

## **Zebrafish embryos can activate caspase-3 prior to the maternal-zygotic transition**

Javier F. Negrón# and Richard A. Lockshin\*

Department of Biological Sciences, St. John's University, Jamaica, New York, 11439,  
USA

#Current address: Skirball Institute of Biomolecular Medicine, New York University  
School of Medicine, New York, New York 10016, USA

\*Corresponding author: tel: 718-990-1854; fax: 718-990-5958; email:  
lockshin@stjohns.edu

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**Abstract:**

Several researchers have noted that developing non-mammalian eggs neither show apoptosis nor can be induced to undergo apoptosis before the maternal-zygotic transition (MZT). We confirmed that this situation applies to the zebrafish. When pre-MZT zebrafish embryos are exposed to cycloheximide or other toxins, they undergo necrosis. They show none of the cytoplasmic or nuclear morphological changes associated with apoptosis; the cells are negative for TUNEL staining and exteriorization of phosphatidylserine; and one cannot demonstrate DNA ladders from cells from these dying embryos. We observed that the mitochondria of these cells depolarize four hrs before the cells lyse and therefore examined the question of activation of caspase 3 in the embryos. We found that caspase 3 is activated in unfertilized eggs approximately 6 hrs after activation and in all fertilized eggs—pre- and post-MZT—approximately 4 hrs after exposure to toxins. Post-MZT eggs undergo apoptosis approximately 2 hrs later. In contrast, pre-MZT eggs lyse within 30 min after the activation of caspase 3. We interpret these results to indicate that pre-MZT eggs may be have the capability of undergoing apoptosis but in the experimental situation lyse before they can do so. There are several possible explanations for the higher vulnerability of the pre-MZT eggs, which we are currently investigating.

## INTRODUCTION

Oocytes and unfertilized eggs can undergo apoptosis, as can cells from embryos that have passed the maternal-zygotic transition (MZT, the period during which the zygotic genome becomes the primary or only source of mRNA in the embryo). However, many authors have argued that cells of pre-MZT embryos other than mammals cannot undergo apoptosis, thus presenting a conundrum and raising many questions (Hensey and Gautier 1999; Hensey and Gautier 1995; Mizoguchi et al. 2000; Yüce and Sadler 2001). Some authors have argued that these young embryos, generally those before the 8<sup>th</sup>-10<sup>th</sup> division (256-1024 cells) are specifically inhibited from undergoing apoptosis, while others consider that the machinery for apoptosis is immature, thus raising the question of why it was lost after fertilization.

We chose to examine the question by exploring the ability of the embryo of the zebrafish *Danio rerio* to undergo apoptosis. As is reported below, we find that embryos between 2 and 256 cells, challenged with toxins, indeed do not undergo apoptosis (as defined by numerous criteria) but instead undergo necrosis. However they activate caspase 3 with kinetics essentially identical to that of older embryos; the difference appears to be that the cells of the younger embryos lyse very shortly after caspase 3 is activated, whereas cells from older embryos persist another two hours after caspase activation and presumably have the time to manifest all the characteristics of apoptosis. We therefore suggest that the difference between pre- and post-MZT embryos is one either in the ability to access or utilize energy, or in the level of protection afforded to specific molecules that define the vulnerability of the cell.

## RESULTS

### Kinetics of survival and death of unfertilized eggs

All unfertilized eggs eventually die, and the zebrafish egg is no exception. Once the eggs are activated by contact with fresh water they can survive approximately 8 hours, but start deteriorating by the 3<sup>rd</sup> hour post activation. Deterioration begins with a patch of cytoplasmic darkening, presumably representing localized denaturation of proteins. This darkening continues to expand until by the 7<sup>th</sup> hour it encompasses the entire cytoplasmic compartment and by 8 hrs (when they should be gastrulating) all unfertilized eggs had ruptured (Fig. 1).

#### [Fig. 1]

Since unfertilized eggs possess only one haploid nucleus, nuclear markers of apoptosis are hard to define, but since the death of an unfertilized egg is biological and precisely timed it is by definition programmed, and some researchers have considered it to be apoptotic (Yüce and Sadler 2001). We therefore evaluated the activity of caspase 3 in unfertilized eggs.

Caspase 3 enzymatic activity is first detected at 5 h and reaches half-maximal activity by 6 h, at which time half the eggs have also lysed (Fig 6). By the criterion of activation of an effector caspase unfertilized zebrafish eggs, like those of other animals, can undergo apoptosis.

### Lack of evidence for apoptosis in pre-MZT embryos

Unfertilized eggs die spontaneously but fertilized eggs do not. Therefore to examine apoptosis in fertilized eggs we exposed them to toxicants such as CHX, ethanol, and staurosporine. If such drugs are applied to embryos of many species after

the embryos have passed the MZT, the embryos undergo apoptosis as defined by all criteria normally associated with apoptosis—margination of chromatin and blebbing and fragmentation of nuclei and cells, internucleosomal degradation of DNA and positive TUNEL assay, activation of caspase 3, exteriorization of phosphatidylserine, and depolarization of mitochondria(Negrón and Lockshin 2004). In contrast, as has been reported for many embryos, embryos exposed to these same toxicants prior to the MZT do not undergo apoptosis. We therefore examined the effect of staurosporine and CHX on pre-MZT zebrafish embryos.

Although germ-ring embryos quickly become apoptotic by morphological criteria as established by either light microscopy (Fig. 2) or electron microscopy (Fig. 3), 64-cell embryos do not, even immediately prior to their lysis. Sixty-four cell embryos, even when examined within 30 minutes of predicted lysis, do not show the condensation of chromatin or blebbing of nuclei that is characteristic of apoptosis (Figs. 2 and 3). At most, an occasional nucleus will show micronuclei (not shown). Micronuclei are considered to be an early form of apoptosis(Negrón and Lockshin 2004). Similarly, the 64-cell embryo is negative for acridine orange staining, used by many researchers to identify apoptotic cells (Fig 4, top).

#### **[Figs. 2-4]**

The 64-cell embryo also displays no evidence of the internucleosomal cleavage of DNA that is characteristic of apoptosis. It is negative for TUNEL labeling (Fig. 4, bottom). TUNEL labeling indicates an abundance of 3' free ends that have not escaped the nucleus—necrotic cells either do not degrade DNA this rapidly, or the small DNA fragments are readily lost from a permeable nucleus, and the cells are most commonly

TUNEL negative. When DNA is isolated for electrophoresis, as noted previously germ ring embryos readily generate fragmented DNA (Fig. 5, lower) but 64-cell embryos do not, even at the time at which they lyse (Fig. 5, upper).

**[Fig. 5]**

Most of the changes that are considered to be hallmarks of apoptosis are directly or indirectly derived from activation of caspase 3. In post-MZT zebrafish embryos this argument likewise appears to be valid, in that caspase 3 activation is seen before permeabilization of the cell and nuclear membranes, condensation of chromatin, or blebbing or fragmentation of nuclei and cells, as we have previously reported (see Fig. 5 in (Negrón and Lockshin 2004). However, when pre-MZT embryos were exposed to these same toxins, the cells showed no shrinkage or blebbing; they were negative for propidium iodide, indicating integrity of the cell membrane, and nuclear morphology by Hoechst 3340 was non-apoptotic).

These results would conventionally be interpreted as indicating a lack of activation of caspase 3. However, as noted above (Fig. 6) the enzyme can be activated in unfertilized eggs. Because of these results, we examined caspase 3 activity at closely-stepped intervals until the embryos lysed. We were startled to realize that caspase 3 is indeed activated in pre-MZT embryos, albeit much closer to the time of lysis than in post-MZT embryos. Caspase 3 is activated between 3.6 and 4.2 h after beginning of exposure in both 64-cell and germ-ring embryos, and a bit later (6.2 h after activation) in unfertilized eggs (Fig. 6). Thus apoptosis—as defined by the activation of caspase 3—can be activated in pre-MZT embryos. The difference between pre- and

post-MZT embryos is that the pre-MZT embryos lyse almost immediately after activation of caspase 3.

### **[Fig. 6]**

Intrinsic-pathway apoptosis is triggered by mitochondrial depolarization and loss of cytochrome c to the cytoplasm, initiating the formation of the apoptosome and activation of caspase 9. We attempted to assess this parameter by using DePsipher®, which measures  $\Delta\psi_m$ . We found that germ-ring embryos retained mitochondrial potential for at least 4 h after beginning of exposure, whereas the mitochondria of 64-cell embryos had depolarized by 4 h (Fig. 7), at which time the embryos were beginning to die (Fig. 8). Lifting off of the embryo occurs approximately 1 h before it does in germ-ring embryos (Negrón and Lockshin 2004).

### **[Figs. 7-8]**

Thus the mitochondria of the 64-cell embryo depolarize earlier, and the embryo dies a little earlier, than the germ-ring embryo. We quantified these results by direct count of the status of the embryos (Fig. 6). The 64-cell embryo is unable to survive as long as the germ-ring embryo. In physiological terms, the difference is substantial: The time to 50% death of 64-cell embryos after exposure to CHX is 4.2 h while that for post-MZT embryos is 6.6 h, a 37% difference in ability to survive.

## **DISCUSSION**

Why do we not see apoptosis in the pre-MZT embryos? An explanation may lie in the dynamics of the fate of these fresh-water embryos. Judging from half-maximal values of caspase activation and survival, germ-ring stage embryos survive 2.4 h after

the activation of caspase 3, whereas unfertilized eggs survive 0.4 h and 64-cell embryos survive 0.6 h (Fig. 8). We therefore suggest that the difference in modes of death derives not from an inhibition of apoptosis but from the failure of the pre-MZT embryo to survive once caspase 3 has been activated. (Since the unfertilized egg has only one haploid nucleus, it is difficult to establish criteria for apoptosis other than the activation of caspase 3.) The egg exists in an environment of fresh water, as does that of *Xenopus*, and it is clear that a very large portion of its energy consumption consists of maintaining ionic and osmotic balance as it matures. The difference between the post-MZT embryo and the pre-MZT embryo and unfertilized egg could be explained by the development of an increased ability to generate energy to maintain ionic pumps or increased efficiency of these pumps. In this regard it is noteworthy that by 4 h the mitochondria of 64-cell embryos are depolarized while those of germ-ring stage embryos are not. Alternatively, it is formally possible that prior to the MZT activated caspase 3 promptly attacks an Achilles' heel in the embryo, for instance a membrane protein, pore protein, or constituent of the energy-generating machinery. This damage would lead to prompt failure of the cell. According to this argument the MZT establishes protection for the Achilles' heel protein, by sequestering or shielding it or creating further, less vital, substrates that compete successfully for the protease. We are currently investigating the energy resources available to the embryos and their ability to maintain energy generation when challenged, and we are attempting to identify endogenous substrates for caspase 3. The first hypothesis is less demanding of special arrangements. Until we find differently, we hypothesize that the inability of pre-MZT

embryos to undergo apoptosis is best explained by their inability to survive long enough for the several consequences of activation of caspase 3 to be manifest.

## EXPERIMENTAL PROCEDURES

Wild type zebrafish embryos (*Danio rerio*) were used for all experiments. Aquaculture methods and staging were standard (Kimmel et al. 1995; Westerfield 1998). Fish were maintained at 14L:10D. Each 40L tank held 30 fish at 28.5°C, and pH 7.0. The ratio of females to males was maintained at 2 to 1 for optimal embryo production. To prevent scavenging of the eggs, 13 mm glass marbles were added to the tank bottoms. Fertilized eggs were collected using a gravity-driven siphon. Siphoned tank water was discarded and replaced with fresh distilled water supplemented with 600 mg/L sea salts. Collected embryos were maintained in embryo rearing medium at 28.5°C (ERM, (Westerfield 1998)). At the germ-ring stage, embryos were isolated from the general population and grouped according to stage of development in fresh ERM. Cultured embryos were viewed with the Olympus SZH10 Research Stereo Microscope and photographed with a NEC NC-15 CCD Color Camera at hourly intervals. All photographs were taken at 52X magnification.

To provoke apoptosis, embryos were placed in ERM containing cycloheximide (CHX, 100µg/ml, Sigma-Aldrich) as noted earlier (Negrón and Lockshin 2004). The embryos were held for up to 8 h, and were either immediately processed after treatment, or groups of 15, 30, or 60 embryos were collected, snap frozen in crushed dry ice, and stored at -80°C.

Embryos were evaluated by several techniques as described in (Negrón and Lockshin 2004) and below. They were stained with acridine orange, propidium iodide, or Hoechst 3340, at a final concentration of 1 µg/ml. The genomic DNA extraction procedure was adapted from an established protocol (Gavrieli et al. 1992). The TUNEL (terminal

deoxynucleotidyl transferase (TdT)-mediated dNTP-fluorescein nick end labeling) assay was adapted from established protocols (Gavrieli et al. 1992; Chan and Yager 1998; Yager et al. 1997; Negrón and Lockshin 2004), using the Fluorescein-FragEL™ DNA FRAGMENTATION DETECTION KIT (Oncogene Research Products, San Diego, CA). The fluorometric enzyme assay for caspase-3 activity was an adaptation from the protocol of Mirkes *et al.* (Mirkes and Little 2000), based on the caspase-3 fluorogenic substrate (DEVD-AFC from Bachem). Fluorescence readings were taken every 15 min, using a 405nm excitation filter and 515nm emission filter. The readings were taken to 75 min and since the responses were very linear, the 60 min reading was plotted. For measurements of living cells, animal pole cells were placed into 100  $\mu$ l ERM with 5  $\mu$ l of 5mg/ml Ac-DEVD-AFC, 1  $\mu$ l of 100  $\mu$ g/ml propidium iodide, and 1  $\mu$ l of 100  $\mu$ g/ml H 33342, and then incubated at 28.5°C for 60 min in the dark and examined by fluorescence microscopy. Mitochondrial transmembrane potential was evaluated using the DePsipher® (Trevigen, Gaithersburg, MD) solutions as indicated by the manufacturer. Phosphatidylserine localization was assayed with the Vybrant Apoptosis Assay Kit, Alexa Fluor 488 conjugated to annexin V (Molecular Probes, Eugene, OR). For electron microscopy, washed embryos were fixed with fresh 3% EM grade glutaraldehyde (Sigma-Aldrich) (v/v) for 3 h at 4°C, rinsed with TBS, immersed in 1% osmium (Sigma-Aldrich) (v/v) for 1 h at RT, and rinsed again. Embryos were then dehydrated in an acetone series, embedded in Araldite, and cut using a glass knife.

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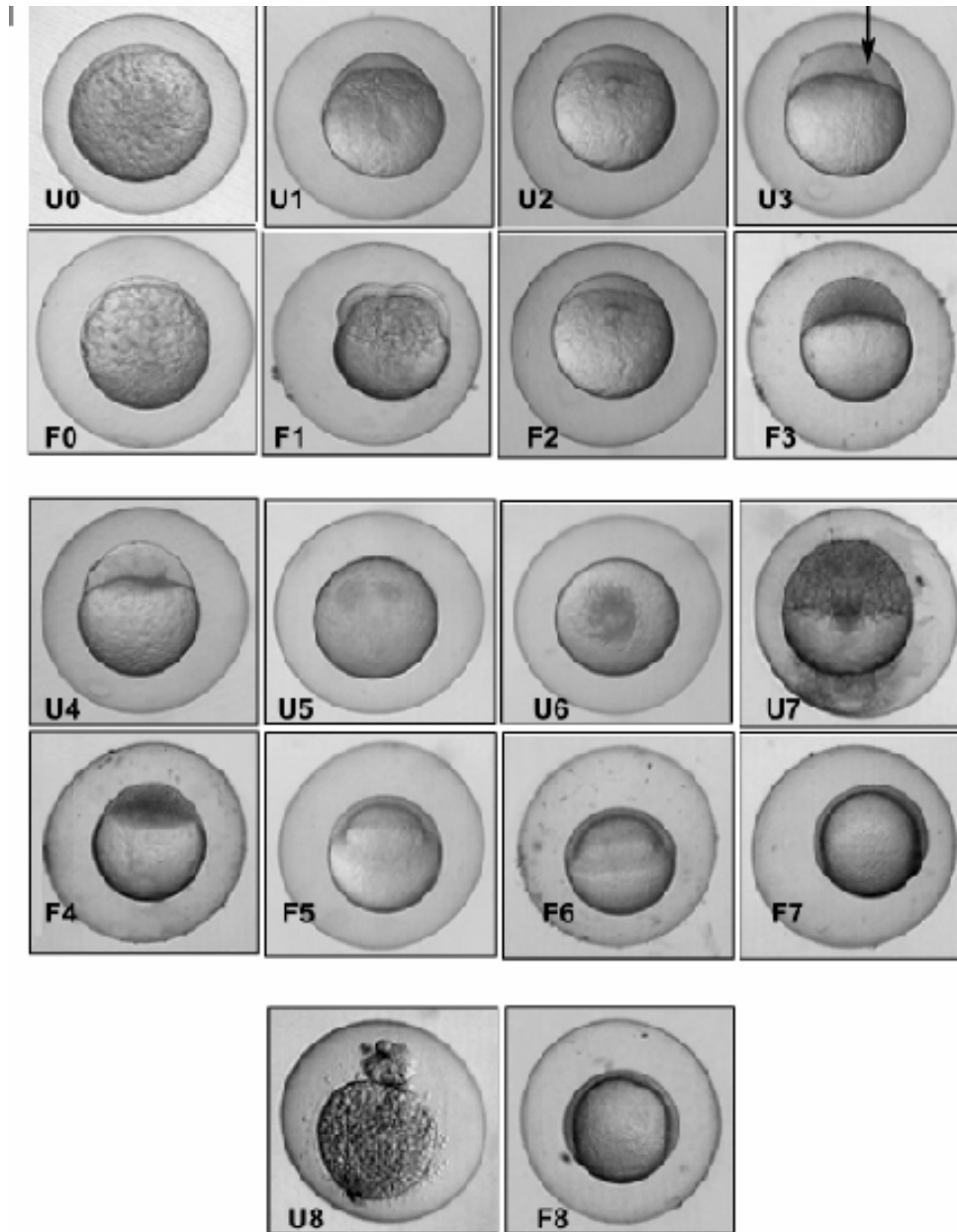


FIG. 1. Death of unfertilized eggs. Unfertilized activated eggs (U—number = hours after activation) undergo cytoplasmic movements similar to those of fertilized eggs (F) but show signs of cytoplasmic deterioration beginning by 3 h (arrow) and are completely lysed by 8 h, at which time fertilized eggs are beginning to segment.

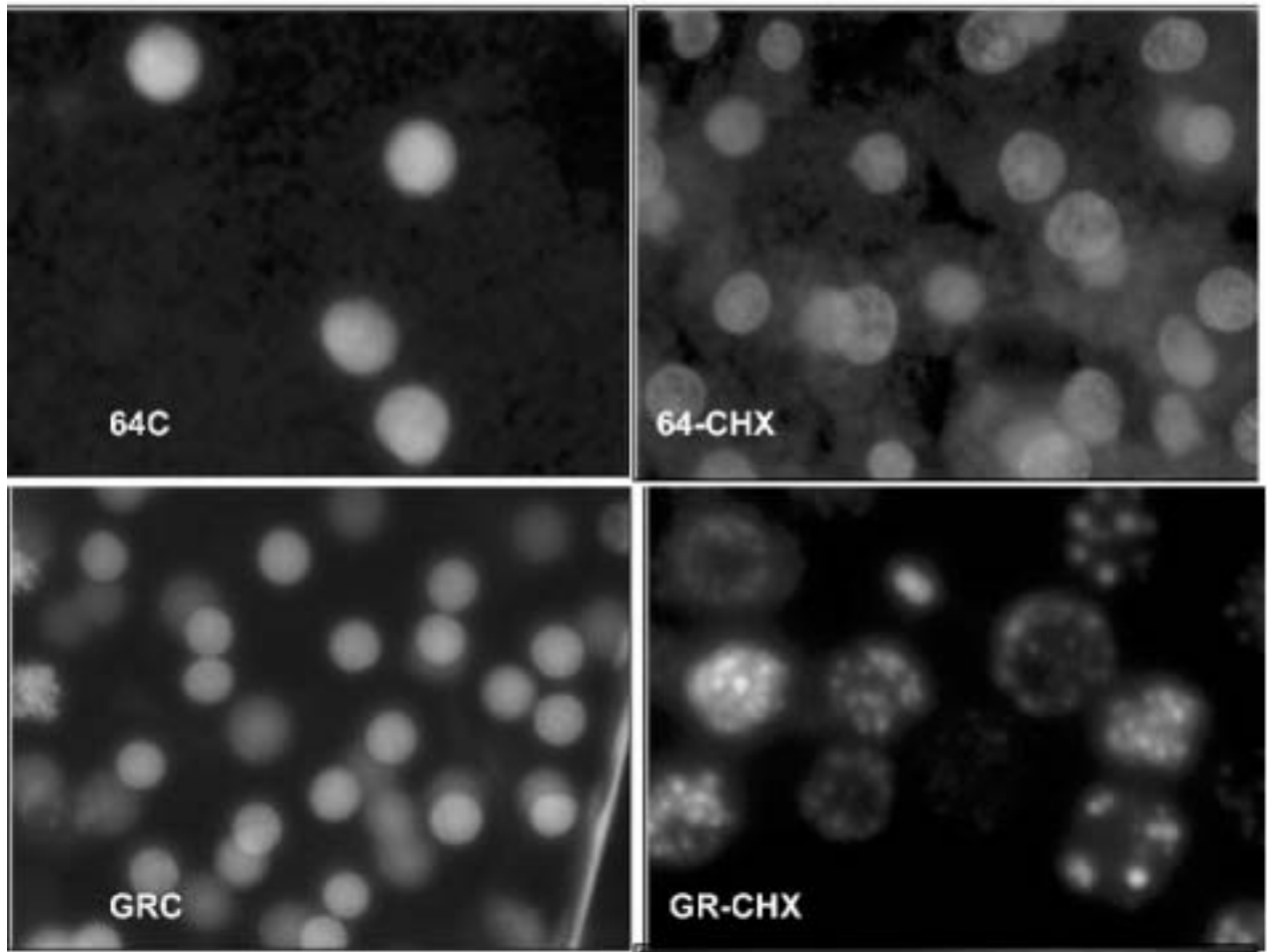


FIG. 2: Lack of apoptosis in 64-cell embryos. Hoechst staining revealing nuclear morphology. All photographs at original magnification of 200X; the cells and hence their nuclei are larger in the younger embryo. Top: 64-cell embryos. Left: control; Right: immediately prior to lysis (4 h exposure; see text). Nuclei show some abnormalities associated with CHX toxicity, but show neither the condensation and margination of chromatin nor the nuclear fragmentation common to apoptosis. Bottom: Germ ring embryos. Left: control; Right: 4 h. These nuclei clearly show chromatin condensation and nuclear fragmentation.

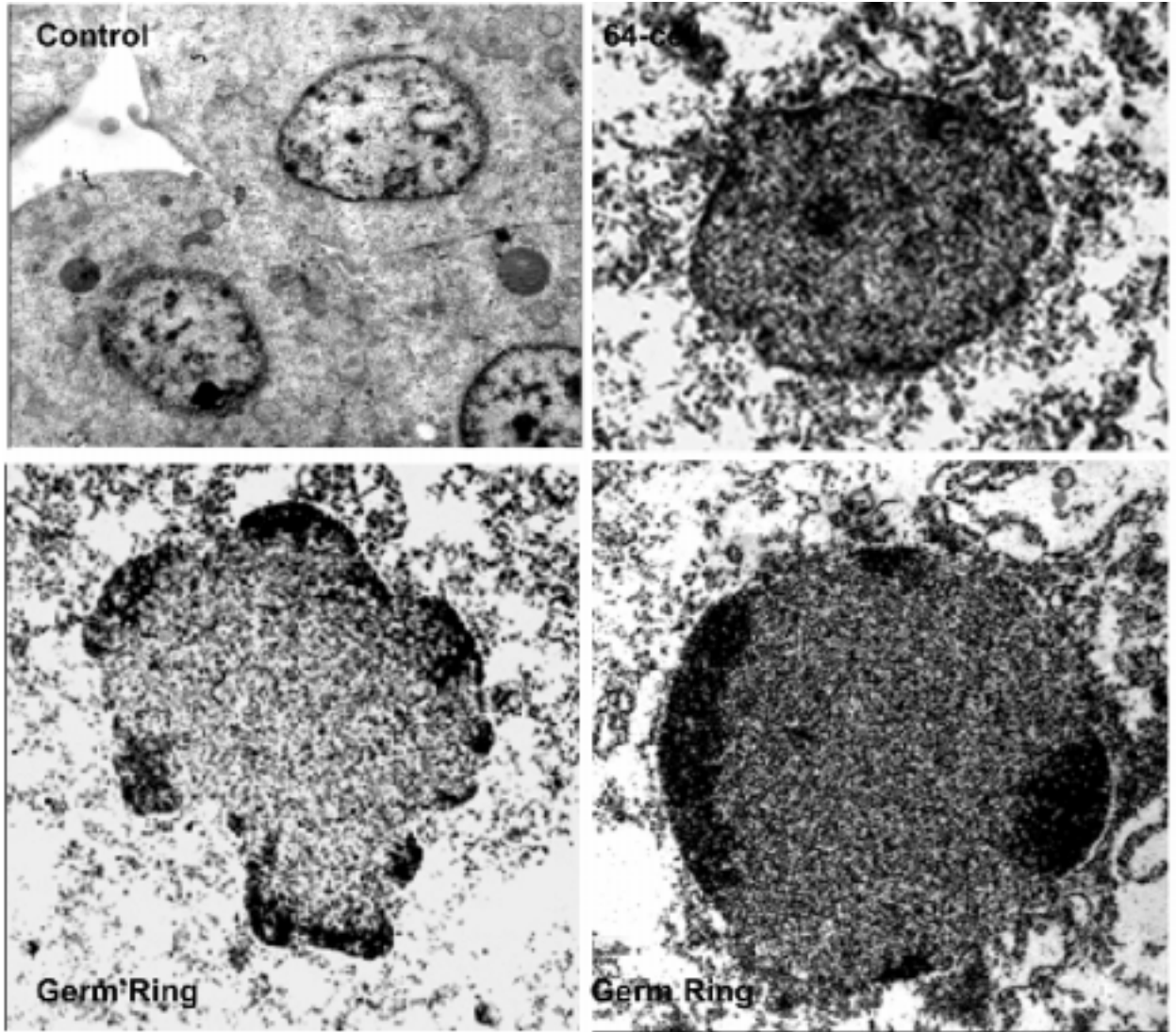


FIG. 3. Lack of apoptosis in 64-cell embryos. Nuclei of animal pole cells from (Top Left): Control; (Top Right): 64-cell embryo, immediately before lysis. The nucleus is denser but shows none of the morphological characteristics of apoptosis. (Bottom): Germ-ring embryos, shortly before lysis. In contrast to the younger embryo, these nuclei show obvious margination and coalescence of chromatin, and the nucleus on the left is beginning to bleb.

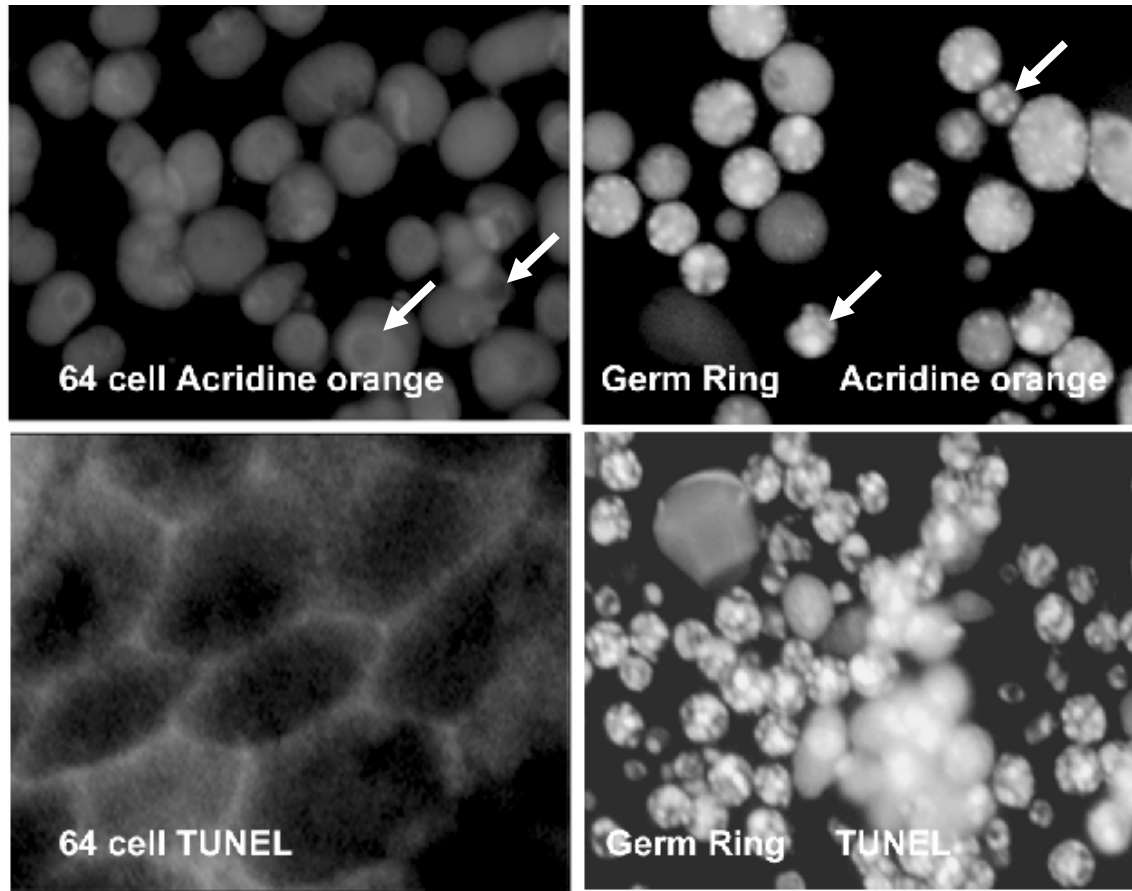


FIG. 4. Lack of evidence for apoptosis in 64-cell embryos, examined just prior to lysis. Top: Acridine orange staining. The 64-cell embryo (Left) shows poor penetration of AO, indicating relatively intact cell membranes, and no penetration into the nucleus (arrows), indicating intact nuclear membrane and no condensation of chromatin. In contrast, the germ ring embryo (Right) shows penetration of AO into the nucleus, nuclear fragmentation (arrows), and chromatin condensation, all characteristic of apoptosis. Likewise, the 64-cell embryo is negative for TUNEL staining (Lower Left) while the germ-ring embryo is uniformly positive for TUNEL staining (Lower Right).

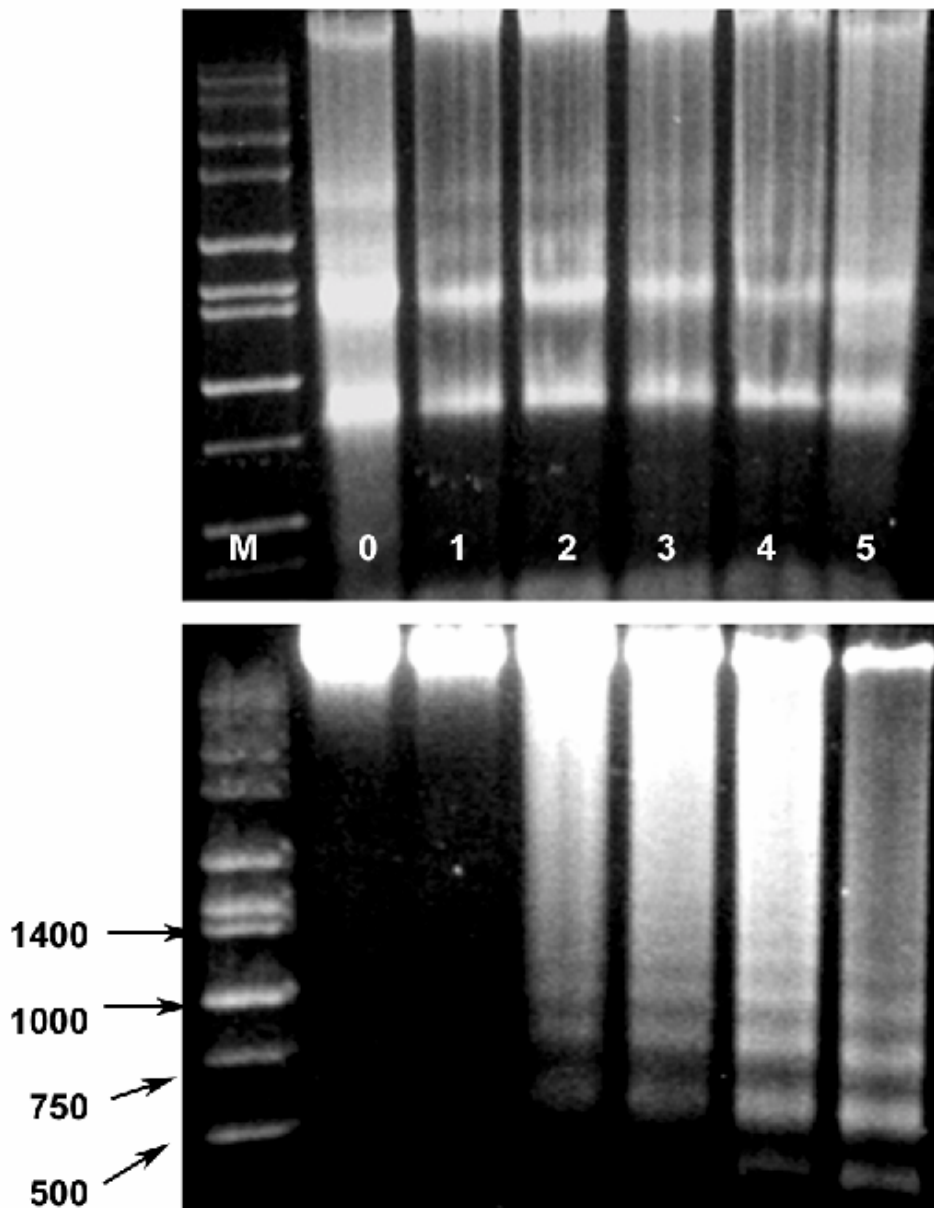


FIG. 5. A. DNA ladder developing in cells from germ-ring embryos after exposure to CHX. Numbers indicate hours from beginning of exposure. Upper: 64-cell embryos. Lower: 1000-cell embryos. In the germ-ring embryos, ladders are seen within 2 h and increase until the embryos lyse at 8 h. In the 64-cell embryos, no ladders are seen. The only relatively low molecular weight staining seen is contaminant ribosomal RNA.

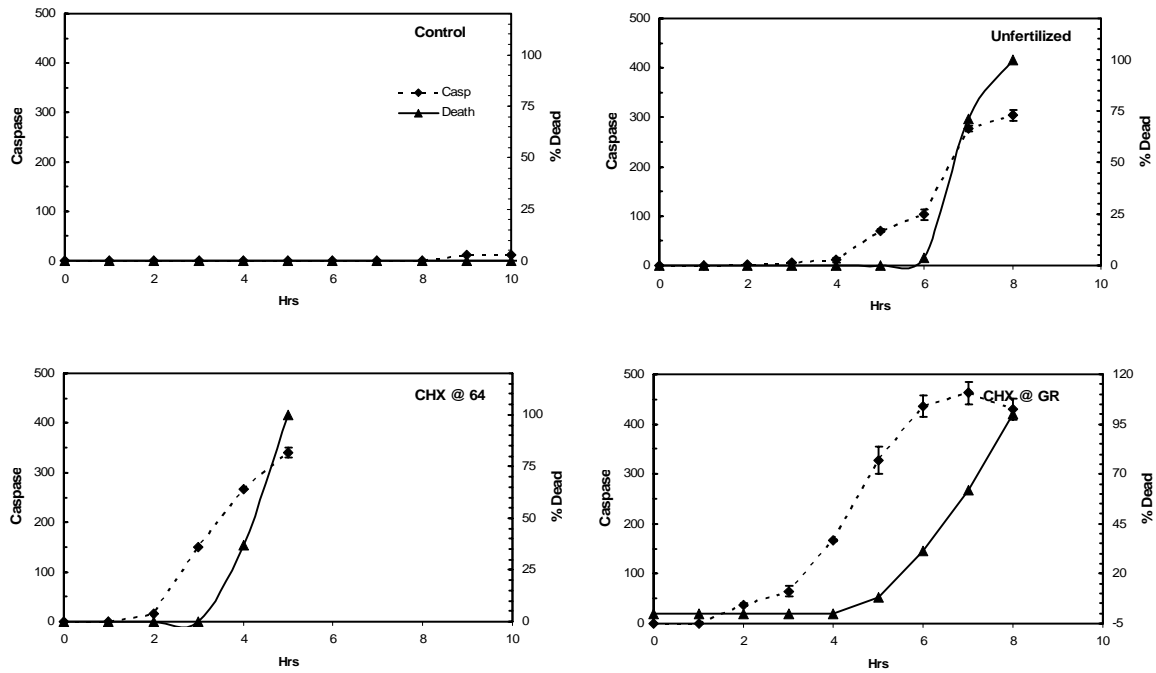


FIG. 6. Relative timing of activation of caspase 3 and death of embryos. Percent death is calculated from at least three pools of at least 50 embryos for each experiment. Caspase activity is measured in fluorescence units as described in Methods. In unfertilized eggs and 64-cell embryos, death follows relatively quickly the activation of caspase 3. Germ-ring embryos survive more than 2 h after the activation of caspase-3.

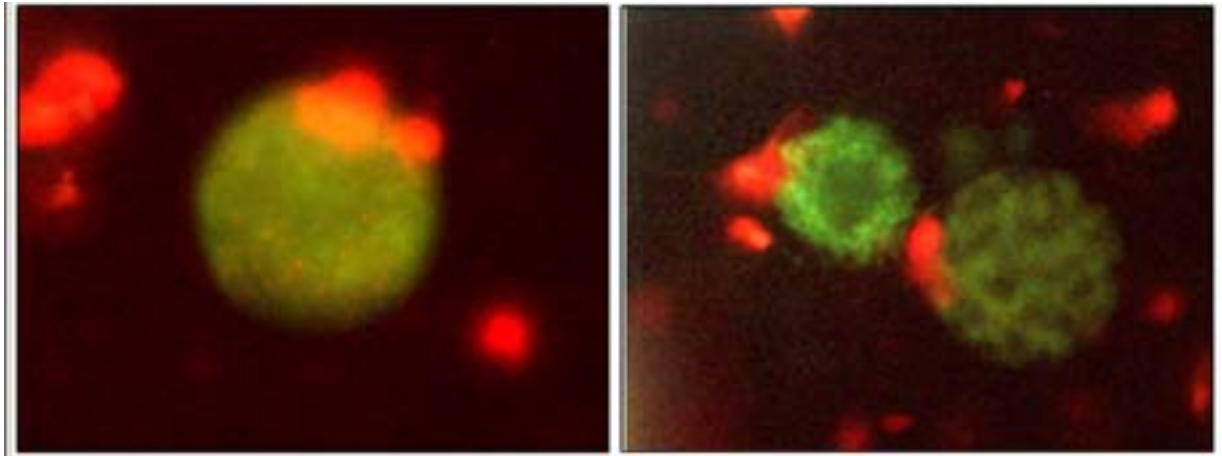


FIG. 7. Mitochondrial transmembrane potential in cycloheximide treated 64-cell stage blastomeres and germ-ring stage embryos, 4 h after beginning exposure to CHX. All magnifications are originally 1000X. The green fluorescence indicates that the dye monomer has penetrated the cells and can be retained, as the membrane is intact. The red punctuate fluorescence in the control embryo (left) indicates dye polymerization indicative of functioning mitochondria. Large red fluorescence is excess crystals of dye. Cells from control 64-cell embryos have many mitochondria with high resting potential, though fewer per-unit-cytoplasm than cells from older embryos. (Negrón and Lockshin 2004) Approximately 4 h after exposure to CHX, the mitochondria from 64-cell embryos are depolarized (right), far earlier than depolarization of mitochondria from germ-ring embryos (Negrón and Lockshin 2004).

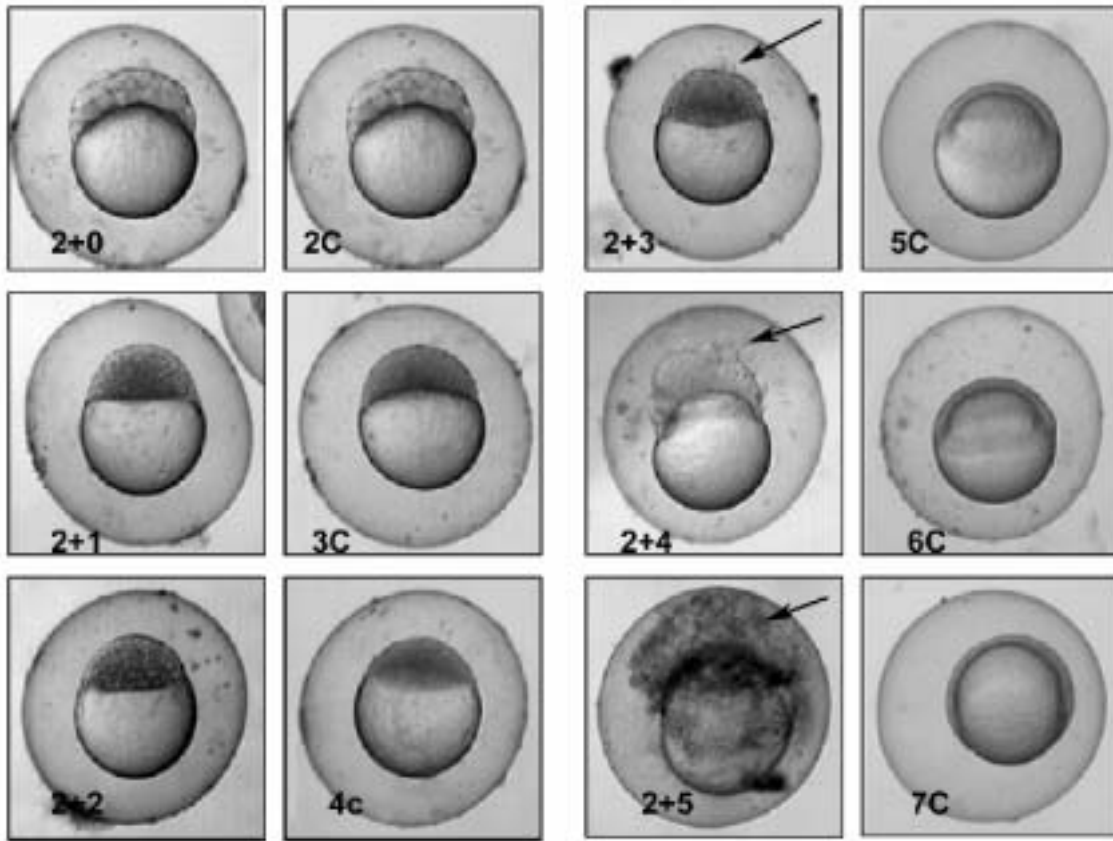


FIG. 8. Fate of CHX- treated 64-cell blastomeres. Numbers indicate age in hours since fertilization (controls, 2c-7c) or cumulative time since beginning of exposure (e.g., 5 h exposure beginning at 2 h = 2+5). All magnifications are 52.5x. Cell cycle arrest is apparent within 1 h; the animal pole begins to contract by 2 h, and the first signs of cell dissociation and shedding are seen by 3 h (arrow in 2+3). The animal pole begins to separate from the vegetal pole by the 4<sup>th</sup> h (arrow in 2+4), and all blastomere cells lyse by the 5<sup>th</sup> h (arrow in 2+5).

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