CLINICOPATHOLOGICAL STUDIES ON HEPATOPROTECTIVE EFFECT OF FUCOIDAN ON CCL4 INDUCED LIVER FIBROSIS IN GUINEA PIGS

El-Boshy, M. E.;** Hussein, S. H. and Fatma, M. A.

* Dept of Clinical Pathology, Fac. Vet. Med., Mansoura University.

** Dept of Pathology, Fac. Vet. Med., Mansoura University.

ABSTRACT

In this study, we investigate the protective and therapeutic effect of fucoidan extract from Laminaria species against liver damage induced by CCl_4 in Guinea pigs by monitoring the hepatic m-RNA expression of TGFβ-1, some serum biochemical parameters and some oxidative stress biomarker. The CCl_4 was orally administrated at dose 1 ml/kg bw twice a week for 8 weeks. Fucoidan orally administrated at a dose rate 200 mg/kg bw/day. We found that fucoidan treatment improved elevated m-RNA expression of TGFβ-1, T.Bil, D.Bil, Ind.Bil. MDA and SOD serum levels induced by CCl_4 at 8^{th} week post treatment. Meanwhile ALT, AST and AP are not improved by fucoidan treatment. Finally, we concluded that crud fucoidan is less effective as hepatoprotective agent, and had a mild effect in the protective treatment at 8^{th} week post treatment.

INTRODUCTION

Liver diseases are some of the fatal disease in the world today, they pose a serious challenge to international public health. Hepatic fibrosis is a wound healing response to chronic liver injury which characterized by net accumulation of extracellular matrix (ECM) including collagen, glycoproteins and protoglycan. Hepatic stellate cell (HSC) which previously known as ito cell that under physiological condition store 80% of retinoids (vitamin A) is the cytological base of the hepatic fibrosis. The quiescent HSC are transformed with progressive injury into myofibroblast like cells that characterized by the appearance of cytoskeleton protein α smooth muscle actin (α SMA) which consider a biomarker for HSC activation **(Yong et al., 2005)**. TGFβ-1 is a key molecule or most fibrogenic cytokines facilitate the activation of HSC and convert it from static HSC into phenotype of myofibroblast to express α SMA and posses the character of contraction **(Semiha et al., 2006)**.

Carbon tetrachloride, CCl_4 is a frequently used chemical to experimentally induced hepatic fibrosis. Depending on the dose and duration the effect of CCl_4 on hepatocytes are manifested histologically as hepatic stetosis, fibrosis, hepatocellular death and carcinogenicity **(Heekyoung et al., 2005)**. The hepatotoxic effect of CCl_4 involved in immediate cleavage of CCl_4 by cytochrome P450

(CYP2E1) in hepatocytes which generate trichloromethyle radical leading to lipid peroxidation and membrane damage subsequently . Activated Kupffer cell produce toxic metabolites (inflammatory cytokines and reactive oxygen intermediates) resulting in the injury of hepatic parenchymal cells **(Chun et al., 2009)**.

Fucoidans, is sulfated polysaccharide extracted from cell wall of brown algae and some marine invertebrates. Firstly isolated by Kylin almost one century ago, contain substantial percentages of L-fucose and sulfate ester groups also called fucan, fucosan or sulfated fucan. Recently fucoidan has been extensively studied due to its numerous biological activities including antiocoagulant, antithrombotic, antitumor, antiviral, anticomplement, antioxidant and anti-inflammatory activities. Also used as immunomodulatory, blood lipid reducing, has activity against hepatopathy, renalpathy and gastric protective effect **(Bo et al., 2008)**. The brown seaweed Laminaria japonica is the most important economic seaweed cultured in China. The utilization of L. japonica as a drug has been documented in Traditional Chinese Medicine (TCM) for over one thousand years. Fucoidan of L. japonica had a hepatoprotective effect **(Ning et al., 2005)**.

This study was aimed to, evaluate hepatoprotective effect of fucoidan on liver fibrosis induced by CCl_4 in guinea pigs through detection of gene expression of TGFβ-1 by RT-PCR., oxidative stress reaction through estimation of MDA, SOD, GSH and CAT enzymes, as well as some biochemical parameters .

MATERIAL AND METHODS

Experimental Animals :

Fifty Guinea pigs of 1-2 month old of both sexes were obtained from Helwan farm of laboratory Animals (Ministry of Public health). The animals were kept in galvanized zinc plate cages under strict hygienic conditions. The animals were ensured free from any infection. The Guinea pigs were maintained on pelleted diet and water ad Libitam. The daily requirement of ascorbic acid (50 mg / liter of drinking water) was supplied allover the experiment accerding to **Sarah and Maggie (2003)**.

Chemicals :

CCl_4 was purchased from ADWIA Co, Egypt., Primer sequences for PCR amplification : Transforming growth factor β1 (TGF-β1).

Obtained from metabion international AG., Lend-Christ-Strasse 44 \ I, Martinsried \ Deutschland.

Gene	Primer sequence		Base pair
TGF- β1	Sense	5\ TAT AGC AAC AAT TCC TGG CG 3\	162
	Antisense	5\ TGC TGT CAC AGG AGC AGT G 3\	

Fucoidan :

Fucoidan exteraet of Laminaria speeies obtained as powder that used as freshly prepared solution dissolved in normal saline. Provided by (Bijing Lilli Agrochemistry CO. LTD, China).

Fibrosis induction and treatment:

Guinea pigs were divided into 5 groups as follow:

Group I (10) served as normal control receive only the vehicle (1ml/kg bw olive oil twiee a week for 8 weeks). **Group II (10)** treatcd with fucoidan (200 mg/kg bw/ day allover the experiment) and olive oil. **Group III (15)** receive 1ml/kg bw of CCl_4 diluted 20% in olive oil twice a week for 8 weeks. **Group IV (10)** pretreated with fucoidan for one week before CCl_4 administration then treated with CCl_4 and fucoidan (protective fucoidan treated group). GroupV (5) after 4 weeks of CCl_4 administration and ensure occurrence of fibrosis (5 animals) further treated with CCl_4 for 4 weeks and at the same time treated with fucoidan (therapeutic fucoidan treated group). At the end of 4^{th} and 8^{th} week of CCl_4 treatment randomly five Guinea pigs were pickcd up from each group. Blood samples were collected individually from heart puncture for serum chemistry, Guinea pigs were then sacrificed and specimen from liver were kept in liquid nitrogen for reverse transcriptase polymerase chain reaetion (RT-PCR) analyses.

RT-PCR analysis:

Total RNA was isolated from Guinea pigs livers using QIAamp extraction mcthod. Preparation of the RNA / primer mix by add the following (RNA template,Forward Primer and Reverse Primer) to a nucleasc-free microtube and mix by pipetting gently up and down. Ineubate the mixture at 70-75°C for 5-10 min and place it at room temperature for 5-10 min for denaturation and primer annealing. Prepare the RT-PCR mix and complete it by adding10 µl RNA/primer mix then pipettc on ice and mix by pipetting gently up and down. Thc thermal profilc that was used consistcd of denaturation at 95°C, anncaling at 55-65°C, clongation at 72°C and Final elongation at 72°C. The PCR product was electrophoresed on 2% agarosc gel electrophoresis. RT-PCR analysis was performed in Biotechnology Center for Service and Researehes (BCSR), Faculty of Vet. Medicine, Cairo University.

Serum biochemical analysis:

Prepared frozen serum samples were analyzed for alanine aminotransferase (ALT), asprtate aminotransferase (AST), alkalin phosphatase (ALP), total (T. Bili.) and direct bilirubin (D.Bili.), glucose, total protein, albumin (Alb), urca, creatinine (Cre.) and some oxidative stress marker malondialdehyde (MDA) superoxide dismutase (SOD), catalase (CAT) and reducd glutathione (GSH) were determined with semi-automatic spectrophotometer (BM-Germany 5010) using commercial test kits (Randox Co. UK and Biodiagnostic, Egypt.) aceording to enclosed pamphlets.

Statistical analysis:

Our results were analyzcd by (ANOVA) using SPSS software statistical program (SPSS for windows (ver.15.00, USA). Two groups were significantly different if P value was statistically lower than 0.05 .

RESULTS & DISCUSSION

The liver plays a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. Importantly, it is the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion **(Joan et al., 2010)**. CCl_4 metabolism is an established model of liver necrosis and fibrosis. The liver damage is created by this metabolism is free radical dependent as CCl_4 is oxidized by cytochrome P450 to highly reactive trichloromethyle (CCl3) radical that being generated by reductive cleavage of CCl_4 bond and generated oxygen radicals and phospholipid peroxides in abundance **(Sreedevi et al., 2006) and (Gopal et al., 2001)**. The generated trichloromethyle free radical caused liver necrosis, destruction of ECM and lipid peroxidation of membranes. Our study showed that TGF-β1 m RNA expression increased as fibrosis developed in CCl_4 induced liver fibrosis in Guinea pigs (82.68 % and 88.66 at 4^{th} and 8^{th} week respectively). As TGF-β1 activity is enhanced by proteolytic release and activation of latent TGF-β1 by HSC also other cell such as kupffer cells, invading mononuclear cells, myofibroblast cells and endothelial cells can synthesis and release TGF-β1 **(Shi-ling et al., 2005)**.

Fucoidans, a family of sulphated polyfucose polysaccharides, exhibit different biological properties. The biological effects of fucoidan are related to their polysaccharide backbones and sulfate content. Recently the antifibrotic activity of fucoidan was reported in an animal model of hepatic fibrosis **(Kyoumi et al., 2009)**. The TGF-β1 m RNA expression was redueed by fucoidan treatment especially in (GP.IV) at 8^{th} week post treatment. The TGF-β1 m RNA expression was 77.32% in GP.IV at 4^{th} week and (41.24% and 48.45%) in (GP. IV and GP.V respectively) at 8^{th} week post treatment with CCl_4 and fucoidan. This is agreeing with **Shinji et al., (2008)** who mentioned that fucoidan treatment attenuate HSC activation by inhibiting TGF-β1.

CCl_4 administration, causes severe liver damage demonstrated by remarkable elevation of serum AST and ALT levels till the end of the experiment as shown in (table.1,2). This elevation may be attributed to the cellular leakage and damaged of structural integrity of the liver eells **(Robert et al., 2009)**. Also CCl_4 treatment induced elevation of serum ALP with high level of total bilirubin, direct and indirect bilirubin (table.1,2). Which are considered indicator of cholestasis and pathological alterations of the biliary flow **(Lalitsingh et al., 2010)**. The high concentration of indirect bilirubin and direct bilirubin in the serum is an indication for increased erythrocyte degeneration rate and liver injury caused by CCl_4 **(Sathesh et al., 2009)**.

In our work fucoidan not have any toxic effect either on liver or kidney function as ALT, AST, ALP, BUN and creatinine level are in normal level in GP.II (table.1,2). The elevated liver enzyme (ALT, AST & ALP) induced by CCl_4 not improved by fucoidan treatment either at 4^{th} or 8^{th} week either in protective (GP.IV) or therapeutic treatment (GP.V) (table.1,2). This is in accordance with **Kyoumi et al., (2009)** who observed that crude fucoidan

extract not improved elevated ALT and AST serum levels in liver injury induced by N-nitrosodiethylamine and concluded that crude fucoidan showed unremarkable anti-fibrogenesis activity. On other hand **Shinji et al., (2008)** reported that administrated fucoidan by I/V injection in chronic liver injury induced by CCl_4 in mice reduce elevation in serum AST & ALT. The differenees may be due to molecular weight of fucoidan that was reported in the anti-tumor activity, pro-angiogenesis and antioxidant activities of fucoidan and rout of administration as anti-fibrogenic effect of fucoidan by oral administration not studied **(Kyoumi et al., 2009)**. The T.Bil. (table.1,2) improved by fucoidan treatment either protective or therapeutic one but Ind. Bili. return to normal level in PF group could be due to decrease erythrocyte damage induced by CCl_4 as fucoidan has strong antioxidant activity **(Bo et al., 2008)**. In the present study, serum glucose (table.1,2) was reduced in CCl_4 treated animal along of the experiment as hepatic glycogen content was decreased reflecting decreased gluconeogenesis by the liver (**Omar et al., 2007**). In addition, **Rui et al., (2002)** recorded that gluconeogenesis and Krebs cycle fluxes are altered in rat livers following CCl_4 intoxication. Meanwhile **Bhatia and Ahujarai, (1984)** suggested that hypoglycemia may be due to depletion of glucose-6-phosphatase in the liver of CCl_4 treated animal. Fucoidan treatment not relive hypoglycemia induced by CCl_4 (table.1,2). TP and albumin blood level insignificantly changed by CCl_4 treatment exeept at 8th week post treatment albumin level was inereased (table.1,2). This is may be due to dehydration as a result of diarrhea caused by CCl_4 treatment. Globulin level in our work was increased by CCl_4 treatment at 4^{th} week but decreased at 8^{th} week post treatment. The increased globulin level occurred due to inflammation and liver disease. Meanwhile CCl_4 significantly decrease globulin at 8^{th} week post treatment which attributed to reduce immunoglobulin IgM, IgA and specially IgG by CCl_4 in a dosc and time dependant manner **(Usama, 2009)**. In fucoidan treatcd groups (GP. II, IV &V) total protcin, albumin, globulin and A/G ratio insignificantly change in comparing with control (table.1,2). Except globulin level increase in (GP.II) at 4^{th} week post treatment. Fucoidan has both humoral and cell-mediated immune responses as it enhance B cell blastogenesis. Therefore, the fucoidan was expected to promote maturation of B cells that might result in the stimulation of antibody secreting activity **(Kyoko et al., 2008)**. Presently administration of CCl_4 to normal Guinea pigs induced renal toxicity revealed by elevation of urea at 4^{th} week post treatment and creatinine level which occurred at 8^{th} week post treatment only (table.1,2). As CCl_4 administration mediated peroxidation of lipid structures and protein conteant of renal tissues, resulting in sub cellular damages **(Muhammad et al., 2009)**. Malondialdehyde is a reactive aldehyde, used as an indicator of the amount of lipid peroxidation **(Shana et al., 2009)**. In the present study, the significant increase in serum MDA concentration observed in the CCl_4 treated group (GP.III) as Lipid peroxidation (LPO), is acccpted to be one of the principal causes of CCl_4 induced liver injury, the reaction resulting from the attack by reactive free radicals on the polyunsaturated fatty acids (PUFAs) to generate different products, including volatile alkanes, aldehydic products that are relatively stable resulting

ultimately in a loss in membrane integrity **(Robert et al., 2009)**.

In fucoidan treated groups (GP.IV & V) MDA (table.3,4) elevated at 4th week post treatment but insignificantly change at 8th week post treatment in comparing with both CCl_4 and control one. This agrees with **Bo et al., (2008)** who reported that fucoidan from L.Japonica had no effect on lipid peroxidation induced by $FeSO_4$ in vitro. Also **Kyoumi et al., (2009)** mentioned that crude fucoidan extract not reduced high MDA level in liver injury induced by N-nitrosodiethylamine In contrast **Kum et al, (2008)** found that I/P administration of fucoidan extract resulted in reduced high MDA level induced by CCl_4 treatment in rats. Antioxidant enzyme such as SOD, CAT and GSH constitute a supportive team of defense against ROS. Our result found an elevation in SOD, CAT and GSH in Guinea pigs treated with CCl_4 at 4th week (table.1,2). The increase in these enzyme activities was probably a response towards the increase in ROS generation **(Gowri et al., 2008)**. The fucoidan treatment correct SOD level in protective group as fucoidan has strong scavenging free radical activity especially against superoxide radical. This is agreeing with **Jing et al., (2008)** who mentioned that fucoidan exhibit radical scavenging activity in vitro and antioxidative activity against oxidative stress in cellular model. Finally we concluded that crud fucoidan is less effective as hepatoprotective agent, and it had mild prophylactic hepatoprotective effect at 8th week post treatment. Further research recommended for more definitive knowledge about efficacy of fucoidan as hepatoprotective agent.

Table (1, 2): Some Serum Biochemical Profiles and Oxidative Stress Biomarkers (Mean ± S.E) at 4th (table, 1) and 8th (table,2) Week Post Treatment with CCl_4, and Fucoidan in Guinea pigs.

(table,1)

Groups	ALT U/L	AST U/L	ALP U/L	T. Bill. mg/dl	Dir. Bil. mg/dl	Indir.Bil.mg/dl	Glucose mg/dl	TP gm/dl	Alb. gm/dl	Glob. gm/dl	A/G	Urea mg/dl	Cre. mg/dl
I (Cont)	20.64^{a} ±1.30	29.11^{a} ±1.62	10.92^{a} ±0.67	0.47^{a} ±0.03	0.22^{a} ±0.02	0.25 ±0.04	134.80^{b} ±2.20	4.58 ±0.23	3.21 ±0.30	1.37^{a} ±0.11	2.49^{b} ±0.43	55.89^{a} ±1.99	0.58 ±0.01
II (F)	19.78^{a} ±1.81	28.00^{a} ±1.34	9.52^{a} ±0.51	0.50^{ab} ±0.05	0.25^{a} ±0.06	0.25 ±0.05	134.60^{b} ±4.52	5.83 ±0.48	3.17 ±0.29	2.67^{b} ±0.35	1.28^{a} ±0.16	60.27^{ab} ±1.12	0.61 ±0.05
III (CCl_4)	34.19^{b} ±2.03	39.70^{b} ±1.46	16.40^{a} ±0.60	0.72^{ac} ±0.1	0.53^{b} ±0.12	0.19 ±0.03	97.20^{a} ±8.53	5.59 ±0.74	2.99 ±0.25	2.59^{b} ±0.74	1.44^{a} ±0.27	71.55^{c} ±2.09	0.67 ±0.06
IV (PF)	33.29^{b} ±2.09	37.30^{b} ±2.54	16^{a} ±0.93	0.75^{c} ±0.09	0.52^{b} ±0.09	0.23 ±0.03	96.60^{a} ±10.54	5.24 ±0.39	3.29 ±0.27	1.95^{ab} ±0.24	1.78^{ab} ±0.26	66.67^{bc} ±3.23	0.66 ±0.02

(table,2)

Groups	ALTU/L	AST U/L	ALP U/L	T. Bill. mg/dl	Dir. Bil. mg/dl	IndLBil. mg/dl	Glucose mg/dl	TP gm/dl	Alb. gm/dl	Glob. gm/dl	A/G	Urea mg/dl	Cre. mg/dl
I (Cont)	19.93^{a} ±0.07	30.98^{a} ±1.20	9.99^{a} ±0.41	0.45^{a} ±0.02	0.24^{a} ±0.03	0.21^{a} ±0.02	133.65^{a} ±2.52	4.76 ±0.13	2.98^{a} ±0.22	1.78^{a} ±0.21	1.86^{a} ±0.35	57.36 ±3.76	0.54^{a} ±0.01
II (F)	20.63^{a} ±0.86	29.89^{a} ±1.61	9.75^{a} ±0.45	0.47^{a} ±0.02	0.17^{a} ±0.03	0.30^{ab} ±0.04	133^{a} ±2.85	5.07 ±0.11	3.35^{a} ±0.13	1.71^{a} ±0.17	2.08^{a} ±0.26	56.92 ±4.24	0.59^{ab} ±0.02
III (CCl_4)	41.76^{b} ±4.19	41.07^{b} ±0.65	16.93^{b} ±0.53	0.80^{b} ±0.06	0.42^{b} ±0.08	0.38^{b} ±0.08	95.60^{a} ±13.57	4.9 ±0.11	3.92^{a} ±0.22	0.98^{a} ±0.15	4.47^{b} ±0.85	59.30 ±7.17	0.71^{c} ±0.02
IV (PF)	36.96^{b} ±2.51	37.60^{b} ±4.09	18.05^{b} ±2.04	0.53^{a} ±0.02	0.31^{ab} ±0.02	0.22^{a} ±0.02	106.40^{a} ±10.06	4.98 ±0.13	3.30^{a} ±0.19	1.68^{a} ±0.28	2.31^{a} ±0.54	59.95 ±9.32	0.73^{c} ±0.04
V(TF)	37.61^{b} ±4.49	37.89^{b} ±2.61	15.84^{b} ±1.94	0.49^{a} ±0.08	0.24^{a} ±0.09	0.25^{ab} ±0.09	93.25^{a} ±9.92	4.91 ±0.10	3.21^{a} ±0.18	1.69^{a} ±0.08	1.95^{a} ±0.19	56.17 ±2.68	0.65^{bc} ±0.06

Cont.(control), F (fucoidan alone), CCl_4 (carbon tetrachloride treatment), PF (protective fucoidan treatment) TF (therapeutic fucoidan)
The same column not followed by the same letter differ significantly ($P<0.05$).

Table (3,4): Some Serum Oxidative Stress Biomarkers (Mean ± S.E) at 4th (table,3) and 8th (table,4) Week Post Treatment with CCl_4, and Fucoidan in Guinea pigs.

(table,3)

Groups	MDA nmol/ml	SOD U/ml	CAT U/L	GSH mg/dl
I (Cont)	7.05^{a} ±0.09	755.60^{a} ±41.01	311.25^{a} ±65.36	0.53^{a} ±0.08
II (F)	8.52^{ab} ±0.64	882.81^{ab} ±104.51	431.25^{ab} ±22.66	0.58^{a} ±0.06
III (CCl_4)	12.45^{c} ±1.62	947.88^{b} ±59.67	572.65^{c} ±57.58	0.77^{b} ±0.03
IV (PF)	10.93^{bc} ±0.85	725.26^{a} ± 26.06	459.98^{bc} ±36.99	0.87^{b} ±0.06

(table,4)

Groups	MDA nmol/ml	SOD U/ml	CAT U/L	GSH mg/dl
I (Cont)	7^{a} ±0.09	744.04^{ac} ±39.58	403.65^{ab} ±59.59	0.55^{a} ±0.06
II (F)	7.03^{a} ±1.14	638.81^{a} ±8.23	393.01^{a} ±37.53	0.59^{a} ±0.19
III (CCl_4)	11.30^{b} ±1.79	715.89^{ab} ±59.51	502.48^{bc} ±40.12	1.49^{b} ±0.18
IV (PF)	9.70^{ab} ±2.19	854.60^{c} ±73.78	448.72^{ac} ±49.19	1.21^{b} ±0.20
V (TF)	10.50^{ab} ±1.29	798.36^{cb} ±15.65	547.58^{c} ±40.31	1.25^{a} ±0.06

Cont.(control), **F** (fucoidan alone), **CCl_4** (carbon tetrachloride treatment), **PF** (protective fucoidan treatment) **TF** (therapeutic fucoidan).

The same column not followed by the same letter differ significantly ($P<0.05$).

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الملخص العربى

دراسات باثولوچية إكلينيكية على الفيوكيدان كعامل وقائى للتليف الكبد المحدث برابع كلوريد الكربون فى خنازير غينيا

أ. د. محمد السيد محمد البوشى* د. حسين سعد حسين**
ط. ب. فاطمه مصطفى عبدالحميد*

قسم الباثولوچيا الإكلينيكية - كلية الطب البيطرى - جامعة المنصورة*

قسم الباثولوچيا - كلية الطب البيطرى - جامعة المنصورة**

أجريت هذه الدراسة على عدد من خنازير غينيا بهدف تقييم مدى فاعلية الفيوكيدان كعامل وقائى لمرض التليف الكبدى المحدث برابع كلوريد الكربون من خلال الفحص الچينى لأنسجة الكبد لتحديد كمية چين ترانسفورمينج - 1 بالإضافة إلى تحديد الاختلافات فى المؤشرات الكيميائية ودلالات الإجهاد التأكسدى، بعد 4 أسابيع من العلاج بالفيوكيدان لم يظهر أى تأثير للدواء على مكونات السيرم الكيميائية ولا على أكسدة الدهون ومضادات الأكسدة ولكن انخفض Gene expression of TGFβ-1 بعد 8 أسابيع من العلاج ظهر له تأثير جزئى من خلال فحص بعض المركبات الكيميائية وخاصة فى المجموعة الوقائية وأدى إلى تقليل كمية چين ترانسفورمينج -1.