Modification of cyto- and genotoxicity of mercury and lead by antioxidant on human lymphocytes *in vitro*

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Mercury (30 μM) induced tenfold higher DNA damage, compared to the same amount of lead as measured by comet assay. This damage could be ameliorated in the presence of free-radical antioxidants like Tempol. Lead-induced olive tail moments were higher for low concentrations and decreased at concentration between 20 and 90 μM. Beyond 90 μM lead, the tail moments increased and remained unaffected by antioxidant treatment. For mercury and lead intoxication, dose-dependent formation of micronucleus (MN) was observed along with increase in apoptotic and necrotic cells. Tempol exhibited inhibition of formation of MN along with reduction in apoptotic and necrotic cells. This inhibition was also more pronounced for mercury than lead.

Keywords: Antioxidants, lead, lymphocytes, mercury, reactive oxygen species, Tempol, toxicity.

HEAVY metals such as mercury and lead are ubiquitous in the environment as contaminants due to anthropogenic activities. These metals up to some levels are utilized by living organisms, though their metabolic function is not known. On accumulation, these metal concentrations can cross the threshold levels of meaningful utility and can become toxic to the living organisms[1]. The toxicities produced by these heavy metals involve neurotoxicity, gastrointestinal toxicity and nephrotoxicity. Specific differences in the toxicities of metal ions may be related to difference in solubility, absorbability, transport, chemical reactivity and ability to form complexes with biologically important molecules[2]. These metals are known to induce toxicity by oxidative damage to biologically important molecules like DNA[3]. The oxidative damage is caused by reactive oxygen species (ROS) via Fenton type or Haber–Weiss-type reactions, which may cause an increase in lipid peroxidation, DNA damage, disturbance in calcium homeostasis, depletion of sulphydryl groups and other detrimental effects leading to carcinogenesis, as well as indirect pro-oxidant action of other metals[4,5].

The results of cytogenetic monitoring in peripherial blood lymphocytes of individuals exposed to elemental mercury or mercury compounds from accidental, occupational or alimentary sources were either negative or borderline or uncertain as to the actual role played by mercury in some positive findings[6–8]. Mercury maybe involved in four main processes that lead to genotoxicity, generation of free radicals and oxidative stress, action on microtubules, influence on DNA repair mechanisms and direct interaction with DNA molecules[9,10].

Lead is a ubiquitous, cumulative and insidious environmental pollutant that induces a broad range of physiological, biochemical and behavioural dysfunctions[11,12]. Cytogenetic monitoring in peripherial blood lymphocytes of individuals exposed to elemental lead in battery and paint industries showed intoxication[13–15]. Lead may induce oxidative stress that may deteriorate biological macromolecules either by increased production of ROS, or by deletion of major cellular antioxidants. However, the exact mechanism of lead intoxication and the dose effect is not known.

As metal-mediated cellular injury is known to be associated with production of ROS, it was reasoned that antioxidants could intervene in metal-mediated injury and confer protection against ROS-induced cellular damage in human lymphocytes. Nitroxides (stable nitroxide radical) have been found to interact with and alter many metabolic processes[16,17]. Tempol (nitroxide) can detoxify ROS and act as superoxide dismutase (SOD) mimic in the cellular system, when there is depletion of naturally occurring antioxidants like glutathione and SOD[18–20]. In the present study tempol was tested for its efficacy in modifying oxidative stress and altering the redox status of tissues (lymphocytes). The present study is aimed to know the difference in the molecular mechanism involved in mercury and lead intoxication, where the external antioxidant could play a significant role.

Comet assay is a highly sensitive technique to study DNA damage caused by metals[21–23] and to detect combined environmental mutagen injuries the micronucleus (MN) assay is also used[24]. In the present work both these techniques have been used to study metal-induced (mercury/lead) intoxication in humans and its modification by antioxidant.

Mercury chloride ($HgCl_2$), lead nitrate $Pb(NO_3)_2$, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), high melting point agarose, low melting point agarose, Ficoll-Histopaque, Na_2-EDTA, Triton X-100, dimethyl sulphoxide (DMSO), Tris-base, ethidium bromide, phosphate buffered saline (PBS; Ca++, Mg++ free), sodium chloride (NaCl) and sodium hydroxide (NaOH) were obtained from Sigma Chemical Co., USA.

Cell viability of the lymphocytes in the absence and presence of mercury or lead was tested after 2½ h exposure by trypan blue exclusion assay using Countess automated cell counter, Invitrogen C10227. Population of cells was also determined using similar technique.

For cytokinesis block micronucleus (CBMN) assay, the method described by Fenech was adopted with some modifications[25,26]. Heparinized human blood from one healthy donor (laboratory volunteer, 24-year-old non-

smoker) was added to RPMI 1640 medium in a volume ratio of 1 : 9 containing 10% (v/v) foetal calf serum (FCS), penicillin, streptomycin sulphate salt (100 μg/ml), phytohemagglutinin (PHA) and 125 mM L-glutamine. Mercury chloride/lead nitrate between 5 and 90 μM was added to the culture medium and incubated along with antioxidant Tempol, of various concentrations at 37°C in CO_2 incubator for 72 h. Twenty-eight hours prior to cell harvest, cytochalasin B (Cyt-B) from *Drechslera dematioidea* (Sigma; 6 μg/ml) was added to the culture. After 72 h, lymphocytes were harvested after brief treatment with 75 mM KCl and immediately fixed in a mixture of methanol : glacialacetic acid (3 : 1).

After three washes, the cells were spread on wet slides and stained with 2% Geimsa in Sorenson's buffer (pH 6.8) for 10–12 min. All slides were coded and evaluated for the frequency of micronuclei in cytokinesis-blocked binucleated (BN) cells, as described by Fenech[25,26]. Cells with chromatin condensation or nuclear fragmentation with intact cytoplasmic boundaries were scored as apoptotic. Cells with intact nuclear structure and irregular nuclear membrane/cytoplasmic membrane were scored as necrotic. A total of 1000 BN cells were analysed from each experimental sample. The same slides were also used for counting apoptotic and necrotic cells according to specifications described by Fenech.

Blood was diluted with PBS in 1 : 1 ratio and layered onto Histopaque in a ratio of 4 : 3 (blood + PBS : Histopaque). Blood was then centrifuged at 400 *g* for 30 min at room temperature. The lymphocyte layer was removed carefully and washed twice in PBS at 250 *g* for 10 min each and then used for further experiments. Mercury or lead salt with or without Tempol was added to the isolated lymphocytes in 1 ml of PBS maintaining the final salt concentration between 10 and 200 μM and incubated for 2½ h at 37°C. Metal-induced DNA damage in the lymphocytes was assayed by SCGE (comet assay) as described by Singh *et al.*[27], with some modifications. The slides were stained with 50 μl ethidium bromide (20 μg/ml) and visualized using a Carl Ziess fluorescent microscope. The images (100 cells/slide) were captured with a high-performance HBO50 colour video camera. The measure of the DNA strand breaks from the stored images was analysed using CASP software, which estimates percentage of DNA in the tail and its length. The product of the two is reported as olive tail moment in the text.

The results represent the mean ± SD from at least four independent experiments (*n* = 4). Differences between (a) control versus metal-treated cultures, (b) control versus Tempol-treated cultures and (c) metal-treated versus metal-plus-Tempol-treated cultures were assessed using one-way analysis of variance (ANOVA) with post hoc testing with Dunnet's multiple comparison test. A *P*-value of less than 0.05 was considered significant.

In the levels of environmental spillage, lead and mercury induced significant injury to lymphocytes. Based on

these observations mercury and lead were tested in the present study in concentration ranges reported for other metals, to evaluate metal-induced toxicity in human lymphocytes[28]. Metal salts such as $HgCl_2$ and $Pb(NO_3)_2$ at concentration 30, 60 and 90 μM, induced a concentration-dependent, statistically significant increase in the percentage of apoptotic (Figure 1) or necrotic cells (Figure 2). It is observed that at higher concentrations of lead (90 μM) the number of apoptotic cells dropped slightly, apparently due to massive occurrence of necrosis. The efficacy of Tempol was studied for its antioxidant properties against metal-induced oxidative stress and cell death (Figures 1–3). It was observed that Tempol between 15 and 45 μM demonstrated anti-apoptotic properties when co-cultured with 30–90 μM of $HgCl_2$ or $Pb(NO_3)_2$. Enhancement in apoptosis at higher concentrations of test metals maybe related to the stoichiometric excess of the metal and partly due the apoptotic activity of Tempol, especially above 60 μM of mercury and 90 μM of lead.

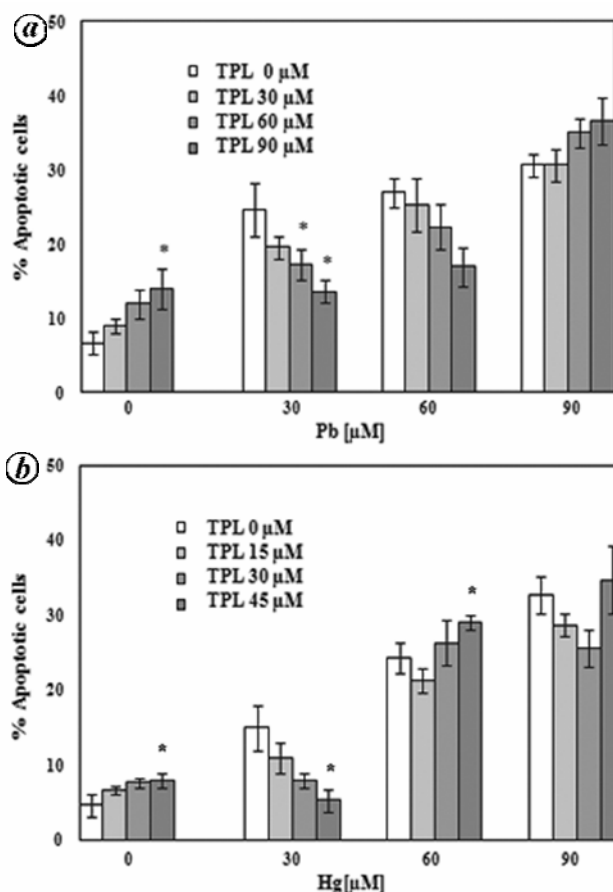Mercury at concentrations as low as 30 μM significantly increased the yield of necrotic cells (~ two fold)

Figure 1. Protection of lymphocytes against metal-induced apoptosis by Tempol. Cells were co-incubated with Tempol and (*a*) $Pb(NO_3)_2$ and (*b*) $HgCl_2$ and inspected after 72 h. SD is shown by ± bars. Three sets of readings were taken for each test and *P* < 0.05 compared with control and metal-treated cultures.
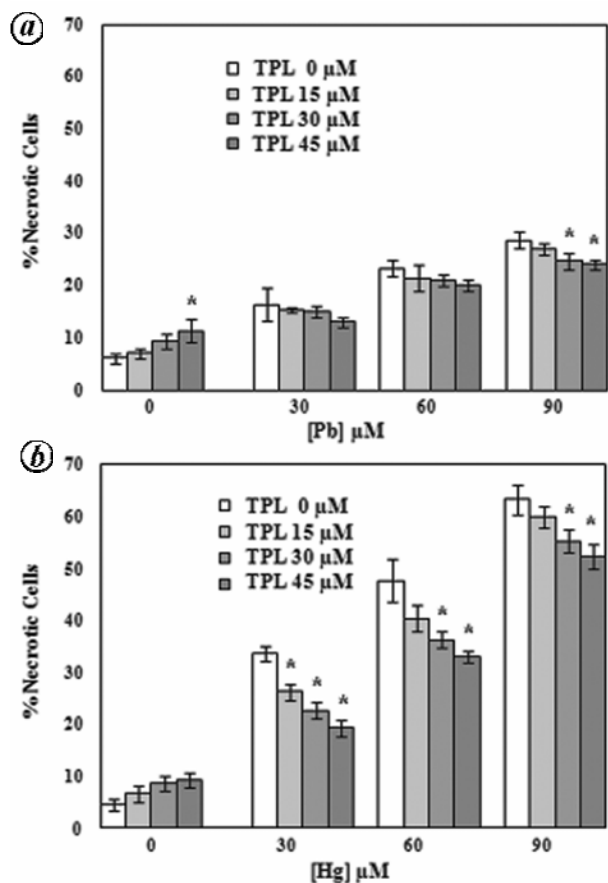
Figure 2. Protection of lymphocytes against metal-induced necrosis by Tempol. Cells were co-incubated with Tempol and (**a**) Pb(NO₃)₂ and (**b**) HgCl₂ and inspected after 72 h. SD is shown by ± bars. Three sets of readings were taken for each test and *P < 0.05 compared with control and metal-treated cultures.

Figure 3. Impact of Tempol on micronucleus formation caused by exposure to (**a**) Pb(NO₃)₂ and (**b**) HgCl₂ and scored after 72 h. SD is shown by ± bars. Three sets of readings were taken for each test and *P < 0.05 compared with control and metal-treated cultures. BN, Binucleated.

compared to lead. A strong and unequivalent protective effect of Tempol (15–45 µM) against metal-mediated necrosis was also observed and it showed highest efficacy at 30 µM mercury.

In the concentration range used along with necrotic and apoptotic cell count, dose-dependent elevation of the MN frequency after exposure to test metals mercury or lead was followed and results are shown in Figure 3. Mercury was observed to be potent MN inducer with 1.5-fold increase in MN compared to lead at 30 and 60 µM concentrations. Tempol efficiently reduced high MN yield for mercury compared to lead, except at very high concentrations; such drastic decrease was not observed for lead. Tempol (45 µM) decreased the MN count by 50% and 25% when co-cultured with 60 µM mercury and lead respectively.

Metal-induced DNA damage was assayed by SCGE and measured by the olive tail moments of their respective comets. Olive tail moment 7.75 reduced to 6.1 when 20 µM lead was co-incubated with 5 µM Tempol, where untreated cells showed tail moment of only 1.83. DNA damage at concentrations of lead between 30 and 90 µM
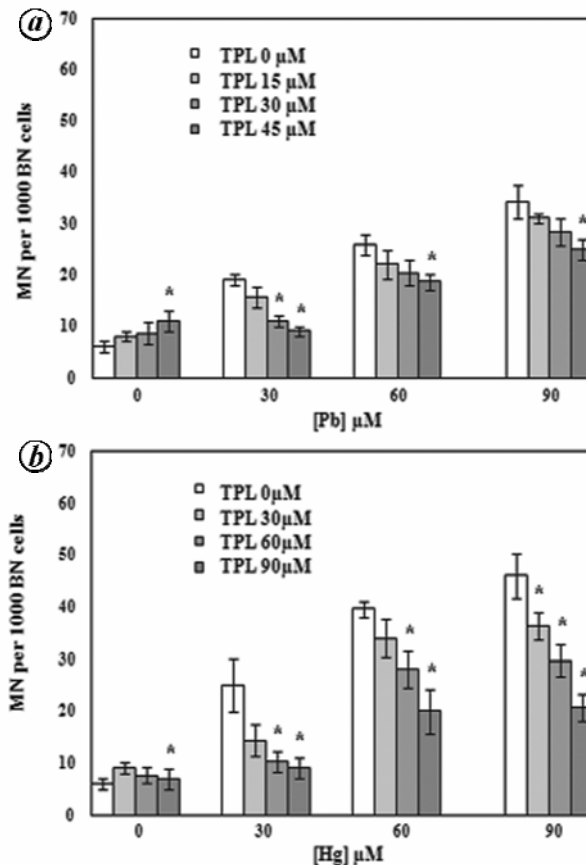
showed lower tail moments compared to 20 µM lead. Beyond 90 µM, the tail moments increased but were not rendered shorter by Tempol, as evident from Figure 4. Mercury induced ten-fold higher DNA damage than lead at 60 µM and shorter comet tails were observed when Tempol was co-incubated with mercury compared to lead.

Increased cell density with decrease in cell viability on incubation with lead between 120 and 180 µM was highly pronounced; threefold increase in population resulted in 16-fold decrease in viable cells at lead concentrations beyond 60 µM. Tempol exhibited no anti-proliferation, nor pro-viability efficacy in these conditions. Cell viability also decreased for mercury; however, no increase in population was observed.

Oxidative stress induced by metals may lead to early apoptosis, necrosis or MN formation and all the three types of cells were scored in our study by CBMN assay. Results (Figures 1 and 2) illustrate that apoptosis is predominant in lymphocytes exposed to lead and necrosis is dominant when exposed to mercury. Necrosis could result from higher production of H₂O₂ under impaired
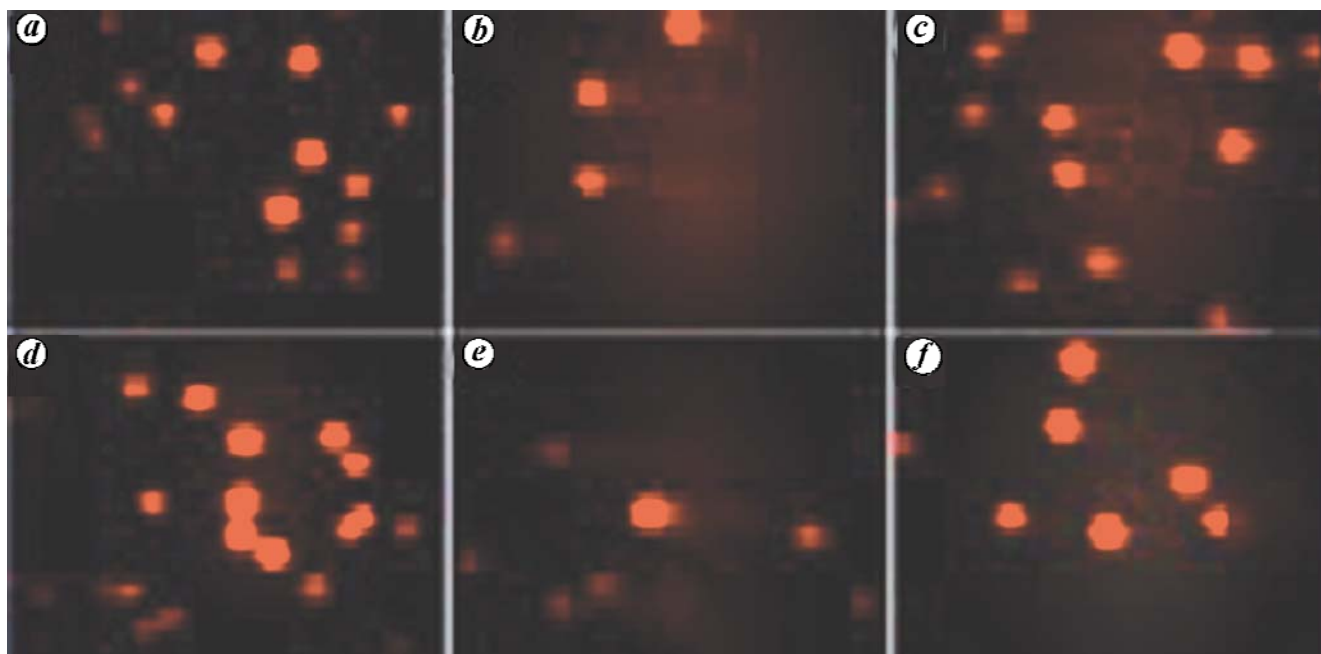
Figure 4. Single-cell gel electrophoresis photomicrographs of peripheral blood cells in healthy volunteers when treated with lead. *a*, Representative comet image of the control group; *b*, 20 μM Pb; *c*, 20 μM Pb and 10 μM Tempol; *d*, 120 μM Pb; *e*, 180 μM Pb and *f*, 180 μM Pb and 80 μM Tempol.

mitochondrial respiratory chain when exposed to mercury[29,30]. Necrosis could also be attributed to the interference with signalling pathway on mercury uptake[31].

Damage leading to necrosis due to higher production of H_2O_2 on mercury exposure can be ameliorated by antioxidant capable of scavenging H_2O_2 and its metabolites. Nitroxides prevent metal ion-catalysed oxidative damage by scavenging ROS[28]. Tempol, a nitroxide, was tested and for anti-apoptotic and anti-necrotic properties for mercury- and lead-induced toxicity. Antioxidant effects were observed for concentrations up to 30 μM of mercury or lead, which turned pro-oxidant at higher Tempol concentration. Tempol is more efficient in ameliorating mercury-induced toxicity compared to lead; and it could only be explained if it is assumed that the mechanism of mercury-induced toxicity is mainly ROS-mediated. Lead is incapable of producing ROS directly[32,33], which renders Tempol inefficient in reducing toxicity.

When cells escape necrosis or apoptosis they undergo MN formation when cell division is blocked in cytokinesis. Leakage of nuclear matter is manifestation of strand breaks, caused due to toxic effects of metals through ROS. Figure 3 shows that lead is a weaker MN inducer in lymphocytes compared to mercury, in agreement with previous reports[24,34]. Tempol demonstrated higher efficacy in reducing MN formation supporting oxidative damage on mercury exposure.

Cell proliferation along with decrease in cell viability was observed on lead exposure as reported earlier[35–37] and Tempol had no effect on it. Decrease in cell viability could be attributed to chromatin condensation at higher concentrations of lead resulting in fall ($P < 0.001$) in cell viability.

Comet assay studies reveal higher olive tail moments and thus higher DNA damage due to mercury exposure compared to lead. Such dose-dependent increase in tail moments has been reported for mercury exposure in bottle-nosed dolphin and rats[21,22]. DNA–DNA and DNA–protein cross-links formed in the presence of lead, manifest chromatin condensation rendering olive tail moments unchanged beyond 100 μM, in accordance with previous findings[37]. Inability of Tempol to significantly reduce DNA damage beyond 90 μM lead suggests that antioxidants have no role as these effects are not ROS-mediated. Tempol, however, significantly reduced DNA damage (olive tail moment for mercury) by its antioxidant activity. Decreased cell viability even when exposed to low amounts of mercury is attributed to the mercury-modulated cellular signalling pathways leading to the suicide of cells resulting in lesser cell viability, even at very low concentrations of mercury. The present results identify mercury to be highly toxic than lead even at low concentrations. The results corroborate the findings where lead is reported to be weakly mutagenic, that too at high concentrations[38] and mercury and methyl mercury to be highly toxic even at low concentrations. Levels of mercury and lead need to be more closely monitored as they could cause serious occupational and environmental hazards leading to irreparable damage even when supplemented with antioxidants.

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