DEVELOPMENTAL HISTOCHEMISTRY OF THE EGG
(1) EPMA SULFUR ANALYSIS OF A RABBIT EGG MEMBRANE

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Received for publication June 17, 1978 and in revised form August 31, 1978

My previous microscopic and autoradiographic observations revealed that the covering membrane of a rabbit's blastocyst was not simply the zona pellucida, and that the zona pellucida was soluble in mercaptoethanol, but the blastocyst membrane was insoluble in mercaptoethanol. In this study, the sulfur content of the zona pellucida and the blastocyst membrane was measured with an EPMA to determine if sulfur changes quantitatively in the egg at various stages of development. Experiments were performed with oviducal and uterine eggs from Japanese white rabbits, 15 hr to 6 days after ovulation, and follicular eggs. The eggs were fixed in glutaraldehyde and OsO₄, dehydrated with alcohol, and dried on carbon or embedded in Epon. The sulfur analysis was performed with an EPMA (JEOL LTD, JMA-50A); the accelerating voltage was 15 kV and the specimen current was 0.03 μ A. A PET crystal was used for diffractive analysis. The sulfur value in the zona pellucida of the follicular eggs and in the uterine eggs were 25 and 75 counts/sec respectively. The amount of sulfur, which is considered a component of the mucoprotein in the blastocyst membrane, was significantly higher than in the zona pellucida.

It is well known that the eggs of mice (3), rats (18), chinchillas (14), guinea pigs (13) and sheep (1) are surrounded, even in the blastocyst phase, by the zona pellucida, which is formed in the ovarian follicle. Some investigators have reported that the rabbit blastocyst membrane consists of only the zona pellucida (4, 10, 17). However, it has also been reported (2, 9) that it consists not only of the zona pellucida but also a mucinous layer containing uterine and oviducal secretions. In previous papers, the authors reported morphological evidence (7, 12) that the main constituents in the membrane of a blastocyst nearing implantation are uterine secretions.

This report describes the results of EPMA analyses conducted to detect the biochemical differences between the blastocyst membrane and the zona pellucida. Since the mucinous layer and the uterine secretions contain sulfonyl, this study primarily concerns the sulfur (S) content in the egg membrane.

MATERIALS AND METHODS

1) Laboratory animals

Eighteen mature female and 5 mature male Japanese white rabbits, weighing

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2.5–4 kg, were placed in aluminum mesh cages (1 rabbit per cage) and permitted free intake of Oriental-Yeast solid feed (ORC4) and water.

The illumination time was from 08:00 to 17:00 and the room temperature was $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with humidity approximately 60%.

II) Egg collection

1) Unfertilized eggs

a) Unfertilized follicular eggs

Five female rabbits were used and eggs were collected by ovariocentesis. Some eggs were surrounded by granulosa cells and others were naturally denuded. The granulosa cells were removed with petri forceps.

b) Unfertilized oviducal eggs.

Two female rabbits were intravenously injected with 100 IU of HCG in one dose (Teikoku Hormone Mfg. Co., Ltd.).

Eggs were obtained by flushing approximately 3 ml of Hank's solution from the oviducts on the 3rd day following injection.

2) Fertilized eggs

a) Fertilized oviducal eggs

Five female rabbits were injected with 100 IU of HCG in one dose immediately after natural mating. Eggs were collected by flushing from the oviducts 18 hr to 3 days after mating.

b) Fertilized uterine eggs

Six females were injected with 100 IU of HCG in one dose immediately after mating. Eggs were collected by flushing approximately 6 ml of Hank's solution from the uterus 4 to 6 days after mating.

III) Preparation of EPMA specimens

Some of the follicular eggs and 6 day-old blastocysts were separated from the egg cytoplasm, and the zona pellucida or blastocyst membrane removed carefully with petri forceps.

Eggs and membrane were rinsed in Hank's solution, fixed for 30 min to two hr with 2.5% glutaraldehyde, and washed for two hr with phosphate buffer solution (0.1 M, pH 7.4). Some of the eggs and egg membrane were dehydrated for 10 to 30 min with 70% ethanol and dried at room temperature on a carbon disk (drying method). The specimens were then coated with carbon by evaporator as many times as necessary for a reference paper to turn from white to greyish-brown, usually 3 or 4 times.

The remaining eggs were postfixed for two hr with OsO_4 , dehydrated with alcohol, and embedded in Epon 812. After resin hardening, the eggs were cut approximately in half by the plane down of a glass knife and the resulting cross section was coated with carbon.

IV) Analyses

Composition, surface, line, and point analyses were performed on a given egg by wave length dispersed EPMA (Model JXA-50A, JEOL Ltd.). The accelerating voltage was 15 kV, the specimen current was $0.03 \mu\text{A}$, beam diameter 10–20 μm , chart speed 20 mm/min, spectrometer speed 4 mm/min, and a PET (pentaerythritol) crystal was used for diffractive analysis.

RESULTS

Compositional analyses revealed that the Epon embedded materials, but not the dried intact eggs, clearly showed an egg membrane and cytoplasm, (Figs. 1, 2). Surface and compositional analyses revealed that the sulfur localization in the egg membrane was consistent in all specimens. The interior of the Epon embedded cross-section in Fig. 3 shows a low S value, but its periphery, which corresponds to the membrane in Fig. 1, has a high S value. Fig. 2 also shows a high S value for the surrounding covered membrane of the dried intact egg. The zona pellucida and 6 day-old blastocyst membrane were approximately 10–20 μm ; point analyses were possible for Epon embedded materials void of egg cytoplasm. It was found that Epon embedded materials had smaller S contents than did dried materials, but both materials had a similar pattern of S content variation according to developmental stage.

I) Analytic results of unfertilized eggs.

1) Unfertilized follicular eggs (Fig. 4-A).

For the follicular eggs, the S value of the naturally denuded eggs was about 7–10 C.P.S. Line analyses on the Epon embedded cross-section showed a high S peak in the periphery, which corresponds to the zona pellucida, and a low peak in the central cytoplasmic part (Fig. 5-A). Furthermore point analyses showed the

FIG. 1. Cross-section of an Epon embedded egg 3 days after mating. The periphery appears like an egg membrane; the interior is cytoplasm.

FIG. 2. Line profile of X-ray S Ka on an egg 3 days after mating. The white straight line on the egg shows the analytic location. The interior of the egg shows a higher sulfur value than in the Epon embedded cross-sections because actually the surrounding membrane is being measured.

counts of artificially denuded dried eggs were about 25 C.P.S. $\pm$ S.D. 8.5 (Fig. 6).

2) Unfertilized oviductal eggs

It is generally known that the rabbit embryo 3 days after mating has a thick mucin layer. A thick oviductal secretion, the mucinous layer was exteriorly attached to the zona pellucida of the 3 day-old eggs obtained by HCG induced ovulation.

The S value in the mucinous layer of the eggs in this stage was almost 150 C.P.S. $\pm$ S.D. 15.8, showing 6 times higher than that in the zona pellucida of the follicular eggs. Increase in egg diameter seemed to result in increase in the S peak width.

II) Fertilized eggs

1) Fertilized oviductal eggs

a) 18 hr after mating (pronuclei fusional stage)

A thin mucinous layer was observed in the periphery of the zona pellucida (Figs. 7-A, 8). Its S value was considerably greater than that in the zona pellucida of the follicular egg (Fig. 5-B).

b) 3 days after mating (morula stage)

A thick mucinous layer was observed (Figs. 7-B, 9). Its S value was about 150 C.P.S. $\pm$ S.D. 15.0, which was about 6 times higher than that in the zona pellucida

FIG. 3. Surface analysis of X-ray S $K\alpha$ over an egg 3 days after mating. The interior of the Epon embedded cross-section specimen shows a low sulfur value, but its periphery, which corresponds to the membrane in Fig. 1, has a high sulfur value.

A. FOLLICLE EGG.

B. OVIDUCAL EGG. (3 DAYS).

FIG. 4. Unfertilized egg.

FIG. 5. S $K\alpha$. Line profiles of follicular egg (A) and an egg 18 hr after mating (B). (A) The interior of the Epon embedded naturally denuded egg shows a low sulfur value, but its periphery which corresponds to the zona pellucida (ZP) shows a high sulfur value. (B) The S value of the thin mucinous layer attached to an ovulated egg is greater than that in the zona pellucida of follicular eggs.

FIG. 6. S $K\alpha$. A point analysis over the zona pellucida of follicular egg (dried material). The artificially denuded follicular egg shows a greater S value than that in the Fig. 5 A naturally denuded egg.

FIG. 7. Fertilized egg. Inner diameter of egg: A = $150\ \mu\text{m}$, B = $150\ \mu\text{m}$, C = $250\ \mu\text{m}$, D = 4 mm.

FIG. 8. 18 hr after mating (pronuclei fusional stage). A thin mucinous layer can be observed in the periphery of the zona pellucida. $\times 400$

FIG. 9. Three days after mating (morula stage). A thick mucinous layer can be observed. $\times 200$

of the follicular eggs. Result seems to be almost the same for unfertilized eggs under identical conditions after ovulation (Fig. 10). The S content of the membranes in each of the above stages is highest in the mucinous layer at 3 days and lowest in the zona pellucida of follicular eggs.

2) Fertilized uterine eggs

a) 4 days after mating (early expanding blastocyst stage) (Figs. 7-C, 11, 12). The S value obtained in this specimen was about 65 C.P.S. $\pm$ S.D. 15.8. Although it seems to be lower than in the 3 day-old eggs, this value indicates that it reaches about 2.5 times the level of the zona pellucida of follicular eggs.

Increase in S peak width seemed to correlate with an increase in egg growth. The interior has a low S value, but its periphery which corresponds to the membrane has a high S value (Fig. 11).

b) 6 days after mating

In the primitive stage a streak usually formed on the embryonic disk. The analytic measurement of the S content of the membrane in this stage, gave a value of 75 C.P.S. $\pm$ S.D. 4.4 (Fig. 13) showing about 3 times higher than that in the zona pellucida of the artificially denuded follicular eggs.

DISCUSSION

There are documents suggesting that the membrane in a rabbit blastocyst is identical to the zona pellucida which is formed in the follicle (10, 17). The author concludes from the following observations that the blastocyst membrane and the

FIG. 10. S K α . Line profile of an egg 3 days after mating (dried materials). The interior of the egg shows a higher sulfur value because actually the surrounding membrane is being measured.

zona pellucide are not identical.

1) Hatching occurs on a rabbit blastocyst cultured *in vitro* but is suppressed *in vivo* by the mucinous layer (8, 12).

2) Histochemical staining revealed (12) that the blastocyst membrane has

FIG. 11. Four days after mating (early expanding blastocyst stage). $\times 200$

multiple layers. Metachromasia was obscure in the zona pellucida and distinct in the mucinous layer.

3) Electron microscopic examination indicated that the zona pellucida of an egg immediately after uterine entry was thin and discontinuous along the inner surface of the mucinous layer (12).

4) When the mucinous layer in the morula was labeled with ^{35}S after transplanting to the uterus, ^{35}S was not localized in the 6 day-old blastocyst membrane (7).

5) Mercaptoethanol solubility tests showed that the zona pellucida was soluble but the blastocyst membrane was insoluble (12).

Present results indicated that Epon embedded materials had smaller S contents than did dried materials, but they showed egg structure (cytoplasm and egg membrane) more clearly than dried material.

Saetersdal *et al.* (15) reported that Ca was lost during the fixing and embedding processes and that it could be detected only in the nonfixed frozen materials.

Flood (5) used chemical methods to prepare muscle containing Ca. He added 0.12 M sodium oxalate to glutaraldehyde and formaldehyde. Epon embedded materials (11) may be contaminated by the diffusion of elements in the resin.

Thus, material preparation remains a problem insofar as accurate quantitative analysis of S concerned. However, the dried and Epon embedded materials had similar S content patterns. The results strongly indicate S content variation by developmental egg stage. The increase in the S content of the unfertilized oviducal eggs corresponds to those of the fertilized eggs.

FIG. 12. S $K\alpha$. Line profile of an egg 4 days after mating. The interior of the Epon embedded cross-section egg shows a low sulfur value, but its periphery which corresponds to the early blastocyst's membrane shows a high sulfur value. The sulfur value is lower than in the 3 day-old egg (Fig. 10).

This supports the hypothesis that the mucinous layer of the egg membrane is due to oviducal secretions rather than fertilization.

Shapiro (16) obtained the same results with electrophoretic testing since the rabbit oviducal secretions are rich in sulfonyl.

FIG. 13. S Ka. A point analysis over a blastocyst membrane 6 days after mating (dried materials) showing about 3 times higher S Ka than in the zona pellucida of the artificially denuded follicular egg (Fig. 6).

The present results indicate that the S content reaches its maximum value in the 3 day-old eggs after ovulation, then decreases in the 4 day-old blastocyst. This also agrees with the results of Shapiro's experiment (16) using antibodies active against oviducal secretion. Shapiro demonstrated that the mucinous layer disap-

peared 3 to 4 days after mating.

When the mucinous layer disappears, the EPMA S analyses indicate that the S content in the blastocyst membrane is about 3 times that in the zona pellucida. The mean mercaptoethanol scission (12) of the membrane's S-S bonds confirmed that the S content not only differed by developmental egg stage but also increased. Therefore, it can be inferred that the blastocyst membrane consists not only of the zona pellucida but also of uterine secretions.

ACKNOWLEDGMENTS

I wish to thank Professor Morio Kato and Dr. Kazuto Nokubi for their kind advice and constant encouragement.

I would also like to thank Mr. Yoshiharu Mukai and Mr. Hiroshi Fujita for their assistance with the EPMA analysis.

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