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Cytostatic Effect of the Cytoplasm of Mature Oocytes in the Newt, *Cynops pyrrhogaster*

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ABSTRACT—In anura, it has been known that the cytoplasm of mature oocytes possesses a specific factor to arrest nuclear division at metaphase. In the present study, we examined a similar effect of the cytoplasm in oocytes of a urodele, *Cynops pyrrhogaster*.

Eggs during the first cleavage were injected various amounts of the oocyte cytoplasm (50–200 nl) into the animal region of one of the two prospective blastomeres. The cytoplasm injection had no effect on the process of the first cleavage, but gave great influence for the subsequent divisions. In many cases, the second division of the cytoplasm-injected blastomeres was considerably delayed or completely arrested. Cytological examination revealed that chromosomes, spindle fibers or astral rays were frequently present in the uncleaved blastomeres.

These results strongly suggested that the mature oocyte of the newt also contained a cytoplasmic factor which could arrest the nuclear division at metaphase.

INTRODUCTION

It is a well known fact that the meiosis of fullymature oocytes is arrested at methaphase II in amphibia, as in many other vertebrates. Several hypotheses have been proposed to account for this phenomenon [1]. For instance, using eggs of anura, *Rana pipiens* [2, 3] and *Xenopus laevis* [4], Masui and his colleagues have shown that the metaphase arrest was caused by some cytoplasmic factor of the mature oocyte [2–4]. Their conclusion was based on the evidence that the nuclear division in recipient zygotes was arrested at metaphase when they were injected with the cytoplasm of unfertilized eggs. The unknown cytoplasmic component responsible for this effect was termed "cytostatic factor " (CSF). Recently, Newport and Kirschner [5] confirmed the existence of CSF in *Xenopus* eggs, and further they showed that CSF inhibited not only cleavage, but also DNA synthesis as well as cyclic changes of cytoplasmic activities such as surface contraction waves.

However, contradicting to these observations, Chulitskaia and Feulgegauer [6] reported negative results of CSF in the eggs of *Rana temporaria* and *Acipenser stellatus* (sturgeon). In the present study, we report examination about the existence of the cytostatic factor in the cytoplasm of mature oocytes of a urodele, *Cynops pyrrhogaster*.

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MATERIALS AND METHODS

Eggs of the newt, *Cynops pyrrhogaster*, were used as the experimental material. Spawning of fertilized eggs was induced by injecting about 80 i.u. of chorionic gonadotropin (Gonatoropin, Teikoku-Zoki Co., Japan) into the abdomen of females every other day. Unfertilized eggs were obtained by squeezing them out of the oviduct of females stimulated by hormone. The jelly coat of the egg was removed by treatment with 1.5% sodium thioglycollate (Wako Pure Chemical Institutes Ltd., Japan) dissolved in Holtfreter's saline solution (pH 10). The eggs with the vitelline membrane but without the jelly coat were put on a small depression of an agar gel (about 3%). They were operated in Ca-free Holtdfreter's saline solution (pH 7.0) and then transferred to the standard Holtfreter's saline solution at room temperature (18–23°C).

Microinjection of the cytoplasm was carried out with a glass micropipette, the one end of which was drawn out into a capillary of about 50 μm inner diameter, and the other end of which was connected to a rubber tubing for applying a negative or positive presure by mouth [7]. The volume of the injected cytoplasm was estimated from the calibration with the predetermined markers on the pipette.

For cytological observation, eggs were fixed in modified Smith's fixative for about one day; solution A (potassium bichromate, 0.5 g; water 87.5 ml) and solution B (40% formalin, 2.5 ml; glacial acetic acid, 10 ml) were prepared, and freshly mixed in the ratio of A:B=9:1 immediately before the application. A modification was made by adding glutaraldehyde up to 3% to the mixed solution. The fixed eggs were washed with running water for one to two days. They were then dehydrated in alcohol series and embedded in paraffin according to the usual way. Serial sections were made about 10 μm thickness. The sections were double stained with 1% acid fuchsin solution and 0.1% amino black which was dissolved in 7% acetate solution.

RESULTS

Eggs undergoing the first cleavage were used as recipients. They were arbitrarily classified into four stages according to the advance of the cleavage furrow (Fig. 1A-D); just after the onset of the formation of cleavage furrow (A), when the cleavage furrow came about half the way (B), when it encompassed the whole circumference of the egg (C), and about 20 min before the onset of the second cleavage (D). At each stage, the cytoplasm from the unfertilized egg was injected into one side of the animal hemisphere separated by the cleavage furrow (one blastomere in the next two-cell stage). In control experiments, eggs were injected with the cytoplasm from fertilized eggs in the same way as described above. The volumes of the cytoplasm injected were approximately 50, 100, 150 or 200 nl. The cytoplasm injection had no effect on the progression of the first cleavage of the recipients, but greatly influenced the subsequent cleavage, especially in the experimental group.

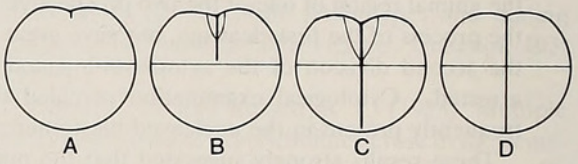

FIG. 1. Stages of recipient eggs classified by the extent of cleavage. (A) just, (B) about 45 min, (C) 90 min and (D) 110 min after the onset of the first cleavage. The stage D is about 20 min before the second cleavage.

In many cases of the group, the second or the third cleavage of the blastomere of the operated side was delayed in various extents compared with that of the unoperated side in which cleavage occurred at the normal time (Fig. 2A, B). Also, in many cases cleavage of the operated side was arrested (Fig. 2C). Disturbances of cleavage were ranked into four categories. To the first category belonged the cases in which the cleavage delay occurred within one cleavage cycle (delay within about 2 hr). These cases were included in the

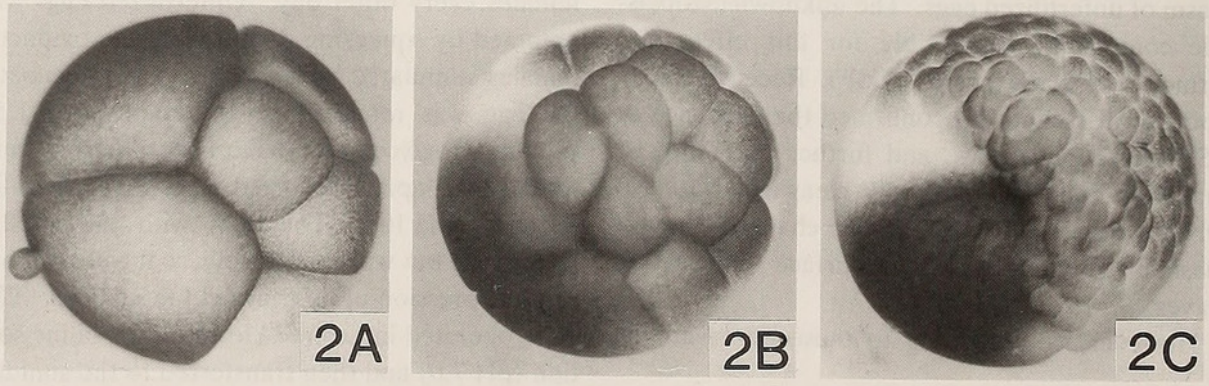

FIG. 2. Delay and arrest of division in the blastomere injected oocyte cytoplasm. (A) Delay of one cleavage cycle (operated blastomere; left 2 cells). (B) Delay of three cycle (left 2 cells). (C) Cleavage arrest. ×23.

TABLE 1. Division of the blastomeres injected with oocyte cytoplasm during the first cleavage cycle

Volume injected (nl)	Stage of recipient	Results: Experiment: Total no.	Normal	Delay	Arrest	% of arrest	Control: Total no.	Normal	Delay	Arrest	% of arrest
50	A	48	25	4	19	40	10	10	0	0	0
	B	26	20	1	5	19	15	13	1	1	7
	C	31	22	2	7	23	18	15	1	2	11
	D	32	23	3	6	19	17	17	0	0	0
100	A	90	39	15	36	40	44	33	5	6	14
	B	116	38	21	57	49	47	31	8	8	17
	C	50	24	8	18	36	18	13	2	3	17
	D	47	24	8	15	31	26	21	2	3	12
150	A	50	16	4	30	60	49	41	2	6	12
	B	67	10	6	51	76	46	31	3	12	26
	C	42	6	5	31	74	34	24	4	6	18
	D	47	18	3	26	55	32	25	2	5	16
200	A	65	14	8	43	66	54	37	9	8	15
	B	85	11	8	66	78	58	47	4	7	12
	C	56	8	6	42	75	38	32	2	4	11
	D	39	12	4	23	59	21	16	1	4	19

See text further explanation.

TABLE 2. The relationship between the stage of recipient zygotes and the time of their cleavage-arrest

Stage of recipents	total no.*	Time of arrest: 2nd cleavage	3rd cleavage	4th cleavage
A	128	119 (93%)	8 (6.3%)	1 (0.8%)
B	179	163 (91.1%)	13 (7.3%)	3 (1.7%)
C	100	77 (77%)	23 (23%)	0
D	70	33 (47%)	37 (53%)	0

*Sum of arrested cases in each stage in the Table 1.

normal category of Table 1, because this slight delay was probably not due to the effect of some cytoplasmic factor but the mechanical perturbation caused by the addition of the cytoplasm. The second category involved cases with the delay longer than one but shorter than three cleavage cycles (about 2–6 hr delay). In the third category, cases with the delay longer than three cycles were included (delay over 6 hr). This extended delay would be regarded as a temporary arrest caused by the effect of a cytoplasmic factor. In the cases of the last category, cleavage was completely arrested during the entire period of observation, at least, until the time when control embryos reached morula to blastula stages. In Table 1, the third and the last groups were consolidated, because these prolonged but temporary arrests as well as complete arrests must be caused by the effect of a cytoplasmic factor. The frequency of cleavage inhibition increased proportionally with the in-

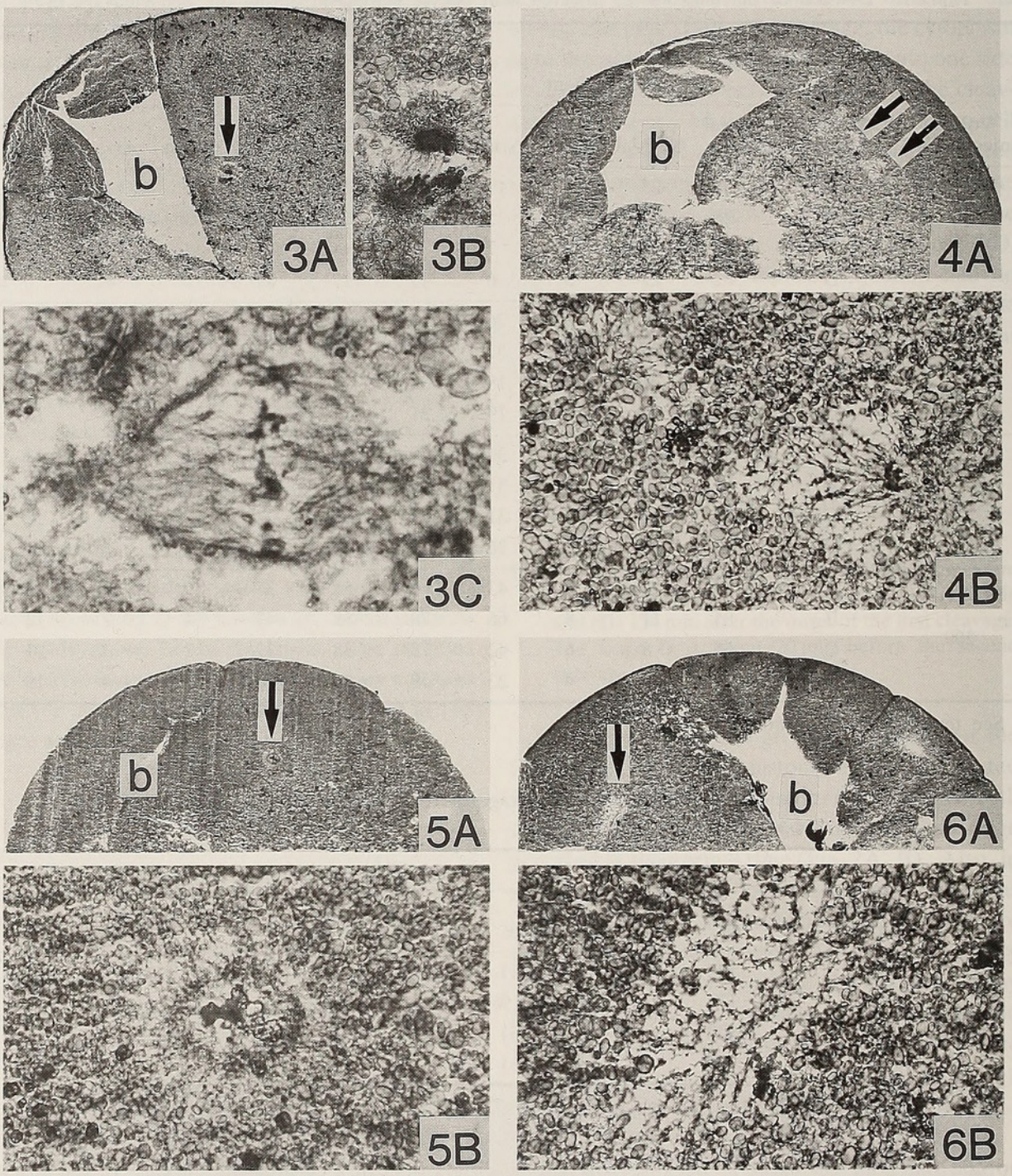

FIG. 3. (A) Anaphase mitotic figure in the cleavage-arrested blastomere (arrow) and (B) its high power observation. (C) Another example of mitotic figure in metaphase. b, blastocoel. A; ×45. B; ×260. C; ×400.

FIG. 4. (A) Aster-like structure in the cleavage-arrested blastomere (arrow) and (B) its high power observation. A; ×36. B; ×280.

FIG. 5. (A) A structure seeming to be degenerated nucleus in the cleavage-arrested blastomere (arrow) and (B) its high power observation. A; ×40. B; ×280.

FIG. 6. (A) Fibrous structures in the cleavage-arrested blastomere (arrow) and (B) its high power observation. A; × 42. B; ×260.

crease of the injected volume of cytoplasm.

Table 2 shows the relationship between the stage of recipient eggs and the time of cleavage arrest. When the injection was carried out in the stage A and B, the arrest occurred at the second cleavage stage in almost all cases. In the injection in the stage C and D, however, the arrest occurred at the second cleavage stage in 77 and 47% of the cases respectively, and in the remaining cases, the arrest took place at the third cleavage stage.

Cleavage-arrested blastomeres were cytologically examined in 31 embryos, 15 examples of which were fixed when the unoperated half developed into 8 to 32 cells (8-12 hr after cytoplasm injection), and 16 examples were fixed at the morula to blastula stages (20–30 hr after cytoplasm injection) of the unoperated one. In 8 cases of the former group, spindle fibers and chromosomes were observed (Fig. 3). Four cases had one nucleus of interphase or telophase which was weakly stained in comparison to the normally dividing nucleus. And in remaining 3 cases, chromatin substance was not observed but fibrous structure resembled to the vestigial asters was observed (Fig. 4). In the latter fixation group, 3 cases involved a structure seeming to be karyomeres in indistinct feature (Fig. 5), 11 cases involved fibrous structures (Fig. 6), but remaining 2 cases involved neither chromatin-like substance nor fibrous structures.

DISCUSSION

The results of the present study strongly suggested that the oocyte cytoplasm of a urodele, *Cynops pyrrhogaster,* involved the cytostatic factor (CSF) which was well known in the oocytes of anura, *Rana pipiens* [2, 3] and *Xenopus laevis* [4].

Concerning the relationship between the amount of injected cytoplasm and the effect of CSF on the recipient egg, the present results good agreed to the previous ones. About 60 nl in *Rana* [2] and 30 nl in *Xenopus* [4] were sufficient amout to arrest cleavage of the recipient in high percentage (about 90%). These quantities represented about 6% of the egg volume of respective species. In the present experiment using *Cynops,* the maximum amount of the injected cytoplasm (about 200 nl) represented roughly 6–7% of *Cynops* egg volume, and arrested cleavage in about 70%.

In the present study, when the injection of the oocyte cytoplasm was made at late stage of the first cleavage, the cleavage arrest did not occur at the second cleavage stage but at the third stage, in half of cases. Similar results had been obtained in the studies with anuran eggs. In such cases, the second nuclear division would already proceeded to anaphase or telophase, and CSF influenced the next division. In fact, the cytokinesis of amphibian eggs could take place even after the mitotic apparatus was removed at metaphase to anaphase of karyokinesis, about 20 min before the appearance of furrow, in *Rana nigromaculata* [8].

In the cytological investigation, the previous experiments with anuran eggs showed that the karyokinesis of cleavage-arrested blastomeres was ceased at metaphase even after unoperated half developed into the blastula or the gastrula stage. In the present works with urodele eggs, mitotic figures were also found in the uncleaved blastomere which was fixed at 8 to 32 cells stage of the unoperated half. In the uncleaved blastomere which was fixed at the morula to the blastula stage, however, nuclei or mitotic figures could not be found. This discrepancy between the anura and the urodele would imply that, in the urodele, the mitotic apparatus of the cleavage-arrested blastomere gradually degenerated after the karyokinesis was ceased at metaphase.

It is also noted that the frequency of the delay or the arrest in the control experiment was about 10–20% in the case of *Cynops,* while its value was several percent in the case of anura. This disagreement might be caused by the difference of the volume of injected cytoplasm between the present experiment and the previous ones. Adding much cytoplasm, probably, gave perturbative effect on cleavage of recipients.

In the previous experiment, the injection of a large amount of the oocyte cytoplasm frequently resulted in cytolysis of the operated blastomere [4]. A similar phenomenon was also observed in the present experiment.

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