewer than in earlier stages, and oocytes with over 40 mitochondria became very few.

Mean numbers of mitochondria per germ cell in mitotic (mitotic and resting stage) and meiotic (leptotene, zygotene and pachytene) division at various days of gestation and age are shown in Table 3. Although there were no significant differences among ages in each stage of mitotic and/or meiotic division, there were significant differences between cell stages at 22, 26 and 28 days of gestation; and at 30 days of gestation and seven days of age, the mean number of mitochondria per germ cell decreased significantly in accordance with the

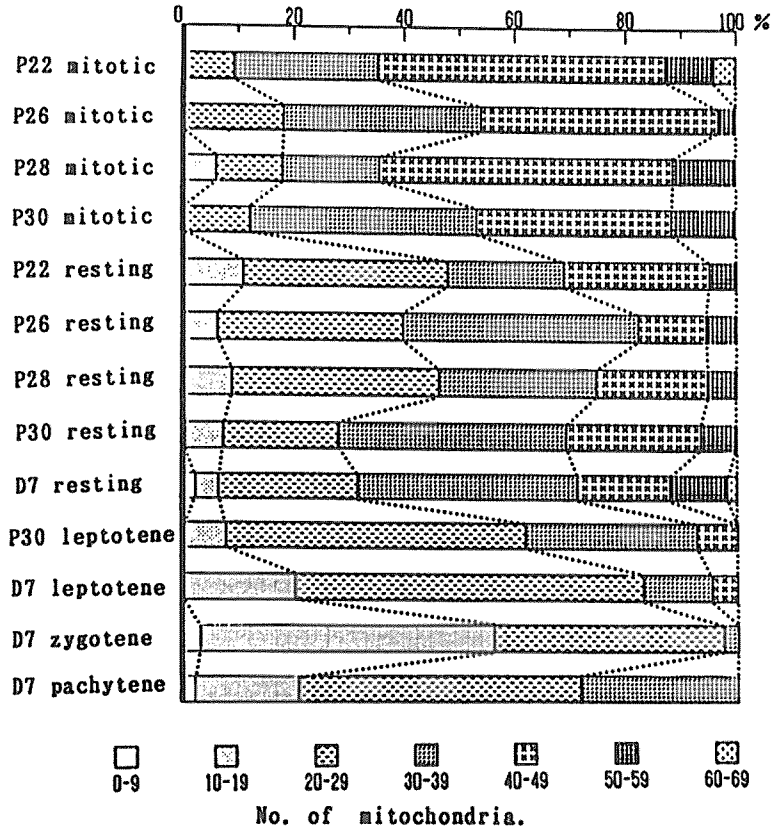
Table 2. Mean number of mitochondria per oogonium in each mitotic stage and resting stage in all fetal rabbit ovaries[1)].

Stage of mitotic division	Number of oogonia counted[2)]	Mean ±S. D.
Mitotic prophase[a]	39	32.82±8.89
Mitotic metaphase[b]	49	40.51±7.00
Mitotic anaphase[b]	36	40.36±8.46
Mitotic telophase[b]	37	39.49±5.76
Resting stage[a]	99	31.65±8.99

1) Mean number of mitochondria observed in specimens in which each nucleus showed maximum size in serial sections.

2) Number of oogonia in which the number of mitochondria was counted.

Significant differences are seen between different letters ([a, b], $p<0.01$).



Text-fig. 1. The distribution of incidences (%) of germ cells according to the number of mitochondria in each stage of mitotic division (mitotic and resting stage) and/or meiotic division (leptotene, zygotene and pachytene) in fetal and infant rabbit ovaries.
P22, P26, P28, P30 and D7 mean 22, 26, 28, 30 days of gestation and seven days of age, respectively.

advance of mitotic and meiotic division ($p<0.05$).

2. *Ultrastructure of mitochondria by transmitting electron microscope*

In general, it seemed that all mitochondria located in oogonia and oocytes were of the orthodox type. However, mitochondria of oogonia in mitotic and resting stage, particularly at 22, 26 and 28 days of gestation, seemed to be pleomorphic, since their appearance was variable; they were elongated-oval, dumbbell-like or spherical in shape. Such mitochondria usually had many cristae traversing the mitochondrial matrix, partially or completely (Fig. 1).

In oogonia in resting stage at and after 30 days of gestation, and in oocytes in leptotene, the ratios of elongated-oval and dumbbell-like mitochondria decreased. Such mitochondria were rare in oocytes in zygotene and pachytene, and most mitochondria had a small spherical shape. So the mitochondria in the oocyte seemed to be spherical at the end of

Table 3. Mean number of mitochondria per germ cell in each stage of mitotic (mitotic and resting stage) and/or meiotic (leptotene, zygotene and pachytene) division at various days of gestation and age.

	Days of gestation				seven days of age
	22	26	28	30	
Oogonium in mitotic stage	42.64± 8.12[a] (23)	37.79±7.51[a] (28)	39.12±10.07[a] (17)	39.00±7.34[a] (17)	—
Oogonium in resting stage	32.00±10.87[b] (19)	32.24±9.69[b] (33)	32.17± 9.78[b] (35)	34.72±9.74[ab] (29)	35.33±11.48[a] (48)
Oocyte in leptotene	—	—	—	29.00±6.75[b] (13)	24.50± 6.53[b] (40)
Oocyte in zygotene	—	—	—	—	21.71± 7.89[b] (39)
Oocyte in pachytene	—	—	—	—	24.64± 6.65[b] (59)

Values indicate mean±S. D.
Significant differences are seen between different letters in the same column ([a, b], $p<0.05$).
Figures in parentheses show the number of germ cells in which the number of mitochondria was counted.

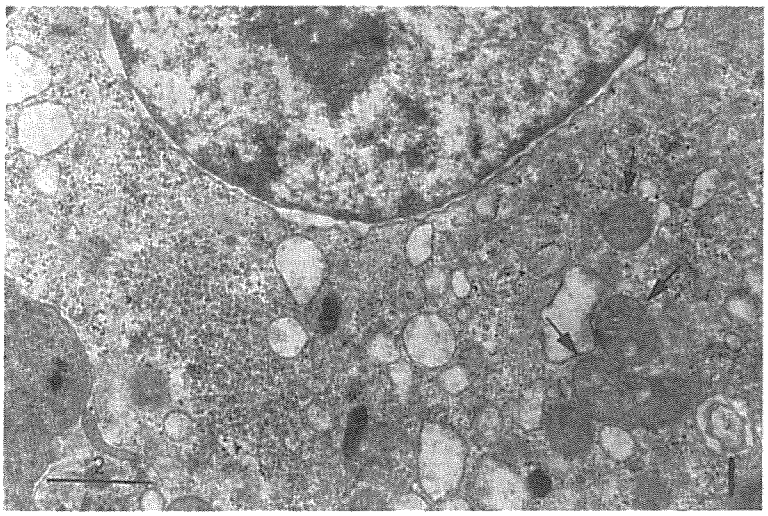


Fig. 1. Electron micrograph of an oogonium in resting stage.
The mitochondria (arrows) show elongated-oval shape and their cristae traverse the organella partially and completely.
Bar indicates 2 μm.

meiotic prophase I. These mitochondria had fewer cristae which projected into the mitochondrial matrix less than those in earlier stages (Fig. 2).

DISCUSSION

Although mean numbers of mitochondria per germ cell did not differ significantly among the various days of gestation and age, they were significantly different among dif-

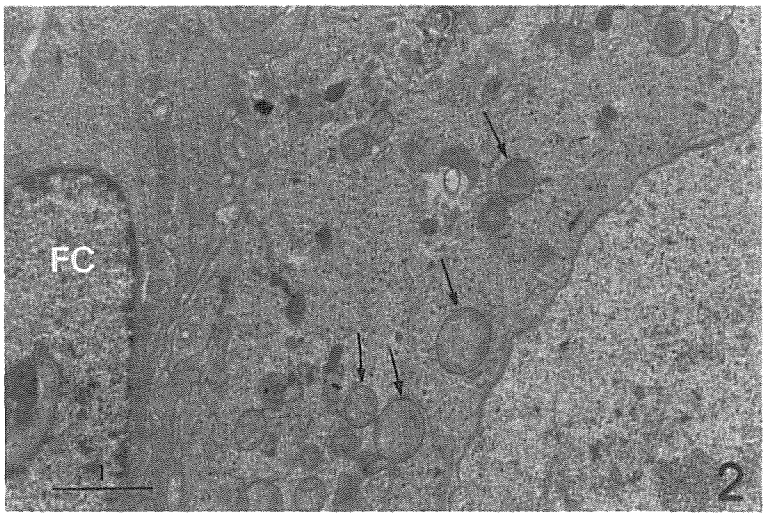


Fig. 2. Electron micrograph of an oocyte in pachytene.
Almost all mitochondria (arrows) show spherical shape, cristae traverse the organella only partially, and the number of cristae decreases. Degree of projection of the cristae into matrix is more incomplete than in germ cells in earlier stages. FC, nucleus of follicular cell.
Bar indicates 1 μm.

ferent stages of mitotic and meiotic division, except for stages at 30 days of gestation and in germ cells transformed from oogonium to oocyte. It is well-known that periodic variation in metabolism according to cell-cycle occurs and that cellular metabolic activities are very high in accordance with high mitotic activities (SAKAI, 1969; YANAGISAWA, 1969). The biological significance of various periodic biochemical variations resulting from the cell-cycle has not been explained yet. However, it is clear that the energy-production system within the cell is activated at the G_2 phase of the cell-cycle, when the replication of nuclear genomic DNA has already completed, and that the mitochondrial DNA is particularly replicated at the end of the G_2 phase. At the M phase, when the nuclear genome is actually separated, the cytoplasmic ATP content remains at the regular level, and consumption rates of O_2 and energy metabolisms are at a minimum for all phases of the cell-cycle (YANAGISAWA, 1969). Although mitotic division is inhibited by deficiency of O_2-supply before replication of mitochondrial DNA, a deficiency of O_2-supply at the other phases of the cell-cycle does not inhibit mitotic division (SAKAI, 1969; YANAGISAWA, 1969). It is generally agreed that the function of mitochondria in high mitotic activities is activated at the resting stage (namely, G_1, S and G_2 phases in the cell-cycle) and that multiplication of mitochondria occurs only at the M phase of the cell-cycle. Table 2 in the present study shows a similar trend. Although mean number of mitochondria per oogonium in resting stage (about 32) was not different significantly from that in mitotic prophase, mean numbers in mitotic metaphase, anaphase and telophase were significant larger as compared with mitotic prophase and resting stage.

This tendency was recognized in the distribution of germ cells according to mitochondrial number (Text-fig. 1). Table 3 and Text-fig. 1 show that the number of mitochondria per germ cell is diminished as the mitotic and/or meiotic division progresses. The ratios of oogonia with over 30 mitochondria were almost stable by mitotic stage (about 85%) and by resting stage (about 50%), respectively. The distribution of oogonia in resting stage on 30 days of gestation and 7 days of age, and of oocytes in pachytene, showed a different pattern, in which mitotic activity was decreased at 30 days of gestation (Table 1) and folliculogenesis started when the oocyte was in the transitory stage between pachytene and the following stage (Sasabe *et al.*, 1989).

Moreover, on 22, 26 and 28 days of gestation, when mitotic activity was steadily proliferating, periodic changes in mitochondrial ultrastructure in oogonia were scarcely found. On the other hand, germ cells indicated transformation into oocytes had spherical and/or oval mitochondria of small size. Those mitochondria contained a few cristae which projected incompletely into the matrix, unlike those of mitochondria of germ cells with high mitotic activities.

Conclusively, the number of mitochondria tends to decrease and their function is declining in the process of division from oogonia in mitotic stage (via resting stage) to meiotic prophase I. These results suggests that the decline of activities of division and of the metabolism of the oocyte is connected closely with the changes in mitochondria.

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胎子および幼若家兎における生殖細胞の減数分裂の開始とミトコンドリアの変化に関する組織学的観察

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減数分裂の前後に起こる生殖細胞の機能的な変化を推定するために胎齢22日から出生後7日までの家兎卵巣内に存在する生殖細胞細胞質内のミトコンドリアの変化を組織学的に観察した。

光顕的に Altmann-Kull 染色を施して観察した結果，体細胞分裂前期および分裂休止期にある卵祖細胞のミトコンドリア数は他の分裂段階にあるものに比べて有意に少ない値を示した。また，各胎齢および日齢ごとに，分裂段階を体細胞分裂期，分裂休止期，第一減数分裂前期の細糸期，接合期および太糸期に区別し，ミトコンドリアの個数別分布割合を比較した場合，体細胞分裂が活発な胎齢22日，26日および28日の体細胞分裂期にある卵祖細胞では一細胞当りのミトコンドリア数が40－49個のものが最も多く，分裂休止期の卵祖細胞では一定の分布傾向は認められなかった。これに対して胎齢30日および出生後7日では体細胞分裂期の核相を呈する卵祖細胞は減少し，分裂休止期の卵祖細胞では，ミトコンドリア数が30－39個のものが最も多く，全体に正規分布状の分布を示しており，また，体細胞分裂期にある卵祖細胞のミトコンドリア数も全体的に減少していた。減数分裂に移行した卵母細胞では，一細胞当りのミトコンドリア数は分裂休止期の卵祖細胞のものに比べて有意に小さい値を示し，ミトコンドリア個数別の生殖細胞の分布割合からもミトコンドリアが全体的に減少する傾向が認められた。

電顕的には，増殖期にある卵祖細胞のミトコンドリアの断面は長楕円または棍棒状を呈するものが多く，杆状であるものと考えられた。これに対して減数分裂に移行した卵母細胞ではミトコンドリアの断面はいずれも小型の円形もしくは短楕円形状を呈し，球状化しているものと考えられた。また，増殖期の卵祖細胞のものよりもクリステのマトリクスへの突出程度が悪くなり，クリステ数の減少が認められた。

以上のことから，減数分裂が進行するにつれてミトコンドリアは数を減じ，また同時にその機能も低下するものと考えられ，卵母細胞の代謝活性が低下するものと考えられた。