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RESEARCH ARTICLE

Effects of Repeated Oral Administration of Lead Combined with Cadmium in Non Lactating Ewes

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ABSTRACT

The aim of the study was to highlight the toxic effects after lead and lead-cadmium repeated oral exposure for nine weeks in ewes. An experiment was conducted using "OuledDjellal" ewes during two periods: before exposure where ewes are considered as controls and during exposure. Ten ewes were randomly divided in two groups of five; the lead group received lead nitrate at 2.5 mg.Pb/kg/day and the lead-cadmium group received lead nitrate at 2.5 mg.Pb/kg/day + cadmium chloride at 2 mg Cd/kg/day orally during 63 days. Both groups were tested for their blood lead levels and hematological and biochemical parameters before and after receiving the treatment. Before exposure, blood lead levels were below the detection limit of 4 µg/l. Blood levels of lead during 9 weeks of exposure varied from 135±57µg/l to 356±147µg/l for the lead group and from 192±75µg/l to 445±294µg/l for the co-exposed group. Mean blood lead levels of lead-cadmium group were more elevated than the ones of the lead group. The transaminases (ALT, AST) are high for the Pb-Cd group during the two last weeks of exposure. The rates of hematocrit and hemoglobin decreased for the Pb-Cd group to reach a value of 28% and 8.9±0.6mg/100ml, respectively. The co-administration of Pb and Cd resulted in a significant reduction in zinc and copper plasma contents.

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INTRODUCTION

Lead and cadmium have been recognized as the most important environmental and industrial pollutants. They are known for their cumulative properties in continental ecological cycling (Rodrigues-estival *et al*., 2012; Javed, 2015). In many circumstances, Cd and Pb pollution co-exists and humans and mammals may be co-exposed to both toxics. It was shown that in blood, Cd and Pb levels may be positively correlated (Xiao *et al*., 2014). The exposure to these metals induces various pathologies in specific target organs following their intestinal absorption and subsequent accumulation. Pb exposure may lead to alterations of some hematological parameters and cause disorders in the trace mineral profile especially by interfering with calcium (Pareja-Carrera *et al*., 2014**)** and zinc metabolisms (Houpert *et al*., 1997). Biochemical modifications at low doses have been reported in humans (Sakai, 2000) and animals (Liu, 2003). Metals levels in blood and urine are used for biological monitoring of exposure and risk (Skerfving and Nilsson, 1993; Khan *et al*., 2014). Lead chronic exposure in cattle and ewes can be monitored by measuring blood lead and blood Zinc-Protoporphyrins levels. (Mehennaoui *et al*., 1997). In addition, several sensitive tests are used to control exposed animals and subsequent effects on a biomarker such haemoglobin concentration, hematocrit rate and hepatic enzymes. Relatively few experimental exposures to Pb and Cd have been carried out in ruminants. The present experiment aimed to highlight the toxic effects of a sub-chronic exposure to lead alone or combined to cadmium in sheep as it may be possible in different countries. To assess the health effects of the chemical mixture compared with single contamination, we focused on the determination of some indicators, biochemical and hematological changes, and the interactions of heavy metals with two elements: zinc (Zn) and copper (Cu).

MATERIALS AND METHODS

The experiment was carried out on ten OuledDjellal non-lactating, two years old ewes, with a mean weight of

40kg. The animals were housed in collective pens, under similar conditions at the sheepfold of the Veterinary Department (University of Batna 1, Route de Biskra, 05 Batna, Algeria). The animals were randomly separated into two distinct groups of five: group 1 (Pb): received Pb; the group 2(Pb+Cd) received Pb and Cd. The study was conducted in two periods; during the period1, for four weeks, the ewes were kept without contamination. The animals during this period acted as the control group for the entire study. In the nine weeks following exposure period, each of the five ewes in group 1 was given a daily single dose of 2.5mg of Pb/kg (as lead nitrate). The ewes in group 2 received a daily single dose of Pb and Cd mixture (as lead nitrate and cadmium chloride), bringing 2.5mg of Pb/kg and 2mg of Cd /kg. Lead and Cadmium oral administration was carried out every morning; metallic salts were enclosed in gelatin capsules and placed on the base of the tongue. The capsules were immediately swallowed. The administration of the capsules was carried without constraints according to animal welfare (Comité Consultatif d'Ethique, Centre de Recherche en Biotechnologie, Constantine). Lead, cadmium, zinc, copper, and calcium levels were determined in hay and granulated feed.

Sampling: Blood samples were collected (4 ml) from the jugular vein into vacutainer tubes guaranteed free of any trace of heavy metals. Sampling was performed before exposure on days 0, 7, 14, 21, and during exposure on days 28, 35, 42, 49, 56, 63, 70, 77, and 84. Blood collection was carried in a similar way during both, pre-treatment and treatment periods and in the Pb and Pb-Cd groups. Four blood samples for each ewe were collected in heparinized tubes, EDTA tubes, citrate tubes and tubes without anticoagulant respectively for the Pb blood, hematology, biochemical parameters, and total proteins analysis.

Elemental analysis: The feed samples were mineralized at 450°C. The white ashes were dissolved in diluted nitric acid (5N). Lead and Cadmium concentrations were estimated by the flameless atomic absorption spectrometry (GF- AAS) and Calcium, Zinc and Copper were measured by flame atomic absorption spectrometry (Perkin Elmer Analyst 100).

Blood lead: The samples were diluted 1:10 in 0.05N ultrapur nitric acid in presence of Triton X at 0.5%. Analyses were performed using of atomic absorption spectrometer (PerkinElmer Analyst100). The lead levels were measured by graphite furnace with deuterium background correction in pyrolytic heated graphite tube, according to the technique described by Mehennaoui *et al*. (1997). The limit of detection was estimated to be 4 µg /l and the limit of quantification 12 µg/l. Calibration standards were prepared from non-exposed sheep blood.

Zinc and copper in plasma: Flame atomic absorption spectrometry (PerkinElmer Analyst, 100) was used for performing zinc and copper analysis in plasma. The determination was on 1 ml sample diluted 1:5 according to the technique described by Mehennaoui *et al*. (1997).

Hematology: Hemoglobin (Hb) and Hematocrit (Hct) were measured. The automated method (Blood Cell Analyzer, Beckman coulter) for the determination of Hb concentrations was used. The Hct values (%) were recorded using a capillary tube reader after centrifugation.

Biochemical parameters: Total serum protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and serum creatinin levels were measured by using Beckman Automatic biochemistry analyzer.

Statistical analysis: Two-way analysis of variance (MedCalc v12.7.1.0 software) was used to discriminate differences over the time (time effect) and between the two groups (treatment effect). When ANOVA was significant, Bonferroni test was used for mean comparaison, significance was assessed at $P<0.05$.

RESULTS

Diet mineral composition and total mineral intake are shown in Table 1. Pb and Cd contents of the diet were below the detection limits. Cu and Zn concentrations in hay were 4.7 and 19mg/kg, respectively. The granulated feed values (barley and maize) were 6.7 and 4mg/kg for Cu and 23 and 27mg/kg for Zn, respectively. Ca values in hay and granulated feed were high.

Before exposure, mean blood lead was below the detection limit in both groups. During the exposure period, the means blood concentration–time profile of Pb, after oral administration of 2.5mg/kg Pb for nine weeks were as shown in Fig. 1 for Pb and Pb+Cd groups. Pb blood concentrations increased during the first weeks to reach in (Pb) group a value of 305±92µg/l in the 63 day whereas the values in (Pb+Cd) group varied from the lowest mean value 184±141µg/l at 70 day to the highest level of 445±293µg /l at 63day. Because ewes had very different Pb blood concentrations within the same group, the group mean lead levels was not sigificantly different among the goups ($P>0.05$).

The mean values for Zn and Cu in controls plasma were 80±17µg/100ml and 85±23µg/100ml respectively (Fig. 2 and 3). Over the first four weeks, after treatment, Zn plasma values ranged from 79 to 96 µg/100ml in (Pb) group and 86 to 100µg/100ml in (Pb+Cd) group (Fig. 2). During exposure, the mean values were significantly decreased ($P<0.001$), in the two groups. The patterns of variations in plasma Cu levels (Fig. 3) were similar to those for plasma Zn levels. Significant ($P<0.01$) decrease of plasma Cu levels was observed in the two groups in the 9th week of exposure. ANOVA did not reveal a significant difference between the two treated groups in plasma zinc concentration whereas for plasma Cu there was a significant effect of treatement ($P<0.01$) (Fig. 3).

Mean values of hemoglobin and hematocrit before exposure were within the physiological values (Table 2). In the (Pb) group a low decrease of Hb values was observed, but no significant change compared to controls ($P>0.05$). However a significant decrease was observed in (Pb+Cd) group on day 84 ($P<0.01$) (Table 2).

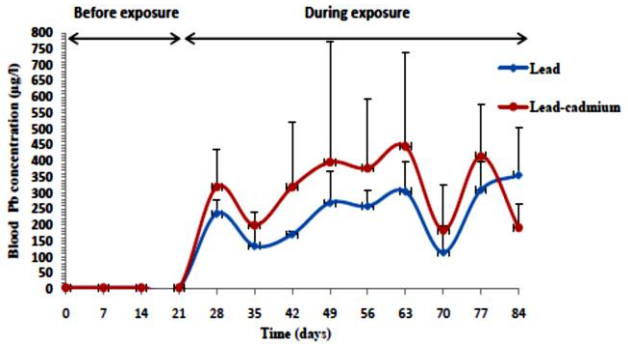

Fig. 1: Blood lead concentration (mean±SD) before and during exposure in the two groups.

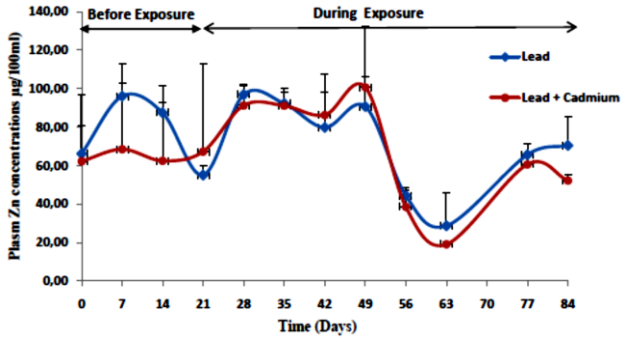

Fig. 2: Plasma Zinc concentrations (mean±SD) before and during exposure in the two groups.

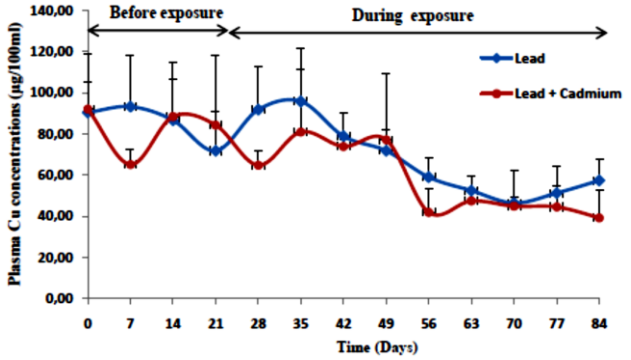

Fig. 3: Plasma Copper concentrations (mean±SD) before and during exposure in the two groups

Table 1: Mineral composition of the foodstuffs

	Hay	Maize	Barely	Bran	Total intake/day
Copper(mg/kg)	4.7	4	6.7	2.6	7.4 mg
Zinc(mg/kg)	19	27	23	51	39.2 mg
Calcium(g/kg)	26	3.8	3.5	6	29 g
Cadmium(µg/kg)	<DL	<DL	<DL	<DL	
Lead (µg/kg)	<DL	<DL	<DL	<DL	

DL: Detection limit.

Mean values of Hct in (Pb) group varied from 32.2±3.4% before exposure to 28.8±1.3% in the 9th week; the decrease was significant (P<0.01). The Hct levels in (Pb+Cd) group were lower than those in the (Pb) group. The mean value in co-exposed ewes was 35±3.3% at the start of treatment (Table 2). We observed a decrease of hematocrit levels to reach the value of 27.3±1.5% at the end of the exposure. ANOVA revealed a significant effect of time and treatment for Hb and Hct (P<0.001). Bonferroni test revealed a significant Htc decrease in (Pb+Cd) group on day 84 (P<0.001) (Table 2).

Before exposure the values of serum protein, ALT, AST and CRE corresponded to the normal physiological range (Table 2). In the Pb group, ALT and AST values ranged from 16.6±5.1 to 34±1.6UI/l and from 57.8±4 to 90.4±7.1UI/l, respectively (Table 2). The increase of ALT level was significant (P<0.01).The Pb-Cd co-exposure caused an intense increase of liver enzymes. ALT levels were 16.7±3.2UI/l before exposure and 37.7±1.5UI/l at the end of exposure. AST levels ranged from 79±25.8 to 188±103UI/l. There was a significant difference between the two groups for AST and ALT levels (P<0.001) (Table 2). The evolution of ALT values were changed and a significant increase was observed (P<0.01) during the period of exposure. AST levels reached a mean level of 188±114 UI /l with great variation in the co-exposed group in 8th week.

Serum creatinin and protein levels in Pb group corresponded to physiological values.We observed a significant increase of serum creatinin levels for the two groups(P<0.05) mainly during the last week of exposure (Table 2). Total proteins ranged from 71.3±1.3 to 70.6±3.6 g/l in Pb group, and from 71.3±2.5 to 68.3±5.9 g/l in co-exposed group (Table 2).

DISCUSSION

We have designed our experimental conditions in order to simulate a repeated low feed exposure to provide detectable Pb blood concentrations and avoid a clinical intoxication : animals received daily low metallic salts doses orally to reproduce a diet exposure. The dosage of 2.5mgPb/kg body weight corresponds to contaminated ruminants forage of 50 mgPb /kg, dose for a daily consumption of 2 kg of forage in sheep.

Lead poisoning is one of the most frequently reported causes of poisoning in livestock at pasture (Pareja-Carrera *et al.*, 2014). Ewes were selected as the experimental animals, as a good model for ruminants; it allows us to perform the necessary blood samplings.

The diet composition revealed that the animal feeding was uncontaminated. Forage in uncontamined area contents of Pb and Cd were below 1 mg /kg of dry matter . Animal feed has not substantially contributed to any change in the exposure doses. Tolerate levels in animal feed is 0.5mg/kg for Cd and 30 mg/kg for Pb (Liu, 2003). Our results showed that blood Pb levels increased in both groups during treatment. These results are in agreement with repeated oral low dose exposure of trace metallic, singly or in combination (Mehennaoui *et al.*, 1997). Gradual increase in blood Pb levels was recorded. In the (Pb+Cd) group, concentrations reached a greater level (445±293µg/l) than that in the (Pb) group (305±92 µg/l). The levels indicative of clinical poisoning (>350µg/l) (Rodrigues-estival *et al.*, 2012) were observed in the (Pb) group in the 9th week and in the (Pb+Cd) group in the 4th week. Our results also showed that in the co-exposed group, Pb blood concentrations have exceeded the threshold base line (about 33% of all samples) while no external signs of apparent toxicity was observed in all corresponding ewes. Indeed, clinical signs are not always correleted to blood Pb concentrations (Waldner *et al.*, 2002). Cattle and sheep tolerated up to 5 mg/kg body weight for at least a year without showing any clinical signs (Payne and Livesy, 2010). Lane *et al.* (2015) reported that some co-exposed cattle to Cd and Pb had a poor general condition.

Table 2: Hematological and biochemical modifications before and during lead and cadmium repeated oral administration in ewes

		Before exposure	During exposure		ANOVA	
Parameters	Groups	Day 28	Day 56	Day 84	Treatment	Time
	Pb	11±0.9	11.2±1.2	9.3±0.6	***	***
Hb (g/100ml)	Pb-Cd	11.2±0.9	9.7±0.4	8.9±0.6		
	Pb	32.2±3.4	34.2±3.2	28.8±1.3	***	***
Hct (%)	Pb-Cd	35±3.3	29.8±1.5	27.3±1.5		
	Pb	16.6±5.1	21.8±1.3	34±1.6	***	***
ALT (UI/l)	Pb-Cd	16.7±3.2	27.8±1.9	37.7±1.5		
	Pb	57.8±4	78.8±8	90.4±7.1	***	NS
AST (UI/l)	Pb-Cd	79±13	188±114	147±20		
	Pb	11±1.9	9.8±1.6	13±1.6	NS	*
Creatinine (mg/l)	Pb-Cd	12.2±0.8	9±2.4	13.7±1.5		
	Pb	71.3±1.3	69.8±4.1	70.6±3.6	***	*
Total Proteins (g/l)	Pb-Cd	71.3±2.5	67.6±7.3	68.3±5.9		

In each group there was 5 animals. Hb=Hemoglobin; Hct=Hematocrit; ALT=Alanine aminoTransferase; AST=Aspartate aminotransferase; NS=Non Significant: P>0.05; *P<0.05; **P<0.01; ***P<0.001.

It is known that the nature of metallic salts changes the metal level in blood. Nitrate as well as acetate had a good solubility which influence positively the bioavailability of Pb. Our results were not in agreement with those found by Mehennaoui *et al*. (1997); the mean concentrations reached 122±36µg/l in the Pb group during the plateau.Those authors used Pb as chloride and lactating ewes as animal model, knowing that the excretion of Pb in milk could influence negatively levels of Pb bood.

Previous studies showed that Pb blood level was significantly reduced with concomitant Cd exposure (Pb+Cd) (Mehennaoui *et al*., 1997; Batool *et al*., 2014). Toxicokinetic interactions were observed at high dose metal mixture exposures and the duration of exposure is critical in evaluating the interactions of metal mixtures particulary at low dose levels (Gensheng and Bruce, 2008), which could explain the elevated blood Pb levels detected in the co-exposed group. Additionally, dietary deficiency of essential trace elements without exogenous supplementation had a positive effect on Pb and Cd absorption, and therefore their toxicokinetics. Pb and Cd have long been known to alter the haematological system. From the 8th week of exposure, the co-exposed group revealed a paleness of the mucous membranes which could correspond to anemia.The anemia induced by lead results primary from both inhibition of heme synthesis and reduction of erythrocyte survival. Shortened life span of red blood cells is thought to be due to increased mechanical fragility of cell membrane (Randa *et al*., 2012). As hematological effects we showed significant (P<0.05) decrease of Hb concentration and Hct mainly in the (Pb+Cd) group in the last week. This report suggests that, the administration of combined treatments of Pb and Cd may be accountable for these hematological effects and in addition the heavy metal accumulation in kidney, spleen, and liver might suppress the activity of these hematopoietic tissues (Yuan *et al*., 2014**)** and lead indirectly to anemia. Co-exposure(Pb+Cd) induced significant elevations in serum transaminases (ALT, AST) levels at the three last weeks of exposure (Table 2). In the Pb group, in spite of the increase of enzyme serum levels, the values were still in the physiological ranges. The levels in the co-exposed group were higher than in the Pb group (P<0.01). The increase in ALT and AST serum levels confirmed the damaging effects of metallic salts on liver cells. Our results are in agreement with other works, which showed that Pb and Cd caused increases of ALT and AST in exposed animals.This increment in enzyme activities is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream. Similar findings of increased ALT and AST were reported by Mehana *et al*. (2012) who found that cell damage exhibited good correlation with the enzyme leakage. Experience also shows that total protein concentration higher than 65g/l is highly indicative of chronic inflammatory reaction when corporal index is low (Braun *et al*., 2010). No change attributable to the administration of Pb and Cd in serum total proteins, in spite of the increase, values still in the standard ranges.This fact suggests that low- metallic trace elements exposure for 9 weeks, singly or combined, had not an adverse effect that could change protein and free amino acids metabolism and their synthesis in liver.

It is well established that kidneys represent the major targets of Pb and Cd toxicity (Ferraro *et al*., 2010; Randa *et al*., 2012). The serum creatinin corresponded to the normal physiological range until the last week where a low increase was detected. Creatinin is known as a good indicator of renal function, i.e., rises in creatinin means an obvious damage to functional nephrons (Yuan *et al*., 2014) but not an early indicator of decreased kidney function (Braun *et al*., 2010).

Relationship between the status of Zn and Cu in blood and the exposure to Pb and Cd was examined**.** Compared with the controls, Pb and Pb-Cd exposures resulted a significant decrease in plasma Zn and Cu levels following 5 weeks of exposure. It was also observed that increasing Pb blood concentration decreases Zn and Cu concentrations.The decrease was found higher for Cu level in the co-exposed group than that in Pb (Fig. 3). Several previous studies demonstrated that Pb and Cd can interact with essential trace element. These interaction can take place at different stages of absorption, distribution in the organism, and excretion of both essential elements and at the stage of Zn and Cu biological functions (Noël *et al*., 2004). Because they have the ability to compete with the divalent metal carriers of Zn and Fe, toxic heavy metals decrease the reabsorption of these essential oligo-metals (Barbier *et al*., 2005). Such competition could induce severe deficiency. Lin *et al*. (2011) reported exposure to Pb and Cd induced increase in urinary Zn and Cu excretion, which may be due to the impaired tubular capacity for the reabsorption and decrease of glomerular filtration. Chronic moderate exposure to Pb decreased serum Zn, calcium and, to a lesser extent, Cu levels

(Pizent *et al*., 2003). At low-dose exposure, impaired metal homeostasis can be an important sensitive indicator of Pb and Cd exposure as this exposure increases the related variations in the hepatic storage of various essential trace element in rats (Noël *et al*., 2004). Up-regulation of divalent metal transporters following chronic ingestion of Pb and Cd was found to disturb the homeostasis for essential metals, thus illustrating the complex interplay between heavy metals and essential metals (Min *et al*., 2008). Renal excretion is one of the most important routes of elimination in essential trace elements and the exposure to Pb and Cd induced an increase in urinary Zn and Cu excretion, which may be due to the impaired tubular capacity for the reabsorption and decrease of glomerular filtration (Lin *et al*., 2011).

Conclusions: The present research shows that elevated blood Pb was accompanied by changes in sensitive biologic markers of Pb poisonning, which also explains its impact on different organs and systems. The adverse effects induced by low oral Pb exposure for 9 weeks were increased with concomitant Cd exposure. Further investigations are needed to correlate the toxic effects when the animals are exposed for a long term and at low doses to more than two metallic traces.

Author's contribution: MS and EB conceived the study. SS, BA and BN executed the experimental design and analyzed the blood and plasma samples. All authors interpreted the data and approved the final version.

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