Preimplantation Development of Embryos and Their Enzyme Activity in Aged Hamsters

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Abstract: *Development and enzyme activities of embryos during the preimplantation period were examined to reveal causes of the age-related embryonic loss in the golden hamster. Embryos were obtained from young (9-12-week old, n=40) and aged hamsters (43–53-week old, n=40) on Days 2, 2.5, 3 and 3.5 of pregnancy and the number and developmental stages were recorded. In order to compare the viability of the embryos, the metabolic activities of mitochondria, microsomes, plasma membrane and lysosomes were examined histochemically. Early embryos from aged hamsters showed delays in their development and transport through the oviduct compared to those from young females. The embryos exhibiting strong activities of succinate dehydrogenase, isocitrate dehydrogenase and Δ^5-3β-hydroxysteroid dehydrogenase were significantly diminished in the aged females. The present results suggest that the reduced metabolic activity may be one possible reason for the retarded development of embryos and that the asynchronous development and delayed migration of the embryos into the uterus may partly account for the embryo loss in the aged hamsters.*

Key words: *Aging, Hamster, Embryo, Development, Enzyme activity*

In female hamsters, aging results in a reduction in litter size, due to an increase in preimplantation loss and in resorption of established implantation sites [1–5]. Our recent studies revealed that about a 30% decline in litter size and delay in ovulation occurred in aged hamsters as compared with young females [6]. In addition, delays in penetration of sperm and male pronuclear development were observed in the eggs from aged females [7], suggesting that such abnormalities early in the process of fertilization are one of the factors causing the preimplantation wastage of embryos in aged hamsters. Delayed development of implants was also observed in aged hamsters [8].

In the present study, therefore, preimplantation development of embryos and their enzyme activities were investigated in young and aged hamsters to compare viability of the embryos. The activities of certain enzymes are known to change in the oocytes from old mice [9]. There is a correlation between development of mouse parthenotes and their enzyme activities [10–12]. Parkening and Soderwall [13] have noticed some differences between embryos from young and old hamsters at the same day of pregnancy in the intensity of succinate dehydrogenase. In the present series of experiments, enzyme activities of the embryos, including the integrity of mitochondria, microsomes, plasma membrane and lysosomes of young and aged hamsters, were histochemically compared.

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Materials and Methods

Animals

Female golden hamsters were kept under controlled conditions, fed *ad libitum* and in a 10-h dark/14-h light schedule. The vaginal discharge of each female was checked prior to mating to assure that the females had normal estrous cycles [14]. A total of 80 virgin females were mated at 9–12 weeks of age and randomly assigned to young (9–12-week-old) or aged groups (43–53 weeks of age). The females in the aged group were mated repeatedly at intervals of 10–12 weeks until examination at 43–53 weeks of age according to our previous studies [6–8]. In the evening of estrus, the females were caged with young fertile males (10–12 weeks of age) and the day when spermatozoa were found in the vaginal smear was designated as Day 1 of pregnancy (Day 1).

Recovery of embryos

The successfully mated hamsters were sedated with ether and killed by cervical dislocation on Days 2, 2.5, 3 and 3.5. Twelve animals each on Days 2, 3 and 3.5, and 4 each on Day 2.5 were examined in the young and aged groups. The reproductive tracts and ovaries were removed and placed in Dulbecco's phosphate buffer solution (Gibco, Grand Island, NY) supplemented with 0.1% polyvinyl alcohol (Sigma Chemical Company, St. Louis, MO; DPBS-PVA). Embryos were recovered by flushing the entire oviduct and/or uterus according to the time of recovery [15]. The number of embryos and their developmental stages, and the number of corpora lutea were recorded.

Detection of enzyme activities

All embryos recovered from young and aged females were subjected to detection of the activities of the following enzymes: cytochrome oxidase (CO) [16], isocitrate dehydrogenase (ICD) [17], succinate dehydrogenase (SDH) [18], Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) [19], adenylate cyclase (AC) [20] and acid phosphatase (ACPase) [21]. CO activity was estimated by the amount of pyrazolon granules formed in the embryos incubated for 10 min at 37°C in 5.0 mM 3,3'-diaminobenzidine tetrahydrochloride, 0.15% cytochrome c, 2.5 mM cobalt (II) acetylacetonate and 2% PVA in 50 mM phosphate buffer. ICD activity was estimated by the amount of formazans formed in the embryos incubated for 10 min at 37°C in 50 mM trisodium DL-isocitrate, 2 mM nicotinamide adenine dinucleotide, 5 mM NaN_3, 0.2 mM phenazine methosulfate and 1 mM tetranitroblue tetrazolium in 0.1 M phosphate buffer. For SDH activity, embryos were placed at 37°C for up to 90 min in 0.1% nitro blue terazolium (NBT) in 0.1 M sodium succinate and 0.1 M phosphate buffer. For Δ^5-3β-HSD, embryos were incubated for 90 min at 37°C in 0.1 M phosphate buffer containing 0.018% dehydroepiandrosterone, 0.04% NAD and 0.02% NBT. The SDH and Δ^5-3β-HSD activities were estimated as described for ICD. For AC activity, embryos were incubated for 60 min at 37°C in 0.5 mM 5'-adenylylimidodiphosphate lithium salt, 2 mM lead nitrate, 2 mM sodium floride, 4 mM magnesium sulfate, 2 mM theophylline, 6% dextran in 80 mM tris maleate buffer and then immersed in 0.1% yellow ammonium sulfide solution. The activity was demonstrated by the amount of lead granules formed in the embryos. ACPase activity was detected by the amount of red granules formed in the embryos placed in 0.16% Naphthol AS-BI phosphate in 0.2 M acetate buffer supplemented with fast red violet LB and $MnCl_2$ at 37°C for 60 min. Embryos incubated in the mixtures devoid of the substrates served as controls. After staining, embryos were rinsed in DPBS-PVA and mounted on glass slides. Under an Olympus microscope (BX50, Olympus Co., Tokyo), the activities of the enzymes were graded into three groups: none, weak and strong, according to the amount of granules stained in the blastomeres and/or whole embryos.

Statistical analysis

Proportion of the embryos exhibiting strong activity of each enzyme was analyzed by Student's *t*-test after the angle transformation of percentage.

Results

Preimplantation development of embryos

The overall mean number of ova ovulated by the young (14.2, n=40) and aged hamsters (14.6, n=40) did not differ significantly. The percentages of eggs recovered were 100% (n=568) and 99.2% (n=579) of the total number of eggs ovulated by the young and aged females, respectively. The developmental stages of preimplantation embryos recovered from the young and aged hamsters are summarized in Table 1. The percentage of degenerated eggs, consisting of those fragmented or developmentally retarded, increased from Day 3 (4.6%) to Day 3.5 (12.2%) in the young hamsters (1.8–3.3% on Days 2–2.5), whereas more degenerated eggs (13.4–24.0%) were found throughout the preimplantation period in the aged hamsters than in the young females. And more unfertilized eggs (67/579) were detected in the aged females than in the young females (24/568).

On Day 2.5, 43.6% of embryos (n=56) reached the 8-cell stage in the young females, but only 1.5% of the embryos were at the same stage in the aged females (n=63). Similarly, 44.4% of embryos (n=173) from the young females on Day 3 developed to morula and further stages, but only 3.8% were at the morula stage in the aged group (n=166). Data in Table 1 clearly demonstrate that embryos from the aged hamsters developed more slowly than those from the young females during the preimplantation period.

In the young females, all embryos/eggs were recovered from the uteri on Day 3 or later. In the aged females, however, 65.7% (109/166) of the embryos/eggs, including molurae, were still recovered from the oviducts on Day 3, suggesting a delay in egg transport through the oviduct.

Table 1. Early development of embryos in young and aged hamsters

Groups	Site of egg recovery*	Number of eggs examined	Percentage (s.e.m.) of eggs at the following stages**:									
			2-Cell	3–4-Cell	5–7-Cell	8-Cell	Morula	EB	ExpB	HB	Deg	UFO
Young												
Day 2	O	178	71.7 (2.0)	22.1 (2.5)							1.8 (0.9)	4.3 (1.2)
Day 2.5	O	56		49.7 (4.4)		43.6 (6.5)					3.3 (1.9)	3.3 (1.9)
Day 3	U	173				42.8 (6.1)	42.1 (5.8)	2.3 (1.7)			4.6 (2.1)	8.2 (2.1)
Day 3.5	U	161						20.4 (5.6)	34.9 (4.4)	32.5 (7.1)	12.2 (1.8)	0
Aged												
Day 2	O	182	67.6 (2.2)								13.4 (3.4)	19.1 (3.5)
Day 2.5	O	63	36.5 (10.3)	25.5 (5.8)	4.7 (2.9)	1.5 (1.5)					16.9 (6.9)	15.8 (5.4)
Day 3	O + U	166		12.2 (5.0)	18.9 (4.4)	39.9 (5.4)	3.8 (2.2)				15.8 (3.1)	9.3 (2.9)
Day 3.5	U	168					44.0 (3.9)	20.1 (2.4)	8.1 (2.5)		24.0 (2.6)	3.8 (1.8)

*, O, Oviduct; U, Uterus. In aged females, 65.7% of the eggs were recovered from the oviduct on Day 3. **, EB, Early blastocyst; ExpB, Expanded Blastocyst; HB, Hatched blastocyst; Deg, Degenerated; UFO, Unfertilized ova.

Table 2. Mean percentages of the embryos exhibiting strong enzyme activity at each developmental stage*

Enzymes	2-Cell		8-Cell		Morula		Blastocyst	
	Young	Aged	Young	Aged	Young	Aged	Young	Aged
CO	52.3[a] (21)	35.4[b] (20)	62.5 (16)	42.7 (12)	41.7 (12)	37.5 (11)	42.1 (19)	41.7 (12)
ICD	82.9[a] (21)	43.8[b] (23)	92.9[a] (14)	41.7[b] (12)	100 (11)	66.7 (12)	95.8[a] (24)	66.7[b] (10)
SDH	70.8[a] (21)	29.2[b] (23)	77.5[a] (17)	45.0[b] (10)	92.9 (14)	58.3 (12)	92.9[a] (14)	55.0[b] (11)
Δ^5-3β-HSD	30.3[a] (21)	5.0[b] (20)	35.0 (14)	65.0 (11)	36.7 (11)	29.2 (17)	94.4[a] (18)	16.7[b] (12)
AC	27.5[a] (22)	10.1[b] (20)	33.3 (10)	41.7 (12)	29.2 (11)	32.5 (16)	44.4 (20)	37.5 (10)
ACPase	55.0[a] (22)	23.6[b] (21)	61.9 (13)	25.0 (12)	56.7 (14)	41.7 (12)	75.0 (20)	70.0 (10)

*, Figures in parenthesis show numbers of embryos examined. [a, b], Means with different superscripts at each developmental stage are significantly different ($p<0.05$).

Enzyme activities of embryos

The percentage of embryos showing signs of strong enzyme activity is shown in Table 2, and representative micrographs are shown in Fig. 1. In all enzymes examined, percentages of 2-cell embryos exhibiting a strong histochemical reaction were significantly lower in aged hamsters than in young females ($p<0.05$). Decreased percentages of embryos showing strong ICD and SDH activities were obvious at the 8-cell and blastocyst stages in the aged females ($p<0.05$). Similarly, the embryos with strong Δ^5-3β-HSD activity were fewer at the blastocyst stage in the aged hamsters ($p<0.05$, photo not shown). In the young hamsters, the enzymes in more than 70% of embryos at the 2-cell to blastocyst stage which exhibited strong activity were ICD (82.9–100%) and SDH (70.8–92.9%).

Discussion

The present study clearly demonstrated that the development and oviductal transport of embryos was more delayed in the aged hamsters than in the young hamsters (Table 1). Developmentally retarded embryos have been described in transplantation studies on aged hamsters [22], mice [23] and rabbits [24]. Blaha [22] reported that in aged hamsters one-sixth of the eggs flushed from uterine horns at 63 to 68 hours after ovulation were still in the 1- to 4-cell stage. Yoshizawa [25] also

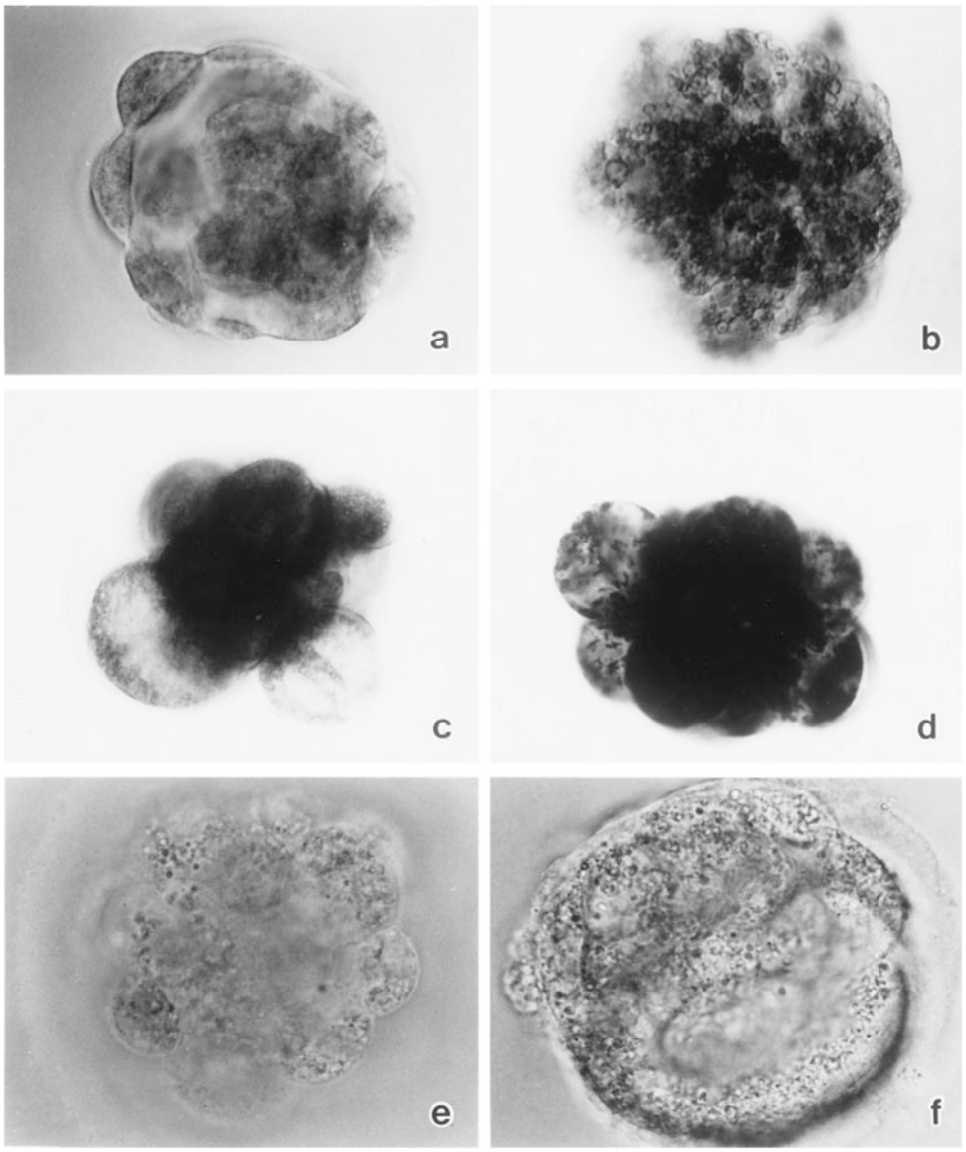

Fig. 1. Representative evaluation of activities of mitochondrial enzymes in hamster embryos. × 660. a, b) A blastocyst showing weak (a) and strong (b) CO activity. c, d) An 8-cell embryo having ICD activity evaluated as weak (c) and strong (d). An embryo having more than 50% of well-reacted blastomeres was evaluated as strong. e, f) A blastocyst showing weak (e) and strong (f) SDH activity.

observed asynchronous development of embryos in old hamsters. Furthermore, Perkeing and Soderwall [26] have noticed a delay in egg transport through the oviduct in old hamsters at 14 to 17 months of age.

It is of interest that enzyme activities decreased at specific developmental stages more frequently in embryos from aged hamsters than in those from young females. In young hamsters, ICD and SDH were the enzymes that exhibited strong activity in the majority of embryos (more than 70%). Significant reduction in the activities of both enzymes was noted at the 2-cell, 8-cell and blastocyst stages in the aged animals as compared to those in young animals. On the other hand, Parkening and Soderwall [13] have reported that SDH activity was not significantly different in blastocysts from young and aged hamsters, but the total percentage of blastocysts from young females exhibiting strong activity seemed to be very low in their report (50.7%) compared to the

results presented here (92.9%). Ishida and Chang [27] also have shown that 78% of blastocysts from young hamsters exhibited a strong SDH reaction. This difference may be attributable to methods of evaluating the intensity of the reaction, or it may be related to the purity of the substrates used. The present results suggest that ICD and SDH may be good makers for demonstrating embryonic viability in relation to maternal aging.

In oocytes of old mice, it was shown that SDH, CO, Δ^5-3β-HSD and AC activities decreased compared to those of young females [9]. There are several reports showing that the enzyme activities in a variety of tissues decrease as the animals grow older: e.g. SDH in muscles of hamsters [28] and CO in renal cells [29] and hepatic cells [30] of rats. Since SDH and ICD are the main enzymes in the Krebs cycle, it is possible that the capacity to maintain basic energy metabolism is impaired in the preimplantation embryos of aged hamsters. In addition, a weak activity of Δ^5-3β-HSD, which relates directly to steroid biosynthesis [19], was detected in blastocysts in aged females. From the results of the present study and those by others [9, 28–30], it is speculated that the embryos developing slowly or having ceased to develop may result from decreased activities of these enzymes. Thereby, asynchrony between the embryonic development and uterine environmental change is considered to arise. Since asynchronous embryo transfer should result in serious deterioration in subsequent development of the embryos [31–33], asynchronous development of the embryos and the delayed migration into the uterus may partly account for the peri-implantation embryo loss reported in aged hamsters [8].

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