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Heavy Metal Interactions on Cadmium-induced Renal Osteodystrophy and Anaemia in Rats

Orinamhe Godwin AGBADUA, Frederick Otunuya OBI, Chukwuebuka Samuel AMADI, Segun Abiodun TAIWO, Daniel Oriobosa EHIKHAMHEN and Glory MALIO-OLISEH*
Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin city, Edo State, Nigeria.

Abstract
The toxicity of cadmium is influenced by several factors among which is the interaction with other metals. The other metals in question co-exist with cadmium as particulate matters in air, water and soil. Exposure of man to cadmium via any of the sources just mentioned means exposure to the co-pollutants as well. Hence this study investigated the effects of iron, lead, zinc, copper, manganese, nickel and chromium which have been reported as co-pollutants of river waters with cadmium, on cadmium-induced chronic kidney disease, osteomalacia and anaemia. Male Wistar albino rats were co-treated with cadmium and the above heavy metals individually and combined at doses found in Warri river, as drinking water daily for 90 days. The control group received heavy metal-free water as drinking water. Zinc reduced the extent of kidney damage and associated anaemia and osteodystrophy caused by cadmium. Iron and nickel reduced the ability of cadmium to cause anaemia in rats besides reduction in kidney cadmium burden. Lead exacerbated hemotoxic effects of cadmium. The findings from this study suggest that nephro- and haemotoxicity of cadmium can be enhanced or impaired depending on which of these metals it co-exists with as pollutants.

Keywords: Osteomalacia, Kidney failure, Haemoglobin, Cadmium

Introduction
The environment in which we claim to "live" in has become a huge reservoir of toxic metals with human health under constant risk of deterioration due to increasing chronic exposure of such metals that adversely affect the quality of life of people (1). Comprehensive and continuous monitoring of the Warri river between 1986 and 1991 showed that the levels of cadmium and other heavy metals were above the maximum allowable limits set by the World Health Organisation (2). Despite environmental and health concerns caused by heavy metals, there is continued exposure in aquatic and terrestrial environments, and is even increasing in some parts of the world, particularly in less developed countries (1,3).

One such heavy metal with a high toxicity found in the earth's crust is cadmium, which is released to the biosphere from both natural processes (such as volcanic emissions and weathering of rocks) and anthropogenic activities (smelting of other metals, burning of fossil fuels and the use of phosphate and sewage sludge fertilizers) (4). Cadmium intoxication has been reported to be nephrotoxic, hepatotoxic, neurotoxic, genotoxic, carcinogenic, teratogenic (5,6), and impairs vitamin D metabolism in the kidney (7), which coupled with calcium malabsorption produces osteomalacia and/or osteoporosis (8). The most extreme example of this process is *itai-itai* disease in Japan, which combines severe pain from osteomalacia with osteoporosis, renal tubular dysfunction, anemia, and calcium malabsorption (9,10).

Although there have been previous studies on cadmium based on the level in the Warri river (11-13), it is worthy to note that cadmium does not exist in isolation but there is continuous interaction with other factors which may affect its toxicity. In all likelihood, cadmium being a divalent cation is accumulated by transport mechanisms similar for essential and heavy metals (14). This study was therefore aimed at determining the individual and combined interaction of the heavy metals found in Warri River on cadmium induced-chronic kidney damage, osteomalacia and associated anaemia in rats.

Materials and Methods
Animals
Fifty albino rats (Wistar strain) weighing between 100-120g, obtained from Biochemistry Department, University of Benin, were used for this study. Rats were housed in the Department of Biochemistry Animal House under standard laboratory conditions (12-hour light/dark cycle, 22-28^{0}C) and veterinary management. They were allowed free access to standard food (growers mash from Bendel Feed and Flour Mill, Ewu, Edo State) and water, and allowed to acclimatize to laboratory conditions for four weeks. All animal experiments were performed in adherence to the NIH animal guidelines.

Chemicals: Cadmium chloride and manganese chloride (Kermel, France); chromium (III) chloride and nickel sulphate (LobaChemie, Mumbai, India); copper (II) sulfate, iron (II) sulphate, lead (II) acetate and zinc sulfate

**Corresponding Author's E-mail: orinamhe.agbadua@uniben.edu*

(Guangdong Guanghua Sci-Tech Co., Ltd, Guangdong, China) were used as sources of heavy metals in the study.

Experimental Design and Treatment

At the end of acclimatization, the rats were re-weighed and thereafter separated into ten groups of 5 rats each as shown below and the treatment in each group lasted for a period of 90 days: Group I (control group) received heavy metal free water as drinking water. Group II received a solution equivalent to 0.229mg cadmium per litre of distilled water as drinking water. Group III received a solution equivalent to 0.229mg cadmium and 1.900mg of iron (Fe) per litre of distilled water as drinking water. Group IV received a solution equivalent to 0.229mg of cadmium and 1.081mg of lead (Pb) per litre of distilled water as drinking water. Group V received a solution equivalent to 0.229mg of cadmium and 0.505mg of zinc (Zn) per litre of distilled water as drinking water. Group VI received a solution equivalent to 0.229mg of cadmium and 0.187mg of copper (Cu) dissolved per litre of distilled water as drinking water. Group VII received a solution equivalent to 0.229mg of cadmium and 0.101mg of manganese (Mn) per litre of distilled water as drinking water. Group VIII received a solution equivalent to 0.229mg of cadmium and 0.534mg of chromium (Cr) per litre of distilled water as drinking water. Group IX received a solution equivalent to 0.229mg of cadmium and 0.964mg of nickel (Ni) per litre of distilled water as drinking water. Group X received a solution equivalent to 0.229mg of cadmium, 1.900mg of iron, 1.081mg of lead, 0.505mg of zinc, 0.187mg of copper, 0.101mg of manganese, 0.534mg of chromium and 0.964mg of nickel per litre of distilled water as drinking water. Rats in each group received 42.86ml per kg body weight of the appropriate solution daily by gavage throughout the duration of treatment to ensure daily minimal water consumption, with the solutions also kept in the water trough. At the end of the treatment period, each rat was weighed and then anaesthetized in a chloroform fume saturated jar. While under anesthesia, each rat's abdominal and thoracic region was opened and exsanguinated by heart puncture using a hypodermic syringe and needle, and blood samples were collected for biochemical and hematology (RBC, Hg and HCT) assays. Samples of the tibia and kidney were also collected for cadmium load estimation.

Biochemical analysis

Serum calcium, phosphorus, urea, creatinine and alkaline phosphatase activity were estimated according to the instructions stipulated in Randox diagnostic kit while sodium, potassium, chloride and bicarbonate levels were estimated by following the instruction of Teco diagnostic kit colorimetrically using a visible spectrophotometer (Model 721G Searchtech Instruments, England). Red blood cell count, haemoglobin and haematocrit were measured using automated haematology analyzers (Sysmex America Inc, USA). The cadmium load in the kidney and bones were measured using an atomic absorption spectrophotometer (BUCK Scientific, Model 210VGP) (15).

Statistical analysis

All data were expressed as means ± standard deviation. Differences between means of various results were assessed for statistical significance by analysis of variance (ANOVA), followed by Dunnett's t–test. A p-value < 0.05 was considered to indicate statistical significance.

Results

Kidney function indices

Serum electrolyte (sodium, potassium, chloride and bicarbonate), urea and creatinine levels in the various experimental groups are shown in table 2.

Table 2: Kidney function parameters of rats after 90 days drinking water treatment

Group	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)	Bicarbonate (mmol/L)	Urea (mg/dl)	Creatinine (mg/dl)
Control	96.95 ± 3.53^{a}	4.40 ± 0.79^{a}	59.08 ± 2.53^{a}	30.48 ± 2.92^{a}	34.11 ± 3.62^{a}	0.53 ± 0.05^{a}
Cd	100.00 ± 4.58^{b}	4.70 ± 0.32^{a}	71.36 ± 2.54^{b}	30.00 ± 1.38^{a}	48.60 ± 4.00^{b}	0.66 ± 0.05^{b}
Cd + Fe	108.15 ± 2.29^{bc}	5.96 ± 0.73^{bc}	79.66 ± 4.64^{c}	31.74 ± 1.00^{a}	49.27 ± 7.14^{b}	0.68 ± 0.05^{b}
Cd + Pb	103.66 ± 2.16^{a}	6.37 ± 0.26^{bc}	78.46 ± 4.05^{bc}	30.92 ± 0.99^{a}	43.57 ± 4.17^{b}	0.58 ± 0.04^{a}
Cd + Zn	100.56 ± 3.80^{a}	6.42 ± 0.60^{bc}	77.37 ± 4.48^{bc}	31.69 ± 1.03^{a}	44.19 ± 2.82^{b}	0.54 ± 0.07^{a}
Cd + Cu	101.01 ± 4.66^{a}	6.32 ± 0.64^{bc}	74.97 ± 3.76^{bc}	30.00 ± 2.32^{a}	47.27 ± 2.93^{b}	0.64 ± 0.04^{b}
Cd + Mn	103.73 ± 1.45^{b}	6.67 ± 0.10^{c}	65.95 ± 3.36^{bd}	30.53 ± 0.74^{a}	47.13 ± 2.67^{b}	0.73 ± 0.07^{b}
Cd + Cr	103.00 ± 2.20^{b}	6.23 ± 0.18^{bc}	65.70 ± 4.96^{bd}	30.68 ± 1.10^{a}	44.14 ± 5.48^{b}	0.65 ± 0.05^{b}
Cd + Ni	99.09 ± 3.63^{a}	5.68 ± 0.58^{b}	72.08 ± 1.78^{bc}	31.38 ± 0.73^{a}	40.63 ± 6.13^{a}	0.67 ± 0.05^{b}
All metals	111.25 ± 4.47^{c}	5.85 ± 0.50^{b}	69.07 ± 2.53^{bd}	29.66 ± 2.15^{a}	44.22 ± 4.50^{b}	0.66 ± 0.07^{b}

Values are expressed in mean ± SD (n = 5)

Values in the same column bearing no superscript common vary significantly ($P < 0.05$).

Relative to the control group, there was a significant increase in the levels of serum urea, creatinine and sodium in rats given cadmium tainted drinking water. Co-treatment with cadmium and iron resulted in potassium and chloride levels significantly higher than rats given cadmium tainted drinking water. Co-administration of cadmium with lead, copper, zinc, nickel and chromium resulted in significant increase in potassium levels and non-significant differences in other electrolytes relative to the cadmium only treated group with a marked decrease in creatinine and urea in rats co-treated with zinc, and significant decrease observed in urea levels of rats co-treated with nickel. Co-administration of cadmium and manganese produced the highest creatinine and potassium levels although only the latter significantly higher than the cadmium. Drinking water containing all the heavy metals led to a significant increase in sodium levels when compared to other treatment groups and a significant increase in potassium when compared to cadmium only group. There was no significant difference observed in the serum bicarbonate levels across the groups.

Osteomalacia indices

The effects of the treatment on the level of serum calcium, phosphorus and alkaline phosphatase activity is shown in table 3.

Table 3: Biochemical indices of osteomalacia in rats after 90 days drinking water treatment

Group	Calcium (mg/dl)	Phosphorus (mg/dl)	Alkaline phosphatase (U/I)
Control	9.30 ± 0.10^{a}	3.90 ± 0.1^{a}	50.89 ± 2.86^{a}
Cd	9.23 ± 0.15^{a}	3.07 ± 0.06^{b}	57.83 ± 1.25^{b}
Cd + Fe	9.10 ± 0.10^{a}	3.13 ± 0.31^{b}	44.22 ± 3.31^{c}
Cd + Pb	9.23 ± 0.06^{a}	3.47 ± 0.46^{ab}	50.24 ± 4.37^{ac}
Cd + Zn	9.43 ± 0.12^{a}	3.73 ± 0.12^{a}	46.79 ± 4.28^{ac}
Cd + Cu	8.83 ± 0.12^{b}	3.53 ± 0.32^{ab}	49.55 ± 2.72^{a}
Cd + Mn	9.20 ± 0.10^{a}	3.57 ± 0.32^{ab}	56.45 ± 4.00^{b}
Cd + Cr	9.20 ± 0.10^{a}	3.70 ± 0.1^{a}	64.04 ± 7.83^{bd}
Cd + Ni	9.00 ± 0.10^{b}	3.33 ± 0.15^{b}	68.14 ± 3.44^{d}
All heavy metals	9.23 ± 0.06^{a}	3.43 ± 0.25^{b}	60.59 ± 4.65^{b}

Values are expressed in mean ± SD (n=4)
Values in the same column bearing no superscript common vary significantly ($P < 0.05$).

While the reduction in level of calcium and the increase in ALP activities were non-significant in rats given cadmium tainted drinking water compared to the control, there was a significant decrease in serum phosphorus levels in the cadmium only group. Co-treatment of cadmium with iron, lead, manganese, nickel and all the heavy metals produced no significant difference in these parameters when compared to rats administered cadmium only. Calcium levels were the highest and alkaline phosphatase levels the lowest in rats given zinc and cadmium tainted drinking water with phosphorus levels significantly higher than rats given cadmium tainted drinking water. Co-administration of cadmium and copper led to calcium levels significantly lower than other treated groups.

Haematology

Red blood cell (RBC) count, haemoglobin (Hb) and haematocrit (HCT) levels in the various groups at the end of the treatment is shown in table 4.

Table 4: Red blood cell count, haemoglobin and haematocrit of rats after 90 days drinking water treatment

Group	Red Blood cells (x 10^6/ul)	Haemoglobin (g/dl)	Haematocrit (%)
Control	9.10 ± 0.49^a	15.05 ± 0.82^{ac}	48.25 ± 2.76^a
Cd	8.84 ± 0.19^a	14.37 ± 0.70^{ab}	47.20 ± 1.81^a
Cd + Fe	8.71 ± 0.19^a	15.48 ± 0.63^{ac}	48.88 ± 1.45^a
Cd + Pb	8.15 ± 0.68^a	13.30 ± 0.61^b	43.23 ± 2.41^b
Cd + Zn	9.01 ± 0.36^a	14.13 ± 0.46^{ab}	45.30 ± 2.30^{ab}
Cd + Cu	8.65 ± 0.10^a	13.93 ± 0.92^{ab}	44.18 ± 2.69^{ab}
Cd + Mn	8.57 ± 0.39^a	13.33 ± 0.82^b	43.50 ± 2.67^b
Cd + Cr	8.42 ± 0.12^a	13.88 ± 0.71^{ab}	43.60 ± 1.54^b
Cd + Ni	8.96 ± 0.50^a	16.10 ± 0.85^c	47.88 ± 2.38^a
All heavy metals	9.05 ± 0.60^a	15.23 ± 1.24^{ac}	46.65 ± 3.25^a

Values are expressed in mean ± SD (n=4)
Values in the same column bearing no superscript common vary significantly ($P < 0.05$).

There was no significant difference observed in the red blood cell (RBC) count among the treated groups. Treatment with cadmium resulted in decreased Hb and HCT concentration. Co-treatment of cadmium with iron and nickel led to an increase in Hb and HCT levels. Co-administration of lead, copper, manganese and chromium with cadmium led to a decrease in the level of these parameters with rats given cadmium and lead tainted drinking water having the lowest RBC, Hb and HCT among all the treatment groups.

Cadmium load

Table 5 shows the organ (kidney and bone) cadmium load of the rats in the various groups at the end of the treatment.

Table 5: Organ cadmium load of rats after 90 days drinking water treatment

Group	Kidney* (µg/g)	Bone** (µg/g)
Control	0.037 ± 0.016^a	0.00
Cd	0.339 ± 0.032^b	0.02
Cd + Fe	0.176 ± 0.027^c	0.02
Cd + Pb	0.251 ± 0.034^d	0.02
Cd + Zn	0.252 ± 0.038^d	0.00
Cd + Cu	0.276 ± 0.024^d	0.02
Cd + Mn	0.256 ± 0.027^d	0.02
Cd + Cr	0.394 ± 0.025^b	0.02
Cd + Ni	0.238 ± 0.021^d	0.00
All heavy metals	0.183 ± 0.022^c	0.00

Values are expressed in mean ± SD
Values in the same column bearing no superscript common vary significantly ($P < 0.05$).
*n=3 **n=1

Rats given heavy metal free water had the least amount of cadmium in the kidneys when compared to other treatment groups. Co-administration of cadmium and chromium had no significant difference in the cadmium load compared to rats given cadmium only tainted water, with both groups having the highest kidney cadmium load compared to all other heavy metal treated groups. Rats in the control group, cadmium and zinc co-treated group, cadmium and nickel co-treated group and given all the heavy metals had no cadmium in the bone after the treatment period.

Discussion

Cadmium intoxication has been reported to lead to chronic kidney failure by numerous reports (16,17,18). Cadmium can accumulate in many organs including the kidney, liver, pancreas and testes and adversely affect the functions of these organs. The chief organ of toxic impact in the human is the kidney, with about 30% of body cadmium is deposited in the kidney tubule region (19,20). Results presented in table 2 showed that cadmium administration at the concentration found in Warri River caused kidney failure at the end of the

treatment period which is in agreement with previous studies (21). Various transporters are responsible for the uptake of cadmium and subsequent deposition (19,22), and this was confirmed by the cadmium load in the kidney of rats given cadmium tainted drinking water.

The major transporters responsible for the absorption of non-heme iron are divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1) (23). Results from various *in vivo* and *in vitro* experiments indicate that both DMT1 and FPN1 are also involved in cadmium transport (24-26) thus, the two minerals compete with each other for uptake (24,27). The cadmium load in the kidney of rats given iron and cadmium drinking water was significantly lower when compared to those that received cadmium tainted water only. Co-treatment with iron and cadmium led to a more pronounced kidney damage despite the low levels of cadmium found in the kidney. The increase in kidney damage could be attributed high concentration of iron above maximum permissible limits, with reported nephrotoxicity associated with iron at high concentrations (28).

Exposure to dietary Zn intake has an important effect on Cd absorption, accumulation and toxicity (29). The Zn status of the body is important in relation to development of Cd toxicity. ZIP proteins have been showed to play a pivotal role in zinc transport across the cellular membrane in the intestine and proximal tubules (30) and studies demonstrated that knockdown of these transporters resulted in significantly reduced cadmium uptake, thus emphasizing the relationship in the uptake of both metals (31-33). The study into the function of zinc transporters on cadmium transport by Barbier and colleagues showed that cadmium uptake could be inhibited by nearly thirty percent in distal convoluted tubules (DCT) by co-injection with a small amount of zinc ion, but no inhibition was observed in proximal tubules (PT) (34). Although zinc transporters are expressed in both DCT and PT, cadmium may have a higher affinity for other transporters in PT segments, the uptake of which might not be inhibited by zinc ions. This can explain the reduction in kidney cadmium load in rats given zinc and cadmium tainted rats with cadmium entry into the kidneys via the PT a possible reason why the cadmium load was not further reduced in this group. Co-treatment with zinc and cadmium significantly reduced blood urea and creatinine levels observed in rats given cadmium only. This corroborates reports on the study by El-Sayed *et al*. (35) who studied the protective effect of zinc against cadmium toxicity on pregnant rats and their fetuses. The result is also in agreement with previous work on zinc on cadmium nephrotoxicity (36-38).

The same observations in the kidney cadmium load were discovered after co-treatment with nickel. The combined effect of Nickel-Cadmium is less toxic than cadmium alone, suggesting antagonism between these toxicants in the tissues of rats (39). The levels of Ni which was almost 50 x the maximum permissible limit might explain why the levels of the kidney parameters were not further reduced. The bicarbonate level across the treatment groups although non-significantly different showed that co-administration of zinc and nickel with cadmium led to increased levels thus suggesting that these metals aided in balancing the metabolic acidosis that occurred as a result of sodium and potassium imbalance. It can therefore further confirm that nickel and zinc possibly reduced kidney damage associated with cadmium.

Co-administration of cadmium and chromium had no significant difference in the cadmium load compared to treatment with cadmium only, suggesting that chromium did not disrupt the uptake and deposition of cadmium in the kidneys, and thus explains why no significant difference was observed in the kidney function biochemical indices when compared to rats given cadmium tainted drinking water only. It is well established that cadmium competes with other metals for transport-mediated entry into the cell. Divalent metal-ion transported-1 (DMT1), not only plays a crucial role in iron homeostasis, but also can mediate transport of essential and toxic divalent metals such as Zn^{2+}, Mn^{2+}, Pb^{2+}, Cd^{2+} and Cu^{2+} (19), and this might explain the non-significant effects of co-treatment with copper and lead on cadmium-induced nephrotoxicity.

Kidney cadmium load showed a decreased uptake of cadmium in rats co-treated with manganese which agrees with findings that the rate of gastrointestinal absorption of cadmium is decreased by supplementation of the drinking water with a 'non-toxic' dose of Mn^{2+} as observed in male rats (39). Co-treatment with manganese and cadmium produced levels of potassium and creatinine significantly higher than treatment with cadmium only, thus suggesting increased nephrotoxicity associated with manganese. This finding could be explained by study of Atessahin et al.(40) who concluded that administration of low dose of Mn^{2+} produced amelioration in biochemical indices of nephrotoxicity with high dose of Mn^{2+} causing an opposite effect on nephrotoxicity induced by gentamicin. Impaired calcium and vitamin D metabolism coupled with kidney failure results in bone disorders such as osteoporosis and/or osteomalacia has been implicated in cadmium intoxication (19). Biochemical abnormalities associated with osteomalacia include hypocalcemia, hypophosphatemia, decreased serum 25-hydroxy vitamin D levels coupled with increased parathyroid hormone (PTH) levels and alkaline phosphatase (ALP) activities (41,42). Zinc is required for the growth, development and maintenance of healthy bones (43) Co-treatment with zinc reversed the biochemical abnormalities caused by cadmium and subsequently reversed the bone cadmium load present in cadmium only treated rats. This is in agreement with previous studies of zinc supplementation on cadmium (44,45). Copper influences bone formation, skeletal mineralization, and the integrity of the connective tissue (46). Copper playing similar roles as zinc in proper bone development should have shown similar changes as zinc but however reduced calcium levels were observed in rats given copper and cadmium co-treatment. Estimation of vitamin D and PTH levels might aid in

explaining this discrepancy as phosphorus levels and ALP activities conformed with results obtained with zinc co-treatment.

No significant effect of lead, iron, manganese and nickel was observed on cadmium-induced osteomalacia. While no significant change was observed in calcium levels of rats given cadmium and chromium, a significant increase in phosphorus levels was observed when compared with cadmium only group. However, this group had a significant increase in serum ALP activities when compared to the other groups. Since the levels of calcium and phosphorus are well within the levels of the control, it is suggestive that the increased ALP activity might be due to chromium-cadmium hepatotoxicity with chromium playing a protective role in bone impairment. While determining the levels of vitamin D and PTH would have aided in determining the individual and general effects of these metals, these analyses were not done due to the unavailability of the techniques (ELISA, RIA or HPLC) required for determining these compounds. Hypophosphatemia and hypocalcemia are due to secondary hyperparathyroidism while increased serum alkaline phosphatase or bone specific alkaline phosphatase activity is classically associated with osteomalacia due to vitamin D deficiency (47), and thus the levels of phosphorus, calcium and ALP are indicative of hyperparathyroidism and vitamin D deficiency.

Anaemia, considered with a decrease in either one or more of red blood cell (RBC) count, haemoglobin (Hb) and haematocrit (HCT), has been associated with chronic kidney disease. Cadmium interacts with iron metabolism and absorption, decreases the hemoglobin and hematocrit concentration, leading to anaemia. This corroborates previous studies on cadmium intoxication on anaemia status (48-50). In rats, iron supplementation corrects the anemia caused by cadmium exposure (51). Thus, co-treatment with cadmium and iron led to an increase in Hb and HCT levels even beyond the control. This corroborates reports that anemia associated with cadmium is as a result of low iron, and thus iron supplementation corrects this (48). Nickel co-treatment also increased the levels of these parameters which agrees with previous reports (52,53). No significant difference was observed with co-treatment with zinc (54). Co-administration of lead, copper, manganese and chromium with cadmium led to a decrease in the level of these parameters. This is in agreement with (54,55) with rats given cadmium and lead tainted drinking water having the lowest RBC, Hb and HCT among all the treatment groups. The effects of cadmium are thus suggested to be magnified by interaction with other toxic metals such as lead.

The combination of all the metals in the drinking water resulted in a greater electrolyte imbalance thus suggesting a greater kidney damage which however cannot be attributed to cadmium alone as most of the other heavy metals present were also above their limits. The cadmium load in rats given drinking water containing all the metals was significantly low in the kidney and absent in the bones thus suggesting competition in the uptake of these metals. Biochemical indices for assessment of osteomalacia and anaemia suggest an interplay of metals that reduced and those that increased the effects of cadmium.

Thus, ingesting cadmium at the concentration found in the Warri river will lead to chronic kidney damage, osteomalacia and anaemia. It is however noteworthy that cadmium rarely exists alone but its toxicity is affected by the interaction with various factors such as the heavy metals and their concentration as present in the Warri river. While the overall effect of these metals led to pronounced kidney damage, decreased toxicity was observed in anaemia and bone disorders. However, the river will still be toxic to fishes and inhabitants around the river using it as a source of water, and the fish a source of food, based on the results obtained.

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