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Cobalt induces alterations in serum parameters associated with bone metabolism in male adult rat

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Abstract: The intention of this study was to investigate the toxic effect of cobalt on the bone metabolism of rats, including calcium, inorganic phosphorus, and bone-specific alkaline phosphatase of serum during short- and long-term experiments. Rats were injected intraperitoneally (IP) with either 16 mg kg^{-1} BW of cobalt for 5 and 10 days or 6 mg kg^{-1} BW for 20 and 40 days. It was found that IP administrations of cobalt could increase all serum parameters associated with bone metabolic processes. The observed effect was greatest for 16 mg kg^{-1} BW when used for 10 continuous days. By using gel filtration chromatography, we found that most of the activity of total serum alkaline phosphatase was related to low molecular weight isoenzymes, which can be used as a biomarker for bone metabolic disturbances. These results suggest that cobalt can induce bone resorption in adult rats and, therefore, behave as an osteoporotic agent.

Key words: Alkaline phosphatase, bone metabolism, bone resorption, cobalt, trace elements

1. Introduction

While it is firmly established that cobalt (Co^{2+}) plays an important role in human and animal biological functions, the toxic role of this trace element is rarely investigated (Kechrid et al., 2006). Co^{2+} can be used for treating a special kind of anemia (i.e. Co^{2+}-containing vitamin B12 is an essential factor for erythropoiesis) (Lison, 2007; Jelkmann, 2012). Due to its advantages, including a high melting point and resistance to oxidation, it is widely used to produce different types of alloys and steel (Lison, 2007). Co^{2+} compounds are now considerably involved in the petroleum and plastic industries, rechargeable battery manufacturing, and also nanoproduction of carbon tubes (Lison et al., 2001; Goc Rasgele, 2013). Anthropogenic activities, such as agricultural fertilizers, mining activities, and smelting, in addition to natural sources of this element, might provide unexpected amounts of Co^{2+} in our environment and pose a threat to animal and human health (Domingo, 1989; Hamilton, 1994). Human and animals may be exposed to Co^{2+} compounds via respiratory, gastrointestinal, and dermal absorptions (ATSDR, 2004; Larese et al., 2004). Furthermore, since Co^{2+} alloys are commonly used as prosthetic materials for long-term bone tissue resurfacing, harmful reactions may occur in the human body due to metal ions released by corrosion (Sun et al., 1997) (for more information, see review by Sansone et al. (2013)). Therefore, Co^{2+}-induced bone loss is more common in patients using Co^{2+}-containing implants and may engender a special type of Co^{2+} toxicity (Campbell and Estey, 2013).

Deleterious effects of overexposure to Co^{2+} have been well documented in different lab animals. In this regard, Co^{2+} exposure has been linked with the impairment of different organs; it is also thought to cause lung cancer and asthma, to interfere with the male reproductive system, and to engender erythrocytosis, polycythemia, heart disease, and goiters (Pedigo et al., 1988; Lasfargues et al., 1992; Barceloux, 1999; Jefferson et al., 2002; Linna et al., 2004). Once Co^{2+} enters human and animal bodies, it is normally distributed throughout the body tissues, mainly the liver, kidney, heart, and bone (Simonsen et al., 2012). Co^{2+} can affect bone health by increasing the number of osteoclast cells (Patntirapong et al., 2009) and also by reducing osteoblast activity (Zijlstra et al., 2012). Previously, it has been postulated that Co^{2+} can affect alkaline phosphatase (ALP) activities in vitro as a bone formation biomarker and also suppress this enzyme expression in osteoblast-like cell lines (Sun et al., 1997). In addition, ALP activity can be elevated following limited oral exposure (i.e. 1.53 μmol 100 g^{-1} BW) to chromium,

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cobalt, copper, magnesium, and nickel in weanling rats. However, the increase in administrated doses up to 15.3 μmol 100 g^{-1} BW resulted in an adverse effect on ALP activity (Yamaguchi et al., 1986).

Since bone tissue is probably exposed to chronic levels of Co^{2+} ion either administrated by external routes (i.e. air, water, and foodborne) or by release from implanted materials (Langton et al., 2008), we aimed to focus on the toxicity of Co^{2+} in bone-related serum parameters, including calcium (Ca), inorganic phosphorus (Pi), and bone-specific ALP (B-ALP) in adult rats to elucidate which parameter(s) can sufficiently point out the metabolic changes of body during the osteogenic process. We also determined low and high molecular weight ALP (LMW-ALP and HMW-ALP, respectively) in the sera of Co^{2+}-treated rats in the presence of Sephacryl S-300 to discern any possible change(s) in ALP isoenzymes following bone metabolic disturbances.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were of reagent grade and were obtained from Merck Chemical Company (Germany) unless otherwise stated. Deionized water was used throughout this project. All glassware was soaked overnight in 3 M HNO_3 and was then washed 3 times with distilled water and lastly with deionized water. Plasticware was also prewashed with 10 mmol EDTA followed by 3 washes with distilled water and finally with deionized water. Stock solutions of Co^{2+} as cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$) were provided and diluted according to appropriate doses of each individual protocol.

2.2. Experimental design

Male Wistar rats were maintained at the animal house until achieving the desired weight. Eight-month-old male Wistar rats weighing 200 ± 10 g were used to carry out this project. These animals were supported ad libitum for food and water. The feed used in this experiment contained 20% protein, 5% starch, 10% cellulose, and 15% lipid. A constant photoperiod was also set at normal light and darkness (12L:12D). The temperature and relative humidity of their environment remained in range of 20 ± 3 °C and 40%–60% respectively during the experiment. The experiment was conducted over separated periods, including 5, 10, 20, and 40 days of Co^{2+} treatments.

To do this, 40 male rats were randomly divided into 8 groups (5 rats in each group), including 4 different Co^{2+} treatments receiving intraperitoneal (IP) injections of 16 mg kg^{-1} body weight (BW) for 5 and 10 days, and 6 mg kg^{-1} BW for 20 and 40 days. These administrated doses were selected according to IP LD_{50} reported for Co^{2+} (90 mg kg^{-1} BW, Sigma, 2006). Four groups were also used as controls (i.e. allotted for each period of time) and were injected with the same volume of normal saline (0.2 mL of NaCl, 9 g L^{-1}) as used for toxicant injection in Co^{2+}-treated animals.

Stock solution was prepared as an isotonic solution for body membrane. Regarding that, cobalt chloride was prepared using the NaCl equivalent method for providing the same osmotic pressure of Co^{2+} as described previously (Sinko, 2006). The stock solution contained the desired amount of Co^{2+} in only 0.2 mL of their solution for final injection to rats with 200 g of body weight.

2.3. Serum biochemical analyses

All rats were anesthetized by diethyl ether and were then sacrificed to withdraw blood from their heart directly using a 5-mL syringe.

Collected blood samples were centrifuged at 2000 rpm (Kubota, France) for 10 min to separate sera for subsequent biochemical tests. Serum Ca was determined by routine colorimetric technique using the o-cresolphthalein complexone method at 570 nm reported by Moorehead and Briggs (1974) and Pi was determined by using ammonium molybdate method (Fiske and Subbarow, 1925). Total ALP activity in the sera was measured at 410 nm and 37 °C by using P-nitrophenyl phosphate as substrate and 2-amino-2-methyl-1-propanol buffer (0.84 mM, pH 10.3) according to a modified method of Bessey et al. (1946) reported elsewhere (Moshtaghie et al., 1995). A Spectronic 50l spectrophotometer was used to determine absorbencies of each individual sample.

For determination of ALP isoenzyme, we applied a heat stability test. The resistances of different ALP isoenzymes are completely different at 56 and 65 °C (Romslo et al., 1975). In this regard, 1 mL of fresh serum was heated in Bain Marie (Memmert) at 56 °C for 10 min and was then transferred to ice bath immediately to stop any reactions. Sera were prepared for ALP activity measurement as previously described. Using this method, we can recognize which fraction of ALP activity is related to bone specific ALP (B-ALP), due to B-ALP's higher sensitivity to temperature.

Additionally, column chromatography (gel filtration) was applied for the separation of HMW-ALP and LMW-ALP isoenzymes. To do this, at the end of experiment, 500 μL of serum samples was added to a column (50 × 0.9 cm) of Sephacryl S-300 and eluted at 10 mL h^{-1} with Tris-HCl buffer (50 mM, pH 7.4). A fraction (1 mL) was finally collected and the activity of ALP in each fraction was determined (see above).

2.4. Statistical test

The values shown in the Table and also all figures are mean ± SD for 5 separated samples. Obtained data were subjected to statistical analysis using SPSS for Windows, version 18. The normality of the data was checked using the Kolmogorov–Smirnov test. Data were analyzed by

independent samples t test for comparing each treatment's mean value with their specified control at P value lower than 0.05%.

3. Results

The effects of varying concentrations of Co^{2+} on serum parameters related to bone metabolism were investigated over short and long periods. During 40 days of IP administration of Co^{2+}, no changes in body weight were observed. The amounts of Ca were determined in the control serum and Co^{2+}-treated rats at specified time points, including 5, 10, 20, and 40 days. The data presented in Figure 1 show that daily injections of Co^{2+} (16 mg kg^{-1} BW) for 5 days did not significantly change serum Ca, while treatment of animals with the same dose of Co^{2+} (i.e. 16 mg kg^{-1} BW) for 10 days led to a significant ($P < 0.001$) increase in serum Ca from 8.8 ± 0.90 to 11.82 ± 0.67 mg dL^{-1} (34.31%). The Ca levels in serum following 20 days' exposure (6 mg kg^{-1} BW) also showed an elevation of 14.16% ($P = 0.026$) when compared with the control group. Further administration of the same dose resulted in an increase in serum Ca by 18.82% ($P = 0.018$) in comparison with untreated animals (Figure 1).

Exposure to Co^{2+} (16 mg kg^{-1} BW) significantly raised the amount of Pi by 20.58% ($P = 0.009$) and 82.53% ($P < 0.001$) following 5 and 10 day IP injection, respectively. The obtained results showed increases in serum Pi by 66.15% ($P = 0.004$) and 57.62% ($P < 0.001$) at days 20 and 40, respectively (Figure 2).

It can be seen from Figure 3 that short-term administrations of Co^{2+} resulted in a significant ($P < 0.001$) increase in total serum ALP activity over the course of 5 days. The activity of ALP was 2.05-fold higher than the control following 10 days of Co^{2+} administration. Twenty days of Co^{2+} exposure had a significant ($P < 0.001$) effect on total serum ALP activity, increasing it by 86.87% in comparison with the unexposed group. Injection of rats with 6 mg Co^{2+} kg^{-1} BW for 40 days resulted in markedly elevated (52.87%, $P < 0.001$) levels of ALP. To determine the effects of Co^{2+} on B-ALP, we used a heat stability technique as described above. The data presented in Table 1 indicate that most activity of total ALP in serum was related to bone-specific isoenzymes. However, significant increases in all Co^{2+} treatments were observed and general trends of each elevation show a similar pattern. Since the experiment was continued for 40 days of Co^{2+} exposure, we conducted gel filtration using Sephacryl S-300 for the separation of H-ALP and L-ALP of serum. Obtained results from fractionations of both Co^{2+} treated and untreated animals showed that the elevation of ALP in the serum was mostly related to LMW-ALP (Figure 4). Serum LMW-ALP in Co^{2+}-injected rats indicated more than 67%

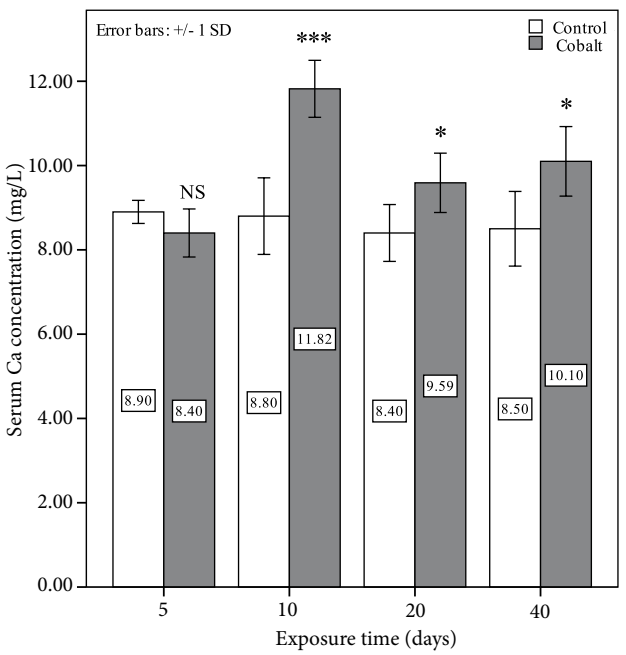

Figure 1. Effects of different doses of Co^{2+} on serum Ca concentrations. Rats were treated with 16 mg kg^{-1} BW of Co^{2+} for 5 and 10 continuous days and 6 mg kg^{-1} BW for 20 and 40 days. All data were expressed as mean ± SD for 5 samples. Asterisks indicate significant differences between treatments and their specified controls.

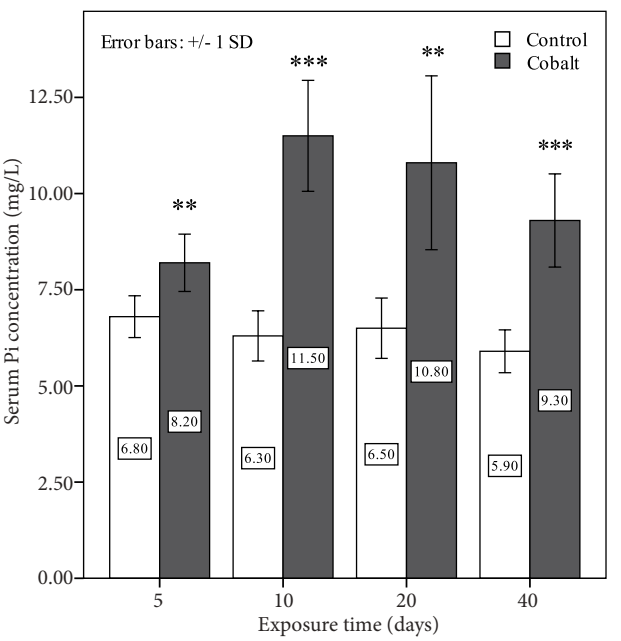

Figure 2. Short- and long-term effects of Co^{2+} on serum inorganic phosphorus are illustrated. All data appear as mean and SD (n = 5). Significances are expressed by asterisks when compared with control treatment.

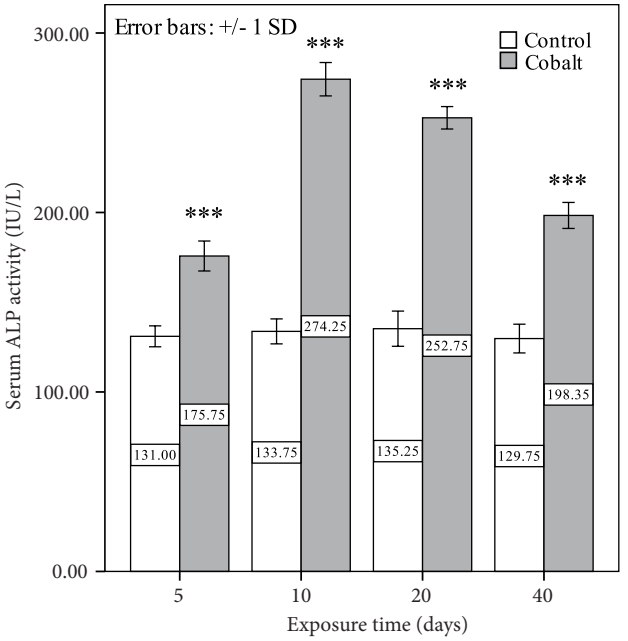

Figure 3. Changes in total serum ALP activities following either 16 mg kg^{-1} BW or 6 mg kg^{-1} BW of Co^{2+} IP injection. Values represent the mean of 5 random samples ± SD. Significances are expressed by asterisks.

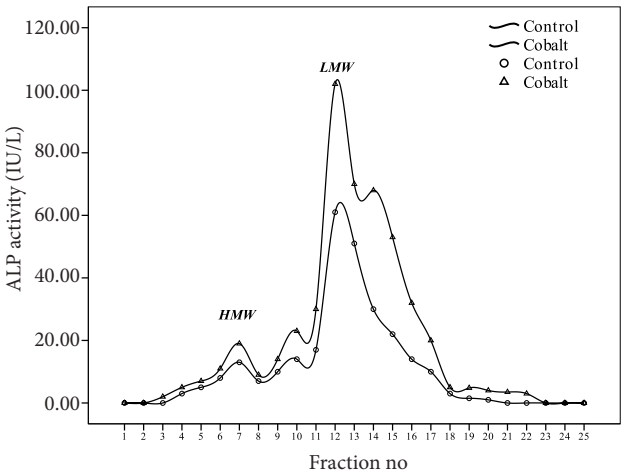

Figure 4. The activities of serum HMW- and LMW-ALP in rats treated by 6 mg kg^{-1} BW of IP Co^{2+} and untreated control group for 40 days. Gel filtration was applied for separation of ALP isoenzymes as explained in the Materials and methods section.

elevation, while the HMW-ALP activity increased slightly from 13 to 19 IU L^{-1}.

4. Discussion

In this study, we examined the effects of Co^{2+} during short- and long-term exposures at concentrations lower than LD_{50} reported previously for this lab animal model. It was found that IP administrations of Co^{2+} affect some serum parameters associated with bone metabolic process. The observed effects were greatest for 16 mg kg^{-1} BW when used for 10 continuous days. We also found that the observed responses also were different with exposure time and do not follow a time-dependent manner.

Co^{2+} can potentially suppress osteoblast synthetic functions and induce histopathological changes in human osteoblast-like cells in vitro (Anissian et al., 2002); the same results were not achieved (Andrews et al., 2011) in concentrations at the clinically observed level following metal-on-metal hip replacements using Co^{2+}-containing prosthetic materials (Langton et al., 2008). Therefore, the authors postulated that the inhibitory effect of these ions (Co^{2+} and chromium) can only be due to the high concentrations mature osteoclasts were exposed to. In contrast to other toxic metals, like aluminum, chromium, cadmium, and lead, which are able to deposit between matrix and calcification front of bone (Boyce et al., 1982; Krishnan et al., 1990; Stepensky et al., 2003; Conti et al., 2012), Co^{2+} does not localize in this area of bone (Stepensky et al., 2003) and hence calcification might not directly be prevented by Co^{2+}.

Previously, Kechrid et al. (2006) reported that if male rats were administrated Co^{2+} orally (383 mg kg^{-1}), a significant increase in serum ALP activity could be observed. They postulated that the high level of serum ALP, in addition to the other liver enzymes, reflected the hepatotoxicity of Co^{2+} and therefore more secretions of liver enzymes from liver cytosol to bloodstream could occur. However, in the present study, we found that total ALP activity of serum was elevated following Co^{2+} exposure. Using a heat stability test, which is described in this manuscript, it was found that 85% of total serum ALP were subjected to the B-ALP (Table 1). Therefore, the authors strongly suggest that Co^{2+} within the mentioned range has a profound effect on bone metabolism. In agreement with our finding, Qureshi et al. (2002) demonstrated that there was a link between Co^{2+} exposure and bone resorption markers. Previously, it has been shown that other elements, including aluminum, cadmium, magnesium, zinc, and lead, could increase total ALP activity in serum (Moshtaghie et al., 2006a; 2006b; Mirhashemi et al., 2009; Malekpouri et al., 2011). In support of this concept, it has been demonstrated that the ALP enzyme in mammals has 2 active sites for zinc- and 1 active site for magnesium-binding. Other elements like calcium, nickel, manganese, and Co^{2+} can be replaced

Table. Effect of Co^{2+} on total serum ALP activity and bone specific ALP.

Exposure time (days)	Cobalt treatment (mg kg^{-1} BW)	ALP activity: total (IU L^{-1}) mean ± SD	ALP activity: residue (IU L^{-1})	ALP activity: bone (IU L^{-1})* mean ± SD	B-ALP activity (%)**
5	16	175.75 ± 8.34	23.8	151.95 ± 3.22	86.9
10	16	274.25 ± 9.30	13.07	261.18 ± 6.12	92.2
20	6	252.75 ± 6.25	18.2	234.55 ± 5.75	92.8
40	6	198.35 ± 7.24	20.7	177.65 ± 2.69	89.6

*B-ALP activities were achieved by using a heat stability test at 56 °C for 10 min. The inactivated part of total ALP can be taken to represent B-ALP.
** Percentage of B-ALP isoenzyme was calculated as the fraction of B-ALP activity to total ALP activity.

and act similarly on osteoclasts. Ciancaglini et al. (1995) showed that Co^{2+} ions can replace either zinc and/or magnesium active sites and can mimic their physiological roles. It is worth noting that the simultaneous replacement of Co^{2+} with both zinc and magnesium active sites can reduce the stability of this enzyme. Therefore, we suggest that the higher levels of activity of this enzyme following 10 days of Co^{2+} exposure might be related to the substitution of Co^{2+} with magnesium; thereby, ALP activity in the presence of zinc could be increased. The gradual decline in ALP activity following 20 and 40 days (compared with day 10) seems to be related to the enzyme saturation and to changes in its stability (for more details about the kinetic change of ALP, see Ciancaglini et al., 1995).

Increasing Ca and Pi levels in serum may suggest that the osteoclast activity was elevated due to an increase in metal concentration (Andrews et al., 2011). This hypothesis has been supported by the histopathological investigations of femoral fracture cases with Co^{2+}-containing orthopedic materials reported elsewhere (Andrews et al., 2011). However, the present study revealed the direct linear relationship (R^2 *linear* = 0.83) between total ALP of serum and the level of Pi (Figure 5). Therefore, the more serum ALP activity there is, which in turn implies bone resorption (explained above), the higher one can expect the serum Pi concentration to be.

Co^{2+} may, however, compete with passive Ca uptake from intestinal cells and therefore result in hypocalcemia in blood. It can be seen from Figure 1 that serum Ca content declined slightly following 5 days of Co^{2+} exposure, although it was not statistically significant. Hereafter, calcium-sensing membrane receptors in the parathyroid gland monitor this deficiency and parathyroid hormone (PTH) will increase the osteoclast number in bone to stimulate bone resorption. Furthermore, this hormone will reduce Ca excretion via kidney tubules and also increase Ca absorption from the intestine. The amount of

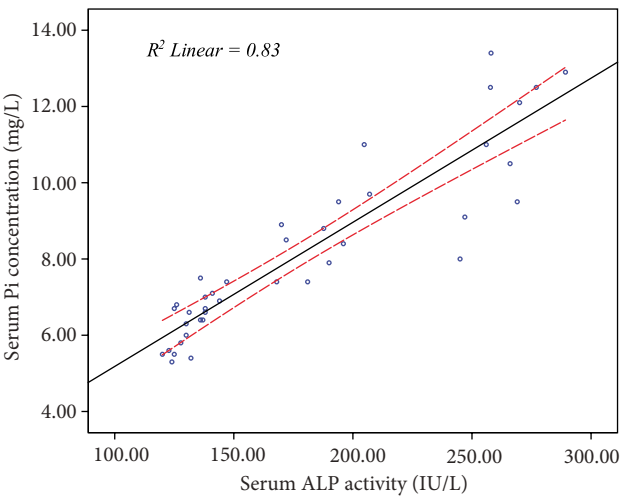

Figure 5: The relationship between total serum ALP activity and serum Pi is exhibited. The black line shows the best-fit line with R^2 of 0.83 and the red dashed lines indicate 95% confidence intervals.

plasma Ca will be thereby increased, following either bone mineral density lessening and/or more Ca reabsorption (Teitelbaum, 2000). The mechanism underlying the PTH activation, described above, will also change the other component of bone mineralization, i.e. Pi. This phenomenon is attributed to the risk of osteoporosis and osteomalacia in people who have been exposed to any kind of bone toxic metals like Co^{2+}. It is worth noting that the increase in the level of PTH does not, however, continue in the long term, i.e. it has been investigated by other trace elements in our lab research activities (unpublished data). This hypothesis can support our finding regarding hypercalcemia occurring following Co^{2+} intoxication.

Moreover, Co^{2+} may impair vitamin D metabolism, which can stimulate calcium-binding protein expression (Gross and Kumar, 1990). This product will regulate the

Ca and Pi uptakes by intestinal cells (Baker and Worthley, 2002). It can be considered another possible mechanism for plasma hypercalcemia and hyperphosphatemia, which is indicated in our experiment. There is no doubt that calcitonin acts as a regulator hormone for plasma hypercalcemia, which can reduce Ca and Pi levels in people with the aforementioned disorders (Baker and Worthley, 2002). It can be, however, supposed that Co^{2+} may alter thyroid gland functions and therefore disturbs calcitonin secretion.

The increase in serum LMW-ALP has been previously proposed to be the result of either bone or intestine cancer, whereas HMW-ALP elevation in serum can be stated as a result of liver cancer (Moshtaghie et al., 1995). In contrast to previous findings, which indicate that magnesium, aluminum, and lead exposure could increase HMW-ALP (Moshtaghie et al., 2006a; 2006b; Mirhashemi et al., 2009) levels in rats, the present work shows that Co^{2+} can increase LMW isoenzymes rather than HMW. In other words, most serum ALP was related to LMW and therefore could be interpreted as a destructive effect of Co^{2+} on bone metabolism.

The data presented in this manuscript might, however, be able to explain any bone disturbances induced by cobalt. Therefore, it could be concluded that Co^{2+} is a potent bone-deleterious agent by increasing all measured, associated parameters, including Ca, Pi, and B-ALP. Co^{2+} can induce bone resorption in adult rats and, therefore, is linked to the producing of osteoporosis like cadmium and copper, and in contrast to zinc, which causes osteomalacia in mammals (Kozuka et al., 1995). However, the exact mechanism by which Co^{2+} can hinder bone metabolism should be examined during future lab and clinical experiments. We think that more investigations are needed to elucidate the possible interaction of Co^{2+} with formation and resorption processes.

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