

Diagnosis of the Problems Which Facing the Researchers during Studying *In vitro* Lead Toxicity

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Abstract

Background: Lead (Pb) is one of the most difficult metals to investigate in laboratory experiments because it is very easily precipitates or forms complex ions. Many experiments were conducted with Pb and observed unique chemical properties of this metal. **Objective:** The present investigation was designed to evaluate the cytotoxicity of Pb exposer on pheochromocytoma (PC12) cells in different condition and media to admit the difficulties facing the researchers in bioavailability of Pb in media and prevent precipitation formation. **Materials and Methods:** Pb from 0.01 to 100 μ M had been used on PC12 cells cultured and treated in different conditions for 6, 12, 24, and 48 h. Resazurin assay was used to assess the cell viability. **Result:** As numerous studies have demonstrated a possible mechanism for Pb-induced neurotoxicity using *in vitro* model, the current study is the first study which explained the complexity of Pb participations in media that facing the authors and what the reason for that and how we can solve this problem to make pb more bioavailable in the media. **Conclusion:** Use of phosphate buffer in experiments with Pb is not acceptable because all Pb can be precipitated. However, it could be possible to use the standard media, but for the time of treatment with Pb phosphates must be removed from the media.

Keywords: Cytotoxicity, free phosphate media, lead Pb, lead phosphate

INTRODUCTION

Lead (Pb) is toxic heavy metals that rise wide range of biochemical and physiological effects that are dose-dependent and partly irreversible.^[1] It is well known as important neurobehavioral toxicant and would interfere with several of developmental processes in the brain leading to variety of impairment of its function.^[2] It has been reported that Pb would be able to induce reactive oxygen species ROS accumulation and to cause inflammation. Pb also would damage nucleic acids and inhibit DNA repair.^[3] So far, no lower threshold for the effects of Pb on nervous system has been establish to avoid that and work to reduce the amount of Pb in the environment to this level.^[4] The real and precise mechanism of Pb-induced toxicity in neuron cells remains unclear. A number of studies have been speculated that Pb induces toxicity-mediated NO formation.^[5]

The mechanism of Pb enter to cells has been studied, and most of the scientists suggested that lead can enter through calcium channels. Potassium, carbachol and veratridine increase the uptake of lead. Lead uptake inhibited by calcium channel blockers.

It is being necessary to identify the exposure level at which Pb could be able to induce cell death in cell culture to avoid this level and to consider this level is lethal for specific cell line. In our study, we tried to identify the toxic level of pheochromocytoma (PC12) cell using different media and different condition to demonstrate under what conditions Pb adversely effects on cell in media.

Even though Pb exposure has been the subject of intensive studies over many decades, the problem of Pb precipitations in media, especially in high does are still poorly understood. In this study, we used many different media and different pH levels with CO₂ or without CO₂ incubator to overcome participation problem and make Pb are bioavailable to the neuronal cells in media. To explore possible of lead-induced cell death, this study was carried out in PC12 cells as a suitable model for neuronal study.

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MATERIALS AND METHODS

PC12 cell line as a model of neuronal cell line and RPMI1640 medium (Cat #30-2001) was obtained from American Tissue Culture Collection (ATCC). Collagen IV was from Sigma-Aldrich (Cat # 9007-34-5), Dulbecco Modified Eagle's media (DMEM), and all other cell culture components such as fetal bovine serum (FBS), penicillin-streptomycin (PS), horse serum (HS), Hank's Balanced Salt Solution, and phosphate-buffered saline were purchased from Invitrogen company (Carlsbad, CA, USA). DMEM-free phosphate medium which purchased from thermo Fisher scientific (Cat #11971025). Lead (IV) Acetate Pb (CH_3CO_2)₄ and Pb (II) Acetate Pb (CH_3CO_2)₂·3H₂O obtained from Sigma-Aldrich. All other chemicals were purchased from Sigma also (St. Louis, MO, USA) unless otherwise noted. PbAc dissolved in distilled water to prepare stock solution daily. For each experiment, there were control samples which remained untreated with lead and received the equal volume of distilled water. Each treatment was performed in triplicate.

Growth media

Nearly 5% bovine serum (FBS) and 10% of HS were added to RPMI1640 medium. In addition, 1% PS added as antibiotic to the media. RPMI1640 with protein are the recommended medium by ATCC. Thus, RPMI media used to grow and sustain on the current cell line.

Measurement of cell viability using resazurin assay

A night before culturing 24-well plate was coated with collagen IV and kept in incubator for 24 h. To investigate the toxicity and bioavailability of Pb in the cell culture, 100,000 cells in 0.3 ml medium per well were seeded in collagen-coated plates and treated with multiple concentrations (0.001–100 μM) of Pb for 6, 12, 24, and even 72 h sometimes. Dimethyl sulfoxide as negative control of the assay was employed in each experiment. Same volume of distal water added to control cells and incubated in the same condition for all the experiments. The cell viability was determined using 5 μM of resazurin in medium. Resazurin solution at 0.5 mM add to the cultured cells for 30 min, and the fluorescence intensity in each well was measured at 535 nm excitation and 590 nm emission wavelengths with a microplate reader (Genios Pro from TECAN, Seestrasse, Switzerland). The principle of the cytotoxicity assay described as the fluorescence intensity which are correlated with the number of living cells in each well. Living cells can metabolize nonfluorescent blue resazurin (oxidized form) to the red, fluorescing resorufine (reduced form). To make the experiment more accurate and to reduce the error each concentration, data point was measured in triplicate wells and each experiment was repeated in three different days. Data are shown as percent of solvent control.

Roswell Park Memorial Institute Medium (PRMI) 1640 media with proteins (fetal bovine serum and horse serum)

After 48 h culturing to the cells in the growth media, same growth media (with proteins and PS additive) was used to expose PC12 cells. PbAc added to the media at 0.001–100 μM

and the cell incubated in CO₂ incubator at 37°C for 6, 12, 24, 48 h.

PRMI1640 media-free proteins

After 48 h culturing to the cells in the growth media. The cells were washed twice with free protein media. Treatment media is RPMI1640 normal medium with only PS additive. PbAc added to the treatment media at 0.01–100 μM and the cell incubated in the treatment media for 6, 12, 24, 48 h in CO₂ incubator at 37°C. Same volume of distal water was added to control cells and incubated in the treatment media without Pb under same conditions for all the experiments.

Dulbecco Modified Eagle's media with protein (fetal bovine serum and horse serum)

DMEM media were used to study the toxicity of PbAc on PC12 cells. Normal DMEM medium with and without proteins and horse serum was used to incubate cells with PbAc exposure. At 6, 12, 24 and 48 h time points were examined to find out if the PbAc will form white crystal precipitation and study the toxicity.

Dulbecco Modified Eagle's media-free proteins

Cells were cultured for 48 h, then normal DMEM media without protein additive were used to study the toxicity of PbAc on PC12 cells (0.001–100 μM) of PbAc with media introduced for 6, 12, 24 and 48 h time points. At the end of each time point, toxicity was evaluated using Resazurin assay.

Adjust pH level

After 48 h culturing the cells in the normal growth media. The cell treated with growth media and PbAc after adjust pH level. HCL was used to adjust pH level from 2.0 to 6.7 and till 4. In each experiment, there is control group without Pb used to compare it with Pb effect on cell viability and to exclude the role of acid in the cytotoxicity. DMEM instead RPMI medium with adjusted pH also used in separate experiment. To reduce the affinity of Pb to protein, two other experiments applied using RPMI1640 and DMEM without proteins.

Complexing Pb with ethylenediaminetetraacetic acid salts

After 48 culturing cells, 0.01–1 and 10 μM of ethylenediaminetetraacetic acid (EDTA) was added to PbAc before adding it to the medium. PRMI or DMEM medium were used; once with the proteins and other without proteins. To increase the solubility of PbAc, the cells were incubated with 0.01–100 of PbAc and EDTA at 6, 12, 24, and 48 h in CO₂ incubator at 37°C. At the end of each point time, evaluate cell availability was conducted.

37°C incubation

After cells cultured and grew for 48 h, exposed to 0.01–100 μM of PbAc are incubated at 37°C without CO₂ incubator for 6, 12, 24, and 48 h. One experiment done using PRMI 1640 media with and without protein; other experiments prepared using DMEM normal media with and without proteins.

Dulbecco Modified Eagle's media free phosphate medium
Special DMEM media was obtained from thermos fisher

to conduct the current experiment. DMEM free phosphate medium was used to expose PC12 cells to PbAc. After 48 h culturing PC12 cells, cells washed out twice with DMEM-free phosphate media to remove of PRMI 1640 (with phosphate) residual. Then, 0.01–100 μM of PbAc added to the cell using DMEM-free phosphate medium. Toxicity of PbAc was measured at each of (6, 12, 24, and 48) time point.

Statistical analysis

Most of the results expressed in mean $\pm$ standard deviation. The significant different was assessed using SPSS with GraphPad Prism version 7.0 software (GraphPad Software, Inc., San Diego CA). the main method was applied using ANOVA two-ways followed by Bonferroni's test. Difference were considered statistically significant if the $P < 0.05$ ($P < 0.05$).

RESULTS

Effect of PbAc using RPMI1640 medium

Over decades the rat PC12 cells has been used to investigate the neuronal cell death. In the current study, we investigate the cytotoxicity using different assays of PbAc even in high does where no work before tried to establish the effect of Pb at high does on PC12 cells line.

After PC12 cells incubated with various concentrations of PbAs (0.01–100 μM) using PRMI1640 media for 6 h, cell viability was evaluated using resazurin assay. The exposure to a different concentration of PbAc showed no statistically significant ($P < 0.05$) comparing to their individual control at different time point [Figure 1]. In addition, white crystal precipitation has been noticed at high concentration (50 and 100 μM). Different trials with changing the condition were used to make the Pb bioavailable. The first trial was conducted without any proteins added to the media; however, there was no statistically different showed with free protein treatments at same time point [Figure 1].

It has been suggested that pH of media or buffer has incredible effect on Pb dissolved in media and PbAc is not stable in solutions with neutral pH because Pb prefers to work in the acid environment. For this reason, the author adjusted pH of the media and the buffer to demonstrate the role of pH

in Pb solubility. Glutamic acid or HCL had been added to the media with monitor pH level using benchtop pH meter from Fisher. Level pH reduced till 4 to keep Pb in solution; however, the white precipitants instantly observed when we add 50 or 100 μM Pb to the media. No any statistic significant different was noticed after changing the pH level at 6 h [Figure 1].

However, it has been suggested that changing the bioavailability of Pb was by enhancing it to EDTA salts before adding it to the media, there was no statistical significant different noticed after 6 h treatment. Incubate cells after treated with PbAc with 37°C without CO₂ nevertheless, no change was witnessed with 6 h exposure [Figure 1].

All the above trials included media with protein, media without protein, adjusting pH, CO₂ and complexing with EDTA repeated with longer incubation duration 12 h. Although increase the time point, there was no significant different were detected [Figure 2].

24 and 48 h also tried in the current paper to investigate the impact of longer incubation. Through this experiment, exactly same conditions used at 6 and 12h were also applied here. The results showed no significant difference even after day and 2 days exposure [Figures 3 and 4].

Effect of PbAc using normal Dulbecco Modified Eagle's media (DMEM)

To investigate the role of PbAc to induce toxicity in Pc12 cells in the present and absent of proteins, normal DMEM medium was utilized. PbAc at different concentrations in the incubation medium displayed no significant different compared to control samples which remained untreated ($P > 0.05$) at 6 h [Figure 5]. White crystal precipitation also noticed in the high does been used in this experiment. To clarify the involvement of level of pH inPbAc dissolved in distal water. pH level was adjusted till 4. There were no significant different in the lead treatment over 6 h incubation among treated and control groups with all the adjustment levels ($P > 0.05$) [Figure 5]. White crystal still been seen with high level of Pb. 0.01–1 and 10 μM of EDTA had been added to Pb before mix it with media but the results appered that there was no statistical significant observed comparing to the control over 6 h incubation [Figure 5]. Change condition of the

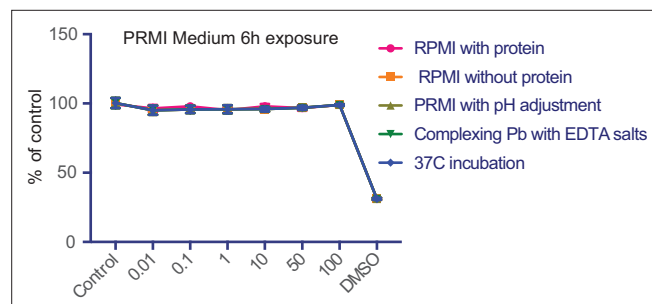


Figure 1: Cell viability after 6 h exposure 0.01–100 μM of PbAc. RPMI used as treatment medium. Dimethyl sulfoxide employed as negative control. No significant change can be seen between treatment comparing with control even at high level

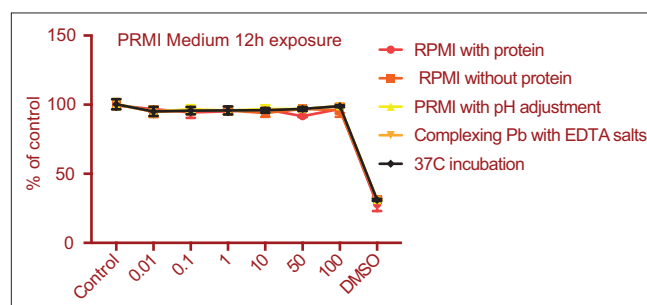


Figure 2: Cell viability after 12 h exposure to PbAc from 0.01 to 100 μM using RPMI as treatment medium. Dimethyl sulfoxide used as a negative control. No significant change can be noticed even at high level

incubation by incubate the cells after exposure to PbAc without CO₂ also do not effect on the toxicity [Figure 5].

All the alternation treatments were used at 6 h with DMEM media repeated and let the cell expose longer to the PbAc. 12, 24, and 48 h attempted, but there was no significant change to treated group comparing to the control [Figures 6-8]. The white crystal still be seen in the bottom of the well after treated with PbAc.

Dulbecco Modified Eagle's media without phosphate

Using DMEM without phosphate in the presence of proteins showed no significant among treatments comparing to the control at each time point suggested in the current

study (the data are not showing here). Utilizing DMEM medium without phosphate in the absence of the proteins with (0.001–100 µM) of PbAc exposure showed for the first time statistical significant at 10, 50, and 100 µM comparing to the control at 6, 12, and 24 h [Figures 9-11]. The significant difference change was even at lower dose with longer incubation 48 h [Figure 12]. Identical adjustment with changing in pH, incubation without CO₂, complexing with EDTA also tried using DMEM without phosphate. In each trial, the a significant different started to be noticed at 10 µM and higher comparing to the control. Surprisingly, No Pb precipitation was observed after using the new media.

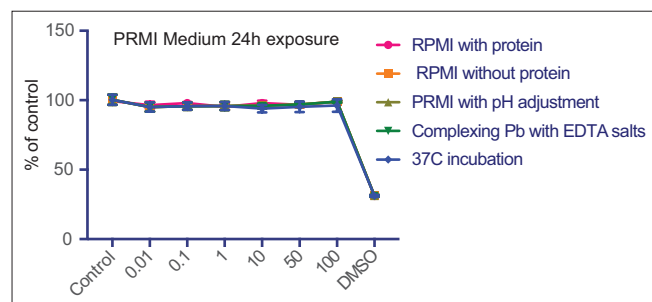


Figure 3: Twenty-four hours exposure to (0.01 to 100 µM) of PbAc continues using RPMI for treatments. Dimethyl sulfoxide used as negative control. No significant change can be remarked between treatment and control even at high level

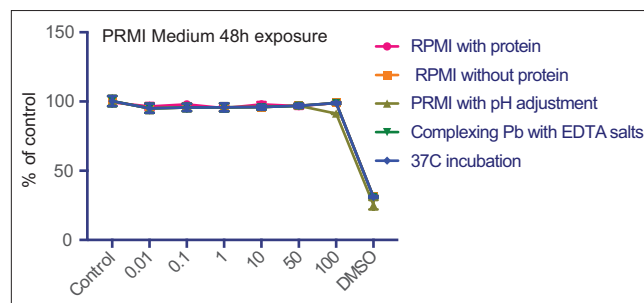


Figure 4: Forty-eight hours exposure to (0.01 to 100 µM) of PbAc continues using RPMI for treatments for longer time. Dimethyl sulfoxide used as negative control. No significant change can be remarked between treatment and control with all the adjustments

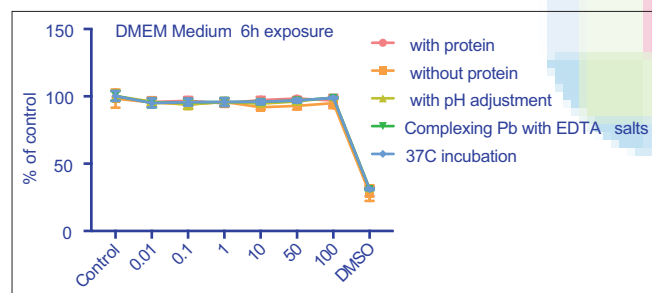


Figure 5: Cell viability after 6 h exposure 0.01–100 µM of PbAc. Dulbecco Modified Eagle's media used as a treatment medium. Dimethyl sulfoxide employed as a negative control. No significant change can be seen between treatment comparing with control even at high level

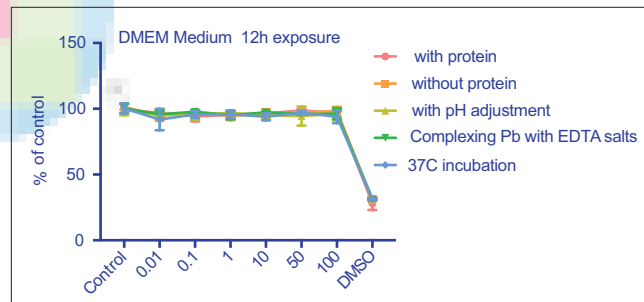


Figure 6: Cell Viability after 12 h exposure to PbAc from 0.01 to 100 µM using Dulbecco Modified Eagle's media as treatment medium. Dimethyl sulfoxide used as negative control. No significant change can be noticed even at high level

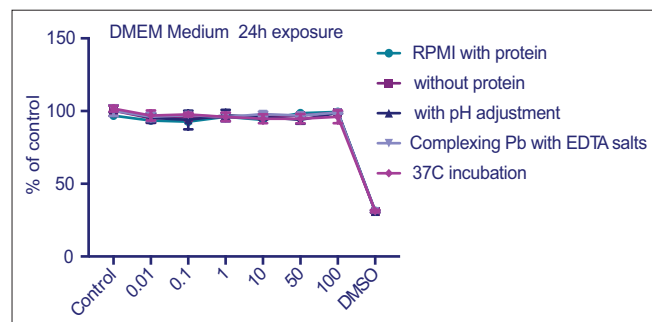


Figure 7: Twenty-four hours exposure to (0.01–100 µM) of PbAc continues using complete Dulbecco Modified Eagle's media for treatments. Dimethyl sulfoxide used as negative control. No significant change can be remarked between treatments and control even at 50 and 100 µM

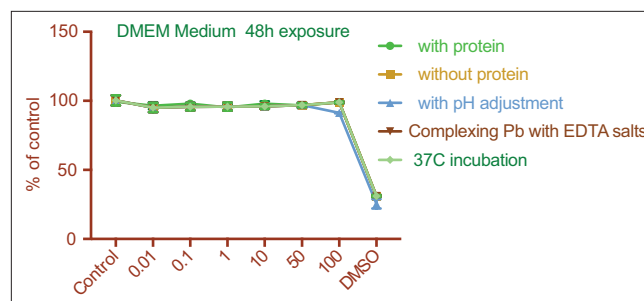


Figure 8: Forty-eight hours exposure to (0.01–100 µM) of PbAc, PbAc introduced using Dulbecco Modified Eagle's media longer time. Dimethyl sulfoxide used as negative control. No significant change can be observed between treatments and control with all conditions change

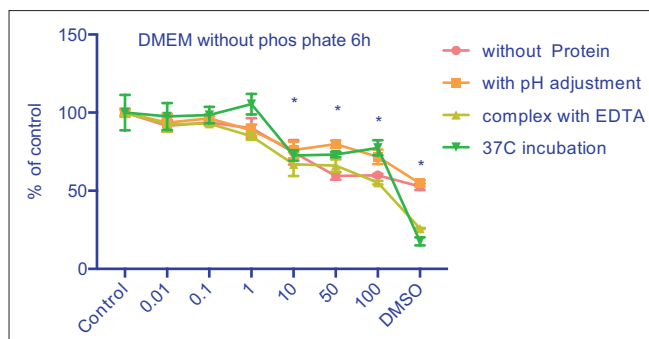


Figure 9: Cell viability after 6 h exposure 0.01–100 μM of PbAc. Dulbecco Modified Eagle's media without phosphate used as treatment medium. Dimethyl sulfoxide employed as negative control. Statistically significant different has been noticed started at 10 μM treatment comparing with control even at high level

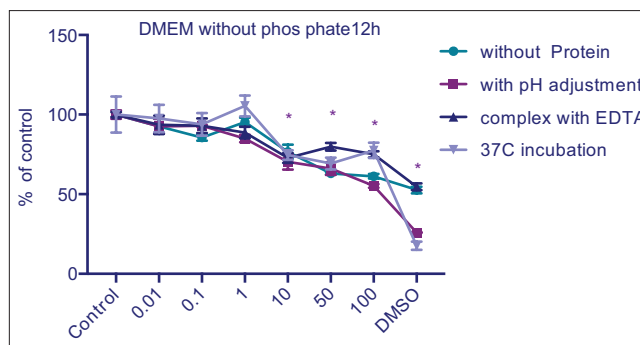


Figure 10: Cell viability after 12 h exposure to PbAc from 0.01 to 100 μM using Dulbecco Modified Eagle's media without phosphate as treatment medium. Dimethyl sulfoxide used as negative control. It easy can be noticed that statistically significant starting from 10 μM

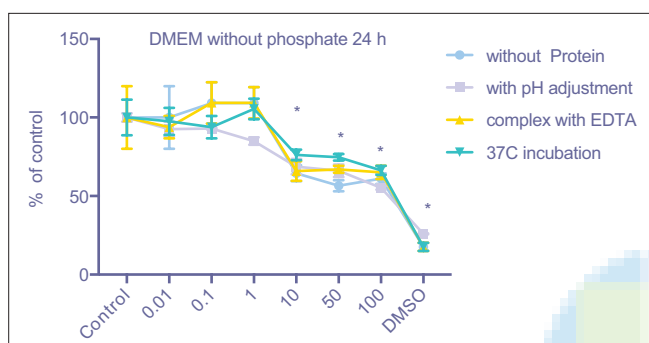


Figure 11: Twenty-four hours exposure to (0.01–100 μM) of PbAc continues using complete Dulbecco Modified Eagle's media for treatments. Dimethyl sulfoxide used as negative control. No significant change can be remarked between treatments and control even at 50 and 100 μM

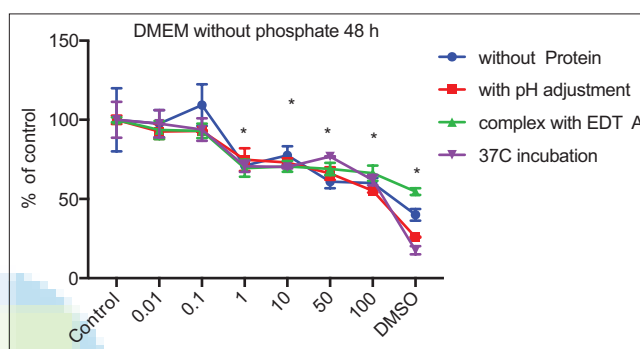


Figure 12: Forty-eight hours exposure to (0.01–100 μM) of PbAc, PbAc introduced using Dulbecco Modified Eagle's media without phosphate for longer time. Dimethyl sulfoxide used as negative control. Statistically different started to be seen at 1 μM comparing to the contro

All the experiments applied above, lead (IV) acetate likewise Pb (II) acetate had been used to investigate the availability of Pb in media. Moreover, PbNO_3 or PbCO_3 also tried to identify if other Pb component may make different. All other Pb components react in similar way, no significant difference was seen with each of the Pb component (the data are not showing here due to limited space).

Moreover, water hardness would be considered a major responsible for the precipitation in the media. Therefore, dilution of Pb using ultrasonic can resolve the problem. In the present study, no change has been noticed even after 15 or 30 min sonication (the result is not showing here).

DISCUSSION

The term of bioavailable is defined chemically as amount of the chemical portion that enters the cell from an administered dosage in the media. The dosage inter to the cells is mostly depend on the physical and chemical properties and associated matrix.^[6] To assess the bioavailability value of Pb, *in vitro* study would offer the advantage of rapid, simple, reliable, and affordability over the expensive and time-consuming *in vivo*

studies.^[7] PbAc chosen in this study because it is highly soluble and greatly toxic form of Pb.^[8]

An effort was made in our study trying to report the influences of bioavailable soluble Pb on its cytotoxicity observed in PC12 cells. It has been reported that expose Pc12 cells to Pb increase cytotoxicity in the same cell line using MTT assay to evaluate the cytotoxicity and they suggested that NO is mediate toxicity of Pb acetate.^[9]

A study tried to investigate the role of PbAc to induce the toxicity in aggregating brain cell culture reported that there was no effect on neuron culture at high concentration 10^{-6} M comparing to the low concentration. The result of the same study also showed that the immature cultures were more sensitive to lead exposure effect than the differentiated culture.^[10]

It has been reported that astrocytes and glial cell are more sensitive and reactive to lead than neurons in cell culture.^[11]

It has been reported that the serum and protein in media would affect negatively on lead induce toxicity in the cell culture.^[12] Thus, in the current study, most of the media used without any protein to increase bioavailability in the media. The result of the recent study which showed there are not different with

impact of Pb on cell using free protein RPMI and DMEM media comparing to present of protein.

Juhasz *et al.* reported that bioaccessibility would be increase with decrease of pH.^[13] *In vivo* studies, investigated the bioaccessibility of PbAc in stomach with the presence of HCL suggested that pH highly affect the bioavailability of Pb with drinking water that approved with comparing to the intestine pH.^[14-16] On the other hands, it has been approved that increase pH would decrease Pb solubility. Results of these studies have not agreed with result of the existing study which showed there are no different can be seen with all the alternation of pH of RPMI and DMEM medium. In concerns of this regards, CO₂ would work to bring the pH up in media, we incubate the cells with PbAc in 37°C instead of CO₂; however, there are no significant different was noticed with change in incubation conditions in RPMI or DMEM medium. Some works suggested that adding EDTA as chelating agent would make PbAc more soluble in media.

Using DMEM without phosphate displayed that present of serum in media decrease the toxicity of PbAc. This result come along with a study tried to investigate the effect on Pb on embryonic mesencephalic primary culture in the present and absent of serum in media. It has been reported that lead exposure in the absence of serum damaged more efficaciously the cultured cells than lead expressed in the presence of serum. It has been suggested that serum would afford delay of cell death may be due to removal of reactive Pb by protein chelate formation which make Pb less available in the cell culture.^[17] These results, however, are difficult to compare it with result from our study because different cells line has been used (primary culture vs. Pc12 cells). Another study demonstrated that lead increase in total SP1 in time depended manner in PC12 cells at low concentration but not in high concentration.^[9]

The result from current investigation exhibited that present of the chelating agent such as EDTA in the PRMI and DMEM medium had no effect on PbAc bioavailability, however, it has been noticed that adding EDTA at three concentrations (1, 5, and 10 mg/kg of contaminated soil) increase total uptake of Pb by shoots of rainbow pink.^[18] In addition, the role of EDTA for enhancing Pb availability proposed in work with artificial contaminated soil.^[19] Tumbleweed plants where grown in agar and liquid media showed that present EDTA in agar or in media would reduce the acquisition of Pb in them and increase extraction efficiency of Pb to the root and leaf.^[20]

The chemical form of Pb is critical importance as factor in the toxic effectiveness of Pb.^[21] In study tried to identified the effects of Pb exposure on neuronal stem cells proliferation and survival, there was no significant increase in LDH release assessed after 24 h exposure to 0.1–100 µM of PbAc.^[22] Consistent with these results, we also observed no change using same rang of concentrations from PbAc.^[21]

Phosphate is rapidly and effectively precipitates lead from solution to generate lead phosphate,^[23] and this comes true

with the result of the current study. When we used free phosphate media, no precipitations have been noticed in the media and effect of the PbAc, and other forms of Pb were significantly different starting of 10 µM. Different studies on Bactria and Alga come over the difficulties of Pb precipitation in the media by removing phosphate from the media. A study carried out on *Chlamydomonas reinhardtii* media demonstrated that cells did not survive when the amount of Pb in the culture exceeded the equivalents of phosphate.^[24] The formation of lead phosphate Pb₃(PO₄)₂ in the media which prevent of lead from been available to the cells. The low solubility of Pb₃(PO₄)₂.

It has previously been shown that adding 1% phosphorus reduced the bioavailability of lead acetate in the soil to 29%. They proofed after comparing the response curves of the control and phosphate-treated soil the effectiveness of the treatment to reduce the bioavailability of Pb in soil.^[25] Using isotope analytical technique, Maddaloni and Graziano performed a study to measure adsorption in fasting human adults. The results from this study displayed that 1% of phosphoric acid added to soil reduced the bioavailability.

Acetic acid and prepare the mixture freshly. The above results indicate that lead acetate and lead nitrate is very effective in free phosphate media. This confirmed in our study, the toxicity of PbAc was maximum at high concentration in free phosphate media. This result was agreed with the analysis of the media and cell samples to measure the concentration of Pb in media and in the cells. Since your sample has already precipitated and the bio-availability will also decrease simultaneously.

CONCLUSION

It is being speculated that Pb induces cell toxicity. Pc12 cells resistant to Pb cytotoxicity include the precipitation of metals as phosphates complex in RBMI and DMEM media even with adjusting of incubation condition such as decrease the PH and adding a chelating agent. Come over on this complex problem for the first time in this paper using DMEM without phosphate media. DMEM without phosphate media showed the real toxicity of PbAc on cells in culture, and this holds true even for the lowest concentration.

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Conflicts of interest

There are no conflicts of interest.

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