Antioxidative and antiapoptotic effects of vitamin A and vitamin C against carbon tetrachloride induced hepatotoxicity in mice

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Abstract

Reactive oxygen radicals play an important role in various forms of liver injury. The present study was a trial to evaluate the efficacy of each of vitamin A&C in its clinical dose (80 mg / Kg ip) on experimental model of chronic liver injury in mice using carbon tetrachloride (CCl4). Animals were subdivided into four groups (control, CCl4 treated, CCl4+Vit.A and CCl4+Vit.C). Vitamin A&C were administrated 2 hours prior interaperitoneal administration of 0.2 ml / Kg CCl4 in mice. A significant decrease in serum Glutathione (GSH) and Superoxide dismutase (SOD) activity along with marked elevation of serum malondialdehyde (MDA), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was recorded in response to CCl4 hepatotoxicity. A notable normalizing to this measured parameters was observed in the groups treated by vitamin A & C. On histopathological basis, hydropic degeneration, steatotic changes and apoptosis was seen obviously in CCl4 treated group but partial improvement in the previous parameters was noted in vitamin A & C treated groups in spite of vitamin C seemed to be less effective as far as vitamin A. These results theorized that vitamin A & C may have a potency to increase the antioxidant and antiapoptotic defense system activity in the CCl4 treated mice.

Introduction

Hepatotoxins namely carbon tetrachloride (CCl4) induces acute hepatic necrosis, fatty degeneration, advanced fibrosis and many other significant alteration in liver enzymes activities (Turkdo et al., 2001 & Nakamoto et al., 2003).

A significant elevation in the activities of ALT, AST, alkaline phosphatase (AP) and serum bilirubin was recorded by Datta et al. (1998) following CCl4 hepatotoxicity.

In addition to the above variables, lactate dehydrogenase (LDH) elevation, significant decrease in total sulphhydryl (-SH) content and catalase activity in hepatic tissues and also a significant increase in lipid peroxidation measured as malondialdehyde (MDA) were considered evidences for CCl4 hepatotoxicity in mice (Mansour, 2000).

CCl4 hepatotoxicity is mainly caused by increase the production of oxygen free radicals that causes lipid peroxidation. Kamataki et al (1977) found that oral administration of CCl4 to rats inhibited microsomal NADPH-dependent lipid peroxidation.

Weber et al. (2003) suggested that CCL4 is activated by cytochrome (cyp) 2E1 or cyp2B2 and possibly cyp3A to form the trichloromethyl radical, CCL3. This radical can bind to cellular molecules (nucleic acid, protein, lipid) impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration (steatosis).

Refere by: Prof. Dr. Yehya Shaker
The protective effects of some antioxidant treatments on CCl4 liver toxicity was studied by many authors (Cajanus indicus derivative (Datta et al, 1998); Colchicines (Mizuoka et al, 1999); Vitamin E (Mustafa Naziroglu et al, 1999); Thymoquinone and Desferrioxamine (Mansour, 2000); Captopril (El-Khatib and Mansour, 2001); PMC, a derivative of alphatocopherol (Hsiao et al., 2001); Vitamin C&E, selenium and Nigella sativa (Turkdo et al., 2001); volatile oil constituent of Nigella sativa, Thymoquinone, Pcyrene and alphapinene (Mansour et al., 2001); total flavonoids of Astragalus (Wang et al., 2001); Salacia reticulata extracts (Yoshikawa et al., 2002); Aquilegia vulgaris extracts (Adamska et al., 2003); Antrodia camphorata extract (Hsiao et al., 2003); and Gossypitrin (Perez et al., 2003).

This study was designed to investigate the antioxidative & antiapoptotic effects of vitamin A and vitamin C on lipid peroxidation, antioxidant enzyme systems, some liver enzymes activities and liver cell apoptosis in carbon tetrachloride induced hepatotoxic mice.

Material and methods

The animal grouping and treatments:

Male Swiss albino mice, weighing 20-25 g kept under good ventilation and balanced diet were used in this study. Animals were subdivided into four groups (15 each). Group 1(control) did not receive any treatment. Group 2 received CCl4 (0.2ml / Kg ip Hewawasam et al 2003) daily for 60 days. Group 3 received vitamin A (80 mg / kg ip Tantcheva et al, 2003) 2hrs prior to CCl4 (0.2ml / kg ip) daily for 60 days. Group 4 received vitamin C (80 mg / kg ip Tantcheva et al., 2003) 2hr prior to CCl4 (0.2ml / kg ip) daily for 60 days. Vitamin A and C were supplied by AL Gomhouria co. for pharmaceuticals and chemical industries.

Biochemical analysis:

Serum samples were prepared through blood centrifugation at 3000 r.p.m for biochemical determinations.

GSH was measured by the procedure of Beutler et al (1963), which is based on extraction of GSH with metaphosphoric acid and then its reaction with dithiobisnitrobenzoic acid (DTNB) to form a yellow color, which is read at 412 nm.

MDA, a product of lipid peroxidation was determined by the method of Begonia et al, (1994) using a saturated solution of thiobarbituric acid and the developed color read at 532 nm.

SOD activity was assayed using a kit obtained from Randox. The method employs xanthine and xanthine oxidase to generate super oxide radicals, which react with 2-(4-iodophenyl)-3(4-nitroph-phenyl)-5 phenyltetrazolium chloride (I.N.T.) to form a red formazon dye. The SOD activity is then measured by the degree of inhibition of this reaction at 560 nm.

ALT &AST were estimated colorimetrically using kits from Bio-Merieux (Marcy I Etoile, France)

Histopathological test:

Mice liver specimens was obtained and fixed in 10% formaline. Sections were prepared and stained by Fulgen stain for histopathological investigation.

Fulgen stain for liver cells:

Slides putted in oven for 10 minutes then putted in xylene for 10 minutes, then in a series of alcohols 100%, 90% and 70% for 5 minutes. The slides were washed by distilled water, rinsed in 5N HCl solution for 50 minutes, and then washed twice by distilled water. Ten drops of schiff
reagent were added and left to act for 10 minutes, the slides rinsed in sodium metabisulphite solution for 2 minutes then re-rinsed in distilled water, again the slides were dehydrated in 70%, 90% and 100% alcohol and finally mounted by xylene.

**Statistical evaluation:**
All calculated data were collected in a database for further statistical evaluation. Using software (SPSS version 9) student “t” test was made to determine the level of significance of difference between different parameters in different groups. Probability values (P < 0.05) were considered significant.

**Results**
Results of the effects of vitamin A and vitamin C administration on serum glutathione (GSH), superoxide dismutase (SOD), Malondialdehyde (MDA), Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) of CCl4 induced hepatotoxicity in mice was recorded in table (1) and represented in fig. (1) and (2).

The results show significant decrease (P < 0.05) in serum glutathione (GSH) level of CCl4 treated groups compared to control group, on the other hand significant increase (P < 0.05) was observed in its level in Vitamin A and Vitamin C administrated groups if compared to CCl4 group.

Statistical analysis shows significant decrease (P < 0.05) in superoxide dismutase (SOD) activity in CCl4 compared to control group. In contrast Vitamin A and Vitamin C administrated groups showed significant increase (P < 0.05) in its activity if compared to CCl4 group and no significant difference with control group.

Serum Malondialdehyde (MDA) was significantly increased (P < 0.05) in CCl4 treated groups compared to control group. Vitamin A supplemented CCl4 group showed significant decrease (P < 0.05) in serum MDA level in comparison with both CCl4 treated group and CCl4+Vitamin C group.

CCl4 induced hepatotoxicity significantly increased (P < 0.05) serum Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT). No significant difference was recorded between CCl4+Vit.A group and control group. Vitamin C markedly decreased the observed elevation in both serum AST and ALT of CCl4 treated groups.

The histopathological effects of CCl4 and vitamin A&C was studied in the liver of mice and the results are shown in table (2) and fig. (3:7).

Control group (fig. 3) showed well-preserved lobular architecture with almost no pathological changes. It is appeared that mice’s treated with CCl4 (fig. 4) had a loss of lobular architecture (100% cirrhotic). Mean percent of cirrhotic cells in CCl4+Vit.A group was 65% while in CCl4+Vit.C group was 80%.

Hydropic degeneration was recorded in all tested samples of CCl4 treated group with the percentage of 70%. It was found in 12 out of 15 animals of CCl4+Vit.A group (fig. 5) with the percentage of 60% and in 10 out of 15 animals of CCl4+Vit.C group (fig. 6) with the percentage of 70%.

A steatotic change was observed in 8 cases out of 15 with the mean percentage of 75% in CCl4 treated group. This changes was also found in 7 out of 15 with the percentage of 50% in CCl4+Vit.A group and in 8 out of 15 in CCl4+Vit.C group with the percentage of 60%.

The apoptotic changes was found in 8 out of 15 cases with the percentage of 20% in CCl4 treated group and the apoptotic bodies were found in hepatocytes adjacent to the porbel
treats. In CCl4+Vit.A group (fig. 7), apoptosis was found to take place in 4 out of 15 cases with the percentage of 10% and in 7 out of 15 cases with the percentage of 18% in CCl4+Vit.C group.

Table (1): Effect of Carbon tetrachloride (CCl4) hepatotoxicity and the possible role of Vitamin A and Vitamin C supplementation on serum Glutathione (GSH), blood Superoxide dismutase (SOD), serum Malondialdehyde (MDA), Aspartate aminotransferase (AST) and Alanin aminotransferase (ALT) of mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment groups</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td>Glutathione (Mg/dl)</td>
<td>Mean</td>
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<td>± S.D.</td>
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<tr>
<td>Superoxide dismutase (U/g Hb)</td>
<td>Mean</td>
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<td>± S.D.</td>
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<tr>
<td>Malondialdehyde (n Mol/ml)</td>
<td>Mean</td>
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<td>± S.D.</td>
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<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>Mean</td>
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<td>± S.D.</td>
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<tr>
<td>Alanin aminotransferase (U/L)</td>
<td>Mean</td>
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<td>± S.D.</td>
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Values given as mean of 15 mice ± SD
(a) Significant at P < 0.05 as compared to control group.
(b) Significant at P < 0.05 as compared to CCl4 group.
(c) Significant at P < 0.05 as compared to CCl4+Vit.A group.
(d) Significant at P < 0.05 as compared to CCl4+Vit.C group.

Table (2): Histopathological changes in the liver of mice treated with CCl4 and supplemented with vitamin A and vitamin C.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Histopathological changes</th>
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<tr>
<td></td>
<td>Lobular architecture</td>
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<td>Control</td>
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<tr>
<td>CCl4</td>
<td>8/15</td>
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<tr>
<td>CCl4 +Vit A</td>
<td>12/15</td>
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<tr>
<td>CCl4 +Vit C</td>
<td>12/15</td>
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</table>

15 animals in each group was investigated, 5 slides per animal and 5 microscopic fields per slide.
Figure (1): Effect of Carbont etachloride (CCL4) hepatotoxicity and the possible role of Vitamin A and Vitamin C supplementation on serum Glutathione, blood superoxide dismutase and serum Malondialdehyde of mice.
Figure (2): Effect of Carbont etrachloride (CCL4) hepatotoxicity and the possible role of Vitamin A and Vitamin C supplementation on Aspartate aminotransferase (AST) and Alanin aminotransferase (ALT) of mice.

Fig. (3): Histological preparation from non-treated control mouse showing normal hepatic architecture and healthy hepatocytes. X400
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Fig. (4) Section of the liver of mouse treated with CC14 showing hepatic fibrosis merging into sinusoid cirrhosis, fibrous strands in between hepatocytes and also shows steatoic changes and hydropic degeneration. X 400

Fig. (5) Section in the liver of mouse treated with CC14 + Vit. C showing steatotic changes (50%), hydropic degeneration (70%) and area of necrosis. X 400
Discussion

Treatment with hepatotoxin namely Carbon tetrachloride (CCl4) induced significant alteration in endogenous antioxidants activity (Glutathione GSH and Superoxid dismutase SOD), hepatic lipid peroxidation measured as malondialdehyde MDA and serum level of aminotransferases AST & ALT.

Animals respond to oxidative stress by maintaining a high antioxidant defense capacity at all times including both elevated activities of antioxidant enzymes and large cellular pools of glutathione (Storey, 1996).

The observed significant decrease in serum glutathione level in mice resulted of CCl4 hepatotoxicity comes in agreement with the finding of El-Khatib and Mansour (2001) and Hewawasam et al. (2003).

Serum Superoxid dismutase (SOD) activity was found reduced significantly by the effect of treatment with CCl4 in agreement with Adamska et al. (2003).

The cell defense mechanisms against oxygen toxicity increases in liver to suppress oxidative imbalance (Sanz et al., 1996), thus serum GSH and SOD levels were significantly decreased.

A variety of different methods have been used to assess the extent of peroxidative damage to lipids (Halliwell and Chirico, 1993). Some aim to quantify the damage done by peroxidation including the formation of conjugated dienes and of various decomposition products such as malondialdehyde (MDA), lipofuscin, isonenes and light emission by Russel reactions (Slater, 1984; Uchiyama and Mihara, 1978).

The significant elevation in MDA of CCl4 treated group, which reflect significant increase in hepatic lipid peroxidation also comes in agreements with the findings of Mansour (2000) and Perez-trueba et al. (2003).

The marked increase in serum transaminase enzymes activity (AST & ALT) in CCl4 treated group was also recorded by many authors (Datta et al., 1998; Mansour, 2000; Al-Katib and Mansour, 2001; Hsiao et al., 2001; Yoshikawa et al., 2002; Hsiao et al., 2003 and Hewawasam et al., 2003).

Treatment with Vitamins A & C were found greatly effective in restoring the values of serum GSH, SOD, MDA, AST and ALT to normal which may be due to free radical scavenging properties of both vitamins.

The results show that vitamin A was found more effective than vitamin C in preventing the dramatic drop in serum GSH level by the effect of CCl4 hepatotoxicity. It was also more powerful in preventing the enhancing of lipid peroxidation which appear in keeping low values of MDA compared to its values in vitamin C treated group.

Some authors studied Cytoprotection against CCl4-induced hepatotoxicity using different antioxidants. Mansour (2000)
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referred the protective effects of Thymoquinone and Desferrioxamine to inhibition of the production of oxygen free radicals that cause lipid peroxidation. El-Khatib and Mansour (2001) revealed that the protective potential of Captopril against the acute hepatotoxicity induced by CCl4 in mice could be attributed at least in part to the free radical scavenging properties of the drug. Vitamin C and Vitamin E was recorded effectively restored the elevated levels of primary and secondary products of lipid peroxidation in mice (Tantcheva et al., 2003). Ascorbic acid exerts its protective role probably mediated via a free radical scavenging mechanism (Hasan et al., 2003). Hsiao (2001) suggested that PMC, (a derivative of alphatocopherol) exerts effective protection in chronic chemical induced hepatic injury in vivo.

In the other hand, in their comprehensive report on the dietary reference intake for vitamin C, vitamin E, selenium and carotenoids Johnson et al. (2003) did not decisively confirmed the role of antioxidants for the prevention of chronic diseases in human. They recommended additional research to define the attributes of antioxidants as studies are in progress progress from in vitro and animal studies to human nutrition.

CCl4 intoxication induced severe histopathological alterations in the liver of mice including great loss of lobular architecture, cirrhoses, hydropic and steatotic changes and finally apoptosis. Similar results were observed by Turkdo et al. (2001) in rabbits and Nakamoto et al. (2003) in rats. The observed partial improvement in the previous parameters in the groups treated with CCl4+Vit.A and CCl4+Vit.C reflect the antioxidative and cellular protective effect of vitamin A&C although vitamin A seemed to be more effective than vitamin C.

It was recorded that Retinoic acid (RA), a form of vitamin A, has been shown to exert antiapoptotic and antioxidative activity in various cells (Shimizu et al 2001). They suggested that RA reduced CCl4 induced oxidative stress and cell death by preventing the decrease in protein levels of superoxide dismutase 1&2 thus supporting the antioxidant defense system. In contrast, in his study on the role of antioxidant vitamins (C and E), selenium and Nigella sativa in the prevention of CCl4 induced liver fibrosis and cirrhosis in rabbit, Turkdo et al (2003) reported that histopathological findings demonstrate that vitamin C seemed to be ineffective in prevention of CCl4 liver fibrosis and cirrhosis in rabbits.

In conclusion our results theorized that vitamin A & C may have a potency to increase the antioxidant and antiapoptotic defense system activity in the CCl4 treated mice.

References
التأثيرات المضادة للأكسدة ولموت الخلايا لكل من فيتامين أ وفيتامين س في مواجهة تسمم الكبد المحدث بفعل مادة رابع كلوريد الكربون في الفناء

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قسم علم الحيوان - كلية العلوم - جامعة الأزهر - القاهرة
قسم الباثولوجي - معهد تيودور بلهارس - القاهرة

تلعب قواعد الأكسجين النشطة دورا هاما في احداث مختلف النماط اصابات الكبد. وقد استخدم العديد من مضادات الأكسدة للتخفيف من أثر هذه الأصابات.

تهدف الدراسة إلى تقييم كفاءة كل من فيتامين أ، سي، من المعطى مسبقا عن طريق الحقن البريتوني بالجرعة المقررة (80 مكغ/كم) على نموذج تجريبي لفتان احدث فيها تسمم الكبد بواسطة مادة رابع كلوريد الكربون.

قسمت الحيوانات الى أربع مجموعات: المجموعة الأولى ضابطة ولم تعط أي معالجات، المجموعة الثانية أعطيت رابع كلوريد الكربون (02 مل/كم) عن طريق الحقن البريتوني، المجموعة الثالثة حقن بالجرعة المقررة من فيتامين أ (80 مكغ/كم) قبل حقنها بربيع كلوريد الكربون (02 مل/كم) بساعتين. أما المجموعة الرابعة فقد حقن فيتامين سي (80 مكغ/كم) قبل حقن براع كلوريد الكربون (02 مل/كم) بساعتين. استمرت المعالجة بالطريقة السابقة يوميا لمدة ستون يوما.

اظهرت النتائج انخفاض ملحوظا في نسبة الجلوتاتيون وكذلك انزيم سوبر أكسيد ديسموتاز في مصل الدم مصاحبا بارتفاع واضح في نسبة مادة مالون دي أشيد وكل من انزيمي أسبرتات أمينو ترانسفيراز والأدين أمينو ترانسفيراز في مصل الدم للفنان المصاب بسمم الكبد بفعل الحقن بحده رابع كلوريد الكربون. وقد لوحظ اتجاه عام نحو عودة قيم المعيار السابقة إلى الحدود الطبيعية بتأثير كل من فيتامين أ وفيتامين سي.

من ناحية أخرى فقد لوحظ تغيرات نسبية تمثلت في ظهور تحلل مائي وتغيرات دهنية وكذلك موت بعض الخلايا في نسيج الكبد نتيجة التسمم الكبدى برابع كلوريد الكربون (وقد ظهر تحسنا جزئيا في هذه المعيار فالمجموعات التي عولجت بكل من فيتامين أ، س بدأ واضح بشكل أكبر في المجموعة المعالجة بفيتامين أ.

ومما سبق نستنتج ان لكل من فيتامين أ وفيتامين سي دورا مضادا للإكسدة ومقاوم لموت الخلايا في كبد الفناء المعرضة لسمية رابع كلوريد الكربون.