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CYTOTOXICITY OF SELECTED CESIUM AND ZINC OXYTHIOMOLYBDATES IN VITRO

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OCCUPATIONAL AND ENVIRONMENTAL HEALTH DIRECTORATE
TOXICOLOGY DIVISION

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FOR THE COMMANDER

JAMES N. McDOUGAL, Lt Col, USAF, BSC
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13. ABSTRACT (Maximum 200 words)

✓ The toxicity of complex oxythiomolybdate (OTM) compounds, solid lubricants under development by the Air Force, was determined. The OTMs investigated were unburned and burned (650°C) cesium oxytrithiomolybdate (COTM), zinc oxydithiomolybdate (ZODM) and zinc oxytrithiomolybdate (ZOTM), respectively. An *in vitro* approach was employed using the rat liver cell line WB 344. Lactate dehydrogenase enzyme leakage (LDH), cellular esterase enzyme activity and DNA synthesis analysis were measured to rank-order the toxicity of the test compounds. The ZODM compounds (at equimolar concentrations; unburned and burned) were the only ones found not to produce any effects on WB 344 cells. While unburned COTM and ZOTM compounds were found to exhibit some cytotoxic effects and depress cellular DNA, the cytotoxic effects and DNA depression were more severe with COTM exposures. The burning of the ZOTM compound did not alter its effects on the WB 344 cells. However, burning of the COTM compound was found to reduce its cytotoxic and DNA effects when compared to the unburned COTM exposures. Based on these results, the relative rank-order of toxicity of the three OTM's tested (unburned and burned) was **COTM > ZOTM > ZODM**.

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PREFACE

This report represents research performed by the Biochemical Toxicology Branch, Toxicology Division, Armstrong Laboratory, from June 1991 to January 1992. The research was performed in support of Project 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Task 630201, "Toxicology of Aerospace Chemicals and Materials, Work Unit 63020174, "Toxicology Screening of Air Force Chemicals".

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SECTION 1

INTRODUCTION

A request was made by WL/POSL to determine the toxicity of several candidate solid lubricants. The candidate solid lubricants are complex oxythiomolybdate (OTM) compounds under development by the Air Force. These OTM compounds included cesium oxytrithiomolybdate (Cs_2MoOS_3 , COTM), zinc oxydithiomolybdate (ZnMoO_2S_2 , ZODM), and zinc oxytrithiomolybdate (ZnMoOS_3 , ZOTM). Very little toxicity data exist on these compounds. Limited toxicity studies were conducted previously using COTM. An acute toxicity study (unpublished) conducted by FDRL in 1982 showed that COTM was non-irritating to the skin, mildly irritating to the eye when not washed out and had little or no dermal toxicity ($\text{LD}_{50} > 2.0$ g/Kg). A letter report summarizing the toxicity of COTM was submitted to WL/POSL by the Toxicology Branch in 1988. This report summarized the finding of two studies. The first study provided solubility and scanning electron microscopy (SEM) data for COTM. In the second study, male and female rats were orally dosed with a single dose of COTM at various concentrations and held for 14 days. Histopathological examination of all tissues examined in male and female rats did not indicate any change. An LD_{50} value of 1.5 g/kg was also determined in this study. This value is similar to the previously determined rat oral LD_{50} for molybdate. The rat oral LD_{50} for cesium hydroxide is reported to be 1.03 g/kg (1). These results suggest that the rat oral toxicity for COTM is not more toxic than molybdate.

Because time and funds are limited, careful selection and prioritization of appropriate studies must be made. In addition, there is a limited amount of OTM samples (4-30 g). Therefore, an in vitro approach to toxicity testing of these samples will be employed. Tasked to rank order these in terms of immediate

cellular toxicity, we conducted experiments to expose living cells to pre-fired (unburned) and post-fired (burned, 650°C) agents and assess impact on several endpoints: cell membrane damage, cell viability as measured by cytoplasmic esterase activity , and cellular DNA integrity. This data can then be used in the decision process of choosing the least toxic OTM to produce in larger quantities. The selected OTM can then be tested in vivo for validation of the in vitro data. This will result in a savings in cost, time and animals because in vivo studies will not have to be conducted on all OTM samples.

SECTION 2
MATERIALS AND METHODS

TEST MATERIALS

Samples of burned and unburned COTM (MW 474.04), ZODM (MW 275.54) and ZOTM (MW 273.64) were supplied by the Lubrication Branch of the Aero Propulsion Directorate (WL/POSL). All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise.

OTM SOLUBILITY

Approximately 100 mg of test material was weighed with an analytical balance. The weight was recorded to the nearest 0.01 mg. Ten mL of solvent (water, saline, or 0.1N HCl) was added to a 15 mL Corning screw cap centrifuge tube containing the weighed sample using a volumetric pipet. Capped centrifuge tubes were mixed mechanically overnight at room temperature. After mixing, tube contents were centrifuged and supernatant saved for chemical analysis. The pellet was dried in air for 72 h, and the tubes (with contents) were weighed.

OTM STOCK SOLUTIONS

Based on the solubility data, 50 mL of Williams E culture medium (WEM; Gibco, Grand Island, NY) was saturated with test agent. Saturated culture medium was then placed in shaker water bath at 37°C and mechanically shaken at 60 rpm for 4 h. After shaking, the saturated solution was refrigerated at 4°C overnight. Following refrigeration, saturated media were aseptically filtered using a 0.22 µm cellulose acetate bottle top filter (Costar, Cambridge, MA). After filtration, 5% fetal bovine serum (FBS) was added to the OTM saturated media. Due to the low solubility of ZOTM, 0.1N HCl (without FBS) was saturated and treated as described above. After filtering the 0.1N HCl, 8.8 mL was removed and added to 41.2 mL of WEM/5% FBS. This medium was then used as the ZOTM stock solution. All stock solutions were serially diluted by two

10-fold dilutions to yield two addition stock solutions (0.1X and 0.01X). All stock media solutions were at a pH of 7.2-7.4.

CELL CULTURE AND EXPOSURE

The WB344 cell line was used throughout these experiments. It is a rat diploid hepatic epithelial cell line which closely resembles the phenotype of mature hepatocytes in culture (2). Culture plates (35mm²) were plated with 300,000 cells per plate into WEM supplemented with 5% FBS and 50 µg/ml gentamicin. Incubation followed under standard conditions in a humidified atmosphere of 5% CO₂ at 37°C. Three to four hours was allowed for cell attachment. After attachment media was aseptically removed from all plates and replaced with the appropriate exposure medium.

Control cultures were treated with fresh WEM. Test cultures were treated with stock solutions of each OTM, respectively (1X, 0.1X, and 0.01X). All plates were incubated at 37°C in a CO₂ incubator for 24 h.

CELL ENZYME LEAKAGE AND VIABILITY

Measurements of lactate dehydrogenase (LDH) activity leaked into the culture medium were made using a DuPont ACA V discrete clinical analyzer (DuPont, Hoffman Estates, IL) as previously described (3).

Cell viability was assessed using the fluorescence Live/Dead Assay Kit™ (Molecular Probes, Eugene, OR). This kit contains the dyes ethidium homodimer (EH) and calcein acetoxymethyl ester (CAE) for staining dead and viable cells, respectively. The general procedure for cell staining is as follows. Cells were released from culture by trypsinization, collected from each plate and placed in appropriately labeled 1.5 mL microcentrifuge tubes. The cells are then centrifuged at 100xg for 5 minutes. After centrifugation, the supernate was aspirated and the cells resuspended in 200 µL Dulbecco's phosphate buffered saline (DPBS). The resuspended cells were then added to a 96-well tissue culture plate. The EH/CAE dye mixture was made by placing 400 µL of EH and

6 μ L of CAM in 10 mL of DPBS. One hundred μ L of this dye mixture was added to each cell suspension in the 96-well culture plate (total vol = 300 μ L). The plate is then incubated for 1 h at room temperature in the dark. After incubation, the fluorescence in the wells of the plate is measured using a fluorescence plate reader (CytoFluor 2300, Millipore Corp., Bedford, MA). The EH and CAE fluorescences were determined at excitation/emission wavelengths of 485/530 and 485/645, respectively.

DNA CYCLE ANALYSIS

Cells were plated and treated as described above. Following trypsinization the cells were suspended in ice cold DPBS + 5% FBS. After centrifugation at 200xg for 10 minutes the cell pellet was resuspended in 200 μ L of cold DPBS and added dropwise to a gently vortexed glass test tube containing 5 ml of 70% ethanol at -20°C . The fixed cells were held in the ethanol for 30 minutes on ice then washed in DPBS. One unit of DNase-free RNase (Boehringer Mannheim, 1119 915) was added to the cell suspension of approximately 10^6 cells. Following incubation in a shaker bath at 30°C for 30 minutes, 5 μ L of propidium iodide (.05 mg/ml stock) was added. The sample was run on a FACScan analyzer (Becton Dickinson, San Jose, CA) using the 488 nm wavelength of an argon laser. Light was collected using a bandpass 585/42 nm (FL2) filter. Cell clumps were removed from singlets by using the doublet discrimination module and displaying the FL2 width (FL2-W) vs. the FL2 area (FL2-A) in a dot display using control samples. Singlet cell events were gated and FL2-A data collected in list mode. Histogram analysis followed using MCYCLE™ software (Phoenix Flow Systems, San Diego, CA). Curves were fitted to the DNA histogram display of FL-A data. Goodness-of-fit was reported as a Chi Square value with < 10 being acceptable and < 5 representing an excellent fit.

STATISTICS

All data were compared by an analysis of variance (ANOVA) using SYSTAT software statistics package (SYSTAT, Inc., Evanston,

IL). Means found significant by ANOVA were compared with the Tukey post hoc test with type 1 error level held at $p < 0.05$.

RESULTS

OTM SOLUBILITY

Previous work had reported the saline solubility of COTM to be 6.47 mg/mL and of ZODM to be 0.402 mg/ml. On that basis, the final concentrations of COTM in the WEM used to expose the cells was 15 mM, 1.5 mM and 0.156 mM, respectively. The final concentrations of ZODM in the exposure media were 1.5 mM, 0.15 mM and 0.015 mM, respectively. The compound ZOTM was insoluble in saline but would enter a 0.1 N HCl aqueous solution at 2.34 mg/ml. The final concentrations of ZOTM in the exposure culture media were 1.5 mM, 0.15 mM and 0.015 mM, respectively.

CELL ENZYME LEAKAGE AND CELL VIABILITY

The total intracellular LDH enzyme in cell cultures used for COTM, ZOTM and ZODM exposures were 96, 132 and 57 U/L, respectively. The percentage of the total LDH enzyme leaked by control cells in COTM, ZOTM and ZODM experiments were 4, 18 and 12 percent. The WB 344 cell cultures exposed to the 15 mM concentration of COTM were completely lysed and no LDH enzyme activity could be detected after 24 h of exposure (Fig. 1). Cell cultures exposed to 1.5 mM and 0.15 mM concentrations of COTM indicated LDH enzyme leakage that was significantly greater than that of control. Only cell cultures exposed to the 1.5 mM concentration of ZOTM indicated significant LDH enzyme leakage when compared to control (Fig. 1). No significant LDH enzyme leakage was found at any of the ZODM exposure concentrations (Fig. 1). Although no LDH enzyme activity could be detected in cell cultures exposed to the 1.5 mM concentration of ZODM, the cells were intact and viable.

Total intracellular LDH enzyme activity in WB344 cell cultures used for burned COTM, ZOTM and ZODM were 92, 219 and 152 U/L, respectively. The percentage of total LDH enzyme leaked by control

cells in COTM, ZOTM and ZODM experimental exposures were 20, 8 and 12 %, respectively. WB344 cells exposed to the highest COTM concentration (15.0 mM) were severely compromised and resulted in low levels of LDH enzyme leakage from surviving cells (Fig 2). Cells exposed to burned COTM concentrations of 0.15 mM and 1.5 mM, respectively, were not found to be significantly different than controls (Fig 2). The LDH enzyme leakage from WB344 cells exposed to ZOTM was only found to be significantly different than control at the highest dose (1.5 mM). The LDH enzyme leakage from cells exposed to 1.5 mM ZOTM was found to be 3-fold greater than that measured in control cultures (Fig 2). No significant cellular LDH enzyme leakage was found at any of the ZODM concentrations tested when compared to control (Fig 2).

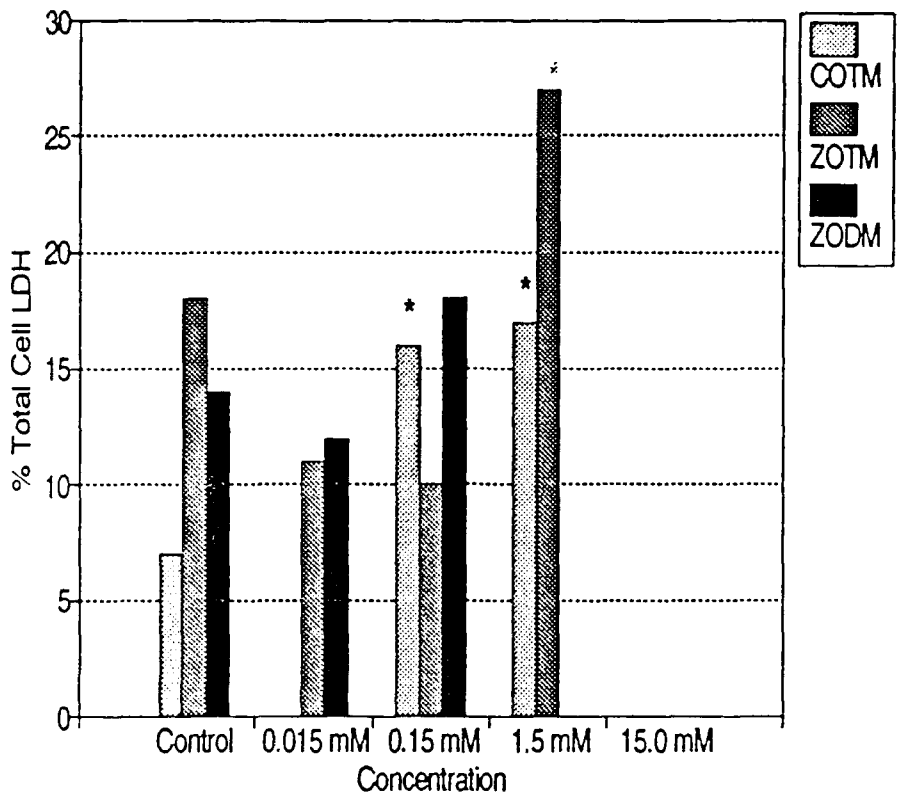


Figure 1. LDH leakage from WB344 cells exposed to unburned solid lubricants. Values are expressed as percent of total intracellular LDH. Each value is the mean of 3 to 4 independent samples. Starred values are significantly different from controls at $p < .05$.

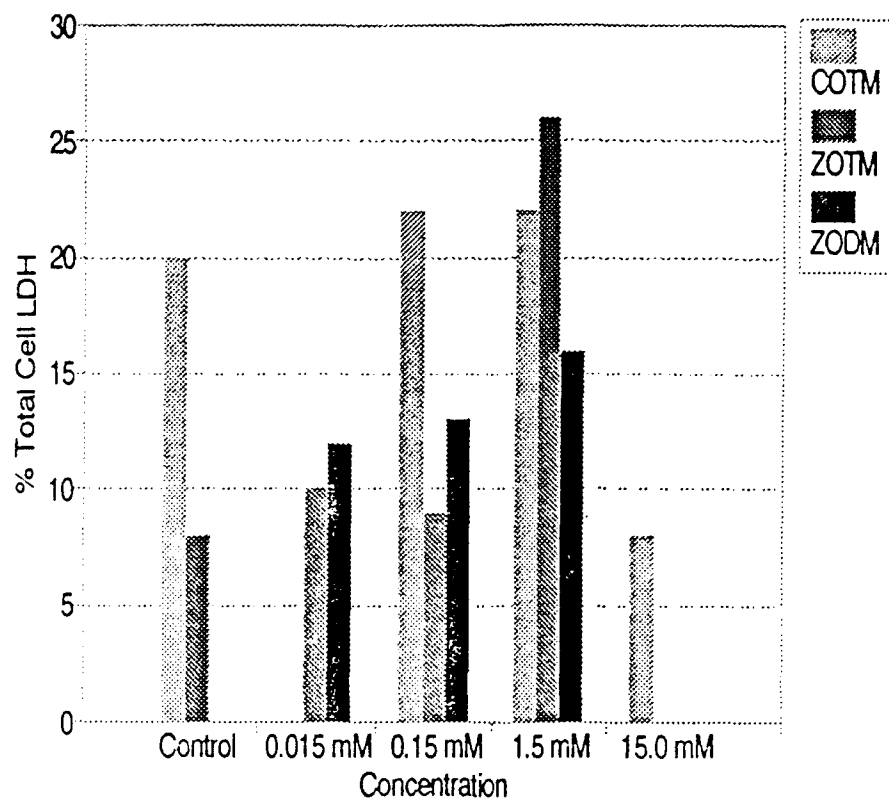


Figure 2. LDH leakage from WB344 cells exposed to burned solid lubricants. Values are expressed as percent of total intracellular LDH. Each value is the mean of 3 to 4 independent samples. Starred values are significantly different from controls at $p < .05$.

CELL VIABILITY/MEMBRANE INTEGRITY

Only the highest concentration of COTM (15 mM) showed a significant difference in cell viability as measured by calcein fluorescence. This concentration was immediately toxic upon addition to the test plates as evidenced by visual inspection of the cultures. Cells began rounding and became detached within two hours.

Cytoplasmic esterase activity for ZODM treatment groups did not differ significantly from controls. Membranes integrity was compromised only in the 1 X concentration group, equivalent to 1.5 mM.

For ZOTM only the most concentrated treatment group, equivalent to 1.5 mM, showed significantly reduced esterase activity. Values in this group were only 35% of control although no obvious cell killing effect was seen. Curiously, the most dilute treatment group at 0.015 mM showed significantly elevated (122% of control) esterase activity. However, examination of the ethidium data indicated that the seeding density in one plate was higher than the others in the treatment group. If that plate is excluded from analysis, the elevated esterase activity is shown to be an artifact. Cell membrane integrity across groups does not differ from control values at the $p < .05$ level.

On the basis of equimolar concentrations for the unburned chemicals it is clear that COTM and ZOTM decrease cell esterase activity more than ZODM, with the former compound being more severe.

Because LDH enzyme leakage and cell viability determinations (fluorescence Live/Dead™ assay described above) correlated very well with unburned OTM exposures, the fluorescence method was not utilized for burned OTMs.

CELL CYCLE/ DNA.

The unburned cesium compound COTM devastated cellular DNA at the highest concentration of 15mM (Fig 3). DNA fragments, indicative of acute cytotoxicity and breakdown, rise to over 50% of the total population at that concentration. DNA synthesis (the "S" phase in fig 3) is correlated to normal cell growth. With both 0.15mM and 1.5mM COTM the S phase is depressed below control values. The apparent increase in S phase seen in the 15mM treatment is artifact which results from the model's inability to discern intact cells when presented with overwhelming debris. The increase in G0/G1, the part of the cell cycle immediately preceding S phase, confirms that cytotoxicity is evident at even the lowest treatment concentration of 0.15mM.

In comparison, burned CTOM shows little evidence of these effects at either 0.15mM or 1.5mM (Fig. 4). Cellular debris is slight across all treatment groups although the 15mM was elevated above control values. Only the 15mM group showed depressed S phase and a concomitant elevated G0/G1 consistent with the cytotoxicity observed in the unburned samples. This supports the conclusion that burning the COTM reduced its acute cytotoxicity at more dilute concentrations.

The dithio-Zn compound, ZODM, did not arrest normal growth patterns as seen in the DNA histogram data (Fig. 5). In fact, the percentage of total cells in S phase is slightly elevated with all treatment concentrations. The G2M compartment is slightly decreased with treatment possibly indicating that cells are transitioning through synthesis and division (mitosis) more quickly. The elevated values for the G0/G1 are consistent with that conclusion. Cell debris levels are low across all treatment groups which indicates a low acute cytotoxicity for unburned ZODM.

The data for burned ZODM is almost identical to its unburned parent (Fig. 6). Low cytotoxicity and rapid cell turnover can be observed for all treatment concentrations. The highest concentration, 1.5mM, does show a lower relative S phase from controls, however both the G2M and G0/G1 compartments are

increased. This may indicate that the burned form of ZODM has a stimulatory effect on cell turnover.

In its unburned form ZOTM has little effect until the highest treatment concentration of 1.5mM (Fig. 7). Depressed S and G2M phases are reflected in the elevated G0/G1 - in effect the cells are "stacking up" in the first phase of the cell cycle. In light of the relatively low debris values and the reduced esterase activity reported previously, this observation is consistent with a true G0/G1 "arrest"., i.e. the cells do not initiate DNA synthesis in a normal manner yet they are not dying. This may be due to a specific depression of critical enzyme systems, a consequence of early cytotoxicity seen only with the higher concentration.

With 1.5mM of the burned ZOTM the arrest in G0/G1 is more pronounced (Fig. 8). The marked elevation in cellular debris supports the conclusion that the effect may result from acute cytotoxicity.

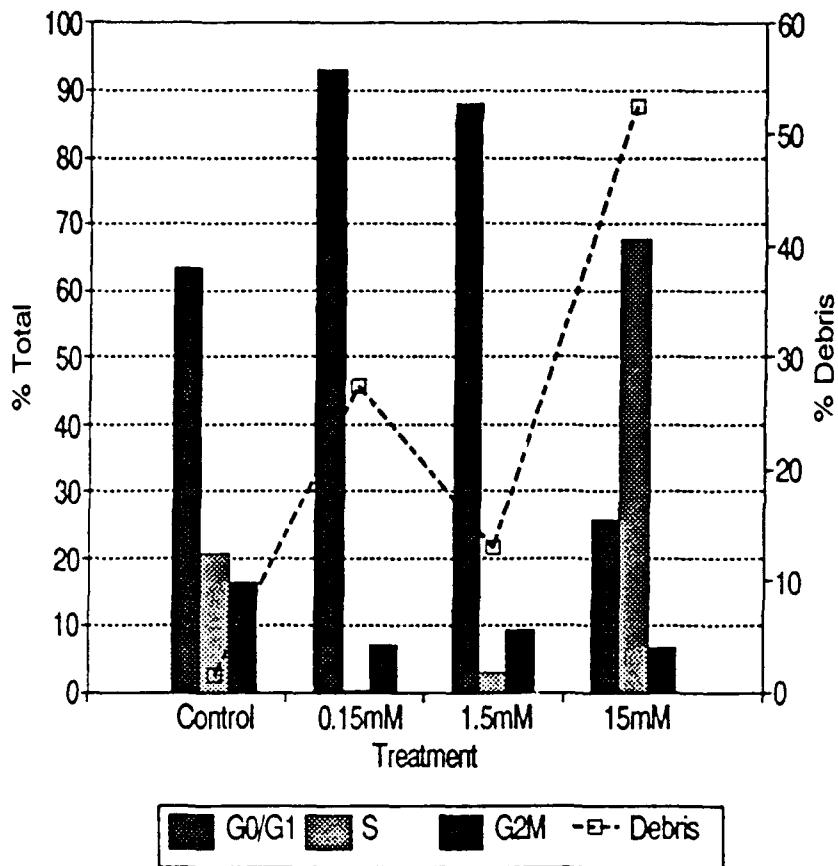


Figure 3. DNA population percentages for unburned COTM. Bars represent the percent of total population (n=20,000) derived from modeled histogram of DNA content vs. cell number (see methods). Chi Square values for goodness-of-fit to the data ranged from 2.4 to 6.2. Dotted line represents the percent of cellular debris for each sample set and is plotted against the second (% Debris) axis.

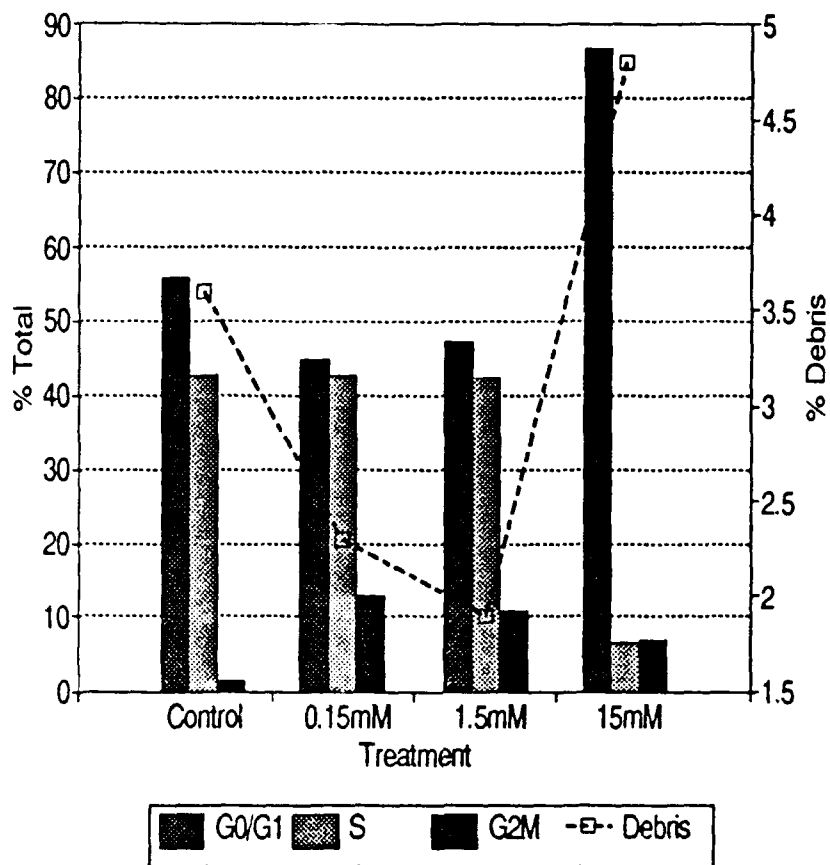


Figure 4. DNA population percentages for burned COTM. Chi square for model goodness-of-fit ranged from 0.9 to 1.3. Each sample histogram listing contained $n = 10,000$ cells.

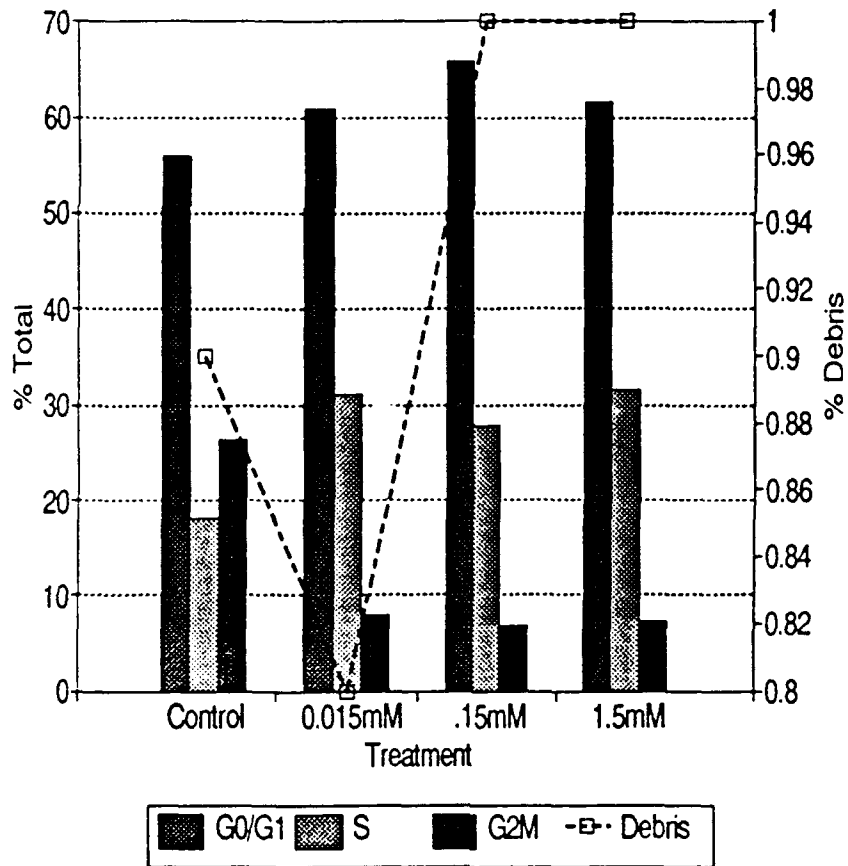


Figure 5. Unburned ZODM DNA histogram populations. DNA histogram data of $n = 10,000$ cells fitted as described in methods. Chi Square ranged from 0.7 to 1.9.

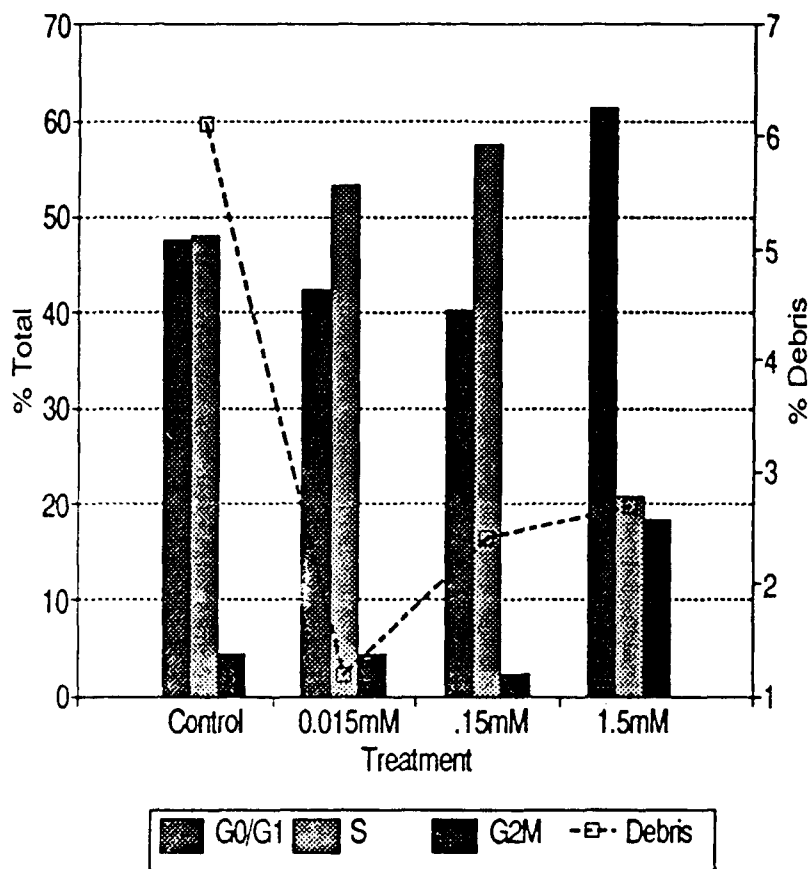


Figure 6. DNA population data for burned ZODM. Chi Square values range from 1.6 to 4.0 for DNA histograms of n = 20,000 cells each.

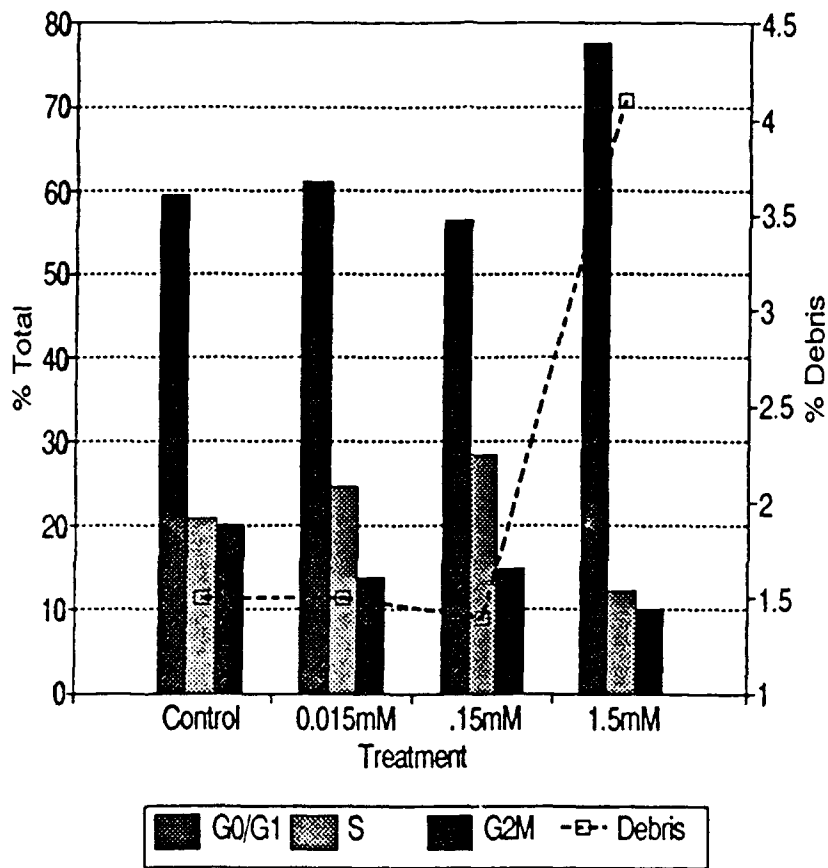


Figure 7. DNA population percentages for unburned ZOTM. Chi Square values range from 0.7 to 6.6 for fitted histograms of n= 10,000 cells each.

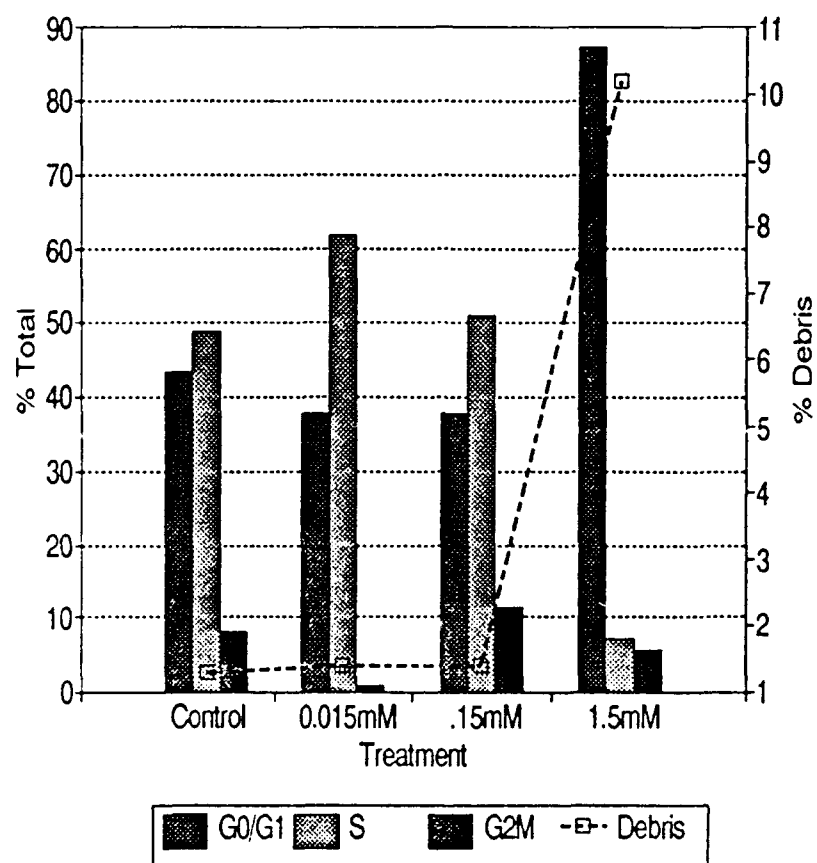


Figure 8. Burned ZOTM DNA population data. DNA histograms of $n = 20,000$ cells each fitted with MCYCLE™ model. Chi Square ranged from 1.1 to 3.2.

SECTION 4 DISCUSSION

On an equimolar basis and using LDH leakage as an endpoint, unburned COTM appears to be the most acutely cytotoxic compound followed by unburned ZOTM. However, historical control values for primary hepatocytes in culture are established at 25% of total intracellular LDH or less. Consequently, the biological significance of the observed LDH elevations must be questioned since the maximum value seen was only 27%, s.d 4. The data from both the cytoplasmic esterase activity and membrane integrity suggests that COTM is not more damaging to cell membranes or esterase activity than either of the other two chemicals in their unburned state.

While it appears that the powdered lubricants tested are not particularly cytotoxic, based on equimolar concentrations and LDH enzyme leakage, there was a noted difference in the level of LDH enzyme leaked between cells exposed to unburned and burned COTM. In contrast to unburned COTM, cells exposed to either 0.15 mM or 1.5 mM burned COTM were not found to be significantly different than controls with respect to LDH enzyme leakage. This data indicated that the burned COTM material was less cytotoxic than the unburned COTM. In contrast to burned COTM, both burned ZOTM and ZODM produced the same effects on exposed cells as the unburned material. It is not clear as to the cause of reduced COTM cytotoxicity after being subjected to a temperature of 650°C, but it appears that the cesium metal is involved because the only difference between the COTM compound and the ZOTM compound is the metal element.

In contrast, the DNA data is striking. At all concentrations tested unburned COTM depressed cellular DNA synthesis and cell cycle progression by a factor of 10. Equimolar concentrations unburned ZODM had no inhibitory effect. Unburned ZOTM did inhibit

DNA synthesis at the highest concentration of 1.5 mM but showed none of the acute cell killing observed with ZOTM. Burning generally decreased the toxicity to cellular DNA for all three compounds.

CONCLUSION

Based on all the data, we would rank these compounds in order of increasing cytotoxicity as follows:

1. ZODM (ZnMoO_2S_2)
2. ZOTM (ZnMoOS_3)
3. COTM (Cs_2MoOS_3)

SECTION 5

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