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Morphology and Behavior of the Sperm Pronucleus in the Frog Egg I. The Fates of Sperm Pronuclei Inactivated by UV-rays and Toluidine Blue

HAJIME SAMBUICHI

Biological Institute, Faculty of Science Yamaguchi University, Yamaguchi 753, Japan Received April 24, 1980

ABSTRACT Through the fertilization course, no difference was found among the behaviors of three kinds of sperm : control, UV-irradiated and toluidine blue-treated sperm. At 60 min after insemination, the sperm pronucleus had already fused with the egg pronucleus in all cases. But, two kinds of inactivated sperm pronuclei could not form a syngamy with the egg pronucleus. Concerning morphological characters, there was a difference between the control sperm and the UV-irradiated sperm. The sectional phase of the latter had a ragged network of chromatin compared with the coarse network of the former. A remarkable difference was also found between the toluidine blue-treated sperm and the control sperm : the toluidine blue treated sperm could not change into the pronucleus and kept an intact form of spermatozoon from its invasion into the egg to the metaphase of the first cleavage. It was concluded that, although the migratory capacity of the sperm was not affected, the morphological characters were influenced by inactivation. (Zool. Mag. 89: 302-307, 1980)

In amphibians, gynogenetic haploid embryos are obtained readily from eggs which were inseminated with genetically inactivated sperm. For the inactivation of sperm, several kinds of radiation and chemicals are used. Rugh (1939) used irradiation of sperm by X-rays to obtain haploid embryos in the frog, Rana pipiens. Pogany (1971) studied in detail, the development of eggs inseminated with UV-irradiated sperm with particular attention to the role of irradiated sperm in the development of eggs. On the other hand, Briggs (1952) obtained gynogenetic haploid embryos from the eggs of Rana pipiens by the insemination of sperm treated with toluidine blue, and clarified the mechanism by which the DNA of sperm nucleus was denatured by this chemical. Nishioka and Kondo (1978) reported a method for the production of gynogenetic haploids by making use of toluidine blue in Rana japonica eggs. Pamera

et al. (1975) examined the functional sterility of sperm of *Xenopus laevis* by treatment with ethylenurea.

Although it is well known that an inactivated sperm can activate the egg but does not participate in the formation of syngamy, the fate of the inactivated sperm in the egg remains equivocal, as pointed out by Pogany (1971) and Pamera *et al.* (1975). The purpose of the present experiment is to clarify the morphology and behavior of the inactivated sperm through the course of fertilization.

Materials and Methods

The eggs and sperm of the frog, *Rana* nigromaculata Hallowell, were used in the present experiment. Mature frogs were collected during the breeding season and reared at 10°C until use. The eggs were obtained after inducing artificial ovulation by the hypodermic implantation of frog pituitaries. The inseminated eggs in the control and experimental groups were kept in temperature-regulated water $(20\pm0.5^{\circ}C)$.

To inactivate the sperm, UV-irradiation and toluidine blue treatment were used. In this experiment, the intensity of treatment was chosen in expectation of complete and incomplete inactivation of sperm in each case. Therefore, the dose applied had an intensity whereby about half of the eggs developed into haploid embryos. A sperm suspension was made by macerating a piece of testis in 2 mm³ of tap water. The testicular debris was removed. The depth of the sperm suspension was kept within 1 mm to insure irradiation of the sperm. The suspension was exposed to UV-rays from a high pressure mercury lamp (HU-2 type, Toshiba Electric Co.) for 60 sec at a distance of 17 cm from the lamp. The energy level was 33.9 erg/mm²/sec at the surface of the suspension. For treatment with toluidine blue, a piece of testis was macerated in 2 mm³ of a 0.0001% solution of the chemical, and the suspension was left for 15 min at room temperature. Immediately after treatment, these suspensions were poured over each batch of eggs.

For cytological examination, eggs were fixed at 20, 40, 60, and 80 min after insemination for 24 hr in Smith's fixative. Following dehydration, eggs mounted in Tissue-mat were sectioned at 12 μ m and stained by the Feulgen reaction and light green. As morphological discrimination between diploid and haploid embryos is reliable, the ploidy of 3-day embryos was determined from their external appearances (cf. Sambuichi, 1964).

Results

Development

To verify genetic inactivation of the sperm, the rates of normal cleft eggs and haploid embryos at 3 days after insemination (stage 18, Shumway, 1940) were examined in the control eggs and two sets of experimentals. As shown in Table 1, most of the eggs inseminated with the normal sperm cleft normally and developed into normal diploid embryos with a few abnormal diploid and haploid embryos. Although the cause of these abnormal embryos is unknown, this occurrence is usually found in the development of artificially inseminated eggs. In the experimental groups, a decrease in the rate of normal cleft eggs was found, and the decrease was greater in the toluidine blue treatment than in UV-irradiation. Several morphological abnormalities appeared in the development of the haploid eggs, and their appearance seems to be due to the haploid number of chromosomes and to the causes described at the haploid per se. From the comparison of the rates of normally cleft eggs and of haploid embryos,

Kinds of sperm	Dosage	No. of	Normally	3 day-embryos*					
	Intensity	Time	eggs	cleft eggs	Diploid	Ab. diploid	Haploid	Ab. haploid	Unknown
Normal sperm			918	875 (95.3%)	845	15	7	3	
					(98.2%)		(1.1%)		- 1
UV-irradiated 3	33.9erg/mm/sec	60 sec	106	95(88.8%)	16	8	42	14	12
					(25.2%)		(58.9%)		14
Toluidine blue-treated	0.0001%	15 min	122	95(77.9%)	8	10	38	21	- 10
					(18.	3%)	(62.	1%)	

Table 1. Development of eggs inseminated with three kinds of sperm.

* The ploidy of 3 day-embryos was discriminated by its external appearance.



Fig. 1. Three kinds of the sperm nuclei at 40 min after insemination. A1, B1, C1: ×360.
A2, B2, C2: ×2100. A1, A2: Normal sperm pronucleus. B1, B2: UV-irradiated sperm pronucleus. C1, C2: Toluidine-blue treated sperm pronucleus.

the chemical treatment affected the sperm more severely than UV-irradiation at the doses used in this experiment.

Behavior of the sperm in the egg

a) Normal sperm

The behavior of the sperm in the egg after its invasion of the cortex was observed. At 20 min after insemination, the sperm was located in the small pigmentless area beneath the pigmented cortex. As shown in Fig. 1 (A1 and A2), at 40 min after insemination, the sperm had already transformed into the pronucleus and moved to the early part of the copulation path. While the sperm pronucleus was moving along this course, the egg pronucleus was also going down from the egg cortex to the fusion site of both pronuclei. At 60 min after insemination, the pronuclei of both the sperm and the egg had already been fused in many cases and 20 min thereafter, a metaphase figure of the first cleavage was observed (Fig. 3, A).

b) Irradiated sperm

The sperm irradiated with UV-rays showed about the same behavior in the fertilization course as the normal sperm. At 40 min after insemination, they also moved to the early part of the copulation path (Fig. 1, B1 and B2). As seen in the control eggs, the nuclei of both the egg and the sperm were in close contact at 60 min after insemination as shown in Fig. 2 (B1 and B2). When the egg reached the metaphase stage of the first cleavage at about 80 min after insemination, the pronucleus of the irradiated sperm was left as a chromatin mass apart from the metaphase plate (Fig. 3, B2). c) Sperm treated with toluidine blue

There was no difference found in the behav-

Morphology and Behavior of the Sperm Pronucleus



Fig. 2. The pronuclei of egg and sperm at 60 min after insemination. A1, B1, C1: ×450. A2, B2, C2: ×2400. A1, A2: Normal egg pronucleus and normal sperm pronucleus. B1, B2: Normal egg pronucleus and UV-irradiated sperm pronucleus. C1, C2: Normal egg pronucleus and toluidine-blue treated sperm pronucleus.



Fig. 3. The metaphases of the first cleavage at 80 min after insemination. A, B, C: ×2400. A: The metaphase of the control egg. B: Female chromosomes at the metaphase (arrow 1) and UV-irradiated male pronucleus as an outsider (arrow 2). C: Female chromosomes at the metaphase (arrow 1) and sperm as swollen head (arrow 2).

ior of pronuclei among the normal, UV-irradiated and the chemically treated spermatozoa. In Figs. 1 and 2, C1 and C2 show the toluidine blue-treated sperm at 40 and 60 min after insemination. Though the sperm moved normally along the copulation path, it could not transform into the pronucleus during these stages. At a subsequent stage, the sperm came into close contact with the egg pronucleus but kept its intact sperm form and could not participate

305

H. SAMBUICHI

in the formation of a syngamy. Eventually it was left out of the cleavage process in the vicinity of the metaphase plate as shown in Fig. 3, C (arrow 2). These observations show there was no difference in the behavior of these three kinds of sperm along the fertilization course.

Morphological characters of the sperm pronucleus

a) Normal sperm

The morphological change from the sperm to the sperm pronucleus usually took place in the early part of the entrance path about 30-40 min after insemination (Fig. 1, A2). The sperm pronucleus was seen as a globular form and its inner structure appeared as a coarse network of chromatin threads in a section. When the sperm pronucleus came into close contact with the egg pronucleus, such character of each pronucleus were seen more clearly (Fig. 2, A2). Of the two pronuclei, the sperm pronucleus might be ascertained by its moving trace from the cortex.

b) Irradiated sperm

In the case of UV-irradiated sperm, the sperm changed into a pronucleus at the early part of the entrance path as the normal sperm did (Fig. 1, B1 and B2). When both the pronuclei were brought into close contact with each other at 60 min after insemination, the morphological difference between the normal and UV-irradiated sperm pronuclei became clearer as shown in Fig. 2 (cf. A2 and B2). While a coarse network of chromatin was seen in the normal sperm pronucleus (A2), the pronucleus of the irradiated sperm showed a disorderly chromatin network (B2). Further in the process of fertilization, the sperm pronucleus was left alone as an irregular mass of chromatins near the metaphase plate (Fig. 3, B, arrow 2). Sperm treated with toluidine blue c)

While the morphological characters of the pronuclei of the normal and the UV-irradiated sperm were about the same, the morphology of the toluidine blue treated sperm was quite different from either of them through all proces-

ses of the fertilization course. The sperm was also found beneath the pigmented cortex of the egg and its shape was the same as that of the normal sperm at 20 min after insemination. At 40 min after insemination, the sperm moved normally along the copulation path, but it could not change into pronucleus as shown in Fig. 1 (C1 and C2), keeping its intact form throughout the subsequent stages. Although the sperm was in close contact with the egg pronucleus at 60 min after insemination, they could not form a syngamy. At this stage, the intact sperm was wrapped in a membrane-like structure in some cases (Fig. 2, C2). In all cases, the sperm finally was left apart from the metaphase plate of the first cleavage as an intact but slightly swollen form (Fig. 3, arrow 1). In this experiment, all cases showed either an intact sperm or a normal pronucleus. There was no intermediate form.

Discussion

Concerning the equivocal fate of the sperm in the gynogenetic eggs, the results obtained in the present experiment showed that the treated sperm activated the egg and moved normally along the fertilization course in spite of the morphological change induced by the treatment. From the fact that the behavior of the genetically inactivated sperm was normal in the cases of UV-irradiation and toluidine blue treatment, it may be said that the dosage applied in the present experiment damaged only the nuclear part of the sperm and that the cytological changes in the pronucleus may indicate the denaturation of its DNA. Pogany (1971) noted the role of protamines in sperm inactivated by UV-rays, and later (1976) he reported that a large dose of UV-rays resulted in a variety of alterations in the DNA of inactivated sperm, i.e. the dimerization of thymine bases and strand denaturation of DNA.

Recently Kawahara (1978) found in the eggs of *Xenopus* the coagulation of nucleoplasm that had occurred in the pronucleus of UV-irradiated sperm. The degree of morphological

change in the sperm pronucleus may depend on the dose applied, and moderate change, i.e. a disordered network of chromatin in the present experiment, seemed to result from the low dose. Although there is no data concerning the relation between the morphological change and the denaturation of DNA in the treated sperm, it may be said that the denaturation of DNA in the treated sperm resulted in morphological change of the pronucleus, and it was also a cause for its inability to form a syngamy with the egg pronucleus.

It is of interest that, while the moving capacity of the sperm treated with toluidine blue was normal as in the control and UVirradiated sperm, it could not change into a pronucleus. It is well known that mature cytoplasm is an essential factor for the transformation of sperm into pronucleus in the eggs of sea urchins, amphibians and mammals (Hiramoto, 1962; Moriya and Katagiri, 1976; Usui and Yanagimachi, 1976). In the present experiment, as the mature egg furnished the cytoplasmic factor for the transformation of the sperm inactivated by toluidine blue, it was clear that the inability of transformation might be due only to the damaged DNA of the sperm. Briggs (1952) studied the mechanism of inactivation of sperm and the results showed that the sperm DNA was bound to the dye, toluidine blue, and that there were very few binding sites among DNA molecules. On other chemical sterilants, such as acriflavine, it was suggested that the intercalation of pigment molecules induced DNA denaturation (Peacocke, 1975). However, what causes the disturbance of syngamy formation and what relation there is between morphological change and DNA denaturation remains to be studied.

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