



PROTECTIVE EFFECT OF FOLIC ACID AGAINST H₂O₂ INDUCED-HEPATIC OXIDATIVE STRESS IN AGED RATS

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Keywords: Folic acid, Hepatic oxidative stress, Liver functions, Superoxide dismutase, Catalase, Lactic acid dehydrogenase

ABSTRACT

The protective effect of folic acid (FA) on hepatic oxidative stress in aged rats exposed to oxidative stress by supply drinking water with 1% H₂O₂ (v/v) was studied. Rats were divided into two groups, the first group was considered as standard one (F1) which was fed on basal diet and administrated 1 mg FA/ Kg body weight (BW) daily by stomach tube without addition H₂O₂ in drinking water. The second group was divided into five subgroups, the first subgroup was the control (F2) which fed on basal diet free from folic acid (FF) with 1 % H₂O₂ in the drinking water, while other groups from F3 to F6 were administrated different concentrations of folic acid (1, 20, 40 and 80 mg FA/ Kg BW), respectively. Weight gain, total feed intake, feed efficiency, liver weight and its relative weight were estimated. Biochemical assay: activity of antioxidant enzymes system such as superoxide dismutase (SOD), catalase (CAT); lipid peroxidation level as malondialdehyde (MDA), hydrogen peroxidase (H₂O₂); and liver functions [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (γ -GT), and lactate dehydrogenase (LDH)], were determined. Additionally, total protein (TP), albumin, globulin, A/G ratio, total bilirubin, direct bilirubin, indirect bilirubin; and kidney functions [creatinine, urea, and uric acid]; and lipid profile as [total cholesterol, HDL-cholesterol and LDL-cholesterol, and triglycerides] were also, carried

out. The obtained results showed accumulated weight gain which significantly increased in rats group administrated 40 and 80 mg/ Kg BW/d folic acid. No changes in relative liver weight among tested rat groups were recorded. No significant difference was observed in lipid profile, LDH and SOD between groups (F1 and F6). Data also indicated that F1 group recorded the best one which was low in MDA and high in CAT, followed by F6. Folic acid showed no effect on kidney functions. No histopathological changes were observed in liver of rat groups administered 40 or 80 mg folic acid / Kg BW/d, thus indicating that supplementation with high doses of FA had a protective effect from the hepatic oxidative stress in liver of tested rats.

INTRODUCTION

Prevention of neural tube defects (NTD) and reducing the risk for cardiovascular disease and colon cancer can be achieved by an adequate folate intake. This can be reached by a high consumption of folate-rich foods, e.g. green vegetables, liver, orange and whole grain foods or by the intake of supplements or fortified food products (Bailey *et al* 2003).

Observational studies, nonrandomized intervention studies and randomized controlled trials have demonstrated that folic acid (FA) taken during the periconceptional period decreases the risk of pregnancies affected by NTD (Milunsky *et al* 1989). In September 1992, the U.S. Public Health Service issued a recommendation that all women of childbearing age capable of becoming consume 400 μ g of folic acid/ d to decrease the risk of hav-

(Received April 10, 2007)

(Accepted June 4, 2007)

ing an NTD-affected pregnancy (**Central for Disease Control, 1992**).

Folic acid is a synthetic form of folate that is a water soluble B vitamin (B9). The active metabolite of folic acid is 5-methyltetrahydrofolate that facilitates the remethylation of homocysteine (Hcy) to methionine. Oral folic acid supplementation has been shown to reduce plasma Hcy levels as well as improve the endothelial function in individuals with mild hyperhomocysteinemia (HHcy) (**Woo et al 1999**). Folate plays a key role in the methylation cycle and in DNA biosynthesis. Folate deficiency has been implicated in HHcy, which result in an increased risk of cardiovascular disease and dementia and in NTD (**Quinlivan, et al 2002**). Homocysteine (Hcy) is a sulphhydryl containing amino acid derived from the demethylation of methionine (**Diez et al 2005**).

Folate nutritional status depends on its intake from food and FA supplements as well as on the bioavailability of the various ingested from of this vitamin (**Wright et al 2005**). Moderate folate deficiency is also associated with an increased risk of age-associated degenerative disease such as occlusive vascular disease, cognitive and neurological dysfunction and cancers eg., colorectal cancer, elevated plasma total Hcy (THcy) concentration in folate deficient subject is associated with cognitive decline and neuropsychiatric disorders such as Alzheimer's disease (**Revaglia et al 2003**). Numerous studies have focused on the Hcy-lowering effect of natural folate and its possible protective effect against degenerative disease (**Racek et al 2005**).

The role of folate in carcinogenesis has been best studied for colorectal cancer. A modest reduction in folate status is sufficient to enhance colorectal cancer risk (**Mason and Choi 2000**). Although adverse effect of folate deficiency on immune functions, including suppressed humoral immunity, have been reported in both human and animals studies (**Green and Miller, 1999**). Also folate deficiency is associated with growth retardation, macrocytic anemia and lymphopenia (**Kim et al 2000**).

Folate is essential in purine synthesis and thymidylate synthesis, and it has been suggested that in folate deficiency, cellular concentration of these essential components of DNA and RNA would be altered and nucleic acid metabolism impaired (**Riggs and Jones, 1983**).

The liver is a key organ in homeostasis. Because of its location between the intestinal mucous and general blood circulation, it receives nutrients,

toxins, and agents of infection. Some of these substances induce hepatocyte death, activating regenerative processes that maintain hepatosomatic equilibrium. There is some controversy concerning the effect of aging on hepatocyte fine structure and function, in both humans and rodents (**Roncales et al 2004**).

The aim of this study was to investigate the effect of dietary folic depletion and different concentration of folic acid on H₂O₂ induced- aged rats to examine effects of supplementation with FA on liver histology and biochemical markers in H₂O₂-induced aged rats compared with control.

MATERIALS AND METHODS

Animal feeding experiments

Animals

Thirty six male Sprague –Dawley strain rats, weighting 300±5 g were obtained from Helwan Station for experiment animals, Helwan, Cairo, Egypt. The animals were housed in stainless steel cages raised in the animal's house of Biochemistry Department, Faculty of Agriculture, Cairo University. The rats were kept under normal healthy laboratory conditions; temperature was adjusted at 25 ± 2°C and 12 hour light-dark. Animals were adapted on free access of water, and fed for one week basal diet before the initiation standard of the experimental (**Roncales et al 2004**).

Experimental design

After adaptation period, rats were weighed and divided randomly into 6 groups of equal mean weight, each one containing 6 rats. The animals were fed on different tested diets for 4 weeks according to the method described by **Roncales et al (2004)**. One group of the experimental rats was fed on the basal diet free from folic acid, without H₂O₂ in water and administered 1 mg FA/ Kg body weight by stomach tube; this group was saved as standard one (F1). The other five groups were feed on basal diet free from folic acid with adding 1 % H₂O₂ in the drinking water; to induce oxidative stress. One of these groups was prevented from folic acid (control group, F2); while, other rat groups from F3 to F6 were administered different concentrations of folic acid (1, 20, 40 and 80 mg FA/ Kg BW/d), respectively. F3 rats group was considered as normal control group with adding 1% H₂O₂ in the drinking water. All different concentrations of folic acid were given to rats by

using stomach tube with 0.5 ml of each tested materials. Rats were given free access to food and water, weighed weekly and examined daily for general conditions and symptoms associated with folate deficiency.

Experimental diet

All animals were fed on basal diet (10 % casein, 10 % corn oil, 4% cellulose, 1 % vitamin mixture free from folic acid, 4 % salt mixture, 70 % corn starch and containing 1% succinylsulfathiazole (w/ w) to prevent folate production by intestinal microflora according to the method described by **Diez et al (2005)**.

Biological parameters

Growth of rats

The rats were weighed twice weekly; total feed intake of each rat was weighed and feed efficiency, (gain weigh of rat / total feed intake, g) was calculated. At the end of the experimental period, rats were weighed and killed by diethyl ether. The carcasses were dissected. Liver was cut off, washed in saline solution (KCl 0.15 mol/L) and weighed. The liver weight percentage was calculated as percentage from body weight.

Biochemical assay

At the end of experimental period, blood samples were collected from the animals from the eye plexuses by a fine capillary glass tubes and placed immediately on ice. Blood serum samples were collected into dry clean centrifuge tubes; the serum was separated after centrifugation for 10 min at 3000 rpm and kept frozen at -20°C until the time of analysis. The activity of lipid peroxidation level (Malondialdehyde,MDA), superoxide dismutase (SOD), catalase (CAT) and hydrogen peroxide of rats blood serum were estimated according to **Meltzer et al (1997)**; **Marklund & Marklund (1974)**; **Luck (1971)**; and **Lacy et al (1998)**, respectively. Alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities were assayed by the method of **Bergmeyer and Harder (1986)**. Alkaline phosphatase (ALP; EC 3.1.3.1) activity was measured at 405 nm by the formation of para-nitrophenol from para-nitrophenylphosphate as a substrate using the method of **Varley et al (1980)**. Gamma -GT was measured according to the method described by **Szasz (1969)**. Also, Lactate dehydrogenase (LDH; EC 1.1.1.27) was determined by the method of **Cabaud and Wroblewski (1958)**.

Serum total protein (TP) was analysed using the method of **Lowry et al (1951)**. Folin and Ciocalteus phenol reagent was used to develop the blue color that was measured spectrophotometrically at 750 nm. Bovine serum albumin was used as a standard. Albumin concentration was determined by the method of **Doumas et al (1977)** Globulin concentration was calculated by the difference between TP and albumin.

Serum total bilirubin and direct bilirubin was measured using the method of **Walters and Gerade (1972)**. Indirect bilirubin was calculated by the difference between serum total bilirubin and direct bilirubin.

Serum total cholesterol, HDL-cholesterol and LDL-cholesterol, triglycerides, creatinine, urea and uric acid were determined by using the methods described by **Watson (1960)**, **Assmann (1979)**, **Wieland & Seidel (1983)**, **Fossati & Prencipe (1982)**, **Larsen (1972)**, **Patton & Crouch (1977)**; and **Caraway (1955)**, respectively.

Histopathological studies

Tissue specimens from rat's liver were fixed in 10% neutral buffered formalin solution. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin then sectioned (4-6 micron) and stained with hematoxyline and eosin according to **Bancroft et al (1996)**. The degree of hepatic injury was estimated using an ordinal scale modified by **Palaa and Charbonneau (1994)**.

Statistical analysis

All values are means \pm S.E.M. obtained from six animals. For statistical analysis, one-way ANOVA with Duncan's variance was used to compare between groups. In all the cases the difference was considered significant when p was ≤ 0.05 according to the method described by **Snedecor and Cochran (1980)**.

RESULTS AND DISCUSSION

Growth of rats administrated various folic acid concentrations for 4 weeks

Table (1) appears that the accumulated weight gain, feed intake, and feed conversion efficiency were significantly differences after feeding period for 4 weeks. F2 and F3 rat groups had a significantly lower body weight. It was interesting to

Table 1. Growth of rats administrated various folic acid concentrations for 4 weeks

Treatments	Initial weight (g)	Accumulated weight gain (g)	Feed intake (g)	Feed conversion efficiency, (F.C.E) (g gain/ g feed)	Liver weight (g)	Relative liver weight
F1 (standard, 1 mg FA/ Kg BW without H ₂ O ₂)	302.32±4.25 ^a	108.77±3.45 ^b	402.84±18.05 ^c	0.27 ± 0.01 ^{ab}	14.19±0.31 ^a	3.46±0.07 ^a
F2 (control, free FA with H ₂ O ₂)	300.42±4.04 ^a	91.35 ±3.76 ^d	351.28±31.63 ^d	0.26 ± 0.02 ^{ab}	12.94±0.27 ^b	3.25±0.12 ^a
F3 (normal, 1 mg FA/ Kg BW with H ₂ O ₂)	301.90±5.03 ^a	97.41±4.86 ^c	389.59±23.36 ^c	0.25 ± 0.02 ^b	12.68±0.67 ^b	3.35±1.37 ^a
F4 (20 mg FA/ Kg BW with H ₂ O ₂)	300.80±6.02 ^a	115.89±4.09 ^{ab}	426.66±17.46 ^b	0.27 ± 0.00 ^{ab}	14.49±1.18 ^{ab}	3.31±3.42 ^a
F5 (40 mg FA/ Kg BW with H ₂ O ₂)	299.65±10.01 ^a	115.46±5.86 ^{ab}	398.13±28.92 ^c	0.29 ±0.021 ^a	13.72±0.90 ^b	3.24±0.29 ^a
F6 (80 mg FA/ Kg BW with H ₂ O ₂)	299.31±5.50 ^a	123.91±6.95 ^a	476.59±26.82 ^a	0.26 ± 0.01 ^{ab}	14.46±0.93 ^a	3.45±0.98 ^a
LSD (P<0.05)	10.89	8.867	22.60	0.03	1.33	0.81

Each value represents the mean ± SE.

The mean values with different superscript alphabets indicate significant differences (P<0.05) using LSD test

note that the decreased food consumption was associated with decreasing weight gain. The reduced weight gain is agreed with **Huang *et al* (2001)**, while contrasted with **Durand *et al* (1997)**. These differences in growth response might be related to the level of folate restriction and length of the experimental periods.

From the obtained data, it could be noticed that no significant difference among the tested rat groups in liver weight and its relative weight. This result is in agreement with **Chanson *et al* (2005)**.

Effect of folic acid on serum lipid peroxidation (MAD), superoxide dismutase (SOD), catalase (CAT), and H₂O₂

The serum MDA, LDH, SOD, CAT and H₂O₂ of aged rats fed on basal diet free from folic (FF) or supplemented with FA without or with 1% H₂O₂-induced oxidative stress in drinking water for 4 weeks are presented in **Table (2)**. Thiobarbituric acid reactions (TBARS) or malondialdehyde (MDA) concentration is a very specific end product of lipid peroxidation (**Cases *et al* 1999**). The degree of lipid peroxidation in rat's serum was examined by measuring the level of MDA. Its level was significantly elevated in group F2 which

fed FF with 1% H₂O₂ in drinking water, reflecting an increased lipid peroxidation in the serum. Serum MDA concentration increased from 11.50 in F1 group without H₂O₂ to 27.29 n mol/ ml in F2 group. This later high value was significantly and gradually decreased by increasing the concentration of supplemented FA from 1 to 80 mg/kg BW/d reaching a value (13.55 n mol) nearby that observed in the standard group F1. These results suggested that NAD(P)H oxidase-mediated superoxide anion generation was responsible for oxidative stress and subsequently increased lipid peroxidation during HHcy (**Woo *et al* 2006**).

SOD and CAT are important antioxidant enzymes that protect intracellular and extracellular membranes from lipid peroxidation via elimination of reactive oxygen species (ROS). Therefore, these antioxidant enzymes were examined. From **Table (2)**, as a folate intake decreased, serum SOD and CAT activity decreased. The F2 group (control FF diet) had significantly lower serum SOD and CAT activity than the F1 group (standard). Supplementation of diet with (1, 20, 40, 80mg FA/ kg BW/d) derived to significant gradual increase in serum SOD and CAT activity. It could be observed that, high concentration of FA was accompanied with elevated activity of antioxidant

Table 2. Malondialdehyde, superoxide dismutase activity, catalase activity and H₂O₂ concentration in serum of rats administrated various folic acid concentrations

Treatments	Malondialdehyde (nmol/ml)	SOD (U/ml)	Catalase (U/ml)	H ₂ O ₂ (mmol/L)
F1 (standard, 1 mg FA/ Kg BW without H ₂ O ₂)	11.50 ± 0.45 ^f	319.53 ± 13.56 ^a	144.00 ± 2.61 ^a	0.15 ± 0.01 ^d
F2 (control, free FA with H ₂ O ₂)	27.29 ± 0.65 ^a	142.42 ± 1.45 ^d	79.34 ± 1.69 ^c	0.42 ± 0.04 ^a
F3 (normal , 1 mg FA/ Kg BW with H ₂ O ₂)	23.92 ± 0.36 ^b	187.74 ± 4.63 ^c	101.67 ± 2.95 ^d	0.34 ± 0.02 ^b
F4 (20 mg FA/ kg BW with H ₂ O ₂)	22.15 ± 0.16 ^c	221.32 ± 5.61 ^b	113.67 ± 5.12 ^c	0.25 ± 0.02 ^c
F5 (40 mg FA/ kg BW with H ₂ O ₂)	17.26 ± 0.88 ^d	300.71 ± 16.88 ^a	120.00 ± 1.18 ^b	0.22 ± 0.01 ^c
F6 (80 mg FA/ kg BW with H ₂ O ₂)	13.55 ± 0.16 ^e	308.86 ± 10.23 ^a	125.67 ± 1.61 ^b	0.15 ± 0.01 ^d
L.S.D (P<0.05)	1.59	30.01	6.13	0.06

Each value represents the mean ± SE.

The mean values with different superscript alphabets indicate significant differences (P<0.05) using LSD test

enzymes. This result was explained by those demonstrated by **Huang *et al* (2001)** and **Woo *et al* (2005)**. They stated that folate deficiency led to increase plasma Hcy concentration; which might play a role in the reduction of antioxidant enzymes.

Table (2) shows the kinetics of serum H₂O₂ (mmol/ L) formation. The highest amount of hydrogen peroxide was found in rats group F2 fed FF diet with 1% H₂O₂ in the drinking water, while it was found in its smaller amounts in F1 group (standard group) and F6 group (80 mg FA/ kg BW/d) than other groups. However, no significant difference could be shown between F6, F1 as well as between F5 and F4. These results indicated that the treatment with 1% H₂O₂ alone without FA increased serum H₂O₂ formation. The generation of H₂O₂ was depended on the FA concentration where, the supplementation with different concentrations of FA decreased serum H₂O₂. Serum H₂O₂ formation are specific indicator of ROS-generation. High amount of plasma H₂O₂ have been response to various oxidative stress.

In conclusion, the presence of FA in the diet led to the reduction of free radicals induced by H₂O₂ as a result of the improvement of antioxidant enzymes activity. The mechanism of antioxidant defense is described by **Arouma (1998)**; he indicated that SOD removes O₂' by greatly accelerating its conversion to H₂O₂. Also, CAT in peroxi-

somes converts H₂O₂ into water and O₂. Other important H₂O₂-removing enzyme in human cells is the glutathione peroxidase. When produced in excess, ROS can cause tissue injury (**Groff and Gropper, 2000**).

Liver functions

Liver functions could be detected the state of liver. High levels of AST, ALT, ALP and Gamma GT in serum are usually indicative of disease and necrosis in the liver of animals (**Manna *et al* 1996**) and humans (**Hassal *et al* 1990**). However, total blood serum bilirubin concentration is a useful measure of liver dysfunction.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gama-GT (γ-GT)

The level of serum ALT, AST, ALP and γ-GT activities are presented in **Table (3)**. Significant increases were observed in these enzymes activity in rats group F2 (fed FF with 1 % H₂O₂). In contrast, supplementation with FA significantly decreased these parameters to the normal values. F2 had significantly higher levels in all the detected enzymes followed by F3 compared to F1 and rats administered different concentrations of FA. AST, ALT and ALP and γ-GT may be released

Table 3. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Alkaline phosphatase (ALP), gamma glutamyl transferase (δ GT) and lactate dehydrogenase (LDH) of rats administered various folic acid concentrations for 4 weeks

Treatments	ALT (U/L)	AST (U/L)	ALP (U/L)	δ GT (U/L)	LDH (U/L)
F1 (standard, 1 mg FA/ Kg BW without H ₂ O ₂)	32.48±2.08 ^b	42.43±3.51 ^b	73.29 ± 2.10 ^{ab}	4.86 ±0.14 ^b	252.75 ± 18.56 ^e
F2 (control, free FA with H ₂ O ₂)	47.84±4.88 ^a	51.08±3.31 ^a	79.50 ±5.12 ^a	8.72 ± 1.6 ^a	827.84 ± 25.45 ^a
F3 (normal, 1 mg FA/ kg BW with H ₂ O ₂)	36.11±2.76 ^b	44.58±4.50 ^b	76.15 ± 1.74 ^{ab}	7.42 ± 0.72 ^a	523.02 ± 24.19 ^b
F4 (20 mg FA/ kg BW with H ₂ O ₂)	33.52±0.84 ^b	40.15±1.54 ^b	73.87±5.71 ^{ab}	4.71 ± 0.68 ^b	451.11 ± 38.9 ^c
F5 (40 mg FA/ kg BW with H ₂ O ₂)	32.49±1.34 ^b	42.57±4.17 ^b	70.97±5.14 ^b	4.44 ±0.92 ^b	340.59 ± 49.69 ^d
F6 (80 mg FA/ kg BW with H ₂ O ₂)	32.70±2.77 ^b	38.66±2.94 ^b	71.44±3.08 ^b	3.99 ±1.2 ^b	244.43 ± 15.39 ^e
L.S.D (P _≤ 0.05)	4.926	5.919	7.349	1.718	55.31

Each value represents the mean ± SE.

The mean values with different superscript alphabets indicate significant differences (P_≤0.05) using LSD test

into blood plasma and increased serum levels of these enzymes due to cellular damage in the liver. Thus, the levels of ALP in blood plasma may be also increase in the early periods of liver damage. Supplementation of folic acid had a protective effect against Hcy-induced liver injury by the reduction of both AST and ALT activities (**Roncales et al 2004**).

Lactic acid dehydrogenase activity (LDH U/L)

Serum LDH activity presented in **Table (3)** show that, LDH was significantly increased by 3.3-fold in the group F2 (administration 1% H₂O₂ with FF diet), while about group F1 this fold was reduced to 2.1, 1.8, 1.3 and 0.9 by supplementation with FA at concentration of 1, 20, 40, 80mg/ kg BW/ d, respectively. It was noticed that there is no significant difference between group F6 and group F1. The obtained data of control rats group fed FF diet indicated that H₂O₂ induced enzymes such as AST, ALT, LDH, ALP, and γ -GT were released into the blood as a result of hepatocyte damage. These values were decreased by supplementation the diet by FA indicating that it prevents damage in the liver, particularly at concentration of 80mg FA/ kg BW. Among free radicals the superoxide anion and hydroxyl radical promote lipid peroxidation and membrane damage by crosslinking to macromolecules (**Maestro et al 1980**). Lipid peroxidation of the cell membrane

leads to increase of membrane fluidity, disturbances of calcium homestasis, and finally, cell death (**Ozdil et al 2004**).

Serum total protein, albumin, globulin, and bilirubin

The data presented in **Table (4)**, showed significant differences among F2 and the tested rat groups in their serum total protein albumin. While, no significant differences were observed between the tested rat groups in blood serum globulin, A/G ratio, total bilirubin, direct bilirubin and indirect bilirubin. The normal range of total blood serum bilirubin of Alderley Park *albino* rats in males is from 0.058 to 0.351 mg/dl, (**Archer and Jeffcott, 1977**). It is known that during liver damage, serum AST, ALT, LDH, and γ -GT activities increase, while albumin and total protein concentration decreased (**Erdogan et al 2005**).

Kidney function

Creatinine, urea and uric acid

Creatinine is the major waste product of creatine metabolism by muscle. In the kidney, it is filtered by the glomerulus and actively excreted by the tubules. Moreover, free creatinine appears in the blood serum, (**Baker et al 1979**). Urea and uric acid are the principal waste products of protein catabolism. They are synthesized in the liver

Table 4. Blood serum total protein (TP), albumin, globulin, A/G ratio, total, direct and indirect bilirubin of rats administrated various folic acid concentrations for 4 weeks

Treatments	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Indirect bilirubin (mg/dl)
F1(standard, 1 mg FA/ Kg BW without H ₂ O ₂)	6.09±0.26 ^{ab}	3.46±0.23 ^{ab}	2.63±0.18 ^{ab}	1.32±0.12 ^a	0.23±0.01 ^{ab}	0.15±0.01 ^{ab}	0.09±0.012 ^a
F2 (control, free FA with H ₂ O ₂)	5.39±0.61 ^b	3.13± 0.21 ^b	2.26±0.42 ^b	1.42±0.23 ^a	0.26±0.01 ^{ab}	0.15±0.02 ^{ab}	0.10±0.012 ^a
F3 (normal, 1 mg FA/ kg BW with H ₂ O ₂)	6.73±0.46 ^a	3.82±0.47 ^a	2.61±0.28 ^{ab}	1.48±0.29 ^a	0.23±0.02 ^a	0.13±0.01 ^b	0.09± 0.01 ^a
F4 (20 mg FA/ Kg BW with H ₂ O ₂)	7.03± 0.42 ^a	4.09±0.56 ^a	2.95±0.47 ^{ab}	1.42±0.35 ^a	0.24±0.01 ^{abc}	0.14±0.02 ^{ab}	0.10±0.015 ^a
F5 (40 mg FA/ Kg BW with H ₂ O ₂)	7.01±0.28 ^a	4.01±0.35 ^a	3.00±0.30 ^{ab}	1.34±0.22 ^a	0.27±0.01 ^c	0.17±0.01 ^a	0.09±0.019 ^a
F6 (80 mg FA/ Kg BW with H ₂ O ₂)	6.95± 0.76 ^a	3.78±0.21 ^a	3.15±0.54 ^a	1.22±0.14 ^a	0.26±0.02 ^{bc}	0.17±0.02 ^a	0.09±0.009 ^a
L.S.D (P<0.05)	0.88	0.59	0.69	0.46	0.03	0.02	0.02

Each value represents the mean ± SE.

The mean values with different superscript alphabets indicate significant differences (P<0.05) using LSD test

from ammonia produced as a result of the deamination of amino acids. The rate of production is accelerated by a high protein diet or by increased endogenous catabolism due to starvation or tissue damage (Young, 1972).

Data in Table (5) show no significant differences in serum creatinine, urea and uric acid among the tested rat groups. It was noticed that these waste products were not affected by neither H₂O₂ intake nor folic acid. These results are in agreement with those observed by Diez *et al* (2005).

Lipid Profile

The effect of supplementation with FA on limiting parameter for lipid metabolism, such as serum total cholesterol, LDL-C, HDL-C and triglycerides of aged rats with H₂O₂-induced oxidative stress in drinking water were studied and the results are shown in Table (6). Rats fed with H₂O₂ without FA in group F2 (control group) had significantly the highest total-cholesterol, LDL-C and triglyceride. Meanwhile, HDL-C was significantly lower, than that obtained in rats fed basal diet without H₂O₂ in drinking water in group F1. On the other hand, supplementations of diet with dif-

ferent concentrations of FA significantly decreased the total cholesterol than that obtained in rats fed the control diet FF (group F2). The maximum percentage of decreasing the T-Chol, LDL, and triglyceride in rat's serum with H₂O₂ were obtained by feeding them with 80mg FA/ kg BW, which showed no significant difference between the obtained values and those of group F1 (standard). In contrary, the concentration of HDL significantly increased by increasing the percentage of FA compared to the control group.

As mentioned above, folate deficiency led to increase plasma Hcy concentration (Woo *et al* 2005). This high level of Hcy caused an activator of several transcription factors in the liver leading increased HMG-CoA reductase and cholesterol biosynthesis (Woo *et al* 2006).

Histopathological study

Histopathological examination of liver indicated that standard rats group showed apparent normal hepatic structure (Fig. 1), while, liver of control rats group revealed vacuolar degeneration of hepatocytes specially around the central vein (Fig. 2). In concerning of tested rat groups, It could be observed that dilatation and congestion of central

Table 5. Serum creatinine, urea and uric acid of rats administrated various folic acid concentrations for 4 weeks

Treatments	Creatinine (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)
F1 (standard, 1 mg FA/ Kg BW without H ₂ O ₂)	0.53 ± 0.04 ^a	21.36 ± 1.41 ^a	4.50 ± 0.46 ^a
F2 (control, free FA with H ₂ O ₂)	0.49 ± 0.04 ^a	21.14 ± 2.40 ^a	4.01 ± 0.33 ^a
F3 (normal group, 1 mg FA/ Kg BW with H ₂ O ₂)	0.47 ± 0.10 ^a	22.80 ± 1.35 ^a	4.42 ± 0.355 ^a
F4 (20 mg FA/ kg BW with H ₂ O ₂)	0.49 ± 0.07 ^a	21.55 ± 2.39 ^a	4.46 ± 0.29 ^a
F5 (40 mg FA/ Kg BW with H ₂ O ₂)	0.53 ± 0.06 ^a	21.52 ± 2.15 ^a	4.29 ± 0.36 ^a
F6 (80 mg FA/ Kg BW with H ₂ O ₂)	0.55 ± 0.06 ^a	21.75 ± 2.85 ^a	4.62 ± 0.38 ^a
L.S.D (P≤0.05)	0.11	3.84	0.65

Each value represents the mean ± SE.

The mean values with different superscript alphabets indicate significant differences (P≤0.05) using LSD test

Table 6. Serum total-cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides of rats administrated various folic acid concentrations for 4 weeks

Treatments	T.cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Triglycerides (mg/dl)
F1 (standard, 1 mg FA/ Kg BW without H ₂ O ₂)	76.47 ± 1.42 ^e	28.77 ± 0.92 ^a	41.64 ± 0.59 ^d	140.93 ± 2.84 ^d
F2 (control, free FA with H ₂ O ₂)	174.59 ± 4.28 ^a	15.03 ± 1.15 ^d	56.50 ± 0.75 ^a	205.62 ± 2.50 ^a
F3 (normal group, 1 mg FA/ kg BW with H ₂ O ₂)	154.12 ± 0.84 ^b	18.49 ± 0.38 ^c	51.56 ± 0.88 ^b	169.32 ± 1.87 ^b
F4 (20 mg FA/ kg BW with H ₂ O ₂)	137.38 ± 5.17 ^c	21.72 ± 0.65 ^b	47.11 ± 0.40 ^c	160.44 ± 1.05 ^c
F5 (40 mg FA/ kg BW with H ₂ O ₂)	101.62 ± 7.12 ^d	22.29 ± 0.82 ^b	47.01 ± 0.91 ^c	142.97 ± 0.74 ^d
F6 (80 mg FA/ kg BW with H ₂ O ₂)	87.07 ± 1.61 ^e	27.53 ± 0.60 ^a	40.71 ± 0.80 ^d	140.93 ± 3.14 ^d
L.S.D (P<0.05)	12.64	2.44	2.85	6.52

Each value represents the mean ± SE.

The mean values with different superscript alphabets indicate significant differences (P≤0.05) using LSD test

veins (**Fig. 3**) of F3 rats group, vacuolization of hepatocytes around the central vein associated with focal leucocytic cells infiltration (**Fig. 4**) of F4 rats group and no histopathological changes was observed in liver of rat groups administered 40 or 80 mg folic acid / Kg bw/d (**Figs. 5 and 6**). It could be concluded that the supplementation with high doses of FA have a protective effect from the hepatic oxidative stress in liver of tested rats. These results indicated that folate supplementation can reduce carcinogen-induced ornithine

decarboxylase and tyrosine kinase activities, both indices of cellular proliferation. Moreover, folate status, in many situations, modifies proliferation rates, increasing proliferation in the livers of folate- or methyl-deficient rats. In this context, the availability of the folate compounds might limit DNA synthesis in both velocity and intensity. For the hepatotropic activity of FA, it was proposed that the transmethylation ability of FA could result in changes in the biochemical structure of the target molecules involved in DNA synthesis.

Fig. 1-6. Histopathological examination of rats liver administrated various folic acid concentrations for 4 weeks

Fig. 1. Standards group , basal diet without H₂O₂, F1
 Fig. 3. Normal group FA 1ppm with H₂O₂, F3
 Fig. 5. FA 40ppm with H₂O₂, F5

Fig. 2. Control group free folic with H₂O₂, F2
 Fig. 4. FA 20ppm with H₂O₂, F4
 Fig. 6. FA 80ppm with H₂O₂, F6

On the other hand, reactive metabolites are produced during biotransformation of H₂O₂ and these metabolites may cause cellular death in the liver **Rohrdanz and Kahl (1998)**.

In conclusion, the obtained results suggest that folic acid had an important role in maintaining good health may extend beyond the cardiovascular system to encompass the hyperhomocysteinemia-associated disorders that involve other organs such as liver.

REFERENCES

Archer, R.K. and L.B. Jeffcott (1977). *Comparative Clinical Haematology*. 2nd Ed. pp. 994 – 998. (Tietz, N.W. ed.), Saunders, Philadelphia, USA.

Arouma, O.I. (1998). Free radicals, oxidative stress and Antioxidants in human health and disease. *JAOCS* **75**: 199-212.

Assmann, G. (1979). A fully enzymatic colorimetric determination of HDL-cholesterol in the serum. *Internist* **20**: 559-563.

Baily, L.B.; G.C. Rampersau and G.P.A. Kaulwell (2003) Folic acid supplements and fortification affect the risk for neural tube defects, vascular disease and cancer: evolving Science. *J. Nutr.* **133**: 1961S- 1968S.

Baker, H.J.; J.R. Lindsey and S.H. Weisbroth (1979). *Clinical Biochemistry. The Laboratory Rats. Biological and Diseases (Volume 1)*. 1st Ed. Ch. 5 pp. 113-118, Academic Press, Harcourt Brace Jovanovicn, New York.

- Bancroft, J.D.; A. Stevans and D.R. Turner (1996).** *Theory and Practice of Histological Techniques*. 4th. Ed. pp. 105-111, Churchill Livingstone, New York.
- Bergmeyer, H.U. and M. Harder (1986).** A colorimetric methods of determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *Clin. Biochem.* **24**: 28 – 34.
- Cabaud P.C. and F. Wroblewski (1958).** Colorimetric measurement of lactate dehydrogenase activity of body fluids. *J. Clin. Pathol.* **30**: 234–240.
- Caraway, W.T. (1955).** Determination of uric acid in serum by a carbonate method. *American Journal of Clin. Pathol.* **2**: 840-845.
- Cases, J.; M. Puig; B. Caporiccio; B. Baroux; J.C. Baccou; P. Besancon and J.M. Rouanet (1999).** Glutathion related enzymic activities in rats receiving high cholesterol or standard diets supplemented with two forms of selenium. *Food Chem.* **65**: 207 – 211.
- Central for Disease Control (1992).** Recommendation for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *Morb. Mortal. Wkly. Rep.* **41**: 1-7.
- Chanson, A.; T. Sayd; E. Rock; C. Chambon; V. Sante-Lhoutellier; G.P. Courcy and P. Brachet, (2005).** Proteomic analysis reveals changes in the liver protein pattern of rats exposed to dietary folate deficiency. *J. Nutr.* **135**: 2524–2529.
- Diez, N.; R. Perez; V. Hurtado and S. Santidrian (2005).** Hyperhomocysteinaemia induced by dietary folate restriction causes kidney oxidative stress in rats. *British J. Nutr.* **94**: 204- 210.
- Doumas, B.T.; W.A. Watson and H.G. Biggs (1977).** Albumin standards and the measurement of serum albumin with bromocresol green. *Clin. Chem. Acta.* **31**: 87–96.
- Durand, P.; M. Prost and D. Blache (1997).** Folic acid deficiency enhances oral contraceptive-induced platelet hyperactivity. *Arterioscler Thromb Vasc. Biol.* **17**: 1939 – 1946 (C.F. *British J. Nutr.* **94**: 204 – 210).
- Erdogan, Z.; S. Erdogan; S. Celik and A. Unlu (2005).** Effect of ascorbic acid on cadmium-induced oxidative stress and performance of broilers. *Biological Trace Element Research*, **104**: 19- 31.
- Fossati, P. and L. Prencipe, (1982).** The determination of triglycerides using enzymatic methods. *Clin. Chem.* **28**: 2077- 2079.
- Green, R. and J.W. Miller (1999).** Folate deficiency beyond megaloblastic anemia: hyperhomocysteinemia and other manifestations of dysfunctional folate status. *Semin. Hematol.* **36**: 47-64.
- Groff, J.L. and S.S. Gropper (2000).** The fat-soluble vitamins: Ch. 10 pp. 359-37, In: *Advanced Nutrition and Human Metabolism*. Library of Congress Cataloging in Publication Data, printed in the United States of America.
- Hassal E.; D.M. Israel and T.M. Gunesakaran (1990).** Halothane hepatitis in children. *J. Paediatr. Gastroenterol. Nutr.* **11**: 553–557.
- Huang, R.F.; Y.C. Hsu; H.L. Lin and F. Yang (2001).** Folate depletion and elevated plasma homocysteine promote oxidative stress in rats livers. *J. Nutr.* **131**: 33 – 38.
- Kim, Y.I.; S. Shirwadkar; S.W. Choi; M. Puchyer; Y. Wang and J.B. Masson (2000).** Effect of dietary folate on DNA strand break within mutation-prone exons of the p53 gene in rat colon. *Gastroenterology* **119**: 151- 161.
- Lacy, F.O.; D.T. Connor; and G.W. Schmid-Schonbein (1998).** Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. *J. Hypertension*, **16**: 291-303.
- Larsen, K. (1972).** Creatinine assay by a reaction-kinetic principle. *Clin. Chem. Acta* **41**: 209- 213.
- Lowry O.H.; N.J. Rosebrough; A.L. Farr and R.J. Randall (1951).** Protein measurement with the Folin Phenol Reagent. *J. Bio. Chem.* **193**: 269–275.
- Luck, H. (1971).** Catalase, In: *Methods of Enzymatic Analysis*, (2 Ed.) pp. 885- 893. Academic Press, New York.
- Maestro, D.F.R.; H.H. Thaw and J. Bijor (1980).** Free radicals as mediators of tissue injury. *Acta Physiol. Scand.* **492**(suppl.): 43-57.
- Manna, Z.; S. Guopei and G.Y. Minuk (1996).** Effects of hepatic stimulator substance herbal medicine selenium/vitamin E and ciprooxacin on cirrhosis in the rat. *Gastroenterology* **1996**; **110**: 1150–1155 (C.F. *Experimental and Toxicologic Pathology* **56**: 59–64, 2004.
- Marklund, S. and G. Marklund (1974).** Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **47**: 469–474.
- Mason, J.B. and S.W. Choi (2000).** Folate and carcinogenesis: developing a unifying hypothesis. *Adv. Enzyme Regul.* **40**: 127- 141.
- Meltzer, H.M.; M. Folmer; S. Wang; Q. Lie; A. Maaga and H.H. Mundal (1997).** Supplementary selenium influences the response to fatty acid in-

- duced oxidative stress in humans. **Biological Trace Element Research** **60**: 51 – 67.
- Milunsky, A.; H. Jick; S.S. Jick; C.L. Bruell; D.S. Maclaughlin; K.J. Rothman and W. Willett (1989). Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. **J. Am. Med. Assoc.** **262**: 2847- 2852.
- Ozdil, S.; S. Bolkent; R. Yanardag and P.A. Pirincci (2004). Protective effects of ascorbic acid, DL- α - tocopherol acetate, and sodium selenate on ethanol-induced liver damage of rats. **Biological Trace Element Research**, **104**: 149- 161.
- Palaa, G.L. and M. Charbonneau (1994). Detection and evaluation of chemically induced liver injury: In: Hayes, A.W. (Ed.), **Principles and Methods of Toxicology**. 3rd. Ed. pp 320- 325. Raven press, New York.
- Quinlivan, E.P.; J. McPartlin; H. McNulty; P.A. Ward; J.J. Strain; D.G. Weir and J.M. Scott (2002). Importance of both folic acid and vitamin B12 in reduction of risk of vascular disease. **Lancet** **359**: 227- 228.
- Racek J.; H. Rusnakova; L. Trefil and K.K. Siala (2005). The influence of folate and antioxidant on homocysteine level and oxidative stress in patients with hyperlipidemia and homocysteinemia. **Physiol. Res.** **54**(1): 87- 95.
- Revaglia G.; P. Forit; F. Maioli; A. Muscari; L. Sacchetti; G. Arnone; V. Nativio; T. Talerico and E. Mariani (2003). Homocysteine and cognitive function in healthy elderly community dwellers in Italy. **Am. J.Clin. Nutr.** **77**(3): 668- 673.
- Riggs A.D. and P.A. Jones (1983). 5-methylcytosine, gene regulation, and cancer. **Adv. Cancer Res.** **40**: 1- 30.
- Rohrdan, E. and A. Kahl. (1998). Alterations of antioxidant enzyme expression in response to hydrogen peroxide. **Free Radical Biological & Medicine**, **24**(1): 27 – 38.
- Roncales M.; M. Achon; F. Manzarbeitia; C. Casas; C. Ramirez; G. Varela-Moreiras and J. Perez-Miguelsanz (2004). Folic acid supplementation for 4 weeks affects liver morphology in aged rats. **J. Nutr.** **134**: 1130- 1134.
- Snedecor, G.W. and W.G. Cochran (1980). **Statistical Methods**. 7th Ed., Oxford and J.B.H Publishing Com. London.
- Szasz, G. (1969). Determination of γ - L-Y-Glutamyl transferase (γ -GT). **Clin. Chem.**, **22**: 124 -136.
- Varley, H.; A. Gewenlock and M. Bell (1980). **Practical Clinical Biochemistry**. Vol.1. 5th Ed. pp. 741- 897. William Heinemen Medical. Books, Ltd. London.
- Walters, M. and H. Gerade (1972). Ultramicro-method for the determination of conjugated and total bilirubin in serum or plasma. **Microchem J.** **15**: 231- 238.
- Wastson, D.A. (1960). Simple method for the determination of serum cholesterol. **Clin. Chem. Acta** **5**: 589-596.
- Wieland, H. and D. Seidel (1983). A fully enzymatic colorimetric determination of HDL-cholesterol in the serum. **J. Nutr.** **109**: 760– 766.
- Woo, C.W.H.; G.A. Prathapainghe; Y.L. Siow and O. Karmin (2006). Hyperhomocysteinemia induces liver injury in rat: Protective effect of folic supplementation. **Biochimica et Biophysica Acta** **10**: 1- 10.
- Woo, C.W.; G.N. Siow; P.C. Pierce; G.Y. Choy; D. Minuk and K.O. Mymin (2005). Hyperhomocysteinemia induces hepatic cholesterol biosynthesis and lipid accumulation via activation of transcription factors, **Am. J. Physiol.: Endocrinol. Metab.** **288**: E1002- E1010.
- Woo, K.S.; P. Chook; Y.I. Lolín; J.E. Sander-son; C. Metreweli and D.S. Celermajer (1999). Folic acid improves arterial endothelial function in adults with hyperhomocysteinemia, **J. Am. Coll. Cardiol.** **34**: 2002–2006.
- Wright, A.J.; P.M. Finglas; J.R. Dainy; C.A. Wolfe; D.J. Hart; S.D.M. Wright and J.F. Gergory (2005). Differential kinetic behavior and distribution for pteroylglutamic acid and reduced folates: a revised hypothesis of the primary site of PeteGlu metabolism in humans. **J. Nutr.** **135**: 619- 623.
- Young, D.S. (1972). Effects of drugs on clinical laboratory tests. **Clin. Chem.** **18**: 430D – 432D.