ORIGINAL ARTICLE

Beneficial Effects of Vitamins (C + E) Supplementation against Nickel-induced Hepatotoxicity in Mice

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ABSTRACT
This study was objected to examine the protective effect of vitamin E and vitamin C on nickel induced impairment of liver function. Forty male mice weighed 42-50 g were divided into four groups of eight animals. Each group was spontaneously drank normal water, except those received vitamin C in their drinking water. Additionally, animals of each group were fed on one of the diet: The first group of animals was fed a basal diet and drank a normal water served as a control, the second group fed a basal diet supplemented with nickel (2.7g NiSO4 6H2O/kg diet), the third group fed the same with drinking water containing vitamin C (1g/l), the forth group fed a basal diet containing supplemented both of nickel and vitamin E (1g/kg diet), the fifth group fed a basal diet with supplemented vitamin E and received vitamin C in drinking water, and the sixth group fed a basal diet with supplemented nickel plus both of vitamins (E+C). All animals were treated for a period of 30 days. Nickel treatment resulted in a significant decrease in body weight and an increase in absolute and relative liver weights. Additionally, serum glucose concentration, serum total protein concentrations, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities were significantly increased in nickel treated mice. As well as, serum total protein concentration was significantly increased, and subsequently, the hepatic protein contents were reduced in Ni(II) treated group. The toxic effect of Ni(II) was also indicated by significantly decreased of catalase (CAT), glutathione peroxidase (GSH-Px) activities and glutathione (GSH) level. Hence, the ultrastructural alterations in hepatic tissue confirmed these observations. Meanwhile, vitamin E and/or vitamin C supplementation has attenuated these hepatotoxic effects.

Key words: Nickel, vitamins (E + C), ALT, AST, ALP, hepatotoxicity, mice.

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INTRODUCTION
Nickel (Ni (II)) is an environmental carcinogenic, nephrotoxic and hepatotoxic heavy metal [1]. Ni (II) is an alloying element for steel and cast iron, yielding alloys and steel increases strength and resistance to corrosion and temperature. Additionally, Ni (II) compounds are used in preparation of Ni (II) alloys, ground coated enamels, in cooling of ceramics and glass, electroplating, Ni (II) iron storage batteries, electronic components and to prepare nickel catalysts [2]. Humans and animals are exposed to Ni (II) via several routes from different occupational and environmental settings, leading to adverse liver and kidney diseases accompanied by histological alterations [4]. Ni (II) cannot be metabolized by the body, but it accumulates especially in kidney [5] and liver [6]. Several studies have reported that heavy metals including Ni (II) are able to induce oxidative stress through reactive oxygen species (ROS) generation, lipid peroxidation and DNA damage in kidney and liver cells [7]. As well as, nickel induced toxicity oxidative threat is associated with depletion of glutathione and other endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) glutathione peroxidase (GPx) and glutathione S-transferase (GST) [8]. The genotoxic effects of nickel might be prevented by some exogenous supplementation of antioxidant compounds which play an important role against the adverse effects of reactive oxygen and nitrogen species [9]. Previous studies have reported, that vitamins E and C are effective antioxidant and scavenger agents against oxidative stress leading to diseases and degenerative processes through reactive oxygen species (ROS) generation, such as free radicals (OH⁺; O₂⁻; COO⁻ etc.). Additionally, vitamins E and C are biologically required for many organism functions, like their
involvement in regulating the immune system and helping lymphocytes to function more effectively [10, 11]. Vitamin E is defined as (alpha-tocopherol) a lipid-soluble element. Meanwhile, vitamin C (ascorbic acid) is a hydro soluble component [12]. Both vitamins E and C are able to reduce hepatotoxicity [13, 14] and nephrotoxicity[15, 16] induced by toxic metals. Therefore, many works have been done on the toxic effects of nickel and the preventive role of ascorbic acid and/or alpha-tocopherol acid in some animal and human tissues [17, 18, 19]. However, their combination effects on nickel-induced liver injury and hepatic enzyme disruptions remain unclear and required further clarification.

MATERIALS AND METHODS

Chemicals
Nickel sulphate (NiSO4 7H2O), vitamin C, vitamin E, 5, 5'dithiobis-(2-nitrobenzoic acid (DTNB) and reduced glutathione were purchased from sigma Chemical Co (St Louis, France) and all other chemicals used in the experiment were of analytical grade.

Animals
Male albino (Wistar) mice weighing between 42 and 50 g were obtained from the Pasteur Institute (Algiers, Algeria). Animals were acclimated for two weeks under the same laboratory conditions of photoperiod (12h light/12 h dark) with a relative humidity 70 % and room temperature of 22 ± 2 C°. Food (Standard diet, supplemented by the ONAB, EL-Harouch, Algeria) and water available ad-libitum.

Experimental design
After 1 week of acclimatization, animals were randomly selected into four groups of eight animals and were named:

- **Group 1: (Control)** Control animals fed a basal diet which's composed of carbohydrate source which is provide by equal amounts of cornstarch 326(ONAB, El Harouche, Algeria) and sucrose 326, protein 168 (egg white solid), lipids 80 (corn oil), fiber 40 (cellulose), vitamin mix 20 (sigma) and mineral mix 40 [20]. Additionally, Control mice were received a normal drinking water.
- **Group 2: Ni(II)** (Basal diet supplemented with 2,7g NiSO4 6H2O/kg diet and received a normal drinking water).
- **Group 3: Ni(II) + Vit C** (Basal diet supplemented with 2,7g NiSO4 6H2O/kg diet + vitamin C (1g/kg diet) in their drinking water).
- **Group 4: Ni(II) + Vit E** (Basal diet supplemented with 2,7g NiSO4 6H2O/kg diet + vitamin E (1g/kg diet) and received a normal drinking water).
- **Group 5: Vit E + Vit C** (Basal diet supplemented with vitamin E (1g/kg diet) and received a normal drinking water).vitamin E (1g/kg diet) and received vitamin C (1g/kg diet) in their drinking water.
- **Group 6: Ni(II) + Vit (C + E)** (Basal diet supplemented with 2,7g NiSO4 6H2O/kg diet + vitamin E (1g/kg diet) and received vitamin C in their drinking water.

The doses of nickel, vitamin C and vitamin E used in our experiment were focused on the previous studies studies [21], [22] and [23] respectively. The experimental procedures were carried out according to the National Institute of Health Guide-lines for Animal Care and approved by the Ethics Committee of our Institution. The treatments of mice continued for a period of four weeks. At the end of the experiment, total body weight was recorded and animals were sacrificed by decapitation without anesthesia to avoid animals stress. At the time of sacrifice, blood was transferred into ice cold centrifuged tubes. Tubes were then centrifuged for 10 minutes at 3000 rpm and serum was used for glucose, total protein, albumin, ALP, AST and ALP assays. Liver was removed immediately and one part was processed immediately for assaying glutathione and antioxidant enzymes activities. The other part was used for histological studies.

Analytical methods

Preparation of tissue homogenate
After animal sacrifices by decapitation, the liver was quickly excised, rinsed with saline, blotted dry on filter paper, weighed and then 10% (w/v) homogenates of tissue was prepared in buffer (0.025 M Tris-HCL buffer (pH 7.4) using a tissue homogenizer. Homogenates were centrifuged at 10000 × g for 15 minutes at 4 C°, and the resultant supernatant was used for the determination of reduced glutathione, protein level, catalase and GSH-Px activities.

Determination of serum glucose, and serum total protein level
Serum glucose (Spinreact, Spain, ref: 41011), and total protein (Spinreact, Spain, refs: 1001291) concentrations were estimated with a commercial kits and determined by enzymatic colorimetric method using spectrophotometer (Jenway 6505, Jenway LTD, UK).

Enzymatic, non-enzymatic antioxidant and marker enzyme estimations
Separate liver homogenates of experimental mice were used for the estimation of following enzymatic and non-enzymatic antioxidants:

1. **Glutathione peroxidase (GSH-Px)** activity was assayed exactly as described elsewhere [24].
2. **Catalase (CAT)** activity was measured as the decrease in H2O2 concentration by recording the absorbance at 240 nm [25].
3. **Glutathione (GSH)**: GSH levels were estimated by the method as described previously [26].
4. **Alanine transaminase (ALT), Aspartate transaminase (AST) and Alkaline and phosphatase (ALP)** activities were carried out by commercially available kits, Spinreact, Spain, refs: ALT-1001161, AST-1001171 and ALP-1001131 respectively.

**Protein Estimation**
The protein content of tissue samples were measured by the method of Bradford [27] using bovine serum albumin as a standard.

**Histological studies**
Histological evaluations were according the method of Bancroft and Stevens [28]. Briefly, liver and kidneys were dissected and immediately fixed in bouin solution for 24 h, processed by using a graded ethanol series, and then embedded in paraffin. The paraffin sections were cut into 5µm thick slices and stained with hematoxylin and eosin for light microscopic examination. The sections were then viewed and photographed.

**Statistical analysis**
Data are given as means ± SE. Statistical significance of the results obtained for various comparisons was estimated by applying one way analysis of variance (ANOVA) followed by Protected Least Significant Difference Fisher’s test (PLSD Fisher) and the level of significance was set at p < 0.05.

**RESULTS**

**Body weight (gain/loss), absolute and relative liver weights.**
The results presented in table 1, showed that body weight of animals exposed to nickel was significantly decreased (P < 0.01) as compared to controls. In addition, a significant increase of absolute and relative liver weights in nickel treated group was noticed (p < 0.001). These physiological paramaters were significantly improved in the combined treatments. In Ni(II)+Vit C and Ni(II)+Vit E, body weight was significantly increased (P < 0.01) as compared to Ni(II) treated group, which by, the absolute and relative liver weights were significantly decreased (p < 0.05; p < 0.01; p < 0.001) in the combined treatments. In the other hand, mice treated with both vitamins showed comparable values to controls regarding body weight, absolute and relative liver weights.

**Table-1** — Body weight (gain(+), loss(-)) absolute, relative liver weights, food consumption and water intake of control and treated mice with Ni(II), Ni(II)+Vit C, Ni(II) + Vit E, Vit (E+C) and Ni(II) + Vit (C+E) after 4 weeks of treatment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experimental groups</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td></td>
<td>(n = 8)</td>
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<tr>
<td>Initial body wt (g.)</td>
<td>46.4±0.16</td>
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<tr>
<td>Body wt gain/loss (g.)</td>
<td>+15.53±0.12</td>
</tr>
<tr>
<td>Absolute Liver wt (g.)</td>
<td>2.11±0.08</td>
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<tr>
<td>Relative liver weight (g.)</td>
<td>0.46±0.04</td>
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<tr>
<td>Food intake (g/kg b.w)</td>
<td>53.7± 8.59</td>
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<tr>
<td>Water intake (ml/day)</td>
<td>57.25±6.00</td>
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Statistically significant differences from control: **p < 0.001,  *p < 0.01,  *p < 0.05;  from Ni(II):  *p < 0.001,  *p < 0.01,  *p < 0.05.

Values are given as mean ± SE, n = number of animals.

**Food intake**
In nickel treated mice, food intake and water consumption were significantly reduced (p < 0.01) as compared to controls. In the (Ni(II)+Vit C), (Ni(II)+VitE) and (NiII)+Vit(C+E)-treated groups, the food...
intake increased significantly (p < 0.05), when compared to Ni(II) group. Hence, Vit (C+E) treated group showed no significant changes, when compared to controls.

**Serum markers of liver damages**

As seen in Table 2, treatment with Ni (II) caused significant increase of serum glucose, serum total protein concentrations, AST, ALT and ALP activities compared to control group (p < 0.001). However, hepatic protein contents were significantly decreased in Ni(II) treated mice (p < 0.01), when compared to controls. The above mentioned biochemical parameters were upregulated in Ni(II) + Vit C, Ni(II)+VitE (p < 0.01 for glucose, ALT and total protein; p <0.05 for ALP and AST), and Ni(II) +Vit(C+E) (p < 0.05 for glucose and total protein, p < 0.01 for ALT and no significant difference for ALP and AST), when compared to controls. Similarly, The glucose level, total protein concentrations, ALP, AST and ALT activities were significantly increased (p < 0.001 and p < 0.01) in Ni(II) + Vit C, Ni(II) + Vit E and Ni(II)+Vit(C+E) treated groups, as compared to Ni(II) alone treated group.

**Table 2**: Changes of biochemical parameters of control and treated mice with Ni(II), Ni(II)+Vit C, Ni(II)+Vit E, Vit (E+C) and Ni(II) +Vit (C+E) after 4 weeks of treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experimental groups</th>
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<tr>
<td></td>
<td>Control (n = 8) Mean±SE</td>
</tr>
<tr>
<td>Glucose (g/l)</td>
<td>1.31±0.024</td>
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<tr>
<td>Total protein (g/100ml)</td>
<td>2.1±0.09</td>
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<tr>
<td>Hep. Protein Cont. mg/100g Tissue wt.</td>
<td>12.8±1±0.36</td>
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<tr>
<td>ALP (U/L)</td>
<td>62.26±4.67</td>
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<tr>
<td>AST (U/L)</td>
<td>39.65±1.1</td>
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<tr>
<td>ALT (U/L)</td>
<td>38.93±0.77</td>
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Statistically significant differences from control: ***p < 0.001, **p < 0.01, *p < 0.05; from Ni(II): ‘p < 0.001, ‘p < 0.01, ‘p < 0.05; from Ni(II)+Vit(E+C). Values are given as mean ± SE, n = number of animals.

**Hepatic oxidative stress parameters**

The results revealed a significant decrease (p < 0.001) of reduced glutathione level (GSH) and the activity of both catalase and GSH-Px in liver of nickel treated mice. Supplementation of vitamin C and/or vitamin E regenerated glutathione peroxidase, catalase activities and glutathione level in Ni(II)+VitC, Ni(II)+VitE and Ni(II)+Vit(C+E) groups when compared to controls and to Ni(II) groups. Additionally, the vitamin C and E in the diet of Vit (C+E) group ameliorated glutathione peroxidase and catalase activities for good, which reached normal values.

**Histological results**

The mentioned biochemical alteration could be referred to the liver histological changes. In fact, liver of the control group had a regular histological structure with a characteristic pattern of hexagonal lobules separated by interlobular septa, traversed by portal veins (figure 2A). Almost, the same histology structure has shown in vitamin (C+E) treatments (figure 3A). In contrast, liver of nickel treated group had shown pathological alterations such as the presence of cellular debris within a central vein and cytological vacuolization. In general some degree of hepatic hypertrophy was evident, with the cytoplasmic structures becoming more and more opaque (figure 2B). However, the combination groups of Ni(II)+Vit C, Ni(II)+Vit E and Ni(II) plus vitamins (C+E) showed prominent recovery in the form of the liver histo-architecture such as the reduced cytoplasmic vacuolization and the normal sinusoidal spaces. Additionally, the lamellar pattern of hepatocytes was restored to almost normal (figures 3C, D and E).
Figure 1. Values of glutathione, catalase and GSH-Px in liver of control and treated mice with Ni(II), Ni(II)+Vit C, Ni(II)+Vit E and Ni(II)+Vit (C+ E) after 4 weeks of treatment. Statistically significant differences from control: ***p < 0.001, **p < 0.01, *p < 0.05; from Ni: pcm < 0.001, p < 0.01, p < 0.05. Values are given as mean ± SE for group of 8 animals each.
**DISCUSSION**

In the present study, we investigated whether the preventive effects of vitamins (C+E) supplementation might prove more effective than vitamin treatment alone on nickel induced oxidative liver injury in mice. Body weights of vitamins(C+E) treated mice showed comparable values to normal control mice, whereas nickel treatment resulted in marked reduction in the body weight which can not be related to low intake of diet consumption of the animals, but possibly due to the overall increased degeneration of lipids and proteins [29]. Findings from this investigation indicated also an increase of absolute and relative liver. This might be explained by the accumulation of nickel in the liver[30] . All these physiological changes have upturned in vitamin C and/or vitamin E plus nickel supplementation. The rise in serum glucose may indicate a disrupted carbohydrate metabolism resulting from enhanced breakdown of liver glycogen, possibly mediated by an increase of pancreatic release of glucagons and subsequently can lead to hypoinsulinemia, through inhibition of insulin release from rat islets:Thus, these alterations were found to cause a drastic drop in the insulin/glucagon plasma ratio [31]. In the other hand, the elevation of blood glucose under nickel toxicity could be explained by the involvement of nitric oxide mediated pathways[32]. Moreover, Hyperglycemia can cause increased production of free radicals via autoxidation of glucose and non-enzymic protein glycation that may lead to disruption of cellular functions and oxidative damage to membranes[33], whereas Co-supplementation of vitamin E and/or vitamin C have attenuated the increased glucose level caused by nickel. Hence, interestingly, both vitamins are biologically important in reducing metal toxicities. As reported previously, vitamin C or ascorbic acid is defined as a free radical scavenger. As well as, it can mediate many intracellular enzymatic reactions. L-ascorbic acid acrosses plasma membrane via the Na+ dependent transporter (SVCT-1 and SVCT-2) systems[34], whereas, the cellular uptake of L-ascorbic acid can be promoted by insulin and inhibited by hyperglycemia [35]. According to our results, Ni(II) + vitamin C treated group have upturned blood glucose levels as compared to controls. This might be due to the role of aldose...
reductase and ascorbic acid in reducing the blood glucose level [36]. In addition, the reduction of glucose level in Ni(II)+Vit E treated group, when compared to control group may be due to an improvement of nickel induced oxidative stress and insulin resistince by α-tocopherol (vit E), thereby boosting insulin sensitivity [37].

**Figure 3.** Effect of vitamin E and/or vitamin C co administrated with nickel on liver mice histology. Vit(E+ C) (C), Ni(II)+Vit E (D), Ni(II)+Vit C (E) and Ni(II)+Vit(E+C) . Sections were stained using the haematoxylin-eosin method (400 x). Vit C and vitamin E coadministred with Ni maintained granular cytoplasm and normal hepatocytes.
In this investigation, it was also found a significant diminution in hepatic protein levels and subsequently, an elevation of serum total protein. Herein, the increased of reactive oxygen species (ROS) formation and lipid peroxidation due to nickel intoxication, are able to induce liver injury and dysfunctions. All these alterations lead to cellular structure and components damages [38]. In nickel treated mice, the activities of serum ALT, AST and ALP were significantly increased, compared to their normal levels. It could be attributed to the hepatic damage resulting in increased release and leakage out of these enzymes from the liver cytosol into the blood stream which gives an indication on the hepatotoxic effect of this metal [39]. Interestingly, the biochemical perturbations seem to be correlated with the liver histological alterations such as the presence of cellular debris within a central vein and a cytoplasmic vacuolization, plasma membrane distruption and cellular hypertrophy. Previous histological studies on liver have documented nickel-induced changes characterized by dilated sinusoids, vacuolization and the appearance of hepatic cells with distorted nuclei [40]. In Ni(II) + Vit C and/or vitamin E treated group, the histological alterations of liver have attenuated, when compared to control normal group. This is agree with some previous studies which have reported the benefecial role of vitamins in scavenging free radicals and enhancement of cellular antioxidant defense [40], [41]. In the current study, Ni(II) induced oxidative stress by affecting the antioxidant defense system, like CAT, GSH-Px and GSH. Our results were agreed with some previous studies which have shown that Ni(II) intoxication induces ROS formation leading to lipid peroxidation and alterations of the antioxidant status of several tissues in rats and mice [42]. The decline of GSH level may be due wether to the higher affinity of sulfhydryl group of cysteine moiety of glutathione to of metals, forming thermo-dynamically stable mercaptide complexes with several metals [43] or that GSH may be oxidized due to the interaction with the free radicals induced by nickel. Therefore, GSH level could be consumed during nickel detoxification [44]. In addition, the decreased activity of hepatic catalase and GSH-Px in nickel treated animals, suggests that there is an interaction between the accumulated free radicals and the active amino acids of these enzymes [45] or due to the direct binding of the metal to the active sites of the enzyme [46]. In (Ni(II)+ Vit C and/or Vit E treated group, the significant improvement of the glutathione level was noticed when compared with Ni(II) treated group. Thus, the observed normalization of GSH levels, GSH-Px, and catalase activities following vitamin C and/or vitamin E treatment could be because these vitamins caused a decline in lipid peroxidation (LPO) accompanied by an increase in the activity of catalase, GSH-Px and GSH level in liver. In other words, these vitamins played an action in reducing the levels and accumulation of oxygen reactive species. However, Vit (C + E) proved more effective as compared to individual vitamin treatments.

In conclusion, our study has clearly confirmed the protective role of vitamin E and/or vitamin C on nickel induced liver damage, through alteration of liver marker enzymes and some major antioxidant enzymes. In the other hand, our data showed that both vitamins have attenuated the toxic effects of nickel for good as compared to either vitamin treatment.

**ACKNOWLEDGEMENT**

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**Conflict of interest**

All of the authors have declared that no competing interests exist.

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