

Effects of Lanthanum on Human Lymphocytes Viability and DNA Strand Break

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Abstract Lanthanum (La) is a rare-earth metal with applications in agriculture, industry, and medicine. Since lanthanides show a broad spectrum of applications there is an increased risk of contamination for humans. We examined the effects of lanthanum in Jurkat cells and human peripheral lymphocytes (HPL), and we found that it was cytotoxic and genotoxic on both cell lines. Additionally, HPL were more sensitive to La treatment than Jurkat cells and necrosis was the pathway by which La induced cytotoxicity. Vitamin E was able to diminish the DNA strand breaks induced suggesting that oxidative stress may be involved in the genotoxic process.

Keywords Lanthanum · Lymphocytes · Cytotoxicity · Genotoxicity

Lanthanum (La) is the first member of the series of rare-earth metals that constitute a group of a 15 transition elements. Lanthanum occurs in ores such as monazite in Brazil and other countries. This element has applications in agriculture, industry and medicine (Palasz and Czekaj

2000). There is an increasing interest in its medical use including the therapeutic application of La as an anticancer agent, proposed by Shi and Huang (2005). In the last 25 years, new technologies in metallurgical, optical, and electronic industries increased the application of artificial lanthanide compounds with special physicochemical properties. Since lanthanides and their compounds show a broad spectrum of industrial applications there is an increased risk of internal contamination for humans and animals. Metallurgy industry utilizes lanthanum to remove oxygen and to enrich steel. Pure lanthanum compounds are used in electronics and optoelectronics to produce luminophores, high-temperature superconductors, lighters, glass additives, ceramics, and batteries (Palasz and Czekaj 2000). These applications increased the chances of accidental exposure, both acute and chronic. The inhalation of aerosols containing La is admittedly the main route of internal contamination for miners exposed to monazite and industrial workers exposed to several chemical forms of La. In addition, lanthanum compounds have been used to increase the productivity of crops and to promote the growth of livestock (Yu et al. 2005). These applications introduce the possibility of contamination of human body with La through food chains. Lanthanum has a higher accumulation rate and lower metabolic rate than other rare earths in the body, which would make lanthanum accumulate more easily than other rare earths and induce more effects on the human body (Feng et al. 2002). These same authors suggest, through rat serum analyses, that $\text{La}(\text{NO}_3)_3$ impairs the liver and kidney and causes irregular enzyme metabolism for animals chronically intaking La. Ions of the lanthanides (Ln^{3+}) series have important biochemical, biological, and pharmacological properties. Many, but not all, of these properties arise from the ability of Ln^{3+} ions to replace Ca^{2+} isomorphously or antagonize Ca^{2+} in a

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variety of cellular and sub cellular reactions (Palasz and Czekaj 2000).

The aim of this work was to determine the cytotoxicity and genotoxicity of lanthanum nitrate in human lymphocytes as well as to clarify the mechanism involved in these processes. Our decision on choosing lanthanum nitrate was based in these facts: (i) lanthanum nitrate is mainly applied in industry (specialty glass and catalyst); (ii) soluble forms of lanthanum have been reported to be more toxic than “insoluble” forms (Lizon and Fritsch 1999); (iii) experiments previously accomplished in our laboratory showed similar toxicology results for lanthanum nitrate and lanthanum chloride (data not show).

Materials and Methods

The $\text{La}(\text{NO}_3)_3$ (SIGMA; CAS-10277-43-7) stock solution (1 M) was obtained by dissolving $\text{La}(\text{NO}_3)_3$ crystals in sterile distilled water (MilliQ). It was then diluted a second time and added to cell cultures at the pre-indicated concentrations, 1% of the culture volume. The maximum concentration used in the experiments, 5 mM, was stipulated based on the limit of pH change.

This project was registered with the Institutional Ethics Committee (Fernandes Figueira Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) under number 013/04. Two cell lines were used: Jurkat cells and primary human peripheral lymphocytes (HPL). The Jurkat cells, a T-cell acute myelogenous leukemia line, were acquired from the Rio de Janeiro Cells Bank (BCRJ no.: CR063) Clementino Fraga Filho University Hospital belonging to Federal University of Rio de Janeiro (UFRJ). Cells were cultured in RPMI 1640 medium (CULTILAB-Brazil), pH 7.2, supplemented with 10% fetal calf serum (CULTILAB-Brazil), 50 mg/L gentamycin and 2 mg/L anfotericin B, under 5% CO_2 atmosphere, at 37°C. Blood withdrawn from a male donor (healthy, non-smoking aged 20–30) was collected into Ficoll-Hypaque (SIGMA-ALDRICH). The samples were then centrifuged at $200 \times g$ at 25°C for 20 min. The isolated lymphocytes (0.3 mL) were cultured in 4.7 mL RPMI 1640 medium including 2% Phytohemagglutinin (PHA–GIBCO).

The different $\text{La}(\text{NO}_3)_3$ concentrations ranged from 0.25 to 5 mM and the culture samples were treated for 48 h with $\text{La}(\text{NO}_3)_3$. Cellular viability, necrosis, and apoptosis were analyzed in three time intervals – 0, 24, and 48 h. For the comet assay, culture samples were incubated for 3 h with $\text{La}(\text{NO}_3)_3$. In a second trial, samples were pre-incubated with 25 μM of water-soluble vitamin E analog (Trolox-CALBIOCHEM) for 30 min (Wozniak and Blasiak 2002), than washed in PBS (phosphate buffer saline–pH 7.4) and incubated in fresh culture medium with the pre-established concentrations of $\text{La}(\text{NO}_3)_3$ for the next 3 h. Vitamin E

was derived from stock solution (100 mM) in sterile distilled H_2O and mixed to cell culture volume (1:100). The cell viability analysis was determined by Trypan blue (TB-0.4% SIGMA-ALDRICH) exclusion assay. The cells were analyzed through microscopic observation to determine the survival ratio (N/N_0). Survival ratio represents the ratio between the number of viable cells after 24 h and 48 h of treatment and the number of viable cells before the treatment. Apoptotic and necrotic cells were evaluated through morphological analysis. One microliter of a 1:1 mixture of 100 $\mu\text{g}/\text{mL}$ acridine orange (AO) and 100 $\mu\text{g}/\text{mL}$ ethidium bromide (EB) was added to 25 μL of cell culture samples. This cocktail was placed on a microscope slide and covered with a 22-mm square coverslip. Cells were than observed and counted using a fluorescence microscope (NIKON filter-495 nm and absorption filter-515 W) at $400\times$ of magnification. At least 200 cells per sample were scored. The classical comet assay was performed under alkaline conditions, $\text{pH} > 13$, according to the procedure by (Singh et al. 1988). Briefly, 1 mL of a freshly prepared suspension of lymphocytes (Jurkat and HPL) ($2.0\text{--}5.0 \times 10^5$ cells/mL) added to 0.5% low melting point agarose dissolved in PBS was spread onto microscope slides pre-coated with 1.5% normal agarose. The cells were then lysed for 1 h at 4°C in a solution consisting of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10. After lyses, slides were rinsed and placed in an electrophoresis chamber. DNA was allowed to unwind for 25 min in the electrophoretic solution consisting of 300 mM NaOH and 1 mM EDTA, $\text{pH} > 13$. Electrophoresis was conducted for 25 min at 25 V and current adjusted to 300 mA. After electrophoresis slides were neutralized with Tris–HCl (0.4 M; pH 7.5) three times for 5 min, fixed in ethanol for 5 min and stained with 50 μL EB (10 $\mu\text{g}/\text{mL}$). Comet images were analyzed under NIKON fluorescence microscope (NIKON filter-546 nm and absorption filter-580 W) by visual scoring of 50 selected images per slide, in triplicate, classifying them into five categories. Each category representing relative tail intensity and thus increasing degrees of damage (Collins et al. 1994). The percentage of damaged DNA was converted to the arbitrary units.

Data for each assay, mean \pm SD of three independent experiments, were statistically analyzed by ANOVA followed by Tukey test. Differences were considered significant when the p -value was less than 0.05.

Results and Discussion

$\text{La}(\text{NO}_3)_3$ effect on cell viability was evaluation with TB exclusion assay in Jurkat cells and HPL cultivated during 24 h or 48 h in the presence of various concentrations of

this compound. After 24 h treatment (Fig. 1a, b) it was already possible to notice a concentration-dependent declining survival of both cell lines.

In order to verify which death pathway (apoptosis or necrosis) could play a role on $\text{La}(\text{NO}_3)_3$ toxicity, apoptotic and necrotic indices were determined by morphological analysis. After testing $\text{La}(\text{NO}_3)_3$ in a range of dose from 0.25 to 5 mM, and 24 h after incubation, both Jurkat and HPL cells revealed an increase of necrotic cells indices (Fig. 1c–f). These results indicated that necrosis is the pathway by which $\text{La}(\text{NO}_3)_3$ induces cytotoxicity.

The genotoxic effect induced by $\text{La}(\text{NO}_3)_3$, measured as DNA strand breaks in alkaline SCGE, was detected since the 0.25 mM $\text{La}(\text{NO}_3)_3$ concentration. $\text{La}(\text{NO}_3)_3$ caused a significant increase ($p < 0.05$) in arbitrary units compared to the unexposed control. We decided to use a pre-treatment with Trolox (a known free radical scavenger) in order to investigate the possibility of ROS involvement in $\text{La}(\text{NO}_3)_3$ genotoxicity. Trolox is a water-soluble Vitamin E analog (capable to pass through cell membrane) and it plays an important role in protection ROS. Liu et al. (2003) reported that La induced cell death in human liver cell line

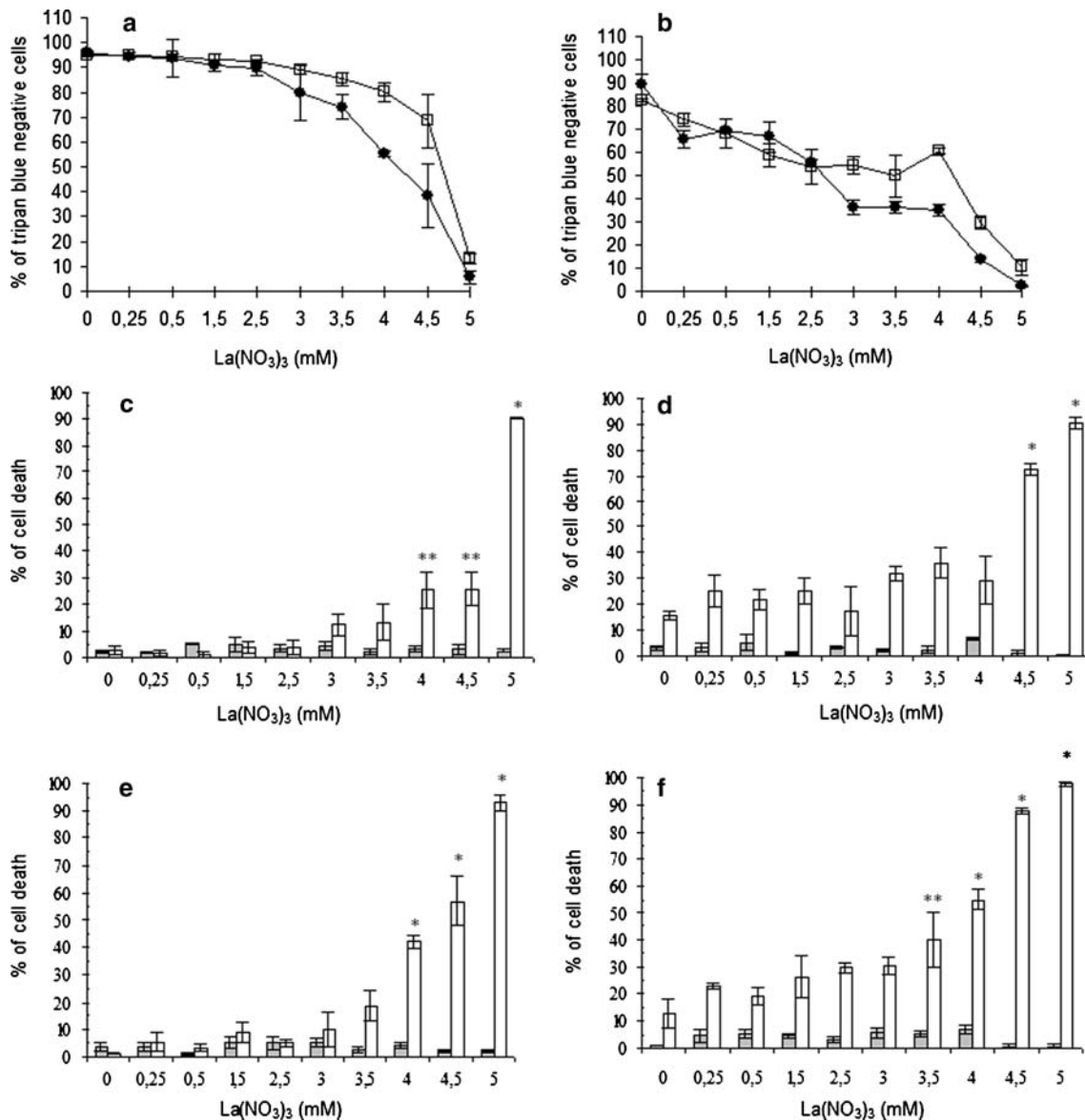
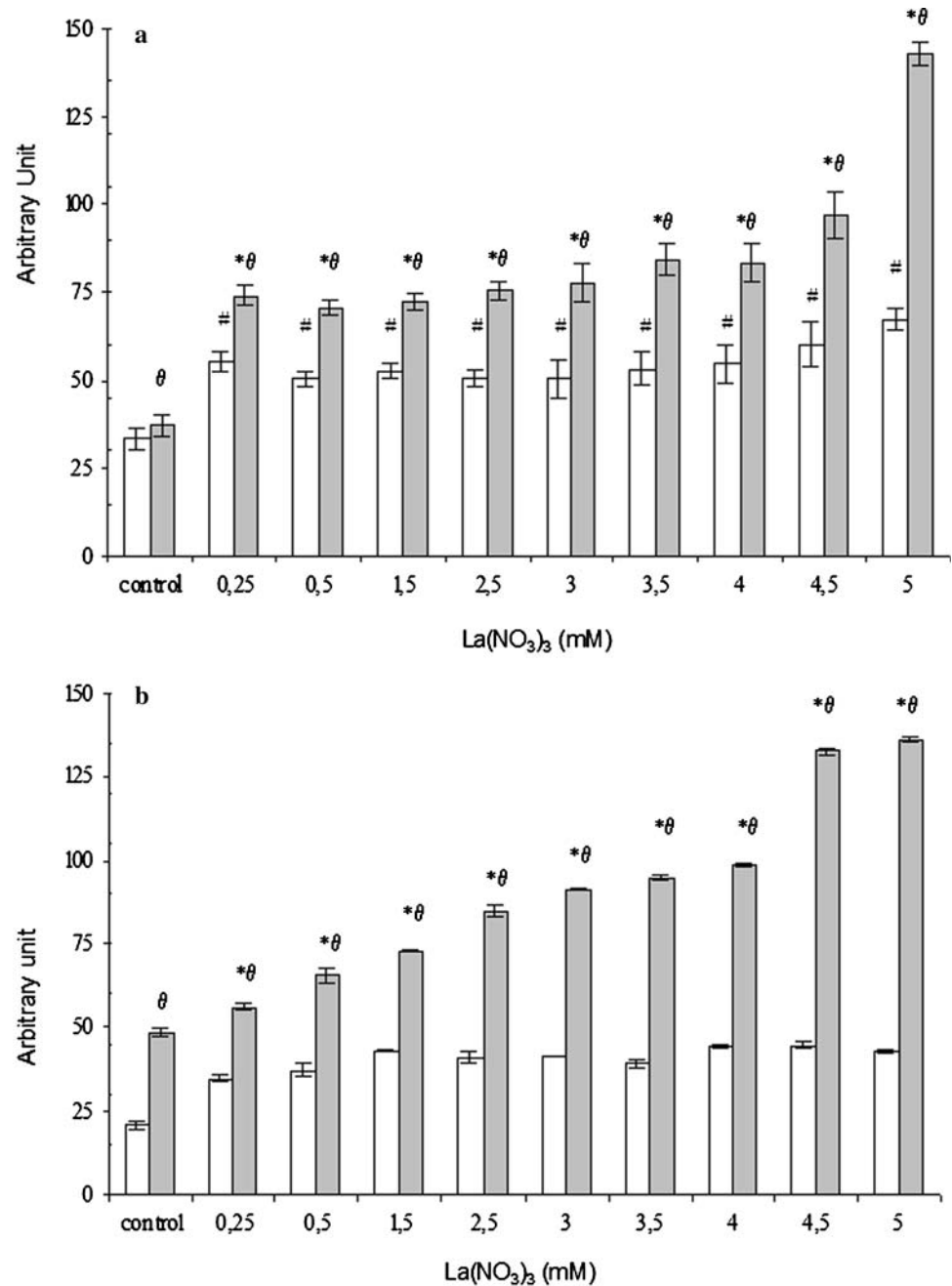


Fig. 1 Viability of Jurkat cells after treatment with $\text{La}(\text{NO}_3)_3$ for 24 h (□) and 48 h (●) (a). Viability of HPL after treatment with $\text{La}(\text{NO}_3)_3$ for 24 h (□) and 48 h (●) (b). Each point represents the mean \pm SD of three experiments. Each point represents the mean \pm SD of three experiments. Error bars indicate standard errors. Apoptotic (gray) and necrotic (white) indices of Jurkat cells (c: 24 h and d: 48 h) and HPL

(e: 24 h and f: 48 h) exposed to $\text{La}(\text{NO}_3)_3$ determined by double staining with AO and EB. Statistically significant differences were determined by ANOVA followed by Tukey test (** $p < 0.05$; * $p < 0.01$). Error bars indicate standard. * or ** indicates statistically significant difference in necrotic index compared to control

Fig. 2 DNA strand breaks of Jurkat cells (**a**) and HPL (**b**) incubated for 3 h at 37°C with $\text{La}(\text{NO}_3)_3$ (gray) and in the presence of vitamin E at 25 μM (white). Statistically significant differences were determined by ANOVA followed by Tukey test ($p < 0.01$). Error bars indicate standard errors. In **a**, * indicates statistically significant difference in arbitrary unit between $\text{La}(\text{NO}_3)_3$ and control (gray). # indicates statistically significant difference in arbitrary unit between $\text{La}(\text{NO}_3)_3$ plus vitamin E (white) and control (white). θ indicates statistically significant difference in arbitrary unit between $\text{La}(\text{NO}_3)_3$ (gray) and $\text{La}(\text{NO}_3)_3$ plus vitamin E (white). In **b**, * indicates statistically significant difference in arbitrary unit between $\text{La}(\text{NO}_3)_3$ and control (gray). θ indicates statistically significant difference in arbitrary unit between $\text{La}(\text{NO}_3)_3$ (gray) and $\text{La}(\text{NO}_3)_3$ plus vitamin E (white)



7701 and that La exposure would be associated with an increase in H_2O_2 within cells. These results may indicate the involvement of ROS in the mechanism of La toxicity. Pre-incubation of lymphocytes with 25 μM Trolox (Wozniak and Blasiak 2002) significantly ($p < 0.05$) decreased DNA damages compared to the control group and cells treated only with $\text{La}(\text{NO}_3)_3$ (Fig. 2a, b) Once free, non-cell bound, vitamin molecules were washed out before incubation of cells with $\text{La}(\text{NO}_3)_3$, the effect of Trolox diminishing DNA damage strongly suggests the involvement of ROS in the genotoxic process.

There is an increasing interest in the study of the biological effect of rare earth elements considering their applications in different fields of activity. Therefore, the chances of accidental acute and chronic exposure are not negligible. However, the biological mechanisms of rare earth metals ions action remain unclear. It has been demonstrated the interactions and effects of Ln, most of it La, with healthy mammal cells (Feng et al. 2002; Zhao et al. 2004). Lanthanum was shown to be cytotoxic to pulmonary macrophage of rats (Palmer et al. 1987), and it was also reported to be cytotoxic increasing micronucleus

frequency, single-strand DNA breaks and unscheduled DNA synthesis (UDS) in peripheral human lymphocytes (Yongxing et al. 2000). Our results indicate that: (i) $\text{La}(\text{NO}_3)_3$ induces cytotoxicity; (ii) Trolox is able to reduce DNA strand breaks induced by $\text{La}(\text{NO}_3)_3$ suggesting that reactive oxygen species (ROS) may be involved in the genotoxic process. Ln^{3+} ions have been reported to replace Ca^{2+} isomorphously or to antagonize Ca^{2+} in a variety of cellular and sub cellular reactions. The high affinity of La^{3+} for the Ca^{2+} sites might result from the combined effect of two factors: an effective radius (1.10 Å) similarity to that of Ca^{2+} (1.06 Å) and a valence higher than that of Ca^{2+} (Lacaz-Vieira and Marques 2004). Ln^{3+} was found to activate a Ca^{2+} -sensing receptor (CaR) and to induce an increase of cellular Ca^{2+} concentration (Shorte and Omiyama 1996). The elevated intracellular Ca^{2+} can result in necrotic cell death. Additionally, Lee and Shacter (1999) demonstrated that, under oxidative stress, human B lymphoma cells are unable to undergo apoptosis and die instead by necrosis. Another study showed that ROS induced necrotic cell death in lymphocytes (Naito et al. 2004). The induction of cellular necrosis derived from the increase of ROS could be dependent on the oxidant concentration (Barbouti et al. 2002). In our study the genotoxic effect was observed for La concentrations equal or higher than 0.25 mM and diminished in the presence of Trolox. In our experiments, the decrease of DNA damage after treatment with Trolox indicates that the genotoxic effect of La is, at least in part, a result of oxidative DNA damage. In this context, it is important to distinguish if those oxidants are generated as a primary action of lanthanum or as a secondary effect promoted by cell necrosis. Calcium-mediated necrosis is likely to be one of the best characterized mechanisms leading to this alternative form of cell death. In that process two major players have an important role, intracellular Ca^{2+} and ROS. The elevated La^{3+} intracellular ions as well as Ca^{2+} ions might induce mitochondrial damage with subsequent ROS overload and DNA damage (Henriquez et al. 2008). Based on these facts we may suggest that oxidants generated by La are due to a secondary effect of generalized cell necrosis. Further investigations will be necessary to determine the contribution of each of those processes in the oxidative DNA damage.

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