Histochemical Studies on Progestagen Production in Mouse Eggs and Embryos during the Early Development

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ABSTRACT: The activities of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) (pregnenolone and 17α-hydroxypregnenolone as the substrates), 20α-HSD (20α-hydroxyprogesterone as the substrate) and 20β-HSD (17α-hydroxyprogesterone and 20β-hydroxyprogesterone as the substrates) were histochemically demonstrated in mouse eggs and embryos, and the progestagen production in those during the early development was examined.

In unfertilized mouse eggs, the activities of such HSDs were observed in 88 to 98% of those. The percentages of embryos showing the activities of such HSDs did not change during the early development. It was also histochemically confirmed that the activity of Δ^5-3β-HSD with DHA as the substrate, which is involved in steroid biosynthesis, was observed in 86 to 100% of the eggs and embryos from the unfertilized to blastocyst stages.

From the present findings, it was suggested that the progestagens such as progesterone, 17α-hydroxyprogesterone, 17α,20β-dihydroxyprogesterone, 20α-hydroxyprogesterone and 20β-hydroxyprogesterone are constantly synthesized in mouse eggs and embryos during the early development.

Key words: mouse embryo, early development, hydroxysteroid dehydrogenase, progestagen production, histochemistry

INTRODUCTION

Recently, biochemical studies proved the presence of progesterone, 17α-hydroxyprogesterone and 20α-hydroxyprogesterone in rabbit blastocysts[1)] and of progesterone in porcine[2)], bovine[3)] and equine[4)] blastocysts. Proved also that the blastocysts of these animals had the ability of synthesis and secretion of these progestagens[2,3,5-9)].

Detection of hydroxysteroid dehydrogenase (HSD), which directly involves progesterone synthesis, in mouse embryos was histochemically attempted[10)]. Namely, the activity of Δ^5-3β-HSD with pregnenolone as the substrate was detected in mouse embryos from the 1-cell to blastocyst stages, and the possibility of progesterone production in those was suggested. On the other hand, NIIMURA and ISHIDA[11-13)], who histochemically demonstrated some progestagen-synthetic HSDs, i.e., Δ^5-3β-HSD (pregnenolone and 17α-hydroxypregnenolone as the substrates), 20α-HSD (20α-hydroxyprogesterone as the substrate) and 20β-HSD (20β-hydroxyprogesterone as the substrate) in hamster, porcine and rabbit embryos, reported that such enzymes were present in all the embryos from the 1-cell to blastocyst stages, and presumed that such facts suggested that progestagens were produced in the embryos throughout their developmental stages. In mouse embryos, however, only Δ^5-3β-HSD was demonstrated among progestagen-synthetic HSDs[10)]. Thus the ability to produce progestagens except progesterone in mouse embryos is unclear.

In the present study, the activities of various kinds of progestagen-synthetic HSDs were histochemically demonstrated in mouse embryos during the early development using as many substrates as possible, in

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Table 1. HSDs investigated, and substrates, solvents and cofactors used for their histochemical analyses

HSDs	Substrates	Solvents	Cofactors
Δ^5-3β-HSD	DHA	Acetone	NAD
	Pregnenolone	Acetone	NAD
	17α-Hydroxypregnenolone	Dimethylformamide	NAD
20α-HSD	20α-Hydroxyprogesterone	Acetone	NADP
20β-HSD	17α-Hydroxyprogesterone	Acetone	NAD
	20β-Hydroxyprogesterone	Acetone	NAD

NAD: Nicotinamide adenine dinucleotide, NADP: nicotinamide adenine dinucleotide phosphate.

order to investigate the ability of progestagen production in mouse embryos.

MATERIALS AND METHODS

Animals

Ninety female mature mice of ICR strain were used in the present study. They were housed in autoclaved metal cages and were given a standard chow (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24℃), under controlled-lighting conditions (14L/10D). These mice were superovulated with 5 i.u. PMSG (Serotropin®, Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan), and with 5 i.u. hCG (Gonatropin®, Teikoku Hormone Manufacturing Co. Ltd.) injected 48 hrs apart.

Collection of eggs and embryos

Unfertilized eggs were obtained from oviducts of superovulated females 14 hrs after the hCG injection, and were immersed in phosphate buffered saline (PBS, pH 7.4)[14] containing 0.1% hyaluronidase (Sigma-Aldrich, NJ, USA) to disperse their cumulus cells. For the recovery of embryos, superovulated females were mated with mature males of the same strain immediately after the hCG injection, and slaughtered 20 hrs after the hCG injection for pronuclear embryos, after 48 hrs for 2-cell embryos, 60 hrs for 4-cell embryos, 70 hrs for 8-cell embryos, 77 hrs for morulae and 96 hrs for blastocysts. Pronuclear to 8-cell embryos were recovered by tearing the excised oviducts immersed in PBS in a watch glass, under a stereomicroscope. Morulae and blastocysts were recovered by flushing the uterine horns with PBS.

Observation of HSD activities

In order to detect the activities of HSDs shown in **Table 1**, the method used by NIIMURA and ISHIDA[10] was employed: eggs and embryos were placed at 37℃ in a solution containing 1.8 mg substrate (Sigma-Aldrich) which had been dissolved in 0.5 ml acetone or dimethylformamide, 4.0 mg cofactor (Sigma-Aldrich), 2.0 mg nitroblue tetrazolium salt (Sigma-Aldrich), and 10.0 ml 0.1 M phosphate buffer solution (pH 7.5). Eggs and embryos incubated in a solution containing the substrate solvent, acetone or dimethylformamide, but devoid of the substrates were observed as negative controls. The incubation time was 60 to 120 min, because this was the period within which unspecified or endogenous dehydrogenase reactions never appeared in the control eggs and embryos incubated in a substrate-free solution. The same procedures for the demonstration of HSDs were applied 3 times at every developmental stage. Atretic eggs and embryos were eliminated from the observation.

As the histochemical reaction of HSDs depends upon the reaction of $NADH_2$ dehydrogenase ($NADH_2$-DH) or $NADPH_2$ dehydrogenase ($NADPH_2$-DH)[15], the demonstration of these enzymes was carried out

according to the BARKA and ANDERSON method[16]. As negative controls, some eggs and embryos were incubated in a substrate-free solution for 60 min at 37℃.

After incubation in the substrate solution, eggs and embryos were transferred into PBS and then a drop of the solution including the eggs and embryos was placed in the center of four Vaseline spots on a slide. A coverslip was then carefully placed on the Vaseline spots and gently depressed, and the eggs and embryos were photographed under a light microscope (OPTIPHOT-2, Nikon, Tokyo, Japan).

RESULTS

Activities of HSDs

When mouse eggs and embryos were immersed in a substrate solution, diformazan granules were found to be deposited in the cytoplasm (**Figs. 1-5**). Since such granules were not observed in the eggs and embryos immersed in a solution containing no substrate (negative control; **Fig. 6**), the granules were considered to represent the activity of HSDs.

With all six substrates, unfertilized eggs and pronuclear embryos showed evenly distributed diformazan granules in the cytoplasm. Some diformazan granules were present in the polar bodies. After cleavage, diformazan granules were found in the cytoplasm of the blastomeres, and were especially abundant at the perinuclear region of the cytoplasm. There was no difference in the amount of granules in the two cell types, inner cell mass and trophoblast, of the blastocyst. Granules were never observed in the zona pellucida. The distribution pattern of diformazan granules as described above did not differ for the three enzymes. The activities of HSDs in mouse eggs and embryos are shown in **Table 2**.

Activities of $NADH_2$-DH and $NADPH_2$-DH

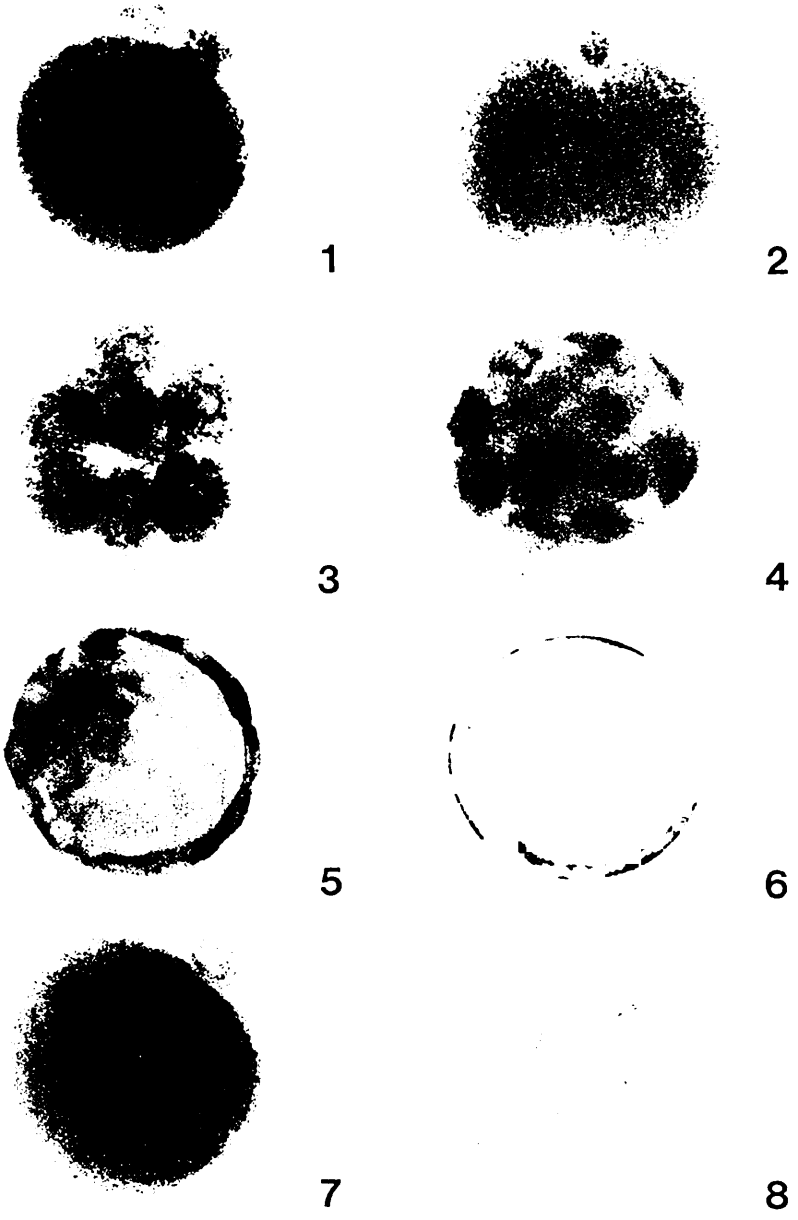
Using the method of BARKA and ANDERSON[16] for the demonstration of $NADH_2$-DH and $NADPH_2$-DH, deposited diformazan granules were found in the cytoplasm of every egg and embryo from each developmental stage (**Fig. 7**). These granules did not appear in the negative control eggs and embryos (**Fig. 8**).

DISCUSSION

Δ^5-3β-HSD, which catalyzes DHA, plays an important role in a steroid metabolic pathway, and the presence of this enzyme activity indicates the presence of the steroid biosynthesis. In the present study, we observed the activity of this enzyme in mouse eggs and embryos in all developmental stages examined, suggesting that steroid biosynthesis in those occurs throughout the early development. The presence of the activity of $NADH_2$-DH also supported the presence of the activity of Δ^5-3β-HSD in mouse eggs and embryos, because $NADH_2$-DH is closely related to the appearance of Δ^5-3β-HSD reaction.

In order to investigate the possibility of progestagen production in mouse eggs and embryos, we then examined activities of various progestagen-synthetic HSDs. In the present study, since the activities of Δ^5-3β-HSD (pregnenolone and 17α-hydroxypregnenolone as the substrates), 20α-HSD (20α-hydroxyprogesterone as the substrate) and 20β-HSD (17α-hydroxyprogesterone and 20β-hydroxyprogesterone as the substrates) were always observed in mouse eggs and embryos from the 1-cell to blastocyst stages. From these results, it is thought that mouse eggs and embryos during the early development have the ability to biosynthesize 17α-hydroxyprogesterone, 17α,20β-dihydroxyprogesterone, 20α-hydroxyprogesterone and 20β-hydroxyprogesterone, in addition to progesterone. Since the production of such progestagens has been considered in hamster, rabbit and porcine embryos[11-13], it is thought that the ability of progestagen production is common to mammalian eggs and embryos.

Although the physiological significance of steroids biosynthesized in eggs and embryos is unclear, progesterone and estrogen are thought to play some role in embryonic development, uterine metabolism or maternal recognition of pregnancy in some mammals[9,17-20]. It is known that the major steroid synthes-



Explanation of Figures

Histochemical evidence of hydroxysteroid dehydrogenases and $NADH_2$-dehydrogenase in mouse egg and embryos (×350). The developmental stages and substrates were as follows.

Fig. 1. A pronuclear embryo, Δ^5-3β-HSD reaction, pregnenolone.
Fig. 2. A 2-cell embryo, 20α-HSD reaction, 20α-hydroxyprogesterone.
Fig. 3. An 8-cell embryo, Δ^5-3β-HSD reaction, 17α-hydroxypregnenolone.
Fig. 4. A morula, 20β-HSD reaction, 17α-hydroxyprogesterone.
Fig. 5. A blastocyst, 20β-HSD reaction, 20β-hydroxyprogesterone.
Fig. 6. A blastocyst, substrate-free medium.
Fig. 7. An unfertilized egg, $NADH_2$-DH reaction, $NADH_2$.
Fig. 8. An 8-cell embryo, substrate-free medium.

Table 2. Activities of HSDs in mouse eggs and embryos during the early development

HSDs	Substrates		Developmental stages					
			1-Cell		2-Cell	4-Cell	8-Cell	Morula	Blastocyst
			Unfertilized	Fertilized					
Δ^5-3β-HSD	DHA*	+	62(98)**	30(100)	25(86)	40(100)	29(100)	27(100)	25(93)
		−	1(2)	0(0)	4(14)	0(0)	0(0)	0(0)	2(7)
	Pregnenolone*	+	61(97)	34(97)	25(83)	30(83)	29(100)	25(96)	21(81)
		−	2(3)	1(3)	5(17)	6(17)	0(0)	1(4)	5(19)
	17α-Hydroxypregnenolone*	+	53(92)	22(100)	31(100)	26(100)	27(93)	33(89)	31(86)
		−	5(9)	0(0)	0(0)	0(0)	2(7)	4(11)	5(14)
20α-HSD	20α-Hydroxyprogesterone*	+	96(92)	30(100)	29(97)	30(97)	25(89)	29(100)	25(86)
		−	8(8)	0(0)	1(3)	1(3)	3(11)	0(0)	4(14)
20β-HSD	17α-Hydroxyprogesterone*	+	75(95)	30(91)	27(93)	34(92)	53(90)	33(92)	32(91)
		−	4(5)	3(9)	2(7)	3(8)	6(10)	3(8)	3(9)
	20β-Hydroxyprogesterone*	+	58(88)	30(100)	29(100)	24(100)	43(100)	25(100)	25(93)
		−	8(12)	0(0)	0(0)	0(0)	0(0)	0(0)	2(7)

*Substrates for enzyme-histochemistry.
+Positive, −negative.
**The number of eggs or embryos with percentages in parentheses.

ized in equine blastocysts is 17α-hydroxyprogesterone, one of progestagens, and this steroid is thought to play a role in embryonic development or maternal recognition of pregnancy[21]. Although further studies are necessary to clarify the significance of the progestagen in mouse embryos, progestagens were thought to play the same physiological role as that in equine embryos.

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発生初期のマウスの卵子と初期胚における プロジェスタジェン合成に関する組織化学的研究

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摘　要

マウスの未受精卵子および前核期から胚盤胞期までの初期胚について、Δ^5-3β-HSD(基質として pregnenolone および 17α-hydroxypregnenolone を使用)、20α-HSD（20α-hydroxyprogesterone）および20β-HSD（17α-hydroxyprogesterone、20β-hydroxyprogesterone）の活性を組織化学的に検出し、プロジェスタジェン合成能を検討した。

未受精期の卵子において、これらHSDの活性は88ないし98%のものに認められた。また、前核期から胚盤胞期までの初期胚においても、83%以上でこれらのHSD活性が検出された。一方、ステロイド生合成の指標となるΔ^5-3β-HSD（DHA）の活性は、86%以上の卵子と初期胚に常に観察された。

これらのことから、発生初期のマウスの卵子と胚は、常にprogesterone、17α-hydroxyprogesterone、17α,20β-dihydroxyprogesterone、20α-hydroxyprogesteroneおよび20β-hydroxyprogesteroneの合成を行っており、これらプロジェスタジェンの合成能は、胚の初期発生に伴って変化しないことが考えられた。

キーワード：マウス胚、初期発生、ヒドロキシステロイド脱水素酵素、プロジェスタジェン合成、組織化学

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