ToxSci Advance Access published May 28, 2003

Thimerosal induces DNA breaks, caspase-3 activation, membrane damage, and cell death in cultured human neurons and fibroblasts.

David S. Baskin, Hop Ngo, Vladimir V. Didenko

Department of Neurosurgery, Baylor College of Medicine

6560 Fannin Suite 944, Houston, Texas 77030

and Veterans Affairs Medical Center, Houston, TX, 77030

Corresponding Author: David S. Baskin, M.D., F.A.C.S.

Department of Neurosurgery, Baylor College of Medicine

6560 Fannin Suite 944, Houston, Texas 77030

(713) 798-4696 (telephone)

(713) 798-3227 (facsimile)

dbaskin@tmh.tmc.edu (email)

Short Title: Toxicity of thimerosal

Abstract

Thimerosal is an organic mercurial compound used as a preservative in biomedical preparations. Little is known about reactions of human neuronal and skin cells to its micro- and nanomolar concentrations, which can occur after using thimerosal-containing products.

A useful combination of fluorescent techniques for the assessment of thimerosal toxicity is introduced. Short-term thimerosal toxicity was investigated in cultured human cerebral cortical neurons and in normal human fibroblasts. Cells were incubated with 125nM - 250µM concentrations of thimerosal for 45 minutes to 24 hours. 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) dye exclusion test was used to identify nonviable cells, and TUNEL to label DNA damage. Detection of active caspase-3 was performed in live cell cultures using a cell permeable fluorescent caspase inhibitor. The morphology of fluorescently labeled nuclei was analyzed.

After 6 hours of incubation, thimerosal toxicity was observed at 2µM based on manual detection of fluorescent attached cells and at 1µM level with the more sensitive GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence. The lower limit did not change after 24-hour incubation. Cortical neurons demonstrated higher sensitivity to thimerosal compared to fibroblasts. The first sign of toxicity was an increase in membrane permeability to DAPI after 2 hours of incubation with 250µM thimerosal. 6-hour incubation resulted in failure to exclude DAPI, generation of DNA breaks, caspase-3 activation, and development of morphological signs of apoptosis.

We demonstrate that thimerosal in micromolar concentrations rapidly induce membrane and DNA damage, and initiate caspase-3 dependent apoptosis in human neurons and fibroblasts. We conclude that a proposed combination of fluorescent techniques can be useful in analyzing the toxicity of thimerosal.

Key Words: thimerosal, active caspase-3, apoptosis, toxicity, neurons, fibroblasts, DNA breaks, membrane damage, DAPI

Introduction

Thimerosal (sodium ethylmercury-thiosalicylate) is an antibacterial and antifungal mercurial compound used as a preservative in biological products and vaccines, in concentrations ranging from 0.003% to 0.01% (30-100 µg/mL) (Ball et al., 2001). Thimerosal contains 49.6 % mercury by weight, and releases ethylmercury as a metabolite. In the body, ethylmercury can be converted to inorganic mercury, which then preferentially accumulates in kidneys and brain (Blair et al, 1975). Inorganic mercury is known to induce membrane and DNA damage (Ferrat et al., 2002; Ben-Ozer et al., 2000), and in cell culture conditions was shown to be mutagenic and generate DNA breaks in concentrations below 500 nM (Schurz et al., 2000). Ethylmercury can significantly increase concentration of inorganic mercury in many organs (Magos et al., 1985). After *in vivo* administration, ethylmercury passes through cellular membranes and concentrates in cells in vital organs including brain, where it releases inorganic mercury, raising its concentrations higher than equimolar doses of its close and highly toxic relative methylmercury (Magos et al., 1985).

However, little is known about acute reactions of various types of human cells following short-time exposure to thimerosal in micro- and nanomolar concentrations.

In this paper, we used a convenient and easily reproducible combination of fluorescent techniques analyzing various markers of DNA and membrane damage, and investigated the toxicity of micromolar and nanomolar concentrations of thimerosal (125nM - 250µM) occurring in the first 24 hours of exposure in cultures of human cortical

neuronal cells and in human fibroblasts.

We found that thimerosal in micromolar concentrations rapidly decreased cellular viability. Within several hours after thimerosal administration, cells lost their capability to exclude the fluorescent dye DAPI, and developed multiple DNA breaks accompanied by caspase-3 activation and apoptotic morphology. Neuronal cell cultures demonstrated higher sensitivity to thimerosal compared with fibroblasts.

Methods

Cell cultures.

HCN-1A Human cerebral cortical neurons (CRL-10442) were purchased from American Type Culture Collection (ATCC, Manassas, VA), and were cultured according to ATCC recommendations. The line was derived from cortical tissue removed from a patient undergoing hemispherectomy for intractable seizures. As recommended by ATCC, the cells were grown in Dulbecco's Modified Eagle's Medium with 4mM L-glutamine, modified to contain 4.5g/L glucose and 1.5g/L sodium bicarbonate, supplemented with 10% fetal bovine serum, and pH adjusted to 7.35 prior to filtration.

Normal neonatal human foreskin HCA 2 fibroblasts (PD32) were obtained from the laboratory of Dr. Smith-Pereira, Ph.D. The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum medium, and pH adjusted to 7.4 prior to filtration. For the experiments, all cells were subcultured in 24-well cell culture plates (Fisher, Pittsburgh, PA). All experiments were reproduced in triplicates. Each of the parallel series yielded identical results.

Thimerosal.

Thimerosal (minimum 97% HPLC), SigmaUltra (Sigma, St. Louis, MO) was added to cell cultures in 30µl of double-distilled water to final concentrations of: 250µM, 50µM, 10µM, 2µM, 1µM, 500nM, 250nM, and 125nM. 1µM-125nM concentrations were

used with neuronal cells only. Control cell cultures received 30 µl of water without thimerosal.

Die exclusion test using 4,6-diamidino-2-phenylindole (DAPI).

DAPI is a non-intercalating DNA-specific dye, with an emission maximum in the blue spectrum (Shapiro, 1985). It is widely used for counterstaining of cellular nuclei in fixed sections, and has been demonstrated to be useful for detection of nonviable cells with compromised membranes in live cell cultures (McCarthy and Hale, 1988; Boutonnat et al., 1999).

The DAPI exclusion test was performed as described (Boutonnat et al., 1999). Briefly, cells were incubated with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO), diluted in cell culture medium at a final concentration of 100 ng/mL for 30 min at 20°C (Boutonnat et al., 1999). Fluorescent signal was monitored and representative images were taken at 45 minutes, and 2, 4, 6 and 24 hours after addition of thimerosal, The DAPI incubation started 30 min before each observation was made (at 15 min, 90 min etc). Images were acquired using an Olympus IX-70 fluorescent microscope equipped with a MicroMax digital camera system (Princeton Instruments, Inc.) containing an RTE/CCD-1300-Y/HS array cooled by a Peltier device. Image acquisition was performed using MetaMorph 4.1 program (Advanced Scientific, Inc.). The micrographs were taken at central parts of wells, where cellular density was most uniform.

T*erminal transferase based Nick-End Labeling (TUNEL)*

Cells were fixed in ice cold methanol, and TUNEL staining for detection of DNA breaks was performed using the ApoTaq Fluorescein and ApoTaq Rhodamine kits for indirect immunofluorescence (Serologicals, Gaithersburg, MD), employing the standard technique recommended by the manufacturer. Following washing, cells were counterstained with the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) (1 µg/mL) for visualization of all cellular nuclei, and were mounted in Vectashield (Vector

Laboratories, Burlingame, CA) for observation by fluorescence microscopy.

Caspase-3 detection.

Detection of active caspase-3 in live cell cultures was performed using an APO LOGIX^{TM} carboxyfluorescein (FAM) caspase detection kit (Cell Technology, Minneapolis, MN). The kit detects active caspases in living cells through the use of a FAM labeled DEVD fluoromethyl ketone (FMK) caspase inhibitor, which irreversibly binds to active caspase-3 (Amstad et al., 2000; Bedner et al., 2000; Smolewski et al., 2001). The inhibitor is cell permeable and non cytotoxic. With lesser affinity, FAM-DEVD-FMK binds to the other caspases participating in apoptosis: caspase-8> caspase-7 > caspase-10 > caspase-6 in the order of decreasing binding affinity (Carcia-Calvo et al., 1998).

The kit was used as recommended by the manufacturer. Briefly, 10 µL of 30X Working Dilution FAM-Peptide-FMK was added to 300µL of cell culture medium/per well, directly in 24-wells cell culture plates after 5 hours of incubation with thimerosal. Cells were incubated for 1hour at 37°C under 5% CO_2, protected from light. Then, the medium was carefully removed, and cells were washed twice with 2 mL/per well of 1X Working Dilution Wash Buffer. Fluorescent signal was observed under Olympus IX-70 fluorescent microscope equipped with a MicroMax digital camera system (Princeton Instruments, Inc.) containing an RTE/CCD-1300-Y/HS array cooled by a Peltier device. Caspase positive cells appeared fluorescing green. Representative images were taken at 45 minutes, 2 hours, 4 hours, 6 and 24 hours after addition of thimerosal. Image acquisition was performed using MetaMorph 4.1 program (Advanced Scientific, Inc.). Positive controls included cultures of cortical neurons treated with 0.5µM staurosporin to induce caspase-3 activation. In several series of experiments, we added DAPI to cell cultures to the concentration of 100 ng/mL for 30 min immediately after 1 hour of incubation with FAM-Peptide-FMK solution. This made the co-localization of active caspase-3 and DAPI signals possible.

Fluorescence measurements using microplate reader

In a separate set of experiments, we measured both active caspase-3 and DAPI signals in co-localization experiments using GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence (Tecan Inc., Research Triangle Park, NC). Neuronal cells were incubated with 1- 250 µM concentrations of thimerosal for 6 hours and processed as described for simultaneous DAPI and active caspase-3 detection. Both FITC and DAPI fluorescence were measured directly in 24-well plates using Chroma Technology bandpass filter set: FITC excitation D490/40, emission 520/10; DAPI excitation D360/40, emission 460/20. The reactions were repeated twice, and yielded the same dose-dependent increase in thimerosal toxicity. Background fluorescence was subtracted from the experimental series, and the results were represented as graphs of average values using Microsoft Excel.

Results

Thimerosal-induced changes in membrane permeability and cell viability.

Changes in cell viability rapidly occurred after administration of thimerosal in all cell cultures, and were detected by the loss of ability to exclude the fluorescent dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). DAPI is classified as a semipermeant dye, which requires a relatively short (30-minute) exposure time of cell cultures to the dye prior to the signal observation in a DAPI exclusion test (Boutonnat et al., 1999). Under these conditions, the dye has been shown to be useful for the detection of nonviable cells, and can be utilized as a selective marker of membrane integrity. Indeed, it is a less toxic alternative to propidium iodide (PI) (Boutonnat et al., 1999).

The results of experiments show a dose and time-dependent increase of membrane permeability to DAPI, first detected after 2 hours of incubation with thimerosal, and resulting in penetration of the dye into the nuclei and DNA staining

(Fig.1-2). Figure 1 presents experiments performed on human cultured cortical neurons (HCN-1A), and shows that after 2 hours of incubation with thimerosal at a concentration of 250 µM, DAPI penetrated through cellular membranes and stained cellular nuclei. Inability to exclude the dye indicates loss of cellular membrane integrity and cell death (McCarthy and Hale, 1988; Boutonnat et al., 1999). After 4 hours of incubation, thimerosal-induced membrane permeability and DNA staining was observed at a concentration of 10µM. After 6 hours of incubation with thimerosal, changes in membrane permeability were detected at concentrations as low as 2 µM, based on the appearance of DAPI stained cells, attached to the bottom of the wells. In control cell cultures, which were treated with DAPI alone, only sporadic dead cells were detected, and their numbers stayed the same 2, 4, and 6 hours after addition of DAPI (Fig. 1). There was no change in cell membrane permeability for DAPI for up to 24 hours if no thimerosal was added.

We performed direct counts of DAPI-positive cells for the initial quantitative assessment of our results. We counted all DAPI positive cells in two 40x fields of view for each of thimerosal concentrations after 6 hours of incubation. All counts were taken in central parts of wells, where cellular density was most uniform. The comparison with the average density of cells in these areas revealed that at 2 µM thimerosal, 11% were DAPI positive, at 10 µM thimerosal, 58% were DAPI positive, at 50µM thimerosal, 61% of cells were DAPI positive, and at 250 µM thimerosal, 100% of neurons had compromised cellular membranes. In controls, less than 1% of cells were DAPI positive, due to cell death naturally occurring in cell cultures.

No changes in membrane permeability and DAPI staining were observed with thimerosal concentrations lower than 2µM at times of incubation up to 24 hours.

Since dying cells disattach from the bottom shortly after death and float in the media, they cannot be counted. This explains similar numbers of DAPI positive cells

counted after 10 and 50 µM thimerosal treatments, and could have affected the sensitivity of the lower limit of toxicity measurements. To address this issue and to take into consideration all DAPI stained cells, we used a fluorescent microplate reader, which detects fluorescence of both attached and floating dead cells (*see* Fig.3). Using GENios Plus microplate reader we detected the lower limit of thimerosal toxicity for neuronal cells after 6 hours of incubation to be at 1 µM concentration of thimerosal.

Experiments with cultured human fibroblasts produced similar results, although when compared with neuronal cells, fibroblasts demonstrated slightly lower sensitivity to thimerosal toxicity by the DAPI exclusion test in terms of the number of DAPI-stained cells (Fig 2).

Similar to neuronal cells, significant numbers of DAPI stained nuclei were first observed after 2 hours of incubation with thimerosal at 250 µM concentration in the fibroblast culture experiments (Fig. 2). After 4 hours of incubation, nuclear staining was detected at 10µM concentration of thimerosal. However unlike neuronal cells, human fibroblasts did not show toxicity at 2µM concentration of thimerosal after 6 hours of incubation.

Detection of thimerosal-induced DNA damage.

We used terminal transferase-based labeling (TUNEL) to detect DNA breaks generated in neurons and fibroblasts after 6 hours of incubation with thimerosal. Following incubation, the cells were fixed, labeled by TUNEL, and counterstained by DAPI, which in these experiments was employed as a fluorescent DNA marker to visualize all cell nuclei in fixed cell cultures.

The results of these experiments are presented in Fig. 4. The figure demonstrates that TUNEL positive cells were detected in all cell cultures after 6 hours of incubation, up to the concentration of 2µM of thimerosal.

To examine if extending the time of incubation with thimerosal at concentrations below 2μM would result in generation of DNA breaks, we extended the time of incubation to 24 hours in a separate series of experiments. After 24 hours, a TUNEL signal was detected in neuronal cells at 1μM concentration of thimerosal (versus 2μM at 6 hours) (not shown). Incubation of neuronal cells for 24-hours with concentrations of thimerosal below 1μM (125nM, 250nM, 500nM) did not produce TUNEL signal.

Detection of apoptotic morphology in thimerosal-treated cells.

We performed a morphological evaluation of the fixed and fluorescently stained cell cultures after thimerosal treatment for the purpose of identification of apoptotic cells. To identify apoptotic morphology, cells were fixed and then stained by DAPI. In this experiment DAPI, was employed not as a vital dye, as in our previous study, but rather as a fluorescent histological nuclear stain. Although DAPI is an important marker used in live cell culture to selectively label nonviable cells (McCarthy and Hale, 1988; Boutonnat et al., 1999), it is also frequently used in fixed cells to visualize nuclear morphology and apoptotic bodies. We used it for this purpose in these tests.

Apoptotic morphology was detected in thimerosal-treated cells. Figure 5 demonstrates that after 6 hours of incubation, both fibroblasts and neurons showed morphological signs of apoptosis, which included chromatin condensation on the nuclear membrane, appearance of characteristic doughnut-shaped nuclei, different stages of apoptotic body formation, and freely positioned apoptotic bodies. After 6 hours of incubation, apoptotic morphology was observed at concentrations as low as 2μM of thimerosal (Fig.5), whereas at 24 hours after incubation, similar apoptotic morphology was observed at concentrations as low as 1μM.

To further confirm the apoptotic nature of cell death induced by thimerosal, we performed detection of active caspase-3, which is a sensitive and specific indicator of apoptosis.

Active caspase-3 in thimerosal-treated cells.

Caspase-3 activation serves as a sensitive marker of apoptosis, developing through caspase-3-dependent mechanisms, which constitutes one of the most frequent apoptotic pathways. We employed visualization of active caspase-3 directly in living cells through the use of a carboxyfluorescein (FAM) labeled peptide caspase inhibitor (FAM-Peptide-FMK) (*See* Methods).

We detected caspase-3 positive neuronal cells after 6 hours of incubation with thimerosal at concentrations ranging from 250µM to 2µM. The intensity of signal was dose-dependent, and much lower at the 2µM concentration compared to higher concentrations, probably due to an earlier stage of caspase-3 activation (Figure 6).

Assessment of 200 cells per well randomly, using the fluorescent microscope, revealed that active caspase-3 was expressed in 20% of cells at 2 µM thimerosal, 26% at 10 µM thimerosal, 83% at 50µM thimerosal and 97% of neurons at 250µM thimerosal concentration. In controls less than 1% of cells was caspase-3 positive, due to cell death naturally occurring in cell cultures.

At 2µM thimerosal, active caspase-3 signal was predominantly observed in the cytoplasm, which represents the early stage of its activation, whereas at higher concentrations of thimerosal, the signal was detected in both cytoplasm and nuclei (Fig.6). (Nuclear localization of active caspase-3 is characteristic for later stages of the apoptotic process.)

When we used a fluorescent microplate reader, which detects signal from the detached cells, we detected active caspase-3 activation at 1µM concentration of thimerosal after 6-hour incubation, probably due to the added contribution from floating dead cells (Fig.3).

When we extended the incubation time with thimerosal from 6 to 24 hours, detectable numbers of attached cells with active caspase-3 were observed at 1µM

concentration of thimerosal (Fig.7). Active caspase-3 signal at 1µM concentration was cytoplasmic, demonstrating an earlier stage of caspase-3 activation. Interestingly, after 24-hour of incubation, the neurons treated with 2µM thimerosal showed migration of caspase-3 from cytoplasm to the nuclei (Fig.7). The majority of caspase-3 positive cells were also DAPI-positive, which indicates membrane damage occurring simultaneously with apoptotic response. However at the higher 250µM concentration of thimerosal a number of cells was only DAPI positive without caspase-3 activation demonstrating necrotic death (Fig.7). We did not detect active caspase-3 at 24-hours of incubation in untreated neurons, or in neuronal cultures treated with lower concentrations of thimerosal (500nM, 250nM and 125 nM).

Discussion

Our data indicates that thimerosal is toxic to human neurons and fibroblasts if applied in micromolar concentrations (1-250µM). An early sign of thimerosal toxicity is a change in cellular membrane permeability to the vital dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), which is associated with the loss of cell viability (McCarthy and Hale, 1988; Boutonnat et al., 1999). This can be detected as early as 2 hours after incubation.

DAPI proved to be useful for analyzing thimerosal toxicity, as it is a sensitive marker of membrane integrity. It is employed as a propidium iodide substitute in cell viability assays, and labels nuclei of dying cells, which lack intact plasma membrane (McCarthy and Hale, 1988; Boutonnat et al, 1999; Robertson et al, 1998; Castro-Hermida et al., 2000). Dual staining experiments using propidium iodide and DAPI co-staining with FACS analysis demonstrated that DAPI stains only dead cells (McCarthy and Hale, 1988). Viable cells that are not stained by PI also exclude DAPI (McCarthy and Hale, 1988).

The nature of cell death labeled by DAPI in the case of thimerosal treatment deserves additional discussion. The DAPI exclusion method relies on the fact that this dye is largely impermeable to cells with an intact plasma membrane. However. when cell membrane integrity becomes compromised, DAPI gains access to the nucleus, where it complexes with DNA, rendering the nucleus highly fluorescent. Early compromised integrity of plasma membranes is a characteristic feature of necrotic cell death, whereas in apoptosis, cellular membranes are compromised at later times. This is why intracellular staining by DAPI (and also by its more toxic substitute propidium iodide) is regularly interpreted as a sign of necrosis (Boutonnat et al, 1999). However, in the case of thimerosal, the changes in membrane permeability coincided with activation of apoptosis-specific caspase-3 (Fig.3). In our opinion, this indicates a separate direct membrane damaging effect of thimerosal, which developed simultaneously with apoptotic changes, such as caspase-3 activation.

In many cases, the importance of caspase-3 activation is related to its connection to specific and extensive apoptotic DNA cleavage (Porter and Janicke, 1999). This DNA fragmentation can be labeled by the TUNEL technique, and is widely used for visualization of apoptotic cells. A caspase-activated deoxyribonuclease (CAD, or DFF 40) is implicated as a direct executioner of the cleavage (Mukae et al, 1998; Liu et al., 1997). Most of the time, the enzyme is kept inactive by the binding of an inhibitor (ICAD, or DFF 45). Activation of the nuclease occurs when the inhibitor is cleaved by activated caspase-3 (Enari et al., 1998; Sakahira et al., 1998). However, the exact sequence of events in case of human brain is likely different from this scheme. In human CNS neurons, other caspase-3-related pathways and possibly the other DNA cleaving enzymes are more important, and the role of the CAD-mediated mechanism is likely limited, as no expression of CAD mRNA was detected in human brain cells (Mukae et al., 1998).

Similar to our results, high cellular toxicity of thimerosal in low micromolar concentrations was recently reported using another cell culture model (Makani et al., 2002). Effects of different concentrations of thimerosal were examined in Jurkat cells. Cells were incubated with 5µM to 0.5 µM concentrations of thimerosal for 24 hours. Concentration-dependent apoptosis was detected and measured by TUNEL. Caspase-3 activation was also detected after 4 and 6 hours of incubation with thimerosal.. The study concluded that thimerosal induced caspase-3 dependent apoptosis in Jurkat cells. This apoptosis was associated with depolarization of the mitochondrial membrane, and release of cytochrome c. In this same study, significantly enhanced generation of reactive oxygen species was also detected, as a result of incubation with thimerosal (Makani et al., 2002). We hypothesize that these elevated levels of free radicals and subsequent oxidation may play role in apoptosis induction, and might also be involved in the direct membrane damaging effects of thimerosal identified in our study.

We showed that the concentrations of thimerosal that induced toxic effects in human cortical neurons ranged from 1µM to 250µM. However, comparisons of the nuclear morphology of dying cells after incubation with higher vs. lower concentrations of thimerosal demonstrate important differences. Although caspase-3 activation was detected in both high and low concentrations of thimerosal, the morphology of dying cells was different in these two situations. The cell bodies of neurons treated with higher concentrations of thimerosal (50-250µM) were swollen, which is more characteristic for necrotic cell death, whereas cells treated with low concentrations (2-10µM) were shrunken, as is typical for apoptosis (Fig.7). Similarly, the nuclei of dying neurons treated with 250µM thimerosal were larger in size and swollen, in contrast to the shrunken nuclei of cells treated with 2µM thimerosal (Fig. 7). Thus, cell death occurring after incubation of neuronal cells with higher concentrations of thimerosal has features of both apoptosis (caspase-3 activation) and necrosis (cell edema and nuclei swelling).

This can be explained by a direct membrane-damaging effect of thimerosal, which rapidly leads to the loss of membrane integrity and cell swelling. This process likely occurs simultaneously with apoptosis induction, and the initiation of the caspase cascade, and activation of caspase-3. At lower concentrations of thimerosal, direct membrane-damaging effects are weaker, and no swelling is observed.

Investigation of thimerosal toxicity is especially important at the present time, because this compound is used in biological products, and can be administered in toxic doses either accidentally or intentionally (Ball et al., 2001).

In our study, the concentrations of thimerosal, which induced toxic effects, ranged from 1µM (405 µg/L) to 250 µM (101 mg/L) that is equivalent to the levels of inorganic mercury from 201 µg/L to 50 mg/L. In clinical cases of accidental or intentional usage in high concentrations, thimerosal was administered in doses from 3 mg/kg to several hundred mg/kg (Ball et al., 2001). Such doses resulted in local necrosis at the application site, and severe central nervous system and kidney injury.

Much lower concentrations are reached during normal vaccination, when thimerosal-containing vaccines are used. In the case of a full series of vaccinations containing thimerosal, up to 403 µg of thimerosal (equivalent to 200µg of mercury) is received by six months of age (calculated from Ball et al., 2001). This results in administration of 200/3.81 = 52 µg/kg; 200/5.22 = 38 µg/kg; 200/6.27 = 32 µg/kg of mercury. These calculations utilize averages of the 5th, 50th, and 95th% weight for females at birth (2.36 kg, 3.23 kg, 3.81 kg) and at 6 months (5.25 kg, 7.21 kg, 8.73 kg) = 3.81 kg, 5.22 kg, 6.27 kg, reported by (Ball et al., 2001) when used in calculating exposure limits for mercury in comparisons of various agencies guidelines.

The lowest toxic concentration of mercury contained in the thimerosal doses in our present study (201 µg/L) is less than four times higher than some of these estimated concentrations. The rapidly developing toxicity of thimerosal in low micromolar

concentrations over short time frames is of concern, and suggests that additional research is necessary to estimate the effects of prolonged exposure to thimerosal in lower doses.

In this paper, we demonstrate that extending the time of incubation with thimerosal from 2 to 6 hours is associated with toxicity that was not seen after a shorter time of exposure. For this reason, further studies of lower concentrations and longer exposure times appear to be warranted. These results indicate that additional research is needed to more fully delineate the dose and time dependent toxicity of thimerosal in sub-micromolar concentrations, and suggests that toxicity may occur at even lower doses than those utilized in these experiments, with longer times of exposure. As mercury can be retained in body organs for months to years, study of longer incubation times is warranted. We also conclude that a proposed combination of fluorescent techniques combining assessment of DNA, membrane damage and active caspase-3 is useful in studying thimerosal toxicity.

Acknowledgments

We are grateful to Katie Fewell and Dr. Benxiao Zhang, M.D. for valuable technical assistance in experiments and in manuscript preparation. This research was supported by grant R01 CA78912-01 from the National Cancer Institute, National Institutes of Health (DSB), by grant 004949-054 from the Texas Higher Education Coordinating Board (DSB & VVD), and by grants from DeBakey Medical Foundation (VVD), Baylor College of Medicine (VVD), The Taub Foundation (DSB), The Henry J.N. Taub Fund for

Neurosurgical Research (DSB)., The George A. Robinson, IV Foundation (DSB), The Blanche Greene Estate Fund of The Pauline Sterne Wolff Memorial Foundation (DSB), and The Seigo Arai and Koppelman Funds of The Neurological Research Foundation (DSB).

References

Amstad, P., Johnson, G., Lee, B., and Dhawan, S. (2000). An in situ marker for the detection of activated caspases. *Biotechnology Laboratory* **18,** 52-56.

Ball, L., Ball, R., and Pratt, R. (2001). An assessment of thimerosal use in childhood vaccines. *Pediatrics* **107,** 1147-1154.

Bedner, E., Smolewski, P., Amstad, P., and Darzynkiewicz, Z. (2000). Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp. Cell Research* **259,** 308-313.

Ben-Ozer, E., Rosenspire, A., McCabe, M., Worth, R., Kindzelskii, A., Warra, N., and Petty, H. (2000). Mercuric chloride damages cellular DNA by a non-apoptotic mechanism. *Mutat. Res*. **470,** 19-27.

Blair, A., Clark, B., Clarke, A., and Wood, P. (1975). Tissue concentrations of mercury after chronic dosing of squirrel monkeys with thimerosal. *Toxicology* **3,** 171-176.

Boutonnat, J., Barbier, M., Muirhead, K., Mousseau, M., Ronot, X., and Seigneurin, D. (1999). Optimized fluorescent probe combinations for evaluation of proliferation and necrosis in anthracycline-treated leukaemic cell lines. *Cell Prolif.* **32,** 203-213.

Carcia-Calvo, M., Peterson, E., Leiting, B., Ruel, R., Nicholson, D., and Thornberry N. (1998). Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J. Biol. Chem*. **273,** 32608-32613.

Castro-Hermida, J., Freire-Santos, F., Oteiza-Lopez, A., and Ares-Mazas E. (2000) Unexpected activity of beta-cyclodextrin against experimental infection by Cryptosporidium parvum. *J. Parasitol*. **86,** 1118-1120.

Enari, M., Sakahira, H., Yokoyama, H., et al. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391,** 43-50.

Ferrat, L., Romeo, M., Gnassia-Barelli, M., and Pergent-Martini, C. (2002). Effects of mercury on antioxidant mechanisms in the marine phanerogam Posidonia oceanica. *Dis Aquat Organ* **50,** 157-160.

Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89,** 175-184.

Magos, L., Brown, A.W., Sparrow, S., Bailey, E., Snowden, R.T., and Skipp, W.R. (1985). The comparative toxicology of ethyl- and methylmercury. *Arch. Toxicol.* **57**, 260-267.

Makani, S., Gollapudi, S., Yel, L., et al. (2002). Biochemical and molecular basis of thimerosal-induced apoptosis in T-cells: major role of mitochondrial pathway. *Genes Immun*. **3,** 270-278.

McCarthy, K., and Hale, M. (1988). Flow cytometry techniques in radiation biology. *Toxicology Lett.* **43,** 219-233.

Mukae, N., Enari, M., Sakahira, H., Fukuda, Y., Inazawa, J., Toh, H., Nagata, S. (1998). Molecular cloning and characterization of human caspase-activated DNase. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9123-9128.

Porter, A., and Janicke, R. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*. **6,** 99-104.

Robertson, L., Campbell, A., Smith. H. (1998). Viability of Cryptosporidium parvum oocysts: assessment by the dye permeability assay. *Appl. Environ. Microbiol*. **64,** 3544-3545.

Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391,** 96-99.

Schurz, F., Sabater-Vilar, M., and Fink-Gremmels, J. (2000). Mutagenicity of mercury chloride and mechanisms of cellular defense: the role of metal-binding proteins. *Mutagenesis* **15,** 525-530.

Shapiro, H. (1985). Practical Flow Cytometry. pp. 84-154. Liss, New York.

Smolewski, P., Bedner, E., Du, L., Hsieh, T., Wu, J., Phelps, J., and Darzynkiewicz Z. (2001). Detection of caspase activation by fluorochrome-labeled inhibitors: multiparameter analysis by laser scanning cytometry. *Cytometry* **44,** 73-82.

Figure legends

Figure 1. Nonviable human cortical neurons in cell culture detected by a DAPI-exclusion test after incubation with various concentrations of thimerosal. All panels have the same density of cells plated on a dish. Only nonviable cells are visualized by nuclear staining with DAPI, when cellular membranes are compromised and cells are either dead or dying. Solitary dying cells can be seen in controls, whereas the majority of cells incubated with thimerosal have compromised membranes. (Bar = 100 µm).

Figure 2. Nonviable human skin fibroblasts (PD 32) in cell culture detected by a DAPI-exclusion test after incubation with various concentrations of thimerosal. All panels have the same density of cells plated on a dish, and represent young fibroblasts, which underwent only 32 population doublings in cell culture conditions. Exclusively nonviable cells are visualized by nuclear staining with DAPI, when cellular membranes are compromised and cells are either dead or dying. (Bar = 100 µm).

Figure 3. Simultaneous detection of caspase-3 activation and nonviable cells (DAPI test) in cultured human cortical neurons after 6-hour incubation with various concentrations of thimerosal.

GENios Plus Multi-Detection Microplate Reader with Enhanced Fluorescence was used for the detection. Labeling of active caspase-3 was performed in live cell cultures using a FAM labeled DEVD fluoromethyl ketone caspase inhibitor (green) with the simultaneously performed DAPI exclusion test (blue), showing dead or dying cells. Fluorescence from both floating and attached nonviable cells was recorded. Chroma

Technology bandpass filter set was used to acquire single color images: FITC excitation D490/40, emission 520/10; DAPI excitation D360/40, emission 460/20. The reactions were repeated twice and averages are shown. For the easier comparisons the caspase-3 signal at 250µM was equalized (multiplied 6.71 times) with DAPI signal. Y-axis - arbitrary GENios Plus readings of fluorescence. Note that DAPI exclusion test reveals the early appearance of cells with compromised membranes and stronger DAPI signal compared to caspase-3 signal.

Figure 4. Detection of DNA breaks by TUNEL in cultures of human cortical neurons (green fluorescence) and human fibroblasts (PD32) (red fluorescence) after 6 hours of incubation with various concentrations of thimerosal. Blue fluorescence - DAPI counterstaining was performed on fixed cells to visualize all cellular nuclei. (Bar -100µm).

Figure 5. Apoptotic morphology in human neurons and fibroblasts after 6 hours of incubation with 2µM and 250 µM concentrations of thimerosal. Cell were fixed and stained by DAPI (1µg/mL) to visualize nuclear morphology and apoptotic bodies. Upper image - DAPI stained neuronal nuclei (blue) after 6 hours of incubation with 2µM thimerosal. Apoptotic doughnut-shaped nuclei with chromatin condensation on the nuclear membrane and apoptotic bodies are seen. Arrows indicate forming and free located apoptotic bodies (Bar - 50 µm). Lower panel - black/white image of DAPI-stained neuronal and fibroblast cells after 6 hours of incubation with 2µM or 250µM thimerosal. Arrows indicate condensation of chromatin on nuclear membrane or formation of apoptotic bodies. (Bar - 10 µm).

Figure 6. Caspase-3 activation in cultured human cortical neurons after 6 hours

incubation with various concentrations of thimerosal. Detection of active caspase-3 was performed in live cell cultures using APO LOGIX^{TM} carboxyfluorescein (FAM) caspase detection kit, which employs a FAM labeled DEVD fluoromethyl ketone (FMK) caspase inhibitor (green fluorescence). The inhibitor irreversibly binds to active caspase-3. Note the primarily cytoplasmic localization of active caspase-3 at lower concentrations of thimerosal, whereas the higher concentrations demonstrate predominantly nuclear localization of active caspase-3, indicating a later stage in progression towards cell death. Blue fluorescence - DAPI counterstaining performed on fixed cells to visualize all cellular nuclei. (Bar -100µm).

Figure 7. Simultaneous detection of caspase-3 activation and nonviable cells in cultured human cortical neurons after 24 hours incubation with various concentrations of thimerosal. Detection of active caspase-3 was performed in live cell cultures using a FAM labeled DEVD fluoromethyl ketone caspase inhibitor (green fluorescence), which irreversibly binds to active caspase-3. Only nonviable cells are visualized by the simultaneously performed DAPI exclusion test (blue fluorescence), showing dead or dying cells with compromised membranes. Note that in this case, DAPI is not used as a counterstain for fixed cells, like in the previous figure, but is employed as an exclusion dye (*see* Methods). 6 hours of incubation with 2µM concentration of thimerosal resulted in cytoplasmic localization of active caspase-3, while after 24 hours, active caspase-3 is now localized in the nuclei, indicating a later stage in cell death progression. Compare the cytoplasmic localization of active caspase-3 at 1µM concentration of thimerosal, to its nuclear localization at 2µM concentration. The majority of caspase-3 positive cells have compromised cellular membranes (Bar - 100µm).

Composite images were created in MetaMorph 4.1 (Advanced Scientific, Inc.) by

overlaying single color images. Chroma Technology bandpass filter set was used to acquire single color images: FITC excitation D490/40, emission 520/10; DAPI excitation D360/40, emission 460/20.

Figure 1

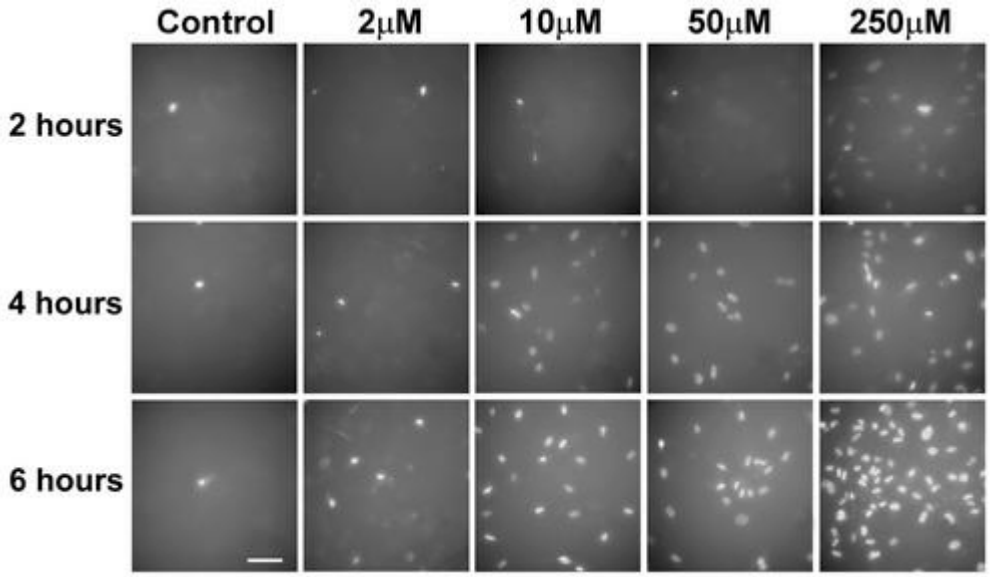


Figure 2

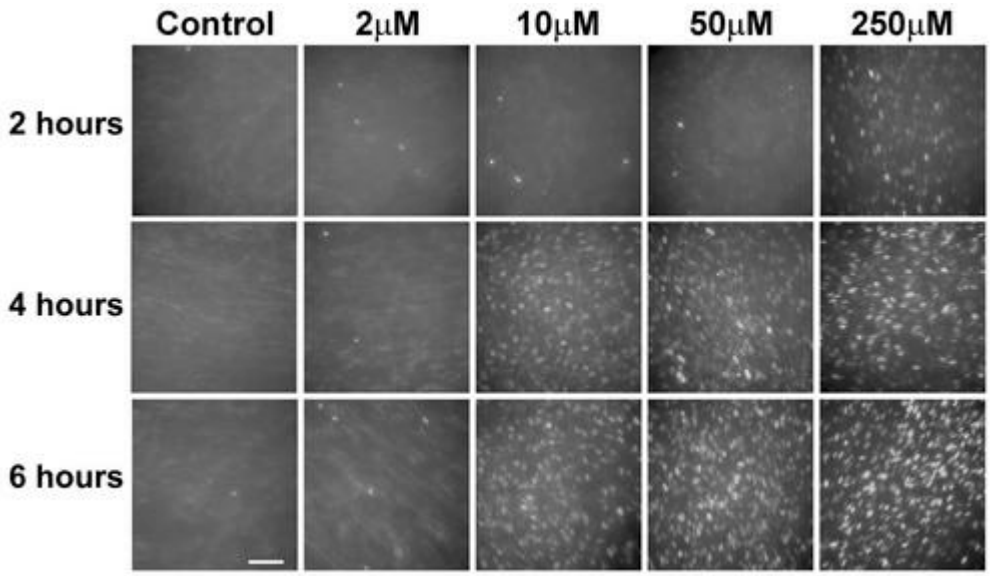


Figure 3

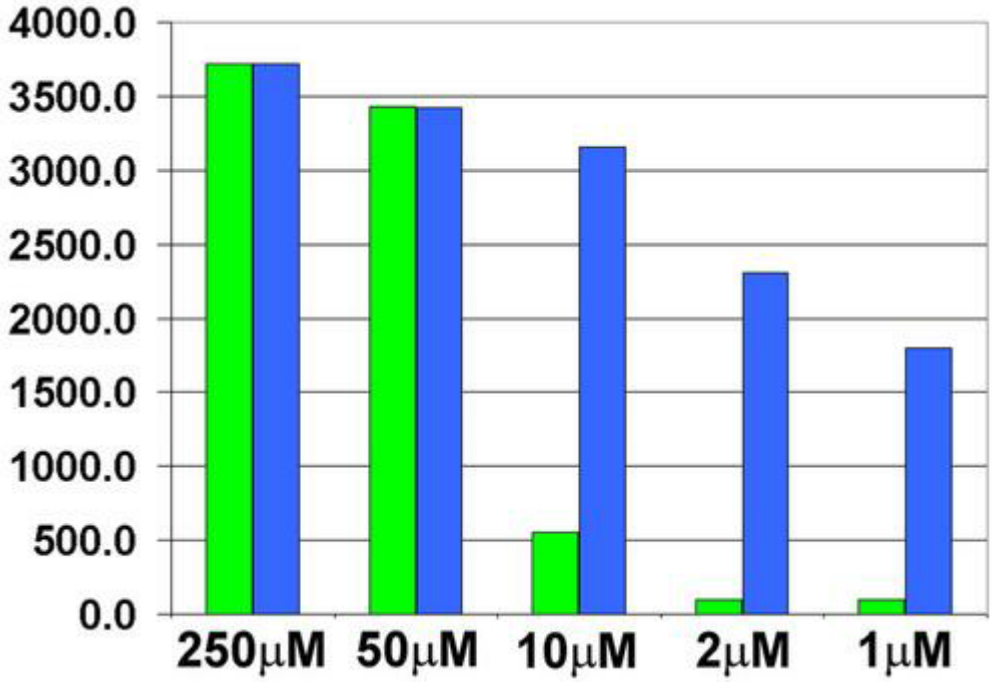

4000.0
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
250μM
50μM
10μM
2μM
1μM

Figure 4

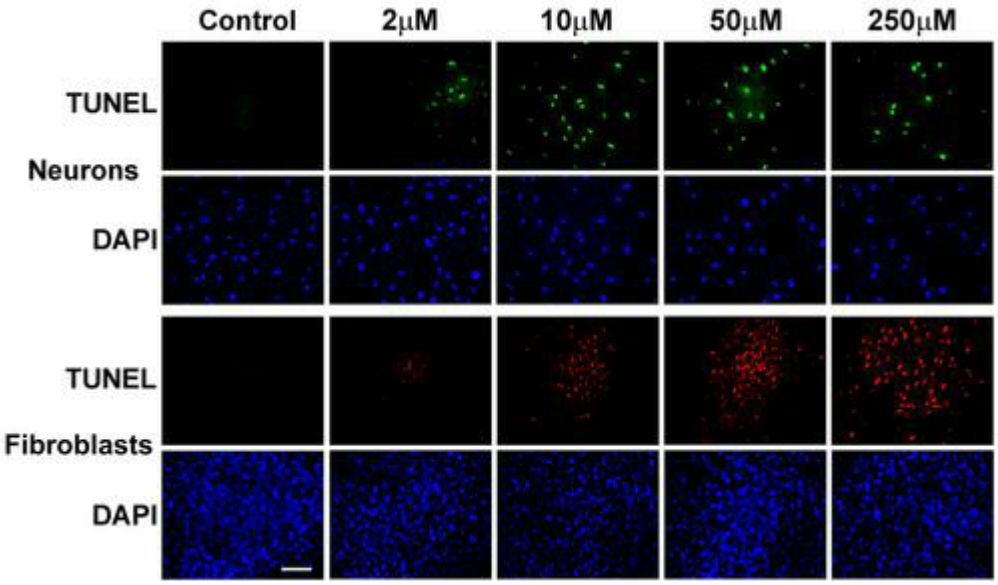
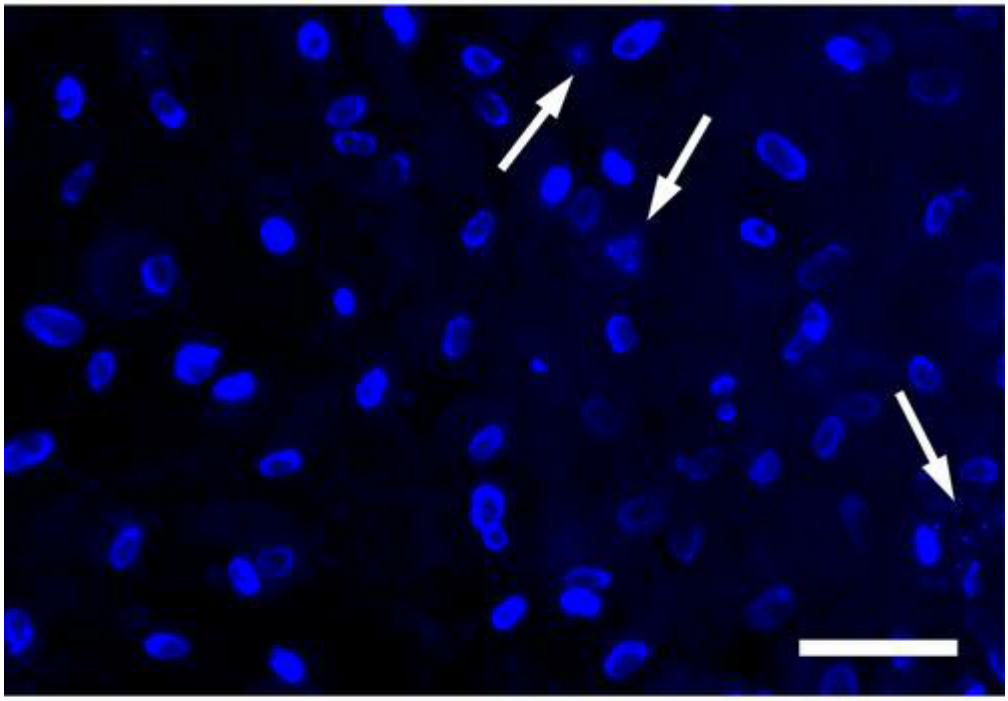

Control
2μM
10μM
50μM
250μM
TUNEL
Neurons
DAPI
TUNEL
Fibroblasts
DAPI

Figure 5



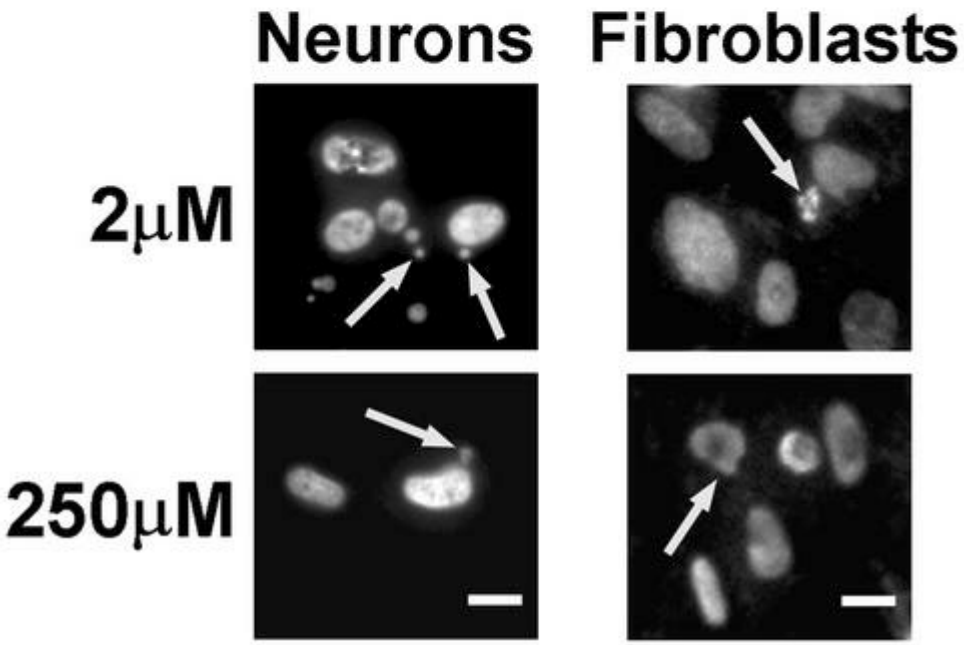


Figure 6

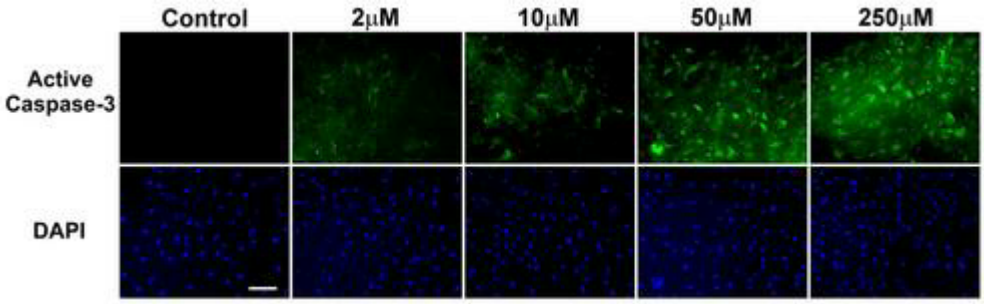
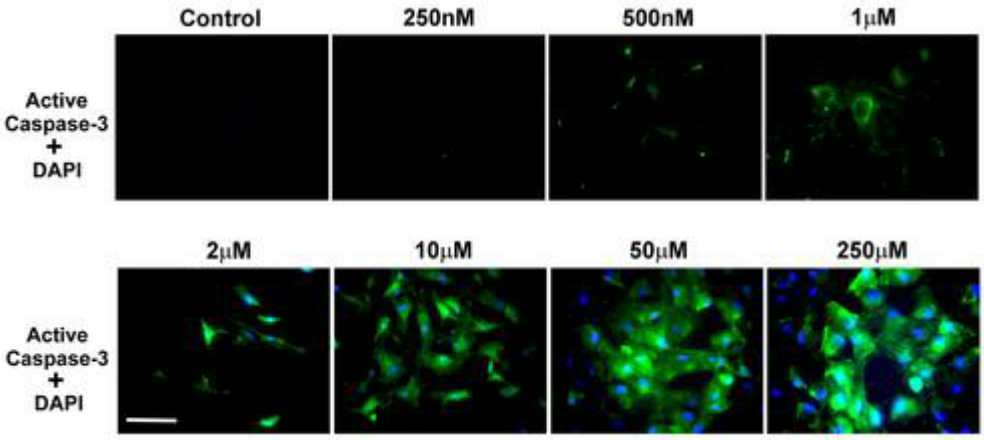


Figure 7


Control
250nM
500nM
1μM
Active Caspase-3 + DAPI
2μM
10μM
50μM
250μM
Active Caspase-3 + DAPI