

Evaluation of Doses-response and Combined Preventive Effects of Zinc and Vitamin D on Liver Toxicity Induced by Carbon Tetrachloride in Wistar Rats

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Abstract Severe hepatic insufficiency caused by a toxic is a strong danger to the individual survival. Zinc and vitamin D are micronutrients involved in the prevention of several diseases. Our study was to evaluate the potential preventive effects of these micronutrients in hepatic toxicity induced by carbon tetrachloride (CCl₄) in rats. One hundred and fourteen (114) male rats were divided into 19 lots of 6 male rats each according to their body weight. Carbon tetrachloride (CCl₄) was used to induce hepatic toxicity in rats. The rats were treated respectively with 25, 50 and 100 mg/kg of zinc and 6, 12 and 24 μg/kg of Vitamin D and also with the combination of zinc and Vitamin D at the same concentrations. There was a significant increase in serum of ALT, AST without changing Total Protein level in the serum of rats treated with CCl₄ compared to rats in the control lots. However, Zinc and Vitamin D supplementation caused a significant decrease in serum of ALT and AST activity without changing Total Protein concentration in the serum of rats. This study revealed that that zinc combined with Vitamin D could be a good protection against hepatic toxicity induced by chemicals.

Keywords Hepatoprotective, Zinc, Vitamin D, Hepatic Toxicity, Rat

1. Introduction

The liver is the organ that fulfills three major groups of vital functions: metabolic functions, detoxification functions and excretion functions [1]. It is therefore an indispensable organ for life. Severe hepatic impairment (acute or chronic) poses a serious risk to the individual with a survival rate of only about 20% [2]. It is reported that the standardized mortality rate from end-stage liver disease (i.e. cirrhosis) is higher than that for cardiovascular disease among patients

with diabetes [3]. Chronic inflammation is involved in the development of hepatic disease [4, 5]. Otherwise, micronutrition can help prevent pathologies, some of which may be a sign of it.

Micronutrition is a recent concept advocated in prevention and in the treatment of acute or chronic pathologies [6]. It consists of "satisfying the micronutrient needs of an individual, through a diversified diet, associated if necessary with a personalized supplementation". In addition, micronutrition, like nutrition, aims to keep people healthy to protect the individual at risk, to optimize other treatments by reducing for example the side effects of medications, and to correct the disorders functional systems [7]. Micronutrients play no role in energy, but they are indispensables body functioning. These are vitamins, trace elements, minerals, essential fatty acids. Both zinc and vitamin D are important micro-nutrients for the body.

Zinc, is an essential component of a large number of enzymes participating in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids as well as metabolism of other micronutrients [8, 9]. It has also been reported that drug therapy or gene therapy that increases the serum zinc concentration can inhibit the progress of hepatic fibrosis [10].

Vitamin D is a fat-soluble, which is an essential micronutrient with major implications for human health. The biologically active form of vitamin D is 1,25-(OH)₂D₃ [11]. Vitamin D receptors are widely distributed in more than 38 tissues [12]. Macrophages and dendrite cells constitutively express vitamin D receptors which indicates plays an important role in regulating the inflammatory response [13]. Vitamin D is widely known for its role in the development and maintenance of bone tissue, as well as in the maintenance of calcium and phosphorus homeostasis. Studies were reported that vitamin D played an important role in decreasing the risk of type 2 diabetes [14], metabolic syndrome [15] and cardiovascular diseases [16]. However,

the study of zinc and vitamin D effects and their combination in the prevention of liver toxicity remains unknown.

The aim of this study was to evaluate the preventive effects of zinc and vitamin D on liver markers in hepatic toxicity induced by carbon tetrachloride (CCl₄) in Wistar rats.

2. Materials and Methods

2.1. Animal Material

The animal species chosen for this study was the male Wistar albino rat. The rats were bred in the animal facility of Higher Normal School (ENS) at room temperature. In these premises, the photoperiod was 12 hours and the animals had free access to water and food. 114 male rats, aged 7-8 weeks, weighing approximately 200 to 230 g were used for the experiments. The selected rats had not been used for previous studies.

2.2. Chemicals

- Carbon tetrachloride was purchased from Sigma, Aldrich (USA).
- Zinc was purchased from Walmark (France, 02-2019) in the form of a bottle containing 30 tablets of 15 mg of Zinc.
- Vitamin D one ampoule (2 ml) of dose 100,000 IU (CRINEX, France, 05-2018)
- NaCl [0,9%] (Baxter, Belgique)
- The products of ALT (Alanine Aminotransferase) and AST (Aspartate Aminotransferase) used were supplied as reagents for "COBAS Integras 400 plus" by Roche Diagnostics® (Mannheim, Germany).

2.3. Methods

2.3.1. Preparation of Solutions

- Carbon tetrachloride (CCl₄) at a dose of 3ml/kg was dissolved in an equal volume of olive oil rather than 50%. It was administered intraperitoneally by injection of intoxication solution [17].
- 15 mg tablet was dissolved in 5 ml distilled water per kilogram bw [18] administered by gavage.
- Vitamin D (one dose) 2 ml were dissolved in olive oil with 12 µg/kg bw [19] administered by gavage.

2.3.2. Animal Treatment

Animals were divided into 19 lots of 6 rats each (n= 6). The duration of experiment was for 14 days. The treatments are carried out every day at the same hour during the experimental period. Liver damage was achieved by injecting 3 ml/kg of CCl₄ intraperitoneally on the 14th day of feeding the animals in groups 4 to 19 with zinc and vitamin D.

2.3.2.1. Treatment of Control Lots

Lot 1: received NaCl 9‰ at 1 ml by gavage over fourteen days (Normal control).

Lot 2: received 0.3mL of olive oil by gavage over fourteen days.

Lot 3: received 1 ml of NaCl 9‰ and 0,3ml of olive oil by gavage over fourteen days.

Lot 4: were administered with CCl₄ only at 3ml/kg body weight of 50% dissolved in olive oil intraperitoneally (ip) the 14th day of treatment (Negative Control).

2.3.2.2. Treatment with Different Doses of Zinc (25, 50 and 100 mg/kg bw) by Gavage over Fourteen Days

Followed by CCl₄ (3ml/kg of 50%) by Intraperitoneal Injection the 14th day

Lot 5: received 25 mg/kg of zinc followed by CCl₄: Zn 25 + CCl₄

Lot 6: received 50 mg/kg of zinc followed by CCl₄: Zn 50 + CCl₄

Lot 7: received 100 mg/kg of zinc by followed by CCl₄: (Zn 100 + CCl₄)

2.3.2.3. Treatment with Different Doses of Vitamin D (6, 12 and 24 µg/kg) by Gavage over Fourteen Days

Followed by CCl₄ (3 ml/kg of 50%) by Intraperitoneal Injection the 14th day

Lot 8: received 6 µg/kg of vitamin D followed by CCl₄: (VD 6 + CCl₄)

Lot 9: received 12 µg/kg of vitamin D followed by CCl₄: (VD 12 + CCl₄)

Lot 10: received 24 µg/kg of vitamin D followed by CCl₄: (VD 24 + CCl₄)

2.3.2.4. Treatment with Combination of Vitamin D and Zn by Gavage over Fourteen Days Followed by CCl₄ (3 ml/kg of 50%) by Intraperitoneal Injection the 14th day

Lot 11: received 25 mg/kg of zinc + 6 µg/kg of vitamin D followed by CCl₄: Zn 25 + VD 6 + CCl₄

Lot 12: received 25 mg/kg of zinc + 12 µg/kg of vitamin D followed by CCl₄: Zn 25 + VD 12 + CCl₄

Lot 13: received 25 mg/kg of zinc + 24 µg/kg of vitamin D followed by CCl₄: Zn 25 + VD 24 + CCl₄

Lot 14: received 50 mg/kg of zinc + 6 µg/kg of vitamin D followed by CCl₄: Zn 50 + VD 6 + CCl₄

Lot 15: received 50 mg/kg of zinc + 12 µg/kg of vitamin D followed by CCl₄: Zn 50 + VD 12 + CCl₄

Lot 16: received 50 mg/kg of zinc + 24 µg/kg of vitamin D followed by CCl₄: Zn 50 + VD 24 + CCl₄

Lot 17: received 100 mg/kg of zinc + 6 µg/kg of vitamin D followed by CCl₄: Zn 100 + VD 6 + CCl₄

Lot 18: received 100 mg/kg of zinc + 12 µg/kg of vitamin D followed by CCl₄: Zn 100 + VD 12 + CCl₄

Lot 19: received 100 mg/kg of zinc + 24 µg/kg of vitamin D followed by CCl₄: Zn 100 + VD 24 + CCl₄

2.3.3. Collection of Blood Samples

All test animals were euthanized 24 hours after the last treatment in 14th day. Also blood of each animal was taken (puncture of the orbital sinus) in a tube without anticoagulant, for metering the biochemical parameters.

2.3.4. Biochemical Study

Blood samples collected in anticoagulant-free tubes were centrifuged at 3000 rev/min for 15 minutes. The collected sera were used to measure the biochemical parameters namely: alanine amino transferase (ALT), the aspartate aminotransferase (AST) and total protein with the *Cobas Integra 400 Plus* (Roche diagnostic, Germany) in Ivory Coast Pasteur Institute.

2.3.4. Statistical Analysis

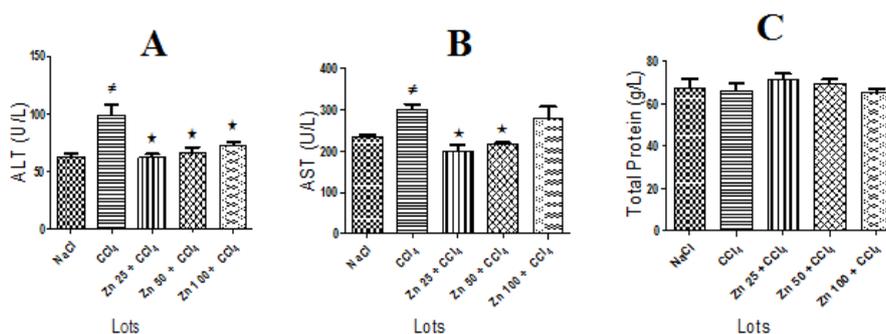
Graph Pad 5.1 was used for statistical analyses. Data were expressed as mean \pm SD. Mean values of the different lots were compared using a one-way analysis of variance (ANOVA) with Dunnett test. If $p < 0.05$, the difference

between the values was considered significant.

3. Results

3.1. Effects of zinc and Carbon Tetrachloride (CCl₄) on Liver Markers

The results in Figures 1A and 1B showed that the administration of CCl₄ induced a significant increase ($p < 0.05$) in the activity of ALAT and ASAT compared to the control lot NaCl 0.9%. However, this high enzyme activity induced by CCl₄ was significantly inhibited ($p < 0.05$) by Zinc concentrations (25, 50 and 100 mg/kg). The values of the enzymatic activity of AST and ALT in rats pretreated with Zn are compatible with those of the rats of the control group (NaCl 0.9%) (Figures 1A and 1B). For the total protein concentration both CCl₄ and zinc pretreatment did not induce a significant variation from the values in rats of the control groups (NaCl 0.9% and Olive oil) as shown in figure 1C.



A: effects on ALT activity

B: effects on AST activity

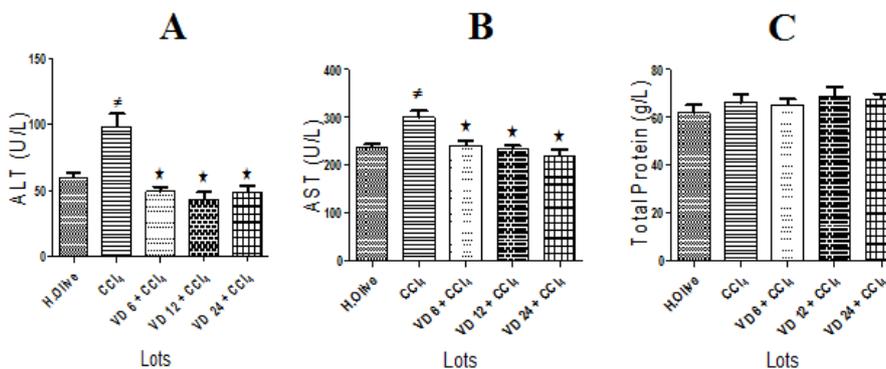
C: effects on Total protein concentration

ALT: alanine aminotransferase, AST: aspartate aminotransferase, Total Protein,

NaCl: sodium chloride, CCl₄: carbon tetrachloride, Zn: zinc, VD: vitamin D, n=6/lot

#, *: ($P < 0.05$): statistically significant #: ($p < 0.05$) vs NaCl *: ($p < 0.05$) vs CCl₄

Figure 1. Effect of Zinc and CCl₄ administration on liver markers level in Wistar rats



A: effects on ALT activity

B: effects on AST activity

C: effects on Total protein concentration

ALT: alanine aminotransferase, AST: aspartate aminotransferase, total protein,

H: Olive: Olive Oil, CCl₄: carbon tetrachloride, Zn: zinc, VD: vitamin D, n=6/lot

#, *: ($P < 0.05$): statistically significant #: ($p < 0.05$) vs H. Olive *: ($p < 0.05$) vs CCl₄

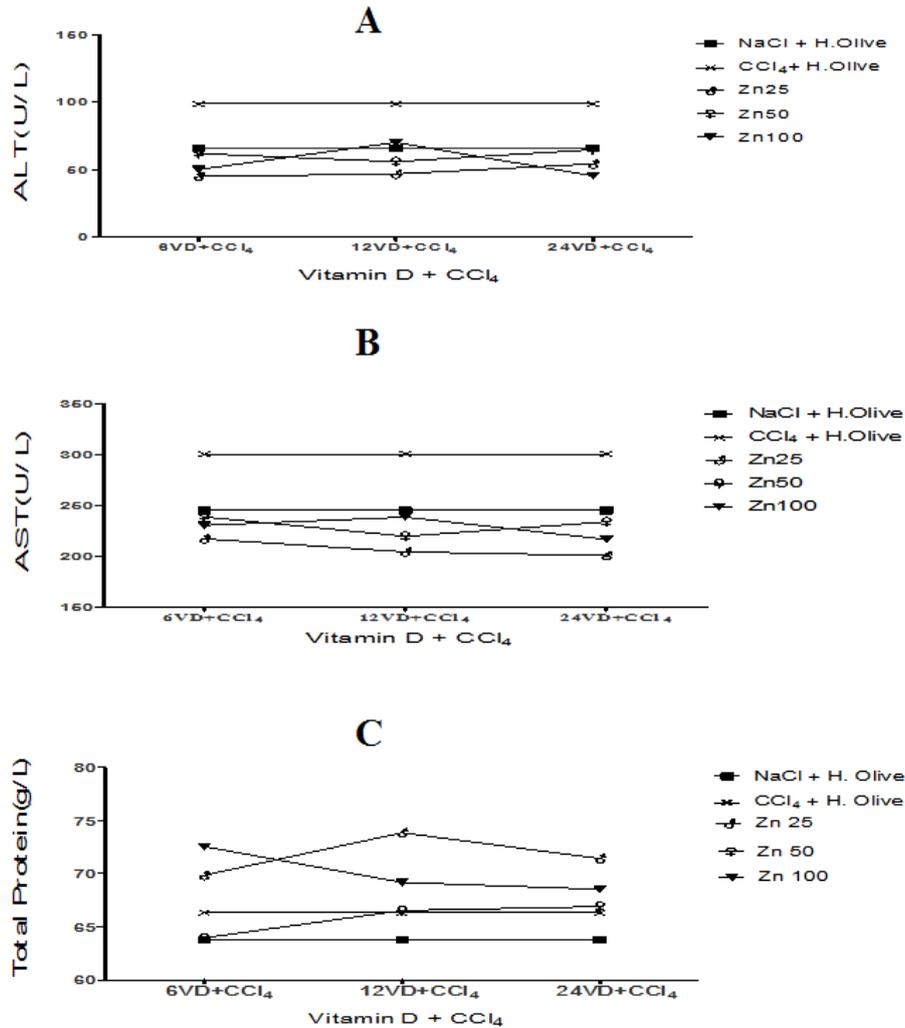
Figure 2. Effect of Vitamin D and CCl₄ administration on liver markers level in Wistar Rats

3.2. Effects of Vitamin D and Carbon Tetrachloride (CCl₄) on Liver Markers

Carbon tetrachloride (CCl₄) administration caused significant ($p < 0.05$) increase in the serum levels of the ALT, AST compared to the control lot Olive oil (figures 2A and 2B). However, treatment with low or high administration dose of vitamin D (6, 12 and 24 $\mu\text{g}/\text{kg}$) caused no significant change on the serum levels of enzymes compared to control lot treated by Olive oil after CCl₄ administration but its induced a significant ($p < 0.05$) decrease in the serum levels of hepatic enzymes compared to control lot treated with only CCl₄ (figures 2A and 2B). There was no significant ($p > 0.05$) change on the serum concentration of Total Protein in the lots administered with low or high dose of vitamin D when compared to the control lots (Olive oil and CCl₄ alone) as shown in (Figure 2C).

3.3. Combined Effects of Zinc and Vitamin D on Liver Markers

The Figure 3 indicated a fixation of each concentration of zinc when a variation of vitamin D concentration was observed as represented in Figure 3. The results showed that 25 mg/kg bw of zinc combined respectively with 6; 12 and 24 $\mu\text{g}/\text{kg}$ of vitamin D was the best combination that reduced the level of parameters ALT and AST. In fact, this combination induced a low activity of hepatic enzymes as compared to others combinations presented by Zn 25 curve below from the others graphs (Figure 3A and 3B). The best association observed on Total Protein concentration was 25 mg/kg of zinc with 12 and 24 $\mu\text{g}/\text{kg}$ of vitamin D. This combination increased in the serum concentration of Total Protein as compared to others combinations represented by Zn 25 curve above others graphs (figures 3C). In addition, liver is protected by association of zinc (25 mg/kg) and vitamin D (12; 24 $\mu\text{g}/\text{kg}$).



A: effects on ALT activity

B: effects on AST activity

C: effects on Total protein concentration

ALT: alanine aminotransferase, AST: aspartate aminotransferase, Total Protein,

NaCl: sodium chloride, H. Olive: Olive Oil, CCl₄: carbon tetrachloride, Zn: zinc, VD: vitamin D, n=6/lot

Figure 3. Effect of Vitamin D and Zinc variation on liver markers in Wistar rats

4. Discussion

The aim of our study was to evaluate the possible protective effects of zinc and vitamin D supplementation on liver functions in experimentally-induced hepatic damage in male albino rats. It was designed to denote the hepatoprotective activity of alimentary micronutrients. Intraperitoneal injection of Carbon tetrachloride (CCl₄) caused hepatic dysfunction. CCl₄ induced a significant increase in liver enzyme activity such as ALAT and ASAT versus non-significant increase in total protein concentration. In fact, liver dysfunction is characterized by alteration in serum levels of liver enzymes and metabolic products [20]. The elevation in the liver marker enzyme was confirmed previous reports on the hepatotoxicity of CCl₄ administration [21]. In this study, a significant elevation of transaminases (ALT and AST) activity in rats treated with CCl₄ alone was observed as compared to normal control lot treated with NaCl. The elevation of marker enzymes reported in this study is the same to the findings of [22] who observed significant hepatic damage in rats treated with single dose of CCl₄.

The efficacy of any hepatoprotective drug is indeed dependent on its capability of either reducing the harmful effects or in maintaining the normal hepatic physiological mechanism which have been imbalanced by a hepatotoxin. It is in this perspective that this study was interested to the preventive effects of zinc and vitamin D supplementation in hepatic damage due to liver toxicity. In fact, Zinc is a known fundamental component of the endogenous enzymatic antioxidant system with antioxidant properties playing an essential role in cell membrane integrity and functions in many aspects of cellular metabolism [23]. Results of the present study showed significant reduction of the ALT and AST activity without significant effect on total protein with the preventive administration of zinc. These results were compatible with those of [24] who reported that after one month of zinc therapy, the mean serum ALT, AST and gamma glutamyl transpeptidase (GGT) levels dropped significantly. It is important to indicate that zinc is not only required for cell-mediated immunity, but also an effective antioxidant and anti-inflammatory agent [25]. It has reported by [26] that antioxidant substances can prevent liver damage induced by thyroid dysfunction.

Vitamin D is one of the fat-soluble vitamins which is activated partly in liver and is from endogenous biosynthesis in skin cells by ultraviolet radiation from sunlight and dietary sources. Carbon tetrachloride (CCl₄) administration induced hepatic damages. These damages were observed through the increasing of transaminases activity level in serum. The increasing evidence suggests that the circulating concentration of vitamin D was negatively associated with the risk of liver disease [27] and with the increasing severity of liver disease, the expression of hepatic pro-inflammatory cytokines also increased [28]. In the present study, rat lots were treated with vitamin D supplementation to prevent for

14 days. In addition, rats treated with CCl₄ alone increased transaminase activity in serum. In the lots pre-treated with vitamin D, the results indicated significant decreasing level in serum of ALT and AST when CCl₄ was administered. These results were confirmed by [29] who showed that serum ALT and AST activity were extremely elevated in rats of diabetic mellitus group (DM) when in the vitamin D group, 1,25-(OH)₂D₃ treatment significantly lowered serum activity of ALT and AST compared with the DM group. All concentrations of vitamin D (6, 12 and 24 µg/kg) tested in the present study reduced these parameters. These vitamin D concentrations can contribute to liver protection. It was reported that 1,25-(OH)₂D₃ has protective effects on liver of diabetic rat by modulating inflammation and lipid metabolism [29].

The present study was realized to prevent liver toxicity. Indeed, it was important to find the best association of zinc and vitamin D which could prevent hepatic damage. To have beneficial effect on liver toxicity, this study has associated micronutrients as zinc and vitamin D. Moreover, the elevation of transaminases activity in serum of rats treated with 100 mg/kg of zinc was observed. Jansen *et al.*, [30] have demonstrated that elevated concentration of zinc (100 mg/kg) induced pancreatic beta-cells destruction of human body. Vitamin D and zinc combination has decreased or attenuated toxic effects of CCl₄ that we have observed. Vitamin D has played a role of modulator in zinc action by regulating zinc effect. Motiwala and Wang [31] have shown that vitamin D modulates lipid metabolism by decreasing the triglyceride level in overweight subjects' serum. Following the possible combinations in this study, the best were 25mg/kg zinc combined with 12µg/kg and 24µg/kg of vitamin D. Moreover, the best association indicated decreasing of liver enzymes (AST, ALT) activities, and then total protein concentration increased. According to [32], preventive hepatoprotective action of *Curcuma longa* (Zingiberaceae) showed that the decreasing activity of liver enzymes elevate by CCl₄ and the increasing concentration of total protein and stress enzymes highly decreased by CCl₄.

These results show that to obtain better effect with high doses of zinc, high concentration of vitamin D must be used because vitamin D attenuate zinc toxicity. Vitamin D and Zinc association is hepatoprotective.

5. Conclusions

At the end of our study, it is important to note that zinc oral supplementation with 25 to 50 mg / kg is beneficial for the intoxicated liver and at 100 mg/kg it has harmful effects on liver. The oral supplementation of Vitamin D which up to 24µg/kg, has a beneficial effect on the liver toxicity. This study has showed that the best combination is obtained with 25 mg/kg of Zinc combined with 12, 24 µg/kg of Vitamin D. Finally, this study suggests that zinc combined with vitamin D provides good protection of liver against hepatic toxicity.

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