MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

EFFECT OF VITAMIN D AND COMBINATION WITH LIV-52 ON ANTIOXIDANT LEVELS IN CARBON TETRACHLORIDE (CCL_4) INDUCED LIVER DISEASE IN WISTAR RATS

K. Ponnazhagan[1*], Ursula Sampson[1], N. Muninathan[2] and J. Sivakumar[3]

[1-3]Department of Biochemistry, Meenakshi Medical College Hospital and Research Institute, Enathur, Kanchipuram, Tamilnadu-631552, India.
[2]Central Research Laboratory, Meenakshi Medical College Hospital and Research Institute, Enathur, Kanchipuram, Tamilnadu-631552, India.

*Corresponding Author: azhaganbio23@gmail.com

History
Received: 1st June 2020
Accepted: 12th August 2020

Keywords:

Carbon tetrachloride, Antioxidants, Vitamin D, Liv 52

Abstract

Background: Liver is the principle organ for maintaining the body's internal environment and metabolizes various compounds that produce free radicals. However, antioxidant system is crucial for human health and survival to maintain the redox homeostasis in the liver. When the excessive ROS is generated in the tissue, the homeostasis will be disturbed, resulting in oxidative stress, which plays a critical role in liver diseases. **Objectives:** The aim of present study was to evaluate both enzymatic and non- enzymatic antioxidant activity of Vitamin D and combination with Liv-52 on Carbon tetrachloride induced liver disease. **Material and Methods:** Male Albino Wistar rats were used in this study. Liver disease was induced in rats by Carbon tetrachloride (CCl4) at the dose of 1ml mixed with 50% of olive oil and administered into experimental animals twice a week for five weeks after conformation of liver disease treated with Vitamin D and Liv-52 for 5 weeks. **Results:** The levels of enzymatic and non-enzymatic antioxidants were significantly increased in vitamin D and Liv-52 treated animals when compared with liver disease animals. Moreover, the levels of Vitamin D and Liv-52 a good indicator of restoring the liver architecture, were also reversed in the damage after treatment. **Conclusion:** we conclude that a combination of Vitamin D and Liv-52 is capable of restoring the liver architecture and can also increase the antioxidants in liver disease rats.

INTRODUCTION

Chronic liver diseases (CLD) cause significant morbidity and mortality worldwide. It is estimated that over 844 million people have chronic liver disease, with a mortality rate of 2 million deaths per year [1]. According to World Health Organization in India 216,865 or 2.44% of total deaths are due to liver diseases and ranked 61 in the world [2]. The main causes of liver disorders are viruses, excessive drug therapy, pollution and alcoholic intoxication.

More than 900 chemicals have been involved in causing liver injury [3-4]. Carbon tetrachloride (CCl4) is one of the xenobiotics that have been reported to induce acute and chronic tissue injuries and is a well-established hepatotoxin [6].The metabolism of CCl4 begins with the formation of trichloromethyl (CCl_3) radical and proxy chloromethyl (O-O- CCl_3) free radicals via the activity of cytochrome P450 oxygenase system in endoplasmic reticulum. The trichloromethyl radical reacts with various important biological substances such as fatty acids, nucleic acids, lipids, proteins, and amino acids [8]. In CCl4-induced liver damages, the balance between Reactive Oxygen Species (ROS) production and antioxidant defense system is disturbed due to oxidative stress which disrupts cellular

functions through some events and causes liver damage and necrosis.

The liver plays a central and important role in regulation of homeostasis, since it is major influence is on the carbohydrate, fat and protein metabolism, synthesis of bile, storage of vitamin and Immunity (the liver contains over half of the body's macrophages). Due to of these high metabolic activity in this organ is an important place for free radicals' generation. There are several enzymes-induced free radicals in the liver, including aldehyde dehydrogenase, diamine oxidase, liver dehydrogenase, tryptophan dual oxidase, and the cytochrome P450 enzyme system [9].Cytochrome P450 enzymes are highly present in the hepatocyte, Kupffer cells, and neutrophils and its responsible for ROS production [10]. ROS may act either positively or negatively on cell functioning depending on the intensity and duration of the oxidative stress produced on the cell.

In mammals, the antioxidant system is crucial for human health and survival to maintain the redox homeostasis in the liver. When the excessive ROS is generated in the tissue, the homeostasis will be disturbed, resulting in oxidative stress, which plays a critical role in liver diseases and other chronic and degenerative disorders [11]. The oxidative stress not only triggers hepatic damage by inducing irretrievable alteration of lipids, proteins and DNA contents and more importantly, modulating pathways that control normal biological functions. Under physiological condition, both enzymatic and non-enzymatic antioxidant system are essential for cellular response in order to deal with oxidative stress. Therefore, antioxidant enzyme such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and non-enzymatic electron receptors such as Glutathione (GSH) are affected and used as indexes to evaluate the level of oxidative stress [12].

The Vitamin D is a group of sterols that have a hormone-like function and it binds with intracellular receptor proteins, receptor complex interacts with DNA in the nucleus of target cells and either selectively stimulates gene expression, or specifically represses gene transcription. Long recognized for its function in regulating calcium–phosphate homeostasis, vitamin D is now involved in an important modulator of the immune response to infection [13] and also been implicated in the cellular proliferation and differentiation mechanisms. It is generally accepted that vitamin D status is related to both innate and adaptive immune system.

There are several evidences supporting the antioxidant activity of vitamin D in the oxidative stress liver disease. The results in some experimental studies implied that vitamin D administration in liver damage mice helps to diminish the ROS formation by the suppression of the gene expression of NADPH oxidase [23]. NADPH oxidase is a main resource of ROS, and its activation contributes as a positive marker for oxidative stress [24]. Vitamin D decreases the lipid peroxidation and improves the antioxidant activity in the mice [25]. The antioxidant enzymes and non-enzymatic are a principal cell defense against free radical attack that protect cellular membrane and cytosolic components against damage mediated by ROS.

Liv.52 natural ingredients contains 8 substance such as, Capparisspinosa, Cichoriumintybus, Solsnumnigrum, Cassia occidentalis, Terminaliaarjuna, Achilleamillefolium, Tamarixgallica and Mandurbhasma [26] and it has exhibit potent hepatoprotective properties against chemically induced hepatotoxicity. It restores the functional efficiency of the liver by protecting the hepatic parenchyma and promoting hepatocellular regeneration. The antiperoxidative activity of Liv.52 prevents the loss of functional integrity of the cell membrane, maintains cytochrome P-450 enzyme system. It has been involved in functional efficiency of the liver by promoting detoxification and thus protecting from harmful food and medication toxins, maintaining healthy levels of liver enzymes.

MATERIALS AND METHODS

Animal Care and Housing

Adult male Albino Wistar rats (150-200 gms) were purchased from Biogen Laboratory Animal Facility, Bangalore, Karnataka and housed in Meenakshi medical college and Research institute, Kanchipuram, Tamilnadu. The animal was housed in polypropylene cages (24 x 14 cm and 6 rats per cage) and maintained in a controlled environmental condition of temperature (23 ± 2°C) and relative humidity (50-70%) on alternatively 12 hr light/dark cycles. All animals were fed standard pellet diet (Gold Mohor rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum.* The rats were acclimatized to laboratory condition for one week before commencement of the experiment. This research work on Wistar rats was obtained and approved by the Institutional Animal Ethical Committee (REG No. 765/03/ca/CPCEA).

Experimental Design

The animals were divided in to six groups, each group consists 6 animals. Group I animals served as control, Group II was the hepatotoxicity group that was given CCl_4 mixed with olive oil (1 mL/kg b.w., 50% CCl_4 in olive oil), twice a week, Group III-IV were administrated Vitamin D and Liv-52 dissolved in distilled water at dose levels of 500 IU and 1 mL /kg b.w., respectively, with CCl_4(as above) daily for five weeks. Group V were administrated both Vitamin D and Liv-52, with CCl_4 (as above) daily for five weeks. Group VI control animals were administrated both vitamin D and liv-52 dissolved in distilled water at dose levels of 500 IU and 1 mL /kg b.w., respectively, daily for five weeks.

After the experimental period of five weeks, the animals were deprived of food overnight and anesthetized by exposing to diethyl ether and then sacrificed by cervical decapitation. 2 ml of the blood were collected from all the

rats through the tail and retro-orbital venous plexus under ether-induced anaesthesia, into plain dry test tube (without anticoagulant) and serum was separated and used for antioxidant (enzymatic and non-enzymatic) assays. Liver tissue was immediately dissected out and washed in ice-cold saline and patted dry and weighed. Around 100 mg tissue from liver was taken and homogenized (10% w/v) with 0.1M Tris-HCL buffer in ice cold condition. The supernatants were separated, stored at 4ºC for one week and used for antioxidant enzymes were determined using spectrophotometric method (Shimadzu UV 1800 spectrophotometer).

Drugs and Chemicals

Carbon tetrachloride, Vitamin D was purchased from sigma chemical, Liv 52 purchased from Himalaya drug company and other chemicals were purchased from SRL chemicals.

Estimation of activity of Superoxide Dismutase (SOD)

This was assayed described as according to the method of Marklund (Marklund et al, 1974) [27]. A mixture containing 2.5 ml of Tris-HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA was prepared. To this mixture, 0.5 ml of pyrogallol was added and the increase in absorbance was read at 420 nm against the blank for 3 min to determine the rate of auto oxidation of pyrogallol. Hundred µl of tissue homogenate and serum sample taken in separate tube was mixed with 2.5 ml of Tris-HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA. To this mixture, 0.5 ml of pyrogallol was added and the increase in absorbance was read at 420 nm using a spectrophotometer against the blank for 3 min. This measurement constituted the rate of inhibition of auto oxidation of pyrogallol brought about by the enzyme present in the tissue homogenate. The reagent blank contained a mixture of 3.1 ml of Tris-HCl buffer, 0.1 ml of EDTA and 0.5 ml of DTPA and this was used to set 100% absorbance.

SOD activity is expressed as Units/mg of protein and it is defined as 50% inhibition of autooxidation of pyrogallol per min by the enzyme.

Estimation of activity of Catalase (CAT)

Activity of catalase was estimated by the method of Sinha (Sinha et al, 1972) [28]. Hundred µl of triplicate samples of tissue homogenate and serum sample taken in separate tubes was mixed with 1.0 ml of phosphate buffer. Five Hundred µl of H_2O_2 was added to these tubes to initiate reaction. The reaction was arrested immediately by addition of 2.0 ml of dichromate-acetic acid reagent at 0, 30- and 60-seconds intervals. The reagent blank was prepared by addition of 1.6 ml of buffer and 2.0 ml of dichromate acetic acid reagent taken in separate tubes. The test and blank tubes were then heated in boiling water bath for 10 min to develop green colour. The tubes were cooled to room temperature and their intensity was measured at 570 nm using spectrophotometer against the blank.

CAT activity is expressed as µmoles of H_2O_2 utilized/min/mg protein of tissue homogenates.

Estimation of activity of Glutathione peroxidase (GPx)

Glutathione peroxidase (Glutathione: Hydrogen peroxide oxidoreductase) activity was estimated by the methods of Rotruck *et al*. (1973). Hundred µl of triplicate samples of liver tissue homogenate and serum sample, taken in separate tubes was mixed with 0.4 ml of phosphate buffer, 0.1 ml each of sodium azide, EDTA and H_2O_2. Then, 0.2 ml of GSH was added to all these tubes and the reaction was arrested by the addition of TCA at 0, 1.5- and 3-min intervals. The tubes were then centrifuged, and 1.0 ml of the supernatant was transferred to fresh tubes. The blank constituted 1 ml of distilled water. The standard glutathione was prepared in separate tubes at a concentration range of 5 to 20 µg in a final volume of 1 ml. To all the above tubes 4 ml of phosphate buffer and 0.5 ml of DTNB was added and the colour developed was read at 412 nm using spectrophotometer against the blank.

GPx activity is expressed as Units/mg protein. One unit of enzyme activity is the amount of the enzyme that converts 1 µmole of GSH to GSSG in the presence of H_2O_2/min.

Estimation of Reduced Glutathione (GSH)

Reduced Glutathione content was estimated by the method of Ellman (Ellman et al, 1959) with little modification (Beutler *et al*., 1963). Two hundred µl of tissue homogenate and serum sample was made up to 1.0 ml by addition of 5% TCA and the protein flocculate formed in the sample was precipitated by centrifugation. Two hundred µl of this protein free supernatant was separated and mixed with, 2.0 ml of DTNB and the final volume was made up to 3.0 ml with phosphate buffer. Its optical density was measured at 412 nm within 60 sec, against blank. The blank tubes constituted 0.2 ml of TCA and 2.0 ml of DTNB, which was made upto 3.0 ml with phosphate buffer. Standard glutathione prepared in separate tubes at a concentration range of 5 to 20 µg were treated with 2.0 ml of DTNB and their volume was made up to 3.0 ml with phosphate buffer. The intensity of absorbance of blank and standard were also measured at 412 nm. GSH content is expressed as µg/gm tissue.

Statistical Analysis

The calculation and statistical analysis were carried out using the Statistical Package for Social Sciences (SPSS) for Windows version 21.0 software, one-way ANOVA method and the group mean were compared by Duncan's Multiple Range Test (DMRT). Statistical probability $P<0.05$ was considered to be significant.

RESULT

Enzymatic and non-enzymatic antioxidant levels in liver are presented in Table 1. SOD, CAT, GPx and GSH were measured as an index of antioxidant status of tissue. All the enzymatic antioxidant (SOD, CAT, Gpx) and non-enzymatic antioxidant (GSH) levels were reduced significantly (P<0.001) in the CCl4 intoxicated rats, when compared with normal rats. In CCl4 + Vitamin D treated animals (G-III) were significantly (P<0.001) increased the enzymatic (SOD, CAT, Gpx) and non-enzymatic (GSH) antioxidants levels when compared with hepatotoxic animals (G-II). In CCl4 + Liv 52 treated animals (G-IV) were significantly (P<0.001) increased the enzymatic (SOD, CAT, Gpx) and non-enzymatic (GSH) antioxidants levels when compared with hepatotoxic animals (G-II). In combination treatment of both vitamin D and liv-52 were significantly (P<0.001) increased the level of enzymatic (SOD, CAT, Gpx) and non-enzymatic (GSH) antioxidants when compared with hepatotoxic bearing animals (G- II). There was no significant difference in the antioxidant's levels between the control rats and the control treated combination of both vitamin D and liv-52 (G-VI).

Table 1: Effect of Vitamin D combination with Liv-52 on Antioxidant Levels in the liver of control and experimental animals

	Group I (Control)	**Group II (CCl4 Induced)**	**Group III (Vitamin D treated)**	**Group IV (Liv-52 treated)**	**Group V (Vit. D and Liv-52 treated)**	**Group VI (Control rats treated with both Vit D and Liv-52)**
SOD (Units/min/mg of protein	4.08±0.38	2.31±0.24[a]	3.38±0.3[b*]	3.75±0.34[b*]	3.86±0.24[b*]	4.2±0.32
CAT (µmoles of H_2O_2 liberated/min/ mg protein)	35.98±2.13	22.93±1.73[a*]	28.36±0.88[b*]	32.45±0.65[b*]	34.5±2.8[b*]	36.73±1.72
GPx (µmoles of GSH oxidised/min/m g protein)	5.96±0.63	2.2±0.86[a*]	5.78±0.29[b*]	5.86±0.3[b*]	5.9±0.38[b*]	6.33±0.17
GSH (µg/mg of protein)	30.07±2.43	9.15±1.3[a*]	27.48±0.86[b*]	28.2±0.69[b*]	29.3±1.2[b*]	31.07±1.8

Each value is expressed as mean ±SD for six rats in each group.
a: as compared with Group I, b: as compared with Group II
Statistical significance: *p<0.001

Enzymatic and non-enzymatic antioxidant levels in serum are presented in Table 2. All the enzymatic antioxidant (SOD, CAT, Gpx) and non-enzymatic antioxidant (GSH) levels were found to be significantly decreased in the hepatotoxic induced group II animals when compared with control (G-I) animals. In treatment with Vitamin D animals (G-III) were significantly increased the enzymatic (SOD, CAT, Gpx) and non-enzymatic (GSH) antioxidants levels when compared with hepatotoxic animals (G-II). In treatment with Liv 52 animals (G-IV) were significantly increased the enzymatic (SOD, CAT, Gpx) and non-enzymatic (GSH) antioxidants levels when compared with hepatotoxic animals (G-II). A combination of both Vitamin D and Liv-52 (G-V) were significantly increased the levels of enzymatic antioxidants (SOD, CAT and GPx) and non-enzymatic antioxidant (GSH) when compared with hepatotoxic bearing rats. There was no significant difference in the antioxidant's levels between the control rats and the control treated combination of both vitamin D and liv-52 (G-VI).

Table 2: Effect of Vitamin D combination with Liv-52 on Antioxidant Levels in the serum of control and experimental animals

	Group I (Control)	Group II (CCl4 Induced)	Group III (Vitamin D treated)	Group IV (Liv-52 treated)	Group V (Vit. D and Liv-52 treated)	Group VI (Control rats treated with both Vit D and Liv-52)
SOD (Units/min/mg of protein)	3.03±0.21	2.04±1.3[a*]	2.82±0.5[b@]	3.12±0.59[b*]	3.22±0.65[b*]	3.11±0.28
CAT (µmoles of H_2O_2 liberated/min/mg protein)	39.13±2.89	26.5±2.31[a*]	31.69±2.7[b*]	32.47±2.57[b*]	33.9±2.97[b*]	39.7±1.5
GPx (µmoles of GSH oxidised/min/mg protein)	11.02±1.0	6.17±0.67[a*]	8.75±0.95[b*]	9.23±0.92[b*]	9.6±0.64[b*]	10.87±0.98
GSH (µg/mg of protein)	16.23±1.28	12.81±1.71[a*]	13.27±1.4[b+]	14.8±2.6[b#]	15.86±1.26[b#]	16.11±1.53

Each value is expressed as mean ±SD for six rats in each group. **a:** as compared with Group I, **b:** as compared with Group II. **Statistical significance:** * $p<0.001$, @ $p<0.004$, # $p<0.003$, + $p<0.04$

DISCUSSION

CCl_4 is considered as a powerful hepatotoxin that can induce toxic liver injury in laboratory animals. This toxicity can be studied under two phases. The initial phase cytochrome P450 metabolizes CCl_4 and thereby CCl_4 gets converted to trichloromethyl radicals ($CCl3^{\cdot}$ and/or $CCl3OO^{\cdot}$). These radicals are involved in causing membrane lipid peroxidation which ultimately leads to cell necrosis [29]. The second phase of CCl4-induced hepatotoxicity involves the activation of Kupffer cells and also the production of proinflammtory mediators [30]. Several other studies revealed gene expression changes which are caused by the CCl_4 toxicity [31]. But these changes are not fully understood.

The liver has an effective mechanism to prevent and neutralize the free radical induced tissue damage. This mechanism is accomplished by some of endogenous antioxidant enzymes, such as SOD, CAT, GPX and GSH. There is balance between a free radical (FR)/ ROS formation and antioxidant defense mechanisms, but if this balance is disturbed, it can produce oxidative stress results, which through a series of events deregulates the cellular functions leading to various pathological conditions. Antioxidant enzymes act against toxic oxygen free radicals such as superoxide and hydroxyl ion in biological system [32]. It is reported that antioxidant enzymes, such as SOD, CAT and the glutathione system, have been known to play important role in alleviating oxidative damage, since they are involved in the direct elimination of reactive oxygen specie [33]. CAT prevents oxidative hazards by catalyzing the formation of $H_2O$and O_2 from H_2O_2 [34]. The lower activities of these antioxidant enzymes are indicative of cellular damage and loss of the functional integrity of the cell membranes in liver which is always associated with oxidative stress [35, 36].

Recently several other experimental studies point towards a direct role of vitamin D in modulating liver inflammation and fibrogenesis and improving hepatic response, likely through the binding to specific VDR expressed on different cell types into the liver. Vitamin D is involved in the decrease of inflammation and fibrosis. Once proinflammatory is activated it transfer signals in monocytes and macrophages to regulate the local metabolism of vitamin D, auto inducing the expression of CYP27B1 and the local production of 1α,25(OH)2D, and thus controlling the excessive inflammatory response. Liver tissue contains 90% of macrophages, which suggests that the liver production of active vitamin D is affected during inflammatory diseases of the liver. Therefore active form of 25(OH)VitD and 1,25(OH)2VitD modulate monocytes and macrophages, and DCs activation in terms of VDR, CYP27B1, (the enzyme degrading 1,25(OH)2VitD), production of soluble mediators, proliferation and maturation, and the expression of cell membrane receptors and through endocrine, paracrine, and intracrine mechanisms [37, 38].

The natural ingredients in Liv.52 exhibit potent hepatoprotective properties against chemically induced hepatotoxicity. It restores the functional efficiency of the liver by protecting the hepatic parenchyma and promoting hepatocellular regeneration. The antiperoxidative activity of

Liv.52 prevents the loss of functional integrity of the cell membrane, maintains cytochrome P-450 enzyme system [39]. Liv.52 is known to improve the functional efficiency of the liver by promoting detoxification and thus protecting from harmful food and medication toxins, maintaining healthy levels of liver enzymes. Liv.52 is also known to support liver's normal ability to burn fat and maintain body's metabolic homeostasis. In the present study proved that, Vitamin D combination with Liv-52 to protect against CCl_4 induced hepatotoxicity and scavenging of free radicals.

The level of antioxidant enzymes like SOD, CAT, GPX and GSH observed in CCl4 treated rat is a clear manifestation of excessive formation of free radicals and initiate lipid peroxidation system resulting in tissue damage. With the treatment of vitamin D and liv-52, the concentration of these constituents was significantly increased and thus indicating protection against liver damage.

CONCLUSION

In the present study, we conclude that the combination of vitamin D and Liv-52 had a more protective effect against CCl_4-induced acute hepatic damage in rats.

ACKNOWLEDGMENT

We wish to thank Meenakshi Medical College Hospital and Research Institute and MAHER University for granting us permission and financial support to carry out the project.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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