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Genetic Consequences of Blocking Polar Body I with Cytochalasin B in Fertilized Eggs of the Pacific Oyster, *Crassostrea gigas:* I. Ploidy of Resultant Embryos

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Abstract. The effect of blocking polar body I (PB1) with cytochalasin B (CB) on the ploidy of embryos was studied in the Pacific oyster, *Crassostrea gigas.* To block the release of PB1, fertilized eggs were treated with CB (1.0 μg/ml) for 15 min beginning at 5 min post-fertilization at 25°C. The CB treatment and its control were repeated in three crosses. Ploidy of 8-h-old embryos was determined with karyological analysis.

In control groups, the majority of the cells (89.3%) had a diploid number of 20 chromosomes, although spontaneous haploids (0.7%), triploids (1.3%) and aneuploids (8.7%) were also encountered. In CB-treated groups, only 4.5% of the cells remained as diploid, and the majority were either triploid (15.6%), tetraploid (19.4%) or aneuploid (57.6%). Despite variation among the three crosses, contingency Chi-square analysis showed that the occurrence of triploids, tetraploids and aneuploids had a significant ($P = 0.0001$) dependence on the CB treatment. The majority of the aneuploids fell into two groups containing either 23–25 or 35–37 chromosomes. The production of triploids, tetraploids and aneuploids in specific distributions suggests that blocking PB1 complicates subsequent chromosome segregation.

Introduction

In most vertebrates, eggs usually mature after the completion of meiosis I. In the Pacific oyster, *Crassostrea gigas,* and many other marine mollusks, mature eggs are arrested at prophase of meiosis I (Ahmed, 1973; Lu, 1986; Strathmann, 1987). Only after fertilization or activation, do the eggs complete meiosis I and II, releasing two polar bodies. Delayed meiosis in eggs of the Pacific oyster provides a unique opportunity for manipulation of both polar body I (PB1) and polar body II (PB2). Although it has been well established that blocking the release of PB2 results in triploidy (reviewed by Beaumont and Fairbrother, 1991), it is still uncertain what ploidy will result from blocking PB1.

Stanley *et al.* (1981) first reported that cytochalasin B (CB) treatment applied during meiosis I resulted in triploid and tetraploid embryos in the American oyster, *Crassostrea virginica,* although only triploids survived to 8 months of age. Similarly, both triploid and tetraploid embryos were produced from blocking PB1 in the blue mussel *Mytilus edulis* (Yamamoto and Sugawara, 1988) and the Pacific abalone, *Haliotis discus hannai* (Arai *et al.,* 1986). In the Pacific oyster, however, only triploids were found when thermal shocks were applied during meiosis I (Quillet and Panelay, 1986). In a later study, blocking PB1 with CB in the Pacific oyster produced tetraploids, not triploids (Stephens and Downing, 1988; Stephens, 1989).

In the Pacific oyster, production of either meiosis I triploids or tetraploids is of interest. Triploid oysters have become an important part of the oyster culture industry because of their sterility (Allen, 1988; Allen *et al.,* 1989). Meiosis I triploids may grow better than meiosis II triploids (Stanley *et al.,* 1984) that are currently produced in commercial hatcheries. Tetraploids may be mated to diploids to produce all triploid progeny. However, available data are inconclusive, if not conflicting, on the ques-

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tion of what ploidy is produced from blocking PB1. The lack of detailed ploidy analyses of early embryos may be partly responsible for this discrepancy. This study used extensive karyological analysis to determine the ploidy of 8-h-old Pacific oyster embryos following blocking PB1 with CB. It was anticipated that results of this study would clarify some of the confusion concerning the genetic consequences of blocking PB1.

Materials and Methods

Gamete preparation

Gametes were obtained by strip-spawning sexually mature Pacific oysters. To obtain eggs, female gonadal tissue was dissected into 2 l of seawater. The egg suspension was first passed through a 100 μm screen (Research Nets Inc.) to remove large tissue debris. Eggs about 50 μm in diameter were collected onto a 20 μm screen. Broken eggs and other smaller debris were washed off. The eggs were then re-suspended in 1 l of seawater, ready for fertilization.

To prepare sperm suspensions, 1 g of male gonadal tissue was completely suspended in 99 g of seawater. The large tissue debris were removed by passing the sperm suspension through a 20 μm screen. For fertilization, 5 ml of the sperm suspension was added to one million eggs suspended in 1 l of seawater. Fertilization, treatment, and embryo culture were all conducted at 25°C.

CB treatment

To block the release of PB1, 1.0 mg CB (Sigma) dissolved in 1.0 ml dimethyl sulfoxide (DMSO) was added to 1 l egg suspension at 5 min post-fertilization for 15 min. CB was removed by collecting and rinsing treated eggs on a 20 μm screen. Eggs were then returned to 0.1% DMSO in seawater for 30 min to remove residual CB. In the control groups, fertilized eggs were treated with only 0.1% DMSO. The embryos were stocked at a density of 20 embryos/ml. The experiment was repeated three times using three pairs of oysters as parents.

Determination of ploidy

Ploidy of the resultant embryos in both control and treated groups was determined by karyological analysis. Samples for karyological analysis were taken at 8 h post-fertilization. Embryos were first treated with 0.01% colchicine for 30 min. After the colchicine was removed, nine parts of a hypotonic solution (0.075 *M* KCl) was added to one part embryo suspension for 25–30 min. The hypotonic solution was then removed as completely as possible, and samples were fixed in a solution of one part glacial acetic acid and three parts of absolute methanol (v/v). About 15 min before staining, the fixative was replaced by 1:1 (v/v) glacial acetic acid and absolute methanol solution.

Embryo suspensions were dropped onto slides and air-dried. Slides were stained with Leishman's stain (Benn and Perle, 1986). The stock solution was made by dissolving 150 mg Leishman's stain (Sigma) in 100 ml methanol. The staining solution was prepared by mixing one part of the stock solution with three parts phosphate buffer (0.025 *M* KH_2PO_4, pH 6.8). The slides were stained for 10–15 min.

Only metaphase plates which showed no evidence of artificial chromosome loss were counted. Only one metaphase plate per embryo was scored and used to represent that embryo. Embryos with more than one countable metaphase were rare. A minimum of 50 metaphase plates from 50 embryos were counted for control and treatment groups of all replicates. Diploid Pacific oysters have 20 chromosomes (Ahmed and Sparks, 1967; Ahmed, 1973). In this study, ploidies were classified as the following: 10, haploid; 20, diploid; 29–30, triploid; 39–40, tetraploid; 49–50, pentaploid. All others were considered to be aneuploid.

Data analysis

For both control and treated groups, homogeneity among the three crosses was tested with Chi-square homogeneity analysis. Differences between the control and treated groups were then tested by contingency Chi-square analysis. The null hypothesis was that the occurrence of ploidies was independent of CB treatment.

Results

In the control groups, PB1 was released around 17 min post-fertilization, and PB2 was released around 35 min post-fertilization. In the CB-treated groups, PB1 was not observed at the time expected, but one polar body was released in most of the eggs between 35 and 45 min post-fertilization. The first mitosis in the treated groups appeared to be normal, except apparently less synchronized than that in the control groups.

At 8 h post-fertilization, all embryos developed to swimming trochophores, and the number of swimmers hatched was high in both treated and control groups (95–100%). However, abnormal trochophores were noticed in the CB-treated groups. The abnormal trochophores were either deformed or constantly swam in a circular motion.

In the control groups, the karyological analysis revealed that 89.3% of all cells examined had exactly 20 chromosomes (Table I). Only 8.7% could be classified as aneuploids, of which, about half (4.7%) had 19 chromosomes, one quarter (2.0%) had 15–18 chromosomes, and one quarter (2.0%) had 21 chromosomes (trisomics). Spon-

taneous haploids and triploids were also observed in the control groups at a low frequency (0.7% and 1.3%, respectively). No tetraploids or pentaploids were found among the 150 cells counted from the control groups.

Compared with the control groups, all three CB-treated groups had dramatic increases in the proportion of polyploid and aneuploid cells; only about 4.5% of all cells remained as diploid (Table I). The majority of cells were either triploid (15.6%), tetraploid (19.4%) or aneuploid (57.6%) (Fig. 1). A low percentage of haploids and pentaploids were also recorded in the CB-treated groups. The mitotic index of CB-treated groups was noticeably lower than control groups.

For both the control and the treated groups, the homogeneity among crosses was accepted at a significance level of 95% for the frequencies of diploids, triploids, tetraploids and aneuploids. Homogeneity was not tested for haploids and pentaploids, where at least one cross had no occurrence of the ploidy tested (Table I). Because the number of haploids and pentaploids observed in all three crosses was low (0–4%), homogeneity among crosses was assumed. Consequently, data from all three crosses were used in a contingency Chi-square analysis to test the null hypothesis that the occurrence of ploidies is independent of the CB treatment. When all ploidies were analyzed in one Chi-square test (df = 5), the null hypothesis was rejected at a confidence level of 95%. When ploidies were tested separately (df = 1), however, the null hypothesis was accepted for haploids and pentaploids (Table I). On the other hand, the occurrence of diploids, triploids, tetraploids, and aneuploids showed a significant ($P = 0.0001$) dependence on whether or not the eggs were treated with CB for blocking PB1.

Although the homogeneity among crosses was statistically accepted, the CB-treated groups from different crosses had noticeable differences in the distribution of the different ploidies induced. For example, in the first cross, CB treatment induced 26% triploids, 24% tetraploids and 45% aneuploids, compared to 11%, 6%, and 72% of respective ploidies induced in cross 3 (Table I).

The distribution of pooled chromosome counts for the control and for the treated groups from the three crosses is shown in Figure 2. As expected, only one peak (at 2n = 20 chromosomes) was present in the control groups (Fig. 2A). In the treated groups, however, four peaks could be identified: triploids, tetraploids, and two aneuploid peaks, corresponding to chromosome counts of 23–25 and 35–37 (Fig. 2B).

Discussion

This study provides an estimate of the frequency of spontaneous aneuploidy in 8-h-old embryos of the Pacific oyster under our experimental conditions. It is unlikely that the aneuploidy in the control groups was caused by DMSO, although this study cannot rule out such a possibility. DMSO is widely used with cultured cells and embryos, and has no known effects on chromosome segregation. Comparable data have not been reported. Since karyological techniques tend to create chromosome losses and overestimate the occurrence of aneuploidy, the 8.7% aneuploids observed in this study may be considered as an upper limit of aneuploidy in the 8-h-old embryos. On the other hand, since the probability of creating trisomics by artifacts is low, the 2% trisomics observed here may provide a conservative low limit.

The formation of aneuploid gametes in mollusks may be common, particularly under environmental stress (Dixon, 1982). Gaffney *et al.* (1990) suggested that heterozygosity deficiency, a common phenomenon in mollusks, could be caused by the occurrence of aneuploids. In juvenile Pacific oysters, aneuploid cells were estimated to range from 4% to 36% (Thiriot-Quievreux *et al.*, 1988). Frequency of spontaneous aneuploids could be very different at different stages of the life cycle.

Table I

Percentage of different ploidies observed from normal fertilization (Control) and blocking PB1 (Treated) in the Pacific oyster

Cross	Group	Sample size	1n	2n	3n	4n	5n	An
1	Control	50	2.0	88.0	0.0	0.0	0.0	10.0
	Treated	54	0.0	5.5	25.9	24.1	0.0	44.5
2	Control	50	0.0	92.0	2.0	0.0	0.0	6.0
	Treated	50	0.0	2.0	10.0	28.0	4.0	56.0
3	Control	50	0.0	88.0	2.0	0.0	0.0	10.0
	Treated	65	1.5	6.2	10.8	6.2	3.1	72.3
Average	Control	(150)	0.7	89.3	1.3	0.0	0.0	8.7
	Treated	(169)	0.5	4.5	15.6	19.4	2.4	57.6
P-value (Chi-square)*			0.5313	0.0001	0.0001	0.0001	0.1639	0.0001

* The null hypothesis of the Chi-square test was that occurrence of each ploidy is independent of the CB treatment.

Ploidy was classified as: 10, haploid (1n); 20, diploid (2n); 29–30, triploid (3n); 39–40, tetraploid (4n); 49–50, pentaploid (5n); and others, aneuploid (An).

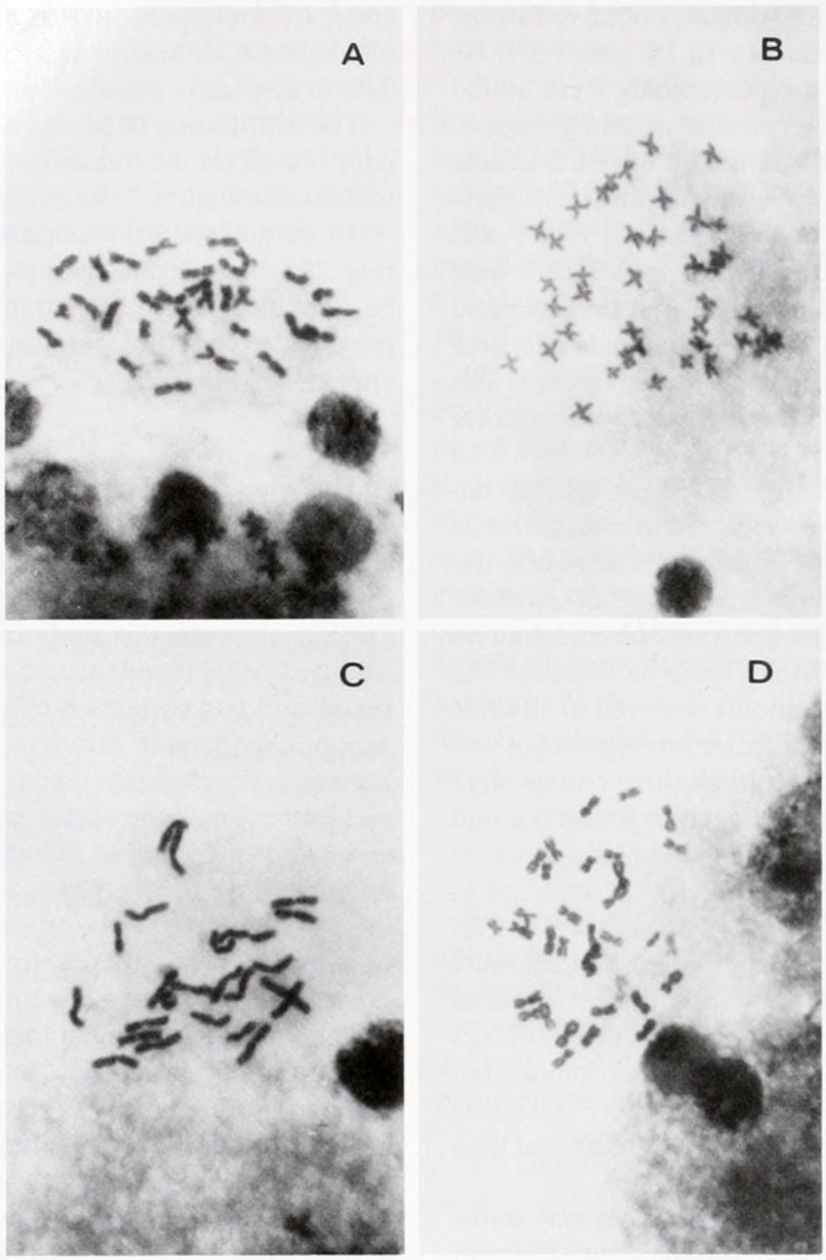

Figure 1. Metaphase plates of triploid (A), tetraploid (B) and aneuploid (C, D) cells produced from blocking polar body I with cytochalasin B in the fertilized eggs of the Pacific oyster.

Results from this study suggest that spontaneous haploids and triploids might occur in the Pacific oyster. Spontaneous triploids have been reported in amphibians and fish and are probably caused by fertilization of unreduced ova by normal sperm (Fankhauser, 1945; Thorgaard and Gall, 1979). Haploids could be caused by the failure of sperm pronucleus incorporation. Again, this study cannot completely rule out the possibility that the haploids and triploids from the control groups were induced by the DMSO.

It is clear that the CB treatment applied during meiosis I was effective in preventing polar body release at 17 min post-fertilization, a time corresponding to the release of PB1. That the polar body released at 40 min post-fertilization (after CB treatment) is PB2 rather than the delayed PB1 has been previously suggested, based on the different fluorescence intensity of the two polar bodies (Stephens, 1989).

The detection of triploids and tetraploids from blocking PB1 in this study agrees with results from the American oyster (Stanley *et al.*, 1981), the blue mussel (Yamamoto and Sugawara, 1988), and the Pacific abalone (Arai *et al.*, 1986). Also, results of this study do not conflict with studies in which only triploid juveniles were observed from blocking PB1 (Stanley *et al.*, 1984; Downing and Allen, 1987). The lack of tetraploids at juvenile or adult stages was probably due to poor viability of tetraploids (Stanley *et al.*, 1981; Stephens, 1989; Guo, 1991). On the other hand, results of this study differ from other studies, in which only triploid embryos (Quillet and Panelay, 1986)

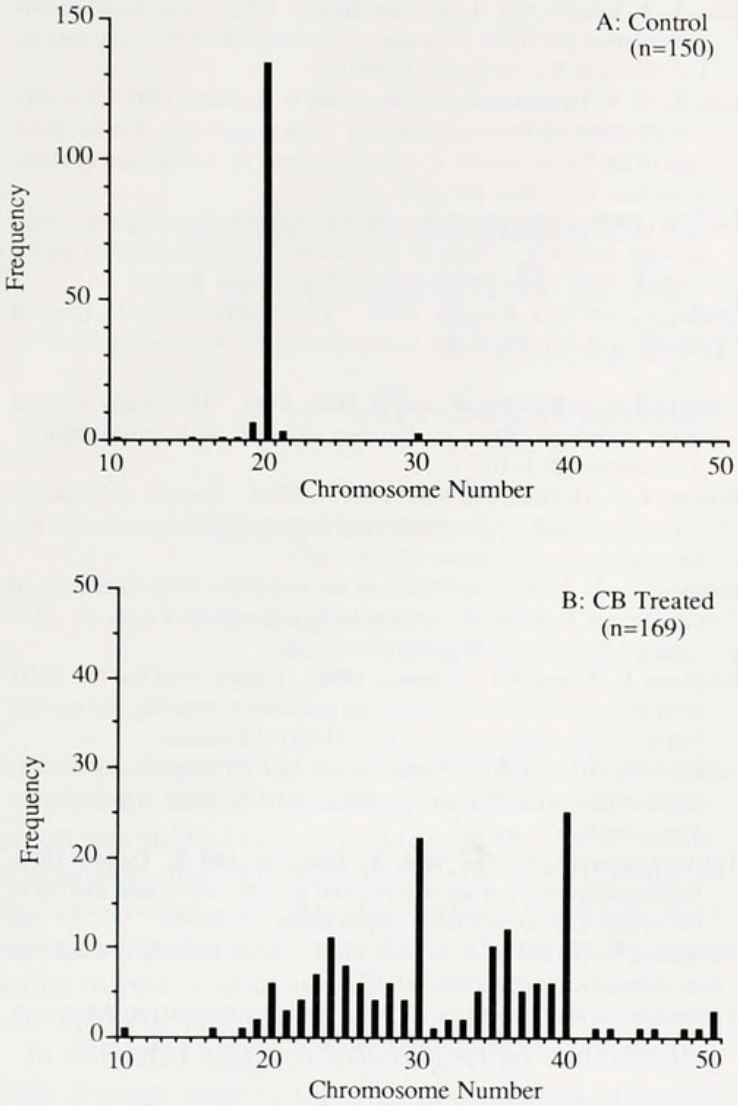

Figure 2. Chromosome counts in embryos resulting from normal fertilization (A:Control) and blocking polar body I with cytochalasin B (B:CB Treated) in the Pacific oyster; combined data from three crosses.

or only tetraploid larvae (Stephens, 1989) were observed from blocking PB1. This variation may be due to differences in experimental methods and conditions. For example, Quillet and Panelay (1986) used temperature shock instead of CB to block PB1, and Stephens (1989) treated zygotes at 18°C instead of at 25°C. Environmental factors, such as temperature, salinity, egg quality, timing and duration of CB treatment, all have effects on meiotic events and triploid induction (Lu, 1986; Downing and Allen, 1987). Variation in those environmental factors was probably also responsible for the different proportions of ploidies observed in the three crosses of this study.

The production of a large proportion of aneuploids from blocking PB1 was unexpected. Further, it was surprising that the aneuploids distributed around two peaks with 23–25 and 35–37 chromosomes. Aneuploids were commonly assumed to be artifacts caused by karyological treatment. In this study, comparison between the control and CB-treated groups clearly demonstrated that the aneuploids were a consequence of CB treatments, not artifacts. Also, the fact that the aneuploids formed two peaks minimized the possibility of artifacts being the explanation. The assumption of artifacts was probably the reason why aneuploids were not reported in previous studies. As a result, aneuploids were grouped into the closest euploid groups. For example, in the blue mussel with a diploid number of 28 chromosomes, Yamamoto and Sugawara (1988) classified cells with 35–48 chromosomes as triploids and those with 49–62 chromosomes as tetraploids. Also, Quillet and Panelay (1986) classified metaphase spreads with "about 20 chromosomes" as diploids, and those with "about 30 chromosomes" as triploids in the Pacific oyster. In studies where ploidy was determined at juvenile or adult stages (Stanley *et al.*, 1981; Downing and Allen, 1987), aneuploids were absent probably because they died in early embryonic development. Aneuploidy often leads to abnormality and death in higher animals. A preliminary study showed that the majority of aneuploids were not viable in the Pacific oyster (Guo *et al.*, 1989).

Survival of the resultant embryos from blocking PB1 was not recorded in this study. It was our experience with the Pacific oyster that blocking PB1 always resulted in higher mortalities than blocking PB2; the survival to D-stage was usually 20–40% for blocking PB1, and 60–80% for blocking PB2 (Guo and Cooper, unpub.). Downing and Allen (1987) also found that early CB treatments in the Pacific oyster produced higher mortalities at D-stage than late treatments. Differential mortality may also occur after D-stage. In the American oyster, the early CB treatment resulted in a higher mortality than the late treatment, as determined at 8 months post-fertilization, although the early treatment produced a larger number of D-stage larvae than the late treatments at 24 h post-fertilization (Stanley *et al.*, 1981). The high mortalities from blocking PB1 could be caused by the poor viability of aneuploids and tetraploids.

The production of triploids, tetraploids and a large proportion of aneuploids in specific distributions suggests that blocking PB1 complicates subsequent chromosome segregation. Mechanisms by which triploids, tetraploids and specific aneuploids are produced from blocking PB1 were investigated in a separate study; the results of that study are presented in the next paper (Guo *et al.*, 1992).

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