REGULAR ARTICLE

Protection against lead contamination by strains of lactic acid bacteria from fermented camel milk

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Abstract

The effectiveness of the fermented milk product for decreasing the absorption of Lead (Pb) in vivo by testing several combinations of different strains and individual strains of LAB isolated from camel milk and shubat (fermented camel milk) was determined. During 4 weeks 0.5 ppm of Pb was given to cavies in fermented milk product and water. Control group and groups treated only by fermented milk products, also, were observed. Faeces, Blood, Heart, Lungs, Liver, Kidneys, and Spleen were analyzed. The lead concentrations in faeces of Control group and lead nitrate treated group were nearly the same. The quantity of Pb in faeces of fermented milk treated groups was higher than in Control and Water Pb groups. In the different cavies' organs of Water Pb group, the higher concentration of heavy metal (ppm) was observed in spleen (1.04), heart (0.65), kidneys (0.58), and blood (0.46) to be compared to 0.82, 0.2, 0.58 and 0.31 respectively in control group. In groups treated with fermented milk without/with Pb, the lead concentration decreased in target organs. Quantity of lead in blood samples of Control group and groups treated fermented milk products without/with Pb is nearly same. Highest concentration of blood Pb was observed for Water Pb group.

Key words: Cavies, Fermented milk product, Lactic acid bacteria, Lead, Target organs

Introduction

Heavy metals are widely responsible of environmental contamination in the world. The quality analysis of drinking water and soil from some areas of Kazakhstan, as mineshafts and nuclear sites, has shown the presence of heavy metals and radionuclides with a content exceeding the permissible value (Kenesariev et al., 2008; Sarsenbayev et al., 2002). This situation should have various adverse effects on a human body (Tuhvatshin et al., 2008). The pollution by lead (Pb) is a health hazard for consumers of dairy products because this metal is concentrated throughout the food chain (Tajkarimi et al., 2007; Dallak, 2009; Kan and Meijer, 2007). High level of lead was observed in the blood samples of individuals inhabiting the polluted areas. This is due to the high content of lead in the soil, drinking water and in the local product (meat, cow milk). The quantity of lead in the blood depends of the age of individuals (Kenesariev et al., 2008; Hallen et al., 1995; Bhagwat et al., 2008).

For long time, it was shown that injected lead, penetrates the cells quickly, being present in all fractions after one hour (Castellino and Aloij (1969).

Lead toxicity results in neurotically disorders; it damages the kidney, affects cardiovascular system and reproduction (Babalola et al., 2005). To avoid a penetration of lead in the food chain, nowadays, there are many methods to remove Pb from different biological matrix. A vast array of biological materials, especially bacteria, algae, yeasts and fungi have received increasing attention for heavy metal removal and recovery due to their good performance, low cost and large available quantities. Biosorbents are cheaper, more effective alternatives for the removal of metallic elements, especially heavy metals from aqueous solution. The capability of some living microorganisms to accumulate metallic elements has been observed at first from toxicological point of view (Volesky and Holan, 1995). Bacteria were used as biosorbents

because of their small size, their ubiquity, their ability to grow under controlled conditions, and their resilience to a wide range of environmental situations.

Halttunen et al. (2006) showed that bacteria could have characteristics which would allow them to fix the toxins in food and water. And to be an efficient and economical alternative compared to methods of classical detoxification. Lactic acid bacteria are capable to eliminate the Cd and Pb from the water. Elimination was quick, influenced strongly by the pH, pointing out a mechanism of ionic exchange.

Fandi et al. (2006) assessed the correlation of probiotic bacteria of camel's milk with the cadmium and the lead. In vitro study is an initial stage of riddling to identify the useful strains for the decontamination of food and to develop the intestinal model.

Camel milk and fermented *shubat*, its derivative product could be contaminated (Konuspayeva et al., 2008, 2009, 2011). The lactic fermentation of *shubat* could reduce the availability of lead in the digestive tract of consumers because lactic acid bacteria (LAB) are able to absorb this metal which is then excreted in the faeces (Dallak, 2009; Al-Hashem, 2009; Akhmetsadykova et al., 2009; Loiseau et al., 2009).

Therefore, the present study was carried out to determine *in vivo* the effectiveness of the fermented milk for decreasing the absorption of Lead Nitrate $(PB_2(NO_3))$ by testing several combinations of different strains of LAB isolated from camel milk and *shubat*.

Materials and Methods
Animals
Female cavies (250-300 g) were purchased from Antigen Ltd, Almaty, Kazakhstan. They were housed in standard metal cages (10 cavies / cage). They were divided into eight treatment groups:

(1) Control (n=10): cavies not receiving lead and used as Control group,

(2) Water Pb (n=10): group with 2 ml of water solution containing Lead Nitrate (0.5 ppm),

(3) 4SF (n=10): cavies treated with 2 ml of milk product fermented by 4 different LAB strains having proved capacity to absorb Pb,

(4) 4SFPb (n=10): cavies treated with 2 ml of milk product fermented by 4 different LAB strains in which the same concentration of Lead Nitrate than group 2 was dissolved.

(5) SF (n=10): cavies treated with 2 ml of milk product fermented by 1 LAB strain having proved capacity to absorb Pb,

(6) SFPb (n=10): cavies treated with 2 ml of milk product fermented by 1 LAB strain in which the same concentration of Lead Nitrate than group 2 was dissolved,

(7) SNF (n=10): cavies treated with 2 ml of milk product fermented by 1 LAB strain without capacity to absorb Pb,

(8) SNFPb (n=10): cavies treated with 2 ml of milk product fermented by 1 LAB strain without capacity to absorb Pb in which the same concentration of Lead Nitrate than group 2 was dissolved.

Fermented cow milk product was achieved by utilization LAB strains isolated from fermented camel milk. $Pb_2(NO_3)$ was used as a source of lead (0.5 ppm) for fermented milk product and water solution for Water Pb group. Cavies were orally administered (2 ml) their respective doses every day for 28 days. Water and standard pellets were provided *ad libitum.*

Sampling
Faeces were collected every 7 days. On the 28th day blood was collected by cardiac puncture (Rader et al., 1981), and following were obtained (a) Heart, (b) Lungs, (c) Liver, (d) Kidneys, (e) Spleen (Schroeder et al., 1964, 1965; Babalola et al., 2010; Chun-Yan et al., 2011). All samples were stored in a freezer at - 20ºC until analysis.

Determination of lead concentration
Samples were mixed by type and by group. At the first step the samples (blood, faeces, and organs) were dried in an oven at 150ºC for 1h and homogenized. Then 1 g of each sample was mineralized by wet mineralization using pure nitric acid 65% of "Carlo Erba Reagents" Ltd. (Italy) by Kjeldahl method "DK6 VELP SCIENTIFICA" (Italy). Lead concentration in the mineralized samples was measured by Atomic Absorption Spectrometry 30 "Carl Zeiss" (Germany) at the Laboratory "KazMekhanObr" (Kazakhstan). Standard solutions of lead were aspirated to calibrate the AAS before the aspiration of the samples.

Statistical analysis
The effect of treatment on the concentration of lead in different organs was assessed by Kruskal-Wallis test. The matrix of correlation for the table treatment x concentration of Pb in the organs was analyzed by Principal Component Analysis (PCA). ANOVA procedure was used to observe the effect of the week on the concentration of lead in faeces. The software used was XLSTAT© (Addinsoft, 2010).

Results

Content of Pb in the faeces

Diet was slightly contaminated: in milk 0.32, water 0.12, and fodder 0.32, HNO_3 0.1 ppm of lead was found. Quantity of Pb in the samples of faeces after 7 days of lead treatment (ppm): Control Gr. 0.43, 4SF 0.54, 4SFPb 0.63, SF 0.4, SFPb 0.5, SNF 0.47, SNFPb 0.68, Water Pb 0.43. The quantity of Pb in faeces of fermented milk treated groups was higher than in Control and Water Pb groups. There was no difference between Control group and Water Pb group for the Pb content in the faeces of cavies, except for 14th day where higher concentration (1.57 ppm) was observed. These results need to be confirmed (Figure 1). The lead concentration in total faeces samples was higher in the groups 4SF and 4SFPb compared to Control group (Figure 2). However, in the 4SF group which was not treated by lead, the quantity of this metal was also higher than in control group. The highest quantity of Pb was observed in 4SFPb group. But the fecal content of lead in those groups changed during the study.
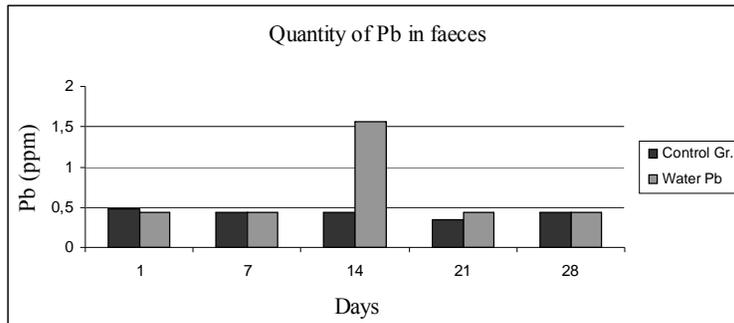

Figure 1. Quantity of lead in faeces of control and water Pb groups during of study.
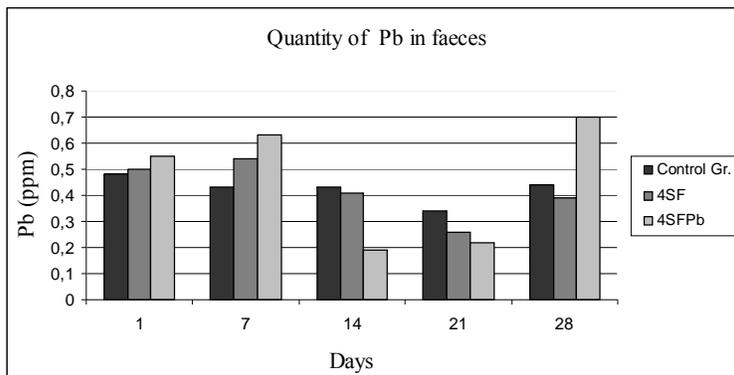

Figure 2. Quantity of lead in faeces of control, 4SF and 4 SFPb groups during of study.
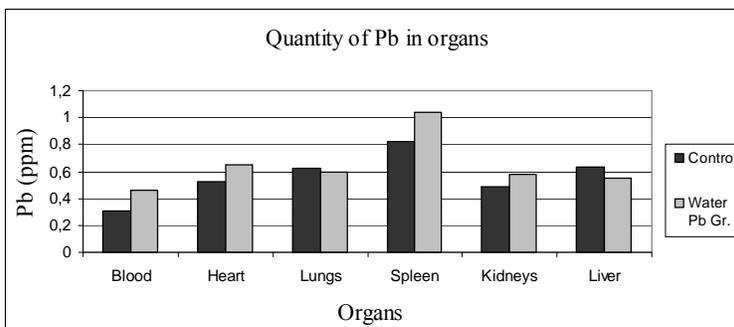

Figure 3. Quantity of Pb in blood and different organs of control and water Pb groups.

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Content of Pb in the different organs

In the different cavies' organs of Water Pb group, the high concentration of heavy metal (ppm) was observed in spleen (1.04), heart (0.65), kidneys (0.58) and blood (0.46) to be compared to 0.82, 0.2, 0.58 and 0.31 respectively in control group (Figure 3). Quantity of kidney lead is the same in Control and Water Pb groups. In groups treated with fermented milk without or with Pb, the lead concentration decreased in target organs (spleen, kidneys, liver and lungs). Mainly, for the SFNPb and 4SFPb groups, quantity of lead in the heart samples was less than in Water Pb group. Also, in the spleen samples of 4SFPb group, concentration of Pb was lower compared to Control and Water Pb groups. The Pb concentration in blood and heart was similar in Control, 4SF and 4SFPb groups (Figure 4) in spite of the lead treatment of the 4SFPb group.

Compared to Control group, in groups treated with fermented milk product diminution of Pb concentration (ppm) in lungs (4SF 0.52; SF 0.41; SNF 0.56 to Control gr. 0.62) and in liver (0.53; 0.49; 0.5 to 0.63) was observed. In the samples of spleen of 4SF and SNF groups the quantity of lead was lower than in Control group, 0.7, 0.6 and 0.82 ppm, respectively. For other tissues Pb quantity was nearly the same.

Quantity of lead in blood samples Control, 4SF, SF groups and in the lead treated group 4SFPb was nearly same. In the blood sample of SFPb group the lowest concentration of lead was observed. On the contrary, in the groups of the strains without capacity to fix Pb the quantity of lead increased and the highest concentration was observed for Water Pb group (Figure 5).

Correlations between lead concentrations in the different organs of different groups

The concentration of Pb in the heart was significantly correlated with that of the spleen. There was no significant correlation between the other organs. However, according to the circle of correlations obtained by PCA, the levels of heart Pb and spleen Pb appeared much higher when the levels in the kidneys and especially in the blood were low. The levels in the lungs and liver were independent from the concentrations observed in other organs. Regarding the correlations with the groups, the high levels in the heart and spleen were closed to the group SFPb. The high levels in the blood were associated with 4SFPb and SNF groups. Control and Water Pb groups were associated by high levels in the liver and lungs. SF group was characterized by a low content of Pb in all organs, SNFPb and 4SF groups by average values in all organs.
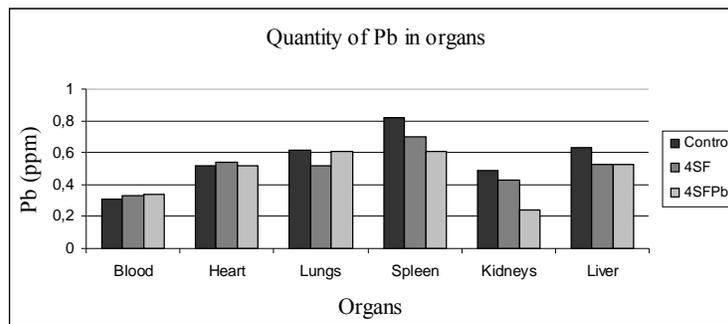

Figure 4. Quantity of Pb in blood and different organs of control, 4SF and 4SFPb groups.
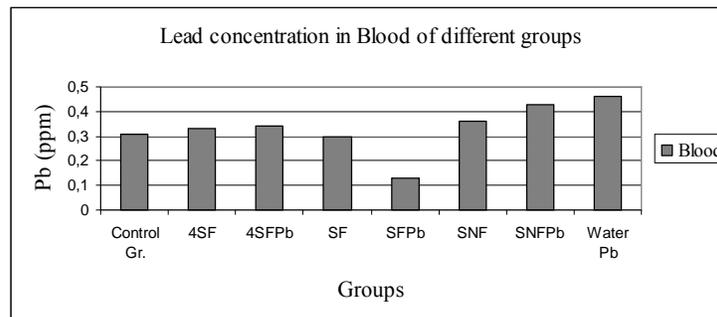

Figure 5. Quantity of Pb in blood of different groups.

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Discussion
Quantity of lead in faeces

According to the literature, several LAB strains are capable of surviving the passage through the gastrointestinal conditions (Corcoran et al., 2007; Guerra et al., 2007; Lambert and Hull, 1996; Takahashi et al., 2004). Many studies about detoxification of water and food by utilization of different microorganisms are available (Halttunen et al., 2006, 2007; Fandi et al., 2006). Many experiences were done with laboratory animals to study lead distribution *in vivo* by treating orally, inhalation or injection (Dallak, 2009; Castellino and Aloij, 1969; Rader et al., 1981; Smith et al., 1992). But, there are no many studies about interaction of LAB and heavy metals *in vivo* and influence for retention or elimination of Pb.

The quantity of faeces lead in groups treated by fermented milk product containing or not lead nitrate was high relatively to Control and Water Pb groups. Such observation means that strains of LAB could increase lead elimination. But, results showed, that elimination of lead from organism was not constant. More lead was excreted in the faeces (35%) than in the urine (15%) (Castellino and Aloij, 1969). Blood lead concentrations were more stable than those in urine (Rabinowitz, 1998).

If the results of fixation test on the nutritive culture and the theory about fixation of Pb by LAB strains during fermentation are right (Akhmetsadykova, 2008), many references show that LAB have capacity to survive and transit through the gastrointestinal tract (Duez et al., 2000; Rochet et al., 2008; Su et al., 2007). Berrada et al. (1991) showed that *Bifidobacterium* strains contained in two different fermented milk behave very differently when exposed to *in vitro* simulated gastric environment. One strain survives very well for 90 min at least (greater than 10^7/g), but the second strain studied was quite less resistant. The results *in vitro*, with slight differences, were confirmed by *in vivo* study in human.

A significant increase in the number of *bifidobacteria* (DN-173010) in faeces was observed during ingestion of fermented milk, and decreased when the ingestion stopped (Collado et al., 2006). Strains which were utilized for fermentation of milk have a good opportunity to survive because some probiotics survives better when investigated in fermented milk (Pochart et al., 1992). Survival rates of *Lactobacillus casei* DN-114 001 ingested in fermented milk were up to 51.2% in the ileum and 28.4% in the feces (Oozeer et al., 2006). Consumption of yogurt and fermented milk

products containing LAB/probiotics influence the increase of their number in fecal samples (Rochet et al., 2000; Yuki et al., 1999). *Streptococcus thermophilus, L. bulgaricus* and *L. casei, Bifidobacteria* were found in the faeces of rats treated with yogurt fermented milk (Djouzi et al., 1997). All these references show that strains can transit in gastrointestinal tract and be eliminated in faeces. Even in groups treated only by fermented milk product, the quantity of Pb in tissues was lower and quantity of Pb in faeces was slightly higher. The presence of lead in the faeces, blood and tissues of the cavies not receiving lead, can be attributed to the metal present in the food given to the animals (Schroeder et al., 1965).

Further, the treatment by strains of LAB decreased total lead quantity in organism. This can be due to beneficial effects of fermented milk product which was as a barrier against lead retention in tissues or because LAB strains absorbed Pb in organism.

Distribution of lead in the different tissues

The half-life of lead in the some tissues was regarded as about 3 months (Rabinowitz et al., 1976). But, the latest data shows that the half-life of lead in these tissues ranges from 40 - 50 days (Babalola et al, 2010).

In the present study, lead was expected to accumulate in the soft tissues within 28 days of exposure. In the Control group spleen Pb and liver Pb were high. Based on the results of Pb quantity in different matrixs of Water Pb group, lead distribution in cavies organism of Water Pb group was achieved: spleen>> blood> heart> lungs> liver> kidney. Many experiments were done with different laboratory animals in this direction. Schroeder et al. (1964) determined lead quantity in the organs of 700 mice after given them 5 ppm Cd, Pb, Cr, Ni or Ti in drinking water. High concentration of lead was noted for spleen and kidneys. These results are nearly same for spleen Pb results, but not for kidney Pb. This may be partly due to a common problem usually encountered in oral exposure method. It is almost impossible to determine accurately the quantity of materials ingested by the animal. Quantity of food and water taken by the animals also affect the amount of the material that will be absorbed by the animal. This observed discrepancy was also true for the concentration of lead in the various organs of the animal ((Rabinowitz et al., 1976). The relatively low quantity of lead (0.5ppm) didn't give clear results about lead distribution. Furthermore, in such

study more high concentration of lead could be taken without fatal effect for animals (O'Tuama et al., 1976; Sierra and Tiffany-Castiglioni, 1992). Only female cavies were used for this study. Thus, according to some references, sex difference was observed for quantity of lead in tissues. Female rats and mice showing significantly higher levels than males (Schroeder et al., 1964; Donald et al., 1987). Also, age influence was described. The young monkeys retained 64.5 and 69.8% of the orally administered $_{210}$Pb at 70 and 150 days of age, respectively, while adult monkeys retained 3.2% of the 210Pb dose (Castellino and Aloj, 1964; Wiles et al., 1977). In general, daily exposure to lead via drinking water resulted in the highest lead content in blood, brain, kidney, and femur for both weanling and adult rats (Rader et al., 1981). Experiments with rats showed the main storage organs: kidneys and bone. Initially, 20% of the dose could be accounted for in the kidneys and the biological half-life was about 100 hours. The level in bone built up rapidly at first and then more slowly. After a week, between 25 and 30% of the dose was present in bone (Castellino and Aloj, 1964; Morgan et al., 1977).

The skeletal lead level is commonly regarded as the best indicator of the cumulative exposure to this element, as more than 90% of the body burden of lead is stored in this tissue. The half-time of lead in finger-bone of humans is about 5 years. However, lead in rats is eliminated faster than in humans (Hac and Krechniak, 1996).

Lead accumulates permanently in bones; consequently, estimation of lead in the teeth can provide a good index of the body burden (Rabinowitz et al., 1976).

The remaining 10% is stored in soft tissues like kidney, liver and brain (Chun-Yan, 2011). Thus, the duration of our experiment being 28 days, Pb had time to be stored in tissues. Castellino and Aloj (1964) studied rats for 14 days after single intravenous injections of 100 μg of lead per rat. ^{210}Pb was rapidly distributed in the tissues, the highest concentrations being in the kidneys, liver, and bones (Castellino and Aloj, 1964).

Chun-yan et al. (2011) exposed male mice to lead nitrate solution (0.1mg/ml). For other target organs, the accumulation of lead was in the order: kidney, liver, spleen, lung, brain and heart. The accumulation of lead in brain gradually increased with lead exposure while the accumulation of lead in the other target organs reached balance after 15-20 days (Chun-Yan et al., 2011).

Usually, the level of lead was determined in blood (as index of current exposure) and in hair, nails (as indices of long-term exposure) (Babalola et al., 2005; Kello and Kostial, 1978). Hair fixes easily elements such as lead and thus provides an accurate and permanent record of exposure of some minutes' duration (Babalola et al., 2005).

It's possible that data of Pb quantity in different matrix weren't clear and difficult to explain because bone, hair simples, brain weren't analyzed.

LAB strain of SNF group determined like strain without capacity to fix Pb by test of fixation *in vitro* (Akhmetsadykova et al., 2009). But diminution of Pb in heart samples was observed. It's possible that this strain fixed better in the fermentation process than on the surface of nutritive culture due to the difference of conditions.

Blood lead quantity

As blood flows through the soft tissues, lead is deposited and bio-accumulated. Some references report that lead concentration in the blood is transient and only represents recent exposure of some days (Rabinowitz et al., 1976). On the contrary, Hilts (2003) showed that lead in whole blood was considered to provide to be the best measure of exposure for 25-35 days. In our experiment, samples of blood were collected for 28 days. Total blood volumes, based on the information given by Schroeder et al. (1965) a rat of 300 g weight contain 18 ml of blood. If this estimation is correct then, on average, 0.7% of the total blood volume was collected. Its means that in Water Pb group having lead concentration of 0.46 ppm in 0.7%, it would be about 66 ppm of lead in total blood volume. Blood becomes a matrix with the highest concentration of lead. On the contrary, Rabinowitz (1998) showed that only 10 mg of 60 mg absorbed lead would remain in the blood, the other 50 mg was thought to be elsewhere in the body, only to leave slowly over a period of many months.

Regarding Pb contained in blood, 98–99% was found in blood cells and 1–2% in blood plasma; 5–8% of the Pb in blood cells was bound to blood cell membranes. None of these parameters varied significantly with age (Willes et al., 1977).

Chun-yan et al. (2011) exposed male mice to lead nitrate solution (0.1mg/ml). The resulting data showed that 98% of lead exposed in blood was accumulated in red blood cells (Rabinowitz, 1998).

But, Schroeder et al. (1965) didn't note lead in red blood cells except for a trace in one female.

Lead is submitted to a rapid elimination from the blood and slow elimination from the bones. Lead bounds reversibly to tissues. The longer term accumulation of lead in the skeleton is regarded as

a future source of blood lead. This release of stored lead is the rate-limiting factor in its clearance of lead from the blood (Rabinowitz et al., 1981).

Correlations between lead concentrations in different tissues

In cavies, significant correlations between the heart lead and spleen lead were found. The lead concentration in kidney was relative to levels in blood. They depend from each other. Smith et al. (1992) showed that elevated concentrations of lead in kidney (fresh weight) relative to levels in blood were consistent with the presence of specific lead-binding sites in the kidney at very low levels of exposure. Rader et al. (1981) observed correlations between blood lead and kidney lead, and correlations between blood lead and femur lead were found only in the rats receiving lead steadily in drinking water.

Conclusion

Effects of fermented milk product containing LAB strains on the retention of lead by cavies' organisms were observed. But, to explain the way of detoxification in the future, a large study should be realized. The main question is in which conditions LAB strains release fixed Pb. This could be achieved by making a test *in vitro* with conditions of gastrointestinal tract, in consideration of difference between human and cavy organisms. In the environment contaminated by heavy metals, it will be more useful to take higher concentration of Pb to see the exact way of the distribution in organism. To have a complete idea of Pb distribution, more possible matrix should be taken to analysis as bone, brain, hair samples which weren't taken in the current study.

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