Dependence of fertilization in sea urchins, *Strongylocentrotus purpuratus,* on microfilament formation and internal calcium concentration

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ABSTRACT

Fertilization in sea urchins, *Strongylocentrotus purpuratus,* is the result of a series of interactions between the egg and sperm. Microfilament formation has been implicated in both the acrosomal process formation and the entry of the sperm into the egg. Changes in internal calcium concentrations have been linked to exocytosis reactions that occur in both the egg and the sperm. Therefore, the dependence of fertilization on microfilament generation and changes in internal calcium concentration were determined by examining the effects of cytochalasin and calcium ionophore A23187 on sea urchin fertilization. These experiments demonstrated the dependence of the fertilization process on microfilament generation and the changes in internal calcium concentration; the inhibition of fertilization by cytochalasin confirmed the anticipated dependence on microfilament generation, while the ability of calcium ionophore A23187 to induce fertilization membrane formation, by increased permeability to calcium, confirmed the importance of calcium as a signaling mechanism for exocytosis.

INTRODUCTION

Fertilization occurs as a result of a successful series of interactions between the egg and sperm. Initially the sperm is attracted to the egg by a chemotactic mechanism connected to the egg jelly layer. Contact with the jelly layer induces the acrosomal reaction, which results in the exocytosis of the acrosomal vesicle and the formation of the acrosomal process. The released enzymes from the acrosomal vesicle allows for the digestion through the jelly and vitelline layers, allowing the sperm to recognize and bind to the egg. Plasma membrane fusion and the entrance of the sperm into the egg complete the fertilization process (Gilbert 2003). Aside from sperm attraction, the egg must act to inhibit polyspermy following the success of a single sperm. The egg employs two mechanisms to achieve this inhibitory action: a large membrane

depolarization to decrease sperm binding ability (Dale & De Santis 1980) and the generation of a fertilization envelope. The fast block to polyspermy, depolarization, is triggered by sperm binding. The fertilization envelope is triggered by an increase in internal calcium, which results in the exocytosis of the cortical granules, which combine with the vitelline layer. An osmotic pressure gradient is responsible for the lifting of the envelope away from the plasma membrane. Following the initial lifting of the fertilization envelope, its surface becomes less permeable due to crosslinking (Matese et al 1997).

Microfilament formation has been implicated in the egg and sperm interactions that occur in the intermediate stages of the fertilization process. The generation of the acrosomal process depends upon the conversion of globular actin, stored beneath the acrosomal vesicle, to filamentous actin to support the process responsible for sperm binding and egg penetration (Dale and De Santis 1980). The surface of the eggs also has microfilament processes suggested to aid in pulling the sperm into the egg (Gilbert 2003). Cytochalsin is a drug known to inhibit the generation of microfilaments from the polymerization of globular actin and thus, has been implicated in the inhibition of successful fertilization in *Paracentrotus lividus* sea urchins (Dale & de Santis 1980). The inability of fertilization to occur in the presence of cytochalasin suggests the sea urchin's dependence on microfilaments for fertilization.

Vesicle exocytosis is also crucial to both the reactions of the sperm and the egg. Exocytosis of the acrosomal vesicle of the sperm is required for the digestion of the jelly layer that eventually leads to contact with the plasma membrane of the egg. The generation of the fertilization envelope to block polyspermy requires the exocytosis of the cortical granules. Intracellular calcium ion concentrations have been implicated in the signaling mechanisms of these reactions (Chambers & Hinkley 1979; Obata & Kuroda 1989). Calcium ionophore A23187

alters membrane permeability and, thus, enables transport of calcium cations across lipid membranes. Release of calcium ions stored within the egg itself have been shown to induce exocytosis of cortical granules. This enablement of calcium permeability has been shown to result in a non-propagated exocytosis of the cortical granules causing either full or partial elevation of the fertilization envelope in sea urchins, *Lytechinus variegates*, depending upon the exposure duration (Chambers & Hinkley 1979). The appearance of the fertilization envelope has been equally observed in seawater with and without calcium present (Obata & Kuroda 1989), which implies that the calcium responsible for the elevation in internal concentration is contained within the egg. The appearance of fertilization envelops under induced changes in membrane permeability to calcium ions suggest the sea urchin's dependence on the change in internal calcium to produce the exocytosis reactions required for fertilization and the prevention of polyspermy.

Based on their solubility properties, both cytochalasin and calcium ionophore A23187 require dissolution in dimethyl sulphoxide (DMSO). Though with low dose administration of cytochalasin and calcium ionophore, the amount of DMSO added amounts to less than 5% of the total volume of the suspension, the possibility of direct interaction between DMSO and the sea urchin gametes exists. With higher dose additions, the percent of the total volume increases, thus increasing the potential interaction. Sperm fertilization ability, mitochondrial function and membrane integrity have been shown to be affected by varying DMSO treatment in experiments designed to explore the possibilities of cryopreservation of sea urchin, *Evenchinus chloroticus,* sperm (Adams et al 2004). The highest post-thaw fertilization of greater than 85% was achieved with DMSO doses ranging from 2.5% to 7.5%, while higher doses inhibited fertilization ability, while maintaining mitochondrial function. Following both treatment with DMSO and

cryopreservation, fertilization ability decreased compared to untreated sperm suggesting that either the DMSO, freezing or a combination results in decreased fertilization capabilities. Incubation in DMSO has also been observed to increase fertilization envelope formation in sea urchins, *Hermicentrotus pulcherrimus* (Fujiwara et al 1987). A slight increase in the number of fertilization envelopes was observed with DMSO concentrations ranging from 1% to 3% and fertilization envelope formation was documented to be 75% greater with doses exceeding 3%. The increased number of fertilization envelopes generated in the presence of DMSO suggests some direct effect on the exocytosis mechanisms responsible for the cortical reactions and lifting of the vitelline membrane.

The dependence of fertilization on microfilament generation and changes in internal calcium concentration were determined by examining the effects of cytochalasin and calcium ionophore A23187 on sea urchin fertilization.

MATERIALS AND METHODS

Animals

Adult purple sea urchins, *Strongylocentrotus purpuratus* were obtained from Pacific Bio Marine. Gamete shedding was induced from urchins by 1mL intracoelomic injection of 1.5M KCl. All handling of gametes was performed at room temperature and dilutions and fertilizations were performed using artificial seawater. Sperm was collected “dry” and diluted 1:100 in seawater within 30 minutes of use. Eggs were collected directly into seawater, and washed in one change of fresh seawater before use.

Fertilization in the presence of cytochalasin

1.0 mg of cytochalasin was dissolved in 1.0mL of DMSO within 1 hour of use; both cytochalasin and DMSO were purchased from Sigma Chemical Company. The effect of

cytochalasin on fertilization was observed by treatment of egg and sperm suspensions with cytochalasin to a concentration of 5µg/mL. The order in which the drug was administered and the eggs and sperm were combined varied as follows: cytochalasin treated sperm was added to untreated eggs, cytochalasin treated eggs were added to untreated sperm, and cytochalasin was added immediately following egg and sperm combination. Following a 5-minute room temperature incubation, the percent fertilization was determined by multiple counts of fertilization envelopes present in each suspension.

Fertilization in the presence of calcium ionophore A23187.

2.0 µmoles of calcium ionophore A23187, purchased from Sigma Chemical Company, was dissolved in 1.0mL of DMSO within 1 hour of use. The effect of calcium ionophore on fertilization was observed by treatment of egg and sperm suspensions with calcium ionophore to a concentration of 20 µM. The order in which the drug was administered and the eggs and sperm were combined varied as follows: calcium ionophore treated sperm was added to untreated eggs, calcium ionophore treated eggs were added to untreated sperm, and calcium ionophore was added immediately following egg and sperm combination. Following a 5-minute room temperature incubation, the percent fertilization was determined by multiple counts of fertilization envelopes present in each suspension.

Dose dependant viability of both sperm and eggs was determined by varying treatment dose from 2.5 µM to 100 µM and observing sperm activity, presence of fertilization envelopes, and high degrees of exocytosis in egg suspensions. Dose dependency of eggs to DMSO was also tested following the same concentration range.

Following overnight incubation of egg and sperm combinations treated with 2.5 µM ionophore, percent cleavage was determined for comparison with percent fertilization envelopes observed following 5 minutes of room temperature incubation.

RESULTS

The normal response of the sea urchin egg to sperm exposure was observed by 1:1 combination of eggs and 1:100 diluted sperm. Between 1 and 5 minutes following suspension combination, the fertilization membrane was formed (Figure 1). This generation of the fertilization membrane was also observed to have a direct effect on sperm behavior; initially sperm actively targeted unfertilized eggs, where as following fertilization membrane formation, the sperm were no longer actively observed on the eggs' surfaces.

Cytochalasin was observed to inhibit fertilization (Table 1). The order of combination of the egg, sperm and cytochalasin was varied to more specifically account for the cytochalasin's action on the egg and sperm individually, though all combinations were allowed to incubate for 5 minutes prior to observation. The control combination of untreated egg and sperm suspensions yielded 60% fertilization (Table 1), thus verifying the viability of both the sperm and the eggs. Following treatment of eggs or sperm prior to combination, however, no fertilization envelopes were observed (Table 1). When cytochalasin was added to a 1:1 egg-sperm mixture immediately after combination, 2.9% of the eggs appeared to be fertilized (Table 1). In the presence of 5 µg/ml cytochalasin the number of fertilization envelopes observed dramatically decreased in comparison to those observed in suspensions of untreated eggs and sperm.

20 µM calcium ionophore A23187 increased the amount of fertilization recognized by fertilization envelope formation (Table 2). The order of combination of the egg, sperm and calcium ionophore was varied to more specifically account for the calcium ionophore's action on

the egg and sperm individually; all combinations were incubated for 5 minutes at room temperature. Combination of untreated egg and sperm suspensions resulted in 90% fertilization (Table 2). Treatment of either eggs or sperm prior to combination increased the number of observed fertilization envelopes to 98% and 97% respectively (Table 2). A treated combination of egg and sperm yielded 91% fertilization (Table 2). Treated eggs observed prior to sperm addition contained 73% eggs with visible fertilization envelopes (Table 2). Calcium ionophore was shown to increase percent fertilization while also inducing fertilization envelope formation in the absence of sperm.

Calcium ionophore A23187 inhibited sperm activity at low dose concentrations (Figure 3); sperm suspensions were treated with calcium ionophore concentrations ranging from 2.5 μM to 100 μM. Sperm activity was observed to significantly decrease to an estimated less than 20% activity in the presence of the 2.5 μM calcium ionophore. 5 μM of calcium ionophore was determined to be lethal to sperm activity, as no movement was observed. At high concentrations 80 μM and 100 μM, aggregation of sperm was observed in clumps approximately 0.5mm to 1.0mm in diameter.

Fertilization envelope formation exhibited a dose-dependant relationship with calcium ionophore A23187 (Figure 4). The appearance on fertilization envelopes (Figure 2, B) of sea urchin eggs increased from 0% to 86% as the calcium ionophore concentration increased from 0μM to 25 μM. However, as doses exceeded 25μM and continued to increase, the number of fertilization envelopes observed decreased while the number of highly exocytosed eggs (Figure 2, C) increased. At high concentrations of 80 μM to 100 μM, an aggregation of all highly exocytosed eggs was observed.

Variation in the administered concentration of DMSO altered egg morphology (Figure 5). In the presence of low concentrations of DMSO, 2.5% (v/v) to 15% (v/v), eggs maintained their normal unfertilized appearance (Figure 2, A). However, as the dose increased from 15% to 80% the number of normal unfertilized eggs decreased while the number of eggs exhibiting high degrees of exocytosis increased (Figure 2, C). Though rare, at doses of 20% and 40% some fertilization envelopes were observed (Figure 2, B); 5% and 8% of the eggs had formed fertilization envelopes at the doses of 20% and 40% respectively. In the presence of greater than 80% DMSO, the number of highly exocytosed eggs exceeded the number of normal unfertilized eggs observed; 91% to 93% of eggs appeared to have undergone multiple exocytosis reactions and had formed clump-like aggregations.

2.5 µM Calcium ionophore treated and untreated egg and sperm combinations were compared after both 5 minute and overnight incubation to differentiate between fertilization and ionophore induced fertilization envelopes by the presence of cleavage (Table 3). No cleavage was observed following an overnight incubation, either in the control combination of egg and sperm or a calcium ionophore treated suspension. The percent fertilization envelopes present in the control remained constant at 13%; in the combinations treated with ionophore, however, the percent fertilization envelopes present decreased from a range of 85% - 91% to a range of 0% - 1% (Table 3). The eggs themselves had a ragged appearance, and fertilization envelopes seemed to have deteriorated.

DISCUSSION

Treatment of egg and sperm suspensions with 5µg/ml cytochalasin revealed a significant decrease in the number of eggs fertilized (Table 2). Results are consistent with the inhibition observed by Dale and De Santis (1981) with the exposure of *Paracentrotus lividus* to

cytochalasin B and D. Inhibition of fertilization by cytochalasin confirms the importance of microfilament formation in the fertilization process. This experiment was unable to determine in what structures the cytochalasin action results in loss of function; however, further experimentation exploring the dose dependency of separate treatments of sperm and eggs could clarify the importance of the microfilament formation in the acrosomal vesicle compared to the microfilamentous aid in sperm entry.

Treatment of egg and sperm suspensions with 20µM increased the amount of sea urchin fertilization documented by fertilization envelopes by approximately 9% (Table 2). The order of individual sperm and egg treatments did not alter the increase in fertilization envelope appearance, however, treatment of a sperm and egg mixture did not elicit a response of the same magnitude. 73% of calcium ionophore treated eggs without sperm addition were observed to have formed fertilization envelopes, suggesting the influx of calcium resulting from changes in membrane permeability is successful in inducing fertilization envelope formation. These initial observations as to the effect of calcium ionophore spawned further investigation of the dose dependency of the egg and sperm to the drug and the distinction between fertilization and drug induced fertilization envelope formation.

Treatment of sperm with calcium ionophore A23187 was shown to have an effect on their fertilizing capacity. Sperm activity was dose-dependant, and the sperm exhibited a high sensitivity to the presence of the ionophore; all sperm activity was lost under 5 µM ionophore conditions (Figure 3). The low lethal dose of calcium ionophore on the sperm suggests that the increase in observed fertilization by the administration of 20 µM calcium ionophore only confirms the ability of calcium ionophore to induce the fertilization envelope without the necessary presence of viable sperm.

Similar to the sperm, the fertilization envelope formation induced by calcium ionophore in sea urchin eggs exhibited a dose dependant relationship: as the concentration of ionophore increased, the percent observed fertilization envelopes increased (Figure 4) up to a concentration of 25 μM. The general dose dependant relationship is consistent with the results observed by Chambers and Hinkley (1979).

The egg morphology also demonstrated a dose dependant relationship with DMSO, the liquid in which the calcium ionophore was dissolved (Figure 5). Though inconsistent with Fujiwara et al's observation that DMSO could induce fertilization envelope formation (1987), an extreme degree of exocytosis was observed when eggs were treated with DMSO doses exceeding 25% (v/v) (Figure 5). Therefore the observed decrease in fertilization envelopes and increased frequency of highly exocytosed eggs at calcium ionophore doses exceeding 25 μM can probably be attributed to the increased DMSO concentration. Aggregation of eggs and sperm under high calcium ionophore doses can also most probably be attributed to an intolerable DMSO concentration.

Calcium ionophore treated and untreated egg and sperm combinations were compared after both 5 minute and overnight incubation to differentiate between fertilization and ionophore induced fertilization envelopes by the presence of cleavage (Table 3). The lack of observed cleavage and deteriorated appearance of the eggs, however, suggests a problem with the experimental protocol. The ragged appearance of the eggs suggests that the overnight conditions were not favorable for cleavage; though the temperature was assumed to remain relatively constant, it was not monitored and, therefore, could be an unseen cause for deterioration. Eggs in the control environment showed intact fertilization envelopes (Table 5), which suggests that either the presence of calcium ionophore or DMSO in the seawater could result in deterioration.

The lack of observed cleavage following overnight incubation does, however, suggest that the rate of action of calcium ionophore A23187 exceeds the rate at which sperm are capable of successfully fertilizing the eggs. In the absence of calcium ionophore, the purpose of the generation of the fertilization envelope is to prevent polyspermy; the fusion of sperm to the egg and the release of the sperm's acrosomal contents results in the exocytosis of the cortical granules, which when mixed with the vitelline layer generate an internally favored calcium ion gradient and osmotic pressure that results in an influx of water and lifting of the membrane (Matese et al 1997). Regardless of the initiation of the alteration in the calcium gradient resulting in fertilization envelope formation, the lack of cleavage confirms the ability of the fertilization envelope to block polyspermy.

These experiments demonstrate the dependence of the fertilization process on microfilament generation and the changes in internal calcium concentration. The inhibition of fertilization by cytochalasin confirmed the anticipated dependence on microfilament generation, while the ability of calcium ionophore A23187 to induce fertilization membrane formation by increased permeability to calcium confirmed the importance of calcium as a signaling mechanism for exocytosis.

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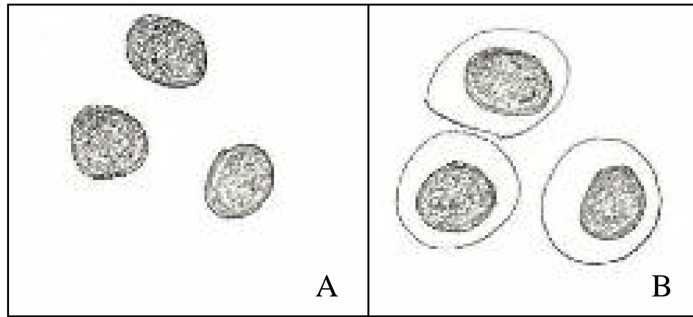

Figure 1. Diagram illustrating the contrast between unfertilized sea urchin eggs (A) and fertilized sea urchin eggs (B) with visible fertilization envelopes resulting from an approximately 5 minute room temperature incubation with a diluted sperm suspension. Eggs are approximately 0.5mm in diameter.

Table 1. The effect of cytochalasin on the cortical reaction of sea urchin, *Strongylocentrotus purpuratus*, during fertilization. Sperm and eggs suspensions were treated with 5 μg/ml cytochalasin, either prior to or following egg-sperm combination. Percent fertilization was determined by multiple counts of each suspension.

Cytochalasin Treatment	Percent Fertilization
Control	60
Treated Sperm	0.0
Treated Egg	0.0
Treated Egg and Sperm Mixture	2.9

Table 2. The effect of calcium ionophore A23187 on the cortical reaction of sea urchin, *Strongylocentrotus purpuratus*, during fertilization. Sperm and eggs suspensions were treated with 20 μM calcium ionophore, either prior to or following egg-sperm combination. Treated eggs were also observed without sperm addition. Percent observed fertilization was determined by multiple counts of each suspension.

Calcium Ionophore Treatment	Percent Fertilization
Control	90
Treated Sperm	98
Treated Egg	97
Treated Egg and Sperm Mixture	91
Treated Egg w/o Sperm Addition	73

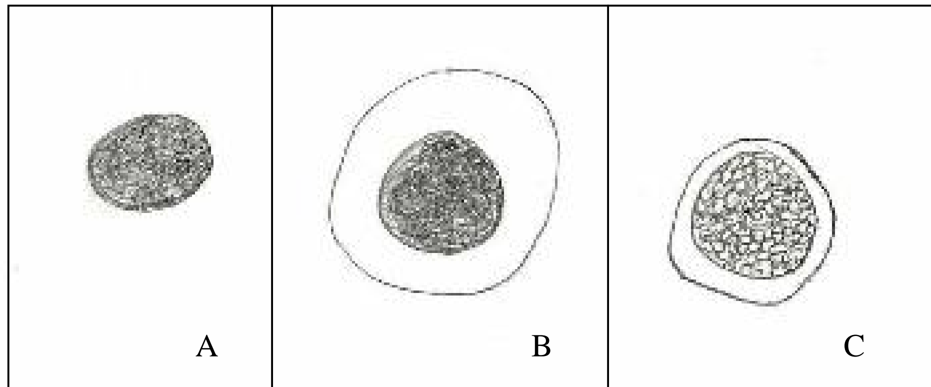

Figure 2. Diagram illustrating the contrast between unfertilized sea urchin eggs (A), eggs with visible fertilization envelopes (B) and highly exocytosed eggs (C) resulting from an approximately 5 minute room temperature incubation with a diluted sperm suspension and treatment with calcium ionophore A23187 in doses ranging from 2.5-100 µM. Eggs are approximately 0.5mm in diameter.

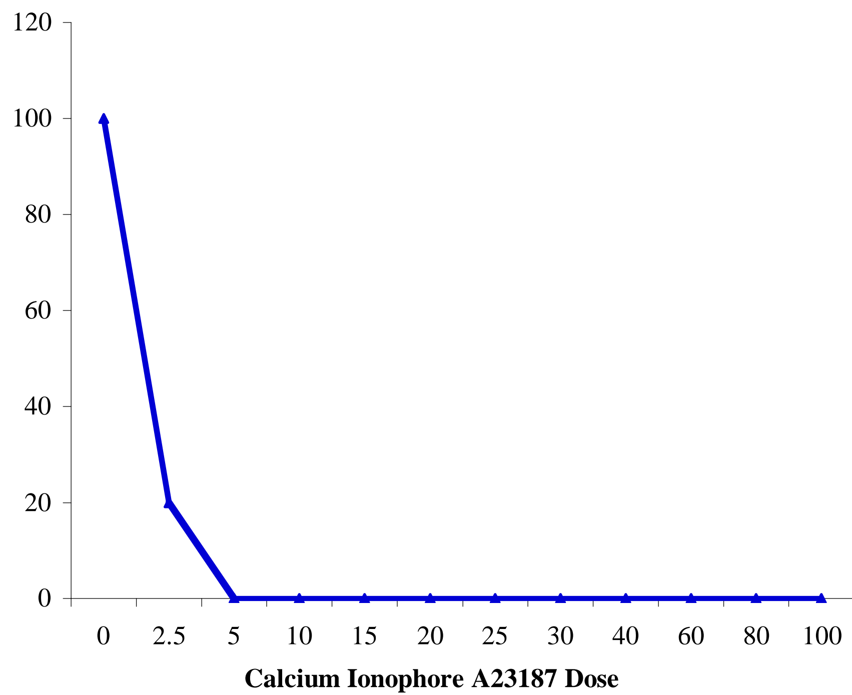

Figure 3. The dose dependant effect of calcium ionophore A23187 on sea urchin sperm. Sperm was diluted 1:100. Percent relative activity was determined by estimation at 10X magnification.

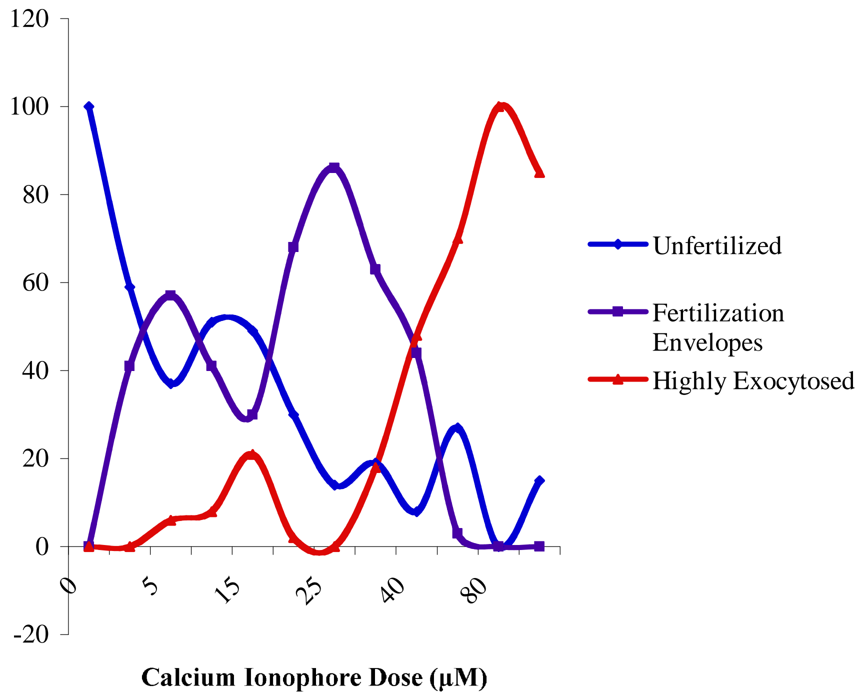

Figure 4. The effect of calcium ionophore dose variation on sea urchin egg morphology. Percents unfertilizatied, fertilization envelopes and highly excytosed eggs were determined by multiple counts.

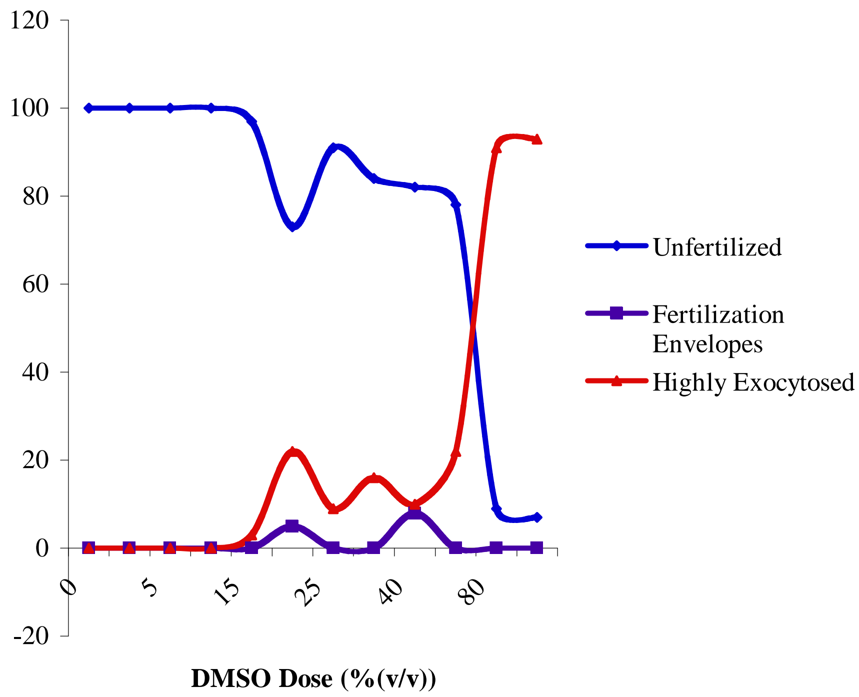

Figure 5. The effect of dimethyl sulfoxide (DMSO) dose variation on sea urchin egg morphology. Percents unfertilizatied, fertilization envelopes and highly excytosed eggs were determined by multiple counts.

Table 3. Comparison between fertilization envelopes induced by egg-sperm interaction and calcium ionophore A23187 in sea urchins, *Strongylocentrotus purpuratus*. Sperm and eggs suspensions were treated with 2.5 µM calcium ionophore, either prior to or following egg-sperm combination. Percent observed fertilization was determined by multiple counts of each suspension after a 5-minute room temperature incubation and again after an overnight room temperature incubation. No cleavage was observed

Calcium Ionophore Treatment	Percent Fertilization Envelopes	
	5 Minutes	Next Day
Control	13	13
2.5 µM Treated Sperm	90	0
2.5 µM Treated Egg	91	1
2.5 µM Treated Egg and Sperm Mixture	85	0