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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds), Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International. pp 181-190.

Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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Journal of Cancer Research and Experimental Oncology

Full Length Research Paper

The prognostic value of IgA/[EBNA1+VCA-p18] on survival of nasopharyngeal cancer patients

Kartika Widayati Taroeno-Hariadi[1], Djohan Kurnianda[1], Jajah Fachiroh[1], Bambang Hariwiyanto[2], Sagung Rai Indrasari[3], Camelia Herdini[3], Ibnu Purwanto[1], Salugu Maesadji[4], Retno Dwi Danarti[4], Mardiah Suci Hardianti[1], Harijadi[5] and Sofia Mubarika Haryana[2]

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Undifferentiated (World Health Organization (WHO) 3) type of nasopharyngeal cancer (NPC) is strongly correlated with Epstein-barr (EBV) virus latent infection. Post-treatment viral reactivation is associated with relapsed or recurrence of NPC. Viral activation can be measured indirectly via plasma IgA responses towards EBV proteins such as EBNA1 and VCA-p18. This study aims at determining the prognostic value of IgA/[EBNA1+VCA-p18] on progression free survival and overall survival of NPC patients. NPC patients aged > 18 years, with locally advanced disease receiving concurrent chemoradiation, with weekly cisplatin 40 mg/m^2 samples for blood plasma before treatment, 3 months post-treatment, and at 12 months after treatment completion or at the time of disease progression, whichever came first. An established enzyme linked immunosorbent assay (ELISA) method was used for evaluation of IgA/[EBNA1+VCA-p18] level reported as optical density 450 nm (OD_{450}) values. Forty six NPC patients, with male predominance and mostly in productive age were included. Twenty seven patients had disease progression or died during study follow up. Mean of pre-treatment IgA OD_{450} was higher in patients with progression compared to those still in remission (2.33 ± 1.08 versus 1.66 ± 1.19, $p < 0.05$). The higher risk serology group ($OD_{450} \geq 1.4$) had shorter time to progression (RR 6.06; $p = 0.014$; median time to progression is 13.47 month). Overall survival was not influenced by plasma IgA. Pretreatment IgA/[EBNA-1+VCA-p18] may predict early progression for NPC

Key words: Nasopharyngeal cancer, prognosis factor, immunological response, IgA/(EBNA1+ VCA-p18).

INTRODUCTION

Nasopharyngeal cancer (NPC) is a common cancer in Southern China, South East Asia and North Africa (Raab-

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Traub, 2000; Chang et al., 2006).. In Indonesia, NPC is the fourth commonest cancer in men and sixth among women (Adham, 2012). More than 90% of NPC in Indonesia comprised of histology type World Health Organization (WHO) III or undifferentiated carcinoma which is strongly correlated with Epstein-barr virus (EBV) infection (Soeripto, 1997; Adham et al., 2012).

Consistent expression of EBV gene products in nasopharyngeal cancer cells, specific immune response to EBV antigen in NPC patients, as well as detection of EBV in premalignant lesion support the pathogenic role of EBV in NPC (Henle et al.,1970; Wolf et al., 1973; Henle, 1976; Ho et al., 1976; Zeng et al 1982, 1983; Yeung et al., 1993; Sam et al., 1994; Pathmanathan et al., 1995; Gulley, 2001; Chien et al., 2001; Middeldorp et al., 2003). Although NPC is sensitive to radiotherapy and chemotherapy, recurrence rate of NPC during the first 2-year post treatment remains high (2-year progression free survival is less than 53% with median time to progression is 17.4 month) in our centre (Taroeno-Hariadi et al., 2005 unpublished observation).

Wildeman et al. (2013) reported that median overall survival of NPC patients in our centre is 21 months (95% CI = 18 to 35) from day of diagnosis. Treatment modality, tumor stage, patient performance status, viral load and viral reactivation may influence recurrence and progression (Farias et al., 2003; Twu et al., 2007; Sham and Choy, 2010; Wu et al., 2012).

IgG (and specifically IgA) response to EBV antigens is the hallmark of NPC (Henle et al., 1970; Henle and Henle, 1976; Ho et al., 1976). With the advent of polymerase chain reaction (PCR) technology, nowadays viral reactivation can be measured more directly by detecting EBV-DNA. EBV-DNA quantification has been reported as sensitive and specific method for NPC diagnosis, treatment monitoring and prognosis (Lo et al., 2000). However, this method is quite expensive to be applied in low income countries such as Indonesia. Fachiroh et al. (2006) have developed serodiagnostic tools based on enzyme-linked immunosorbent assay (ELISA) to measure IgA antibody response to combination of EBV immunodominant epitopes [EBNA1+VCA-p18] in one assay to diagnose NPC.

This method had a reported sensititivity of 85.4% and specificity of 90.1%. Sensitivity, a specificity of IgA/[EBNA1+VCA-p18] is better than either IgA EBNA-1 or IgA VCAp-18 alone (Fachiroh et al., 2006). The application of this serodiagnostic tool to predict survival or recurrence in NPC requires further clinical evidence. This is the first study reporting the potential role of IgA/[EBNA1+VCA-p18] as predictor of progression or survival.

MATERIALS AND METHODS

This preliminary prospective study was held in Dr Sardjito Hospital Yogyakarta Indonesia from January, 2007 to October, 2010 and included all newly diagnosed, locally advanced NPC. Diagnosis of NPC was confirmed by histology examination and clinical staging was performed by a Multi Slice Computed Tomography scan of head and neck region for primary tumor, by abdominal ultrasonography, chest x-ray and skeletal survey for detecting metastases. Patients with stage III, IVA and IVB as designated by American Joint Commitee of Cancer (AJCC), 7th edition, aged above 18 year-old and have performance status of WHO 0, 1, 2, 3, were included in this study. Patients had normal complete blood count and blood chemistry results as requirements to receive chemoradiation with weekly low dose cisplatin (40 mg/m^2) for 8 cycles concurrently with radiotherapy for 70 Gy in 35 fractions. Hemoglobin was > 10 g/dl, white blood cell (WBC) count > 4,000/L or absolute neutrophil count > 1,500/L, platelet > 100,000/mmk and creatinin clearance ≥50 ml/mnt. Alanine aminotransferase (ALAT) or aspartate aminotransferase (ASAT) ≤ 2 × upper limit of normal and bilirubin ≤ 2 × upper limit of normal.

Patients who received less than 80% of planned treatment, patients with severe infection or co-morbid ilnesses were excluded from the study. Plasma samples for IgA/[EBNA1+VCA-p18] ELISA were taken at pre-treatment, at the time of tumor assessment (12-weeks post-treatment) and at 12 months after treatment completion, or at the time of disease progression, whichever came first. Treatment responses were assessed at 12-weeks after treatment completion. Responses were catagorized as: complete response (CR) = disappearance of all target lesions (any pathological lymph nodes must have reduction in short axis to < 10 mm), partial response (PR) = at least a 30% decrease in the sum of diameters of target lesions, stable disease (SD) = neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD and progression disease (PD) = at least 20% increase in the sums of diameters of the target lesions, according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria version 1.1.

IgA/[EBNA1+VCA-p18] ELISA

The method was described in previous publication (Fachiroh et al., 2006) by the use of EBNA1 and VCA-p18 peptides (Cyto-Barr, Zuidhorn, The Netherlands, kindly provided through KWF funding) in one ELISA well. All OD_{450} values were normalized by subtracting the value for 1:100-diluted EBV-negative sera used in duplicate in each ELISA run. The receiver operating characteristic analysis was done to predict the cut-off value of IgA, giving best sensitivity and specificity to predict progression (Fachiroh et al., 2006).

Statistical analysis

IgA/[EBNA1+VCA-p18]-level at pre-treatment, at 12-weeks post-treatment, and at 12-months post-treatment or at the time of disease progression were calculated as mean of OD_{450} ± standard deviation and grouped according to the treatment responses. The difference of mean IgA according to treatment response were analyzed with student t-test. Association of IgA reactivity and treatment response were analyzed with χ^2 test. Kaplan-Meir plots of overall survival and event-free survival were established for patients group of different serological groups. Log rank tests were performed to assess survival probabilities between patients subsets (high risk serological group versus low risk).

Ethics

The study protocol was reviewed and approved by the institutional review board of the Faculty of Medicine, Universitas Gadjah Mada, and all patients were required to fill in written informed consent before participation.

Table 1. Characteristic of subjects.

Character	N (%)
Sex	
Male	32 (69.6)
female	14 (30.4)
Mean of age: 45.2 year-old ± 12.3	
< 45 year-old	19 (41.3)
≥ 45 year-old	27 (58.7)
Clinical Performance	
0, 1	17 (37)
2	24 (52.2)
3	5 (10.9)
Histology type	
WHO 2	3 (6.52)
WHO 3	43 (93.5)
Tumor size	
T1	5 (10.9)
T2	13 (28.2)
T3	15 (32.6)
T4	13 (28.2)
Lymphnodes metastasis	
N0	10 (21.7)
N1	10 (21.7)
N2	11 (23.9)
N3	15 (32.6)

RESULTS

Characteristic of subjects

Forty six patients were eligible for this study as shown as Table 1. Most of them were men at productive age, with moderate performance status. Patients were characterized by larger tumor size (60.8%) and extensive neck lymphnodes involvement (56.5%). Ninety five percent subjects completed their treatment according to protocol. Pre-treatment serology data were available from all subjects, while in post treatment, serology data was missing for 13.3% patients due to early progression before the scheduled sample collection. Treatment response could be assessed in 95.6% patients. Twenty one patients achieved complete response (47.7%), 19 patients were in partial response (43.2%), 2 patients were stable (4.5%), and 2 patients (4.5%) were in disease progression. A follow-up was done during 36 months. Median time to progression was 10.81 ± 11.8 months, with 27 events of death or progression during follow up.

Serology test IgA/[EBNA1+VCA-p18] reactivity and treatment response

Dynamic fluctuation of plasma IgA/[EBNA1+VCA-p18] levels was observed during follow up. Patients with complete response had lower pre-treatment OD_{450} IgA/[EBNA1+VCA-p18] than those with partial response or stable disease; but did not reach statistical significance (1.85 ± 1.18 versus 2.40 ± 0.98, p = 0.113; 95% CI: 1.22 to 0.135). Patients with complete response had more often declined post-treatment plasma IgA/[EBNA1+VCA-p18] levels than other groups (χ^2 = 12.25; p = 0.016). Pre-treatment plasma IgA/[EBNA1+VCA-p18] were higher in those who had disease progression or who died compared to those who achieved good clinical response (OD_{450} = 2.33 ± 0.2 versus 1.66 ± 0.27; p < 0.05) as shown in Figure 1.

Pre-treatment IgA/[EBNA1+VCA-p18] plasma as prognostic marker of disease progression

To determine the OD_{450} level that can define progression risk, a calculation based on receiver operating characteristic (ROC) was done and cut-off value for IgA/[EBNA1+VCA-p18] was determined at OD_{450} 1.44 with 81.3% of sensitivity and 58.9% of specificity. Kaplan-Meir analysis was performed to estimate survival difference based on high risk serology (IgA/[EBNA1+VCA-p18] OD_{450} ≥1.4) and low risk serology (OD_{450} < 1.4). Twenty seven of 46 NPC patients (58.1%) had disease progression or died during follow-up. The estimation of survival difference by serology yielded 30 patients at high risk and 16 at low risk. Disease progression or death were more frequent in high-risk serology group (22 of 30 subjects or 73%), with median time to progression of 13.5 month. In low risk serology group (n = 16), 5 patients had progression (31.3%) while median time of progression was not reached. There were significant differences in progression free-survival (PFS) according to serology risk (p = 0.014) as shown in Figure 2A. In high risk serology group, median overal survival (OS) was 17.3 month, whereas in low risk serology group, median OS was not reached (p = 0.114) as shown in Figure 2B.

Serology IgA/[EBNA1+VCA-p18] changes and disease relapse

The changes of serology level in this study failed to indicate a difference between those who had poor outcome (disease progression or relapse) and good outcome (remission and stable disease, without progression). During follow-up, patients with disease progression showed elevated serology, with mean elevation of OD_{450} 0.73 ± 1.00; whereas those without progression had mean

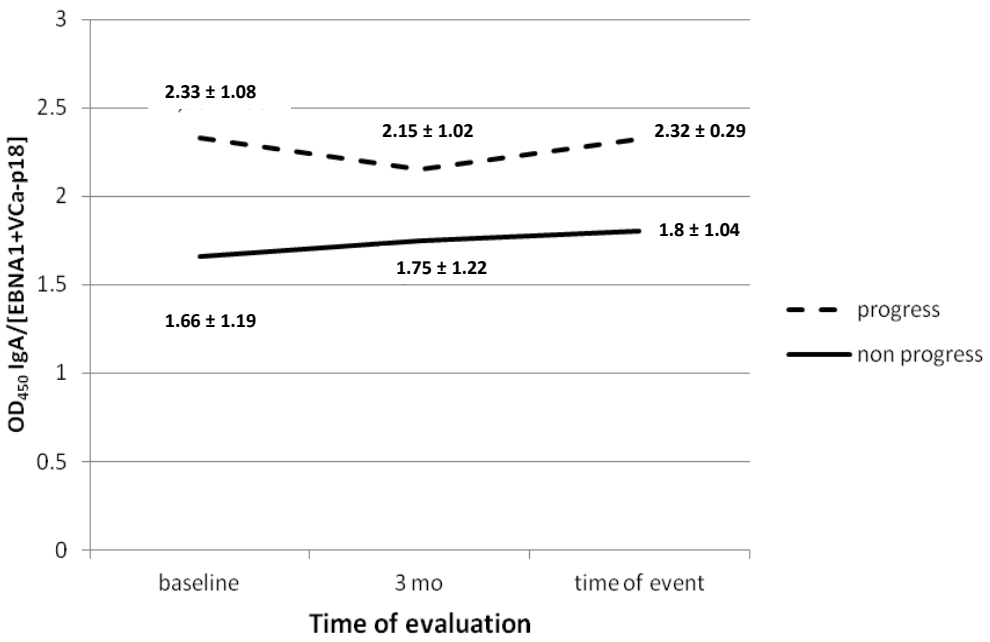


Figure 1. Dynamic changes of OD $_{450}$ plasma IgA/[EBNA1+VCA-p18] based on time of evaluation between NPC groups that progressed (n=27) and non progressed (n=19). Evaluation was performed at baseline (pre-treatment), 3-month or 12-week post-treatment, and at 1-year post-treatment or at the time of disease progression ($p<0.05$).

mean elevation OD_{450} 0.50 ± 0.89 ($p = 0.49$; 95% CI: 0.45 to 0.90). Eighteen out of 28 patients (64.3%) with stable or elevated IgA within 1 year had disease progression; whereas 2 out of 7 patients (28.6%) with decreased IgA during the same period had progression ($X^2 = 3.85$; $p = 0.14$).

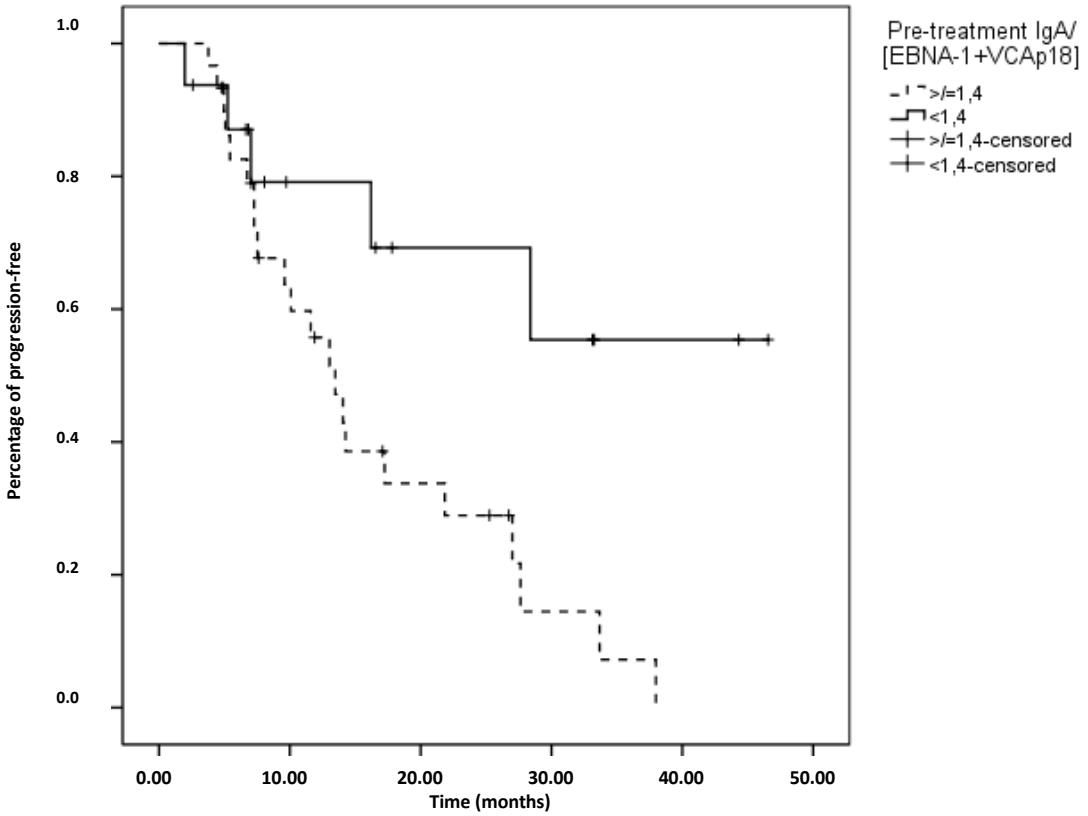
DISCUSSION

People infected with EBV will develop specific antibody response including IgM against viral capsid antigen (VCA) during acute primary infection, followed by IgG against VCA and EBV nuclear antigen 1 (EBNA1) that persist for life (Tsuchiya, 2002; Hess, 2004). Aberrant level of antibody response against EBV has been evident in various EBV-related malignancies (Tao et al., 2006). Nasopharyngeal cancer patients often shows increase in antibody response of IgA and IgG against VCA, EA, EBNA1 and transcription activator Zta and Rta, as well as other EBV lytic cycle protein (Henle and Henle, 1976; Fachiroh et al., 2004). Elevation of antibody responses to EBV may precede onset of clinical manifestation of NPC by 1 to 5 year (Yip et al., 1994; Ji MF et al., 2007). Combined EBV serological biomarkers could improve diagnostic value of NPC (Neel and Taylor, 1990; Fachiroh et al., 2006; Liu et al., 2012; Ai et al., 2013; Chang et al., 2013).

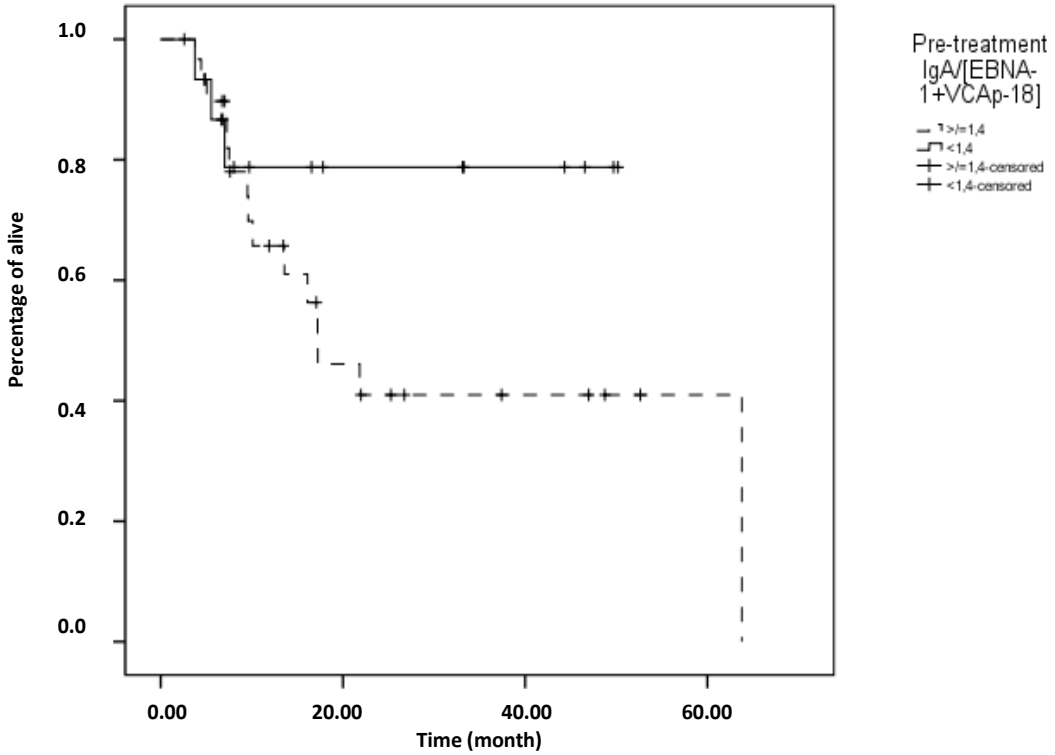
Furthermore, dynamic fluctuation of antibody level after treatment of NPC raised the possibilities of humoral response to be used as prognostic marker (Yip et al., 1994). Specific antibody responses to EBV proteins have become a powerful tool to detect reactivation of this virus in human body. Previous studies reported various serological biomarkers as prognostic factors with inconsistent results (Karray et al., 2005; Liu et el., 2004; Neel and Taylor, 1990; Yip et al., 1994; de Vathaire et al., 1988). Fan et al. (2004) reported the use of IgA early antigen (EA) serology to predict post treatment outcome. IgA/EA was still detected in 44% of patients and IgG/EA was detected in 94% NPC patients in remission; whilst EBV DNA became undetectable during remission.

This led to conclusion that the role of EA serology was less important than viral EBV DNA load (Liu et al., 2004; Twu et al., 2007). Similar findings was reported by Adham et al. (2013) among NPC patients in Indonesia. Adham et al. (2013) reported that there was no significant reduction at 2-months post-treatment of IgA EBV either IgA EBNA1 or IgA VCA-p18.

On the contrary, Ling et al. (2009) reported that pretreatment IgA/VCA serology test had prognostic value. Patients with higher IgA/VCA level had shorter survival. In this study, pre-treatment IgA/[EBNA1+VCA-p18] serology level could discriminate risk for progression. To our knowledge, there had not been a report of IgA/[EBNA1+VCA-p18] as a prognostic marker of NPC. This preliminary finding may help us to adjust treatment plan for high risk group for recurrence or progression. Higher dose of chemoradiation may be recommended to NPC patients with higher level of pre-treatment



(A)



(B)

Figure 2. Survival function based on pre-treatment OD 450 IgA/[EBNA1+VCA-p18] groups (low risk OD450 <1.4 vs. high risk OD450 ≥ 1.4). (A) is progression free-survival and (B) is overall survival.

IgA/[EBNA1+VCA-p18]. This study measured combined EBV serological biomarkers for prognostic use, added to its diagnostic capacity (Fachiroh et al., 2006; Ai-di et al., 2009). Only pre-treatment serology IgA/[EBNA1+VCA-p18] showed prognostic role for progression. Elevation of IgA/EBV titer within 1-year post-treatment tend to be followed by disease progression, however, this did not reach the statistical significance. Some patients had disease progression or died before the serology test (missing post-treatment serology). Hence it may contribute to this result. Another possible explanation for incapability of post-treatment IgA/EBNA as prognostic factor was that EBV might be harbored not only in nasopharingeal tumor cells, but also in activated infiltrating T-cell, B lymphocytes and epithelial cells which are able to produce EBV-related antigen. This may result in elevated IgA/EBV even after remission of disease.

Our unpublished data showed that IgA/[EBNA1+VCA-p18] level was not an independent prognostic factors for survival because of its dependency on patient's clinical performance. The prognostic role of serology IgA/[EBNA1+VCA-p18] to predict progression was strong enough (HR 3.19; 95% CI: 1.09 to 9.37; p = 0.03) without adjusting to clinical performance. Clinical performance of the patients declined continously even after treatment cessation due to difficulty in swallowing. Poor nutrition status affects the overall health status and furthermore may impair immune response. Antibody response to EBV antigen may decline after treatment and raise at the time of progression (Ai-di et al., 2009) but unfortunately this dynamic fluctuation is obscured by the declining of clinical performance, immunity and nutritional status.

Immunocompromised host responded inadequately to antigens so the reactivation of EBV might not be detected by measuring antibody response in this situation (Verschuuren et al., 2003). This may explain that only pre-treatment IgA/[EBNA1+VCA-p18] has a role to determine risk for progression. Post-treatment IgA-EBV serology failed to determine prognostic difference among NPC patients. This is consistent with the previous studies done by Adham et al. (2013) and Ai et al. (2013). Direct quantification of viral EBV DNA, therefore, might lend a hand to support the notion that viral reactivation play important role in disease progression and survival (Hassen et al., 2011; Wang et al., 2013; Yip et al., 2014). EBV DNA load from nasopharyngeal brushings and whole blood showed significant reduction at 2-month after treatment, which was not reflected by EBV-IgA serology (Adham et al., 2013).

Conclusion

Pre-treatment serology IgA/[EBNA1+VCA-p18] can predict progression of NPC. High risk serology group (OD_{450} IgA > 1.4) progresses earlier. The role of IgA/[EBNA1+VCA-p18] to replace EBV DNA load for disease monitoring is less convincing.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effects of omega-3 fatty acids against Ehrlich carcinoma-induced hepatic dysfunction

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Omega-3 essential fatty acids (ω-3 FATs); found in the highest concentrations in fish oil, claim a plethora of health benefits. The present study aims to evaluate the biological effects of ω-3 FATs supplementation against Ehrlich carcinoma (EC) induced inflammation, oxidative stress, biochemical and histopathological alterations in the liver tissue of albino mouse. ω-3 FATs were orally administered via gavage to mice for a period of 30 consecutive days at a dose of 300 mg/kg body weight. On the 7th day of ω-3 FATs administration, female mice were subcutaneously injected with 0.2 ml of Ehrlich ascite carcinoma for solid tumor induction. The present study revealed that, subcutaneous injection of Ehrlich solid tumor led to hepatic oxidative stress (as significant increase in lipid peroxidation (thiobarbituric acid reactive species, TBARS), concomitant with a significant decrease in glutathione and antioxidant enzymes), systemic inflammation (significant increases in C-reactive protein, tumor necrosis factor-alpha and leukocyte counts) and biochemical alterations (as increase in liver function enzymes)). While in the tumor tissue, significant increase in tumor TBARS content and non significant changes in glutathione and antioxidant enzymes were observed. Histopathological studies showed that EC cells metastasis caused fatty degeneration, enlargement of liver cells nuclei and presence of necrosis. Pretreatment of animals with ω-3 FATs significantly reduced tumor size and markedly improved most of the biochemical parameters associated with the inoculation of EC. It could be concluded that ω-3 FATs administrated to mice, reduce tumor size, inhibit systemic inflammation, improving liver function profile, modulating lipid peroxidation and augmenting antioxidant defense system in EC bearing mice.

Key words: Ehrlich carcinoma (EC), omega-3 fatty acids, liver.

INTRODUCTION

Malignancy is one of the most serious diseases afflicting mankind today. The cancer burden in developing countries is increasing as a result of increasing ageing and growth population, and adaptation of cancer-associated lifestyle choices including smoking, physical inactivity and 'Westernized diet' (Preetha et al., 2008). Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Mantovani, 2005). Chronic inflammation and the metabolic products of phagocytosis are often accompanied by the excessive formation of reactive oxygen and nitrogen species that are potentially damaging to DNA, lipoproteins, and cell membranes (Schottenfeld and Beebe-

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Dimmer, 2006). Inflammatory cells also release metabolites of arachidonic acid, or eicosanoids, including prostanoids or prostaglandins and leukotrienes (O'Byrne and Dalgleish, 2001). Animal bearing experimental Ehrlich carcinoma (EC)revealed low activity of antioxidant system (Bhattacharya and Haldar, 2011). In addition, free radicals, particularly oxygen radical, play an important role in the complex course of multistep carcinogenesis (Marnett, 2000). First, an oxidative stress can induce DNA damages that lead to genomic instability and possibly stimulate cancer progression (Oberley, 2002). Secondly, elevated reactive oxygen species (ROS) levels are responsible for constant activation of transcription factors and the progression of the disease (Gupta et al., 1999).

Resistance to conventional anticancer therapies in patients with advanced solid tumors (Patyar et al., 2010); in addition to the side effects that patients experience from conventional chemotherapy, number of studies demonstrated that oils rich in omega-3 essential fatty acids (ω-3 FATs) decrease the tumor weight and metastasis number (Espada et al., 2007). ω-3 FATs; found in the highest concentrations in fish oil, claim a plethora of health benefits. Fish oil-derived ω-3 FATs seem to prevent cancer by influencing the activity of enzymes and proteins related to intracellular signaling and, ultimately, cell proliferation (Bartsch et al., 1999).

These fatty acids are known to have pleiotropic effects, including effects against inflammation (Browning, 2002), platelet aggregation and hyperlipidemia (Hu, 2001).

The main purpose of the current study is to explore the major complications that occur due to experimental EC implantation in female mice, and to explore the potential role of ω-3 essential fatty acids for prevention of tumor progression and disturbances induced in the antioxidants status and metabolic profile.

MATERIALS AND METHODS

Because Ehrlich ascites carcinoma (EAC) cells were reported to show greater initial growth and total cell count in female than male mice (Vincent and Nicholls, 1967), the present study used female mice as experimental subjects. Adult female Swiss albino mice weighing 22 to 25 g purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Cairo) were used in this study. The animals were maintained on a commercial standard pellet diet and tap water *ad libitum*. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All procedures described were reviewed and approved by the University Animal Ethical Committee.

ω-3 FATs treatment

ω-3 FATs capsules were purchased from Kirkland Signature Company (Kirkland, Washington, USA). The product is supplied as transparent soft-gelatin capsules filled with light-yellow oil tablets of 300 mg ω-3 FATs (eicosapentaenoic acid (EPA): 180.0 mg/g and docosahexaenoic acid (DHA): 120.0 mg/g). It was suspended in doses of 300 mg/kg body weight/day during 30 successive days(An et al., 2009).

Tumor transplantation

A cell line of EAC supplied through the courtesy of Dr. Gklien, Amsterdam Holland was maintained in experimental female Swiss albino mice by weekly intraperitoneal injection of 2.5×10^6 cells per mouse (El-Gawish, 2003). The solid form was done by inoculating 2.5×10^6 cells per mouse subcutaneously between scapulae in the neck region. After 7 to 8 days from EAC cell inoculation, solid tumor was observed.

Experimental design

Healthy Swiss albino mice were randomly assigned into four experimental groups (10 mice/group), which were classified as follows: control group (1), the mice in this group were orally administered 1 ml water/mouse daily and served as control group for one months; EC group (2), each mouse in this group was injected subcutaneously (SC) in the neck region with 0.2 ml of EAC which contained 2.5×10^6 cells for solid tumor induction; ω-3 FATs group (3), the mice in this group were orally treated with omega 3 (300 mg/kg body weight) daily for 30 days from the 1st day of the experiment; ω-3 FATs + EC group (4), the mice in this group were orally treated with ω-3 FATs (300 mg/kg body weight) daily for 30 days, on day 7 each mouse was injected subcutaneous with 2.5×10^6 EAC cells for solid tumor induction.

Monitoring of tumor size

The effects of the ω-3 FATs on tumor growth were evaluated through monitoring of tumor growth for each experimental group. Tumors were measured individually using a caliper. Tumor size was determined by the following formula (Jia et al., 2005):

$$\text{Tumor size} = \text{length} \times \text{width}^2 \times 0.52$$

After 24 h of the last dose of ω-3 FATs treatment and 16-h fasting, animals of each group were sacrificed. Blood samples were collected and serum obtained by centrifugation at 3000 rpm for 10 min for biochemical analysis. Samples of liver and tumor tissue were excised. Parts of the excised liver and tumor tissues were used for the histopathological examination, while the other part was used for the biochemical analysis.

Biochemical assays

The activities of aspartate and alanine transaminases (AST and ALT) were assayed by the kinetic method using available commercial kits (Spinreact, Spain) according to the method described by Young (2001), while alkaline phosphatase (ALP) activity was assayed depending on the method of Roy et al. (1970) using Biodiagnostic kit. The activities of lactate dehydrogenase (LDH) in serum were assayed by the kinetic method using available commercial kits (Spinreact, Spain) according to the method described by Young (2001). The levels of tumor necrosis factor-alpha in serum were assayed by the standard sandwich enzyme-linked immune-sorbent (ELISA) assay technique using ELISA kit (K0331186, KOMABIOTECH, Seoul, Korea) following the manufacturer's instructions. The levels of Serum C-reactive protein (CRP) were assayed by the latex-agglutination test described by Hanson et al. (1997). The white blood cells (WBCs) count was determined according to the improved Neubauer method using the haemocytometer.

Lipid peroxides, measured as thiobarbituric acid reactive species (TBARs), mainly malondialdehyde (MDA) were measured according warm distilled water and administered via gavages to the mice at

Table 1. Effect of ω3 FATs administration on serum tumor necrosis factor alpha (TNFα), reactive protein (CRP) levels and total leucocytes count (TLC) in different animal groups.

Group	TLC (10^3/ml)	TNF-α (Pg/ml)	CRP
Normal control	7.15±0.11	86±4.95	>6
EC	18.08±0.47[a]	223±8.13[a]	18[a]
ω-3	7.37±0.19[b]	71±1.63[b]	>6
ω-3+EC	12.7±0.44[ab]	76.75±3.64[b]	>6

Each value represents the mean of 6 records ± SE. [a]Significant differences versus normal control group. [b]Significant differences versus Ehrlich carcinoma (EC) bearing animals group.

Table 2. Effect of ω3 FATs on liver function enzymes of control or Ehrlich carcinoma (EC) bearing mice.

Group	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)
Normal control	59.0±3.07	34.5±2.45	78±6.78	336±14.75
EC	160±6.79[a]	94±4.18[a]	136±5.28[a]	490±5.89[a]
ω-3	63±2.62[b]	35±2.45[b]	78±4.58[b]	355±14.29[b]
ω-3+EC	126±8.14[ab]	66±5.21[ab]	105±9.18[ab]	439±13.44[ab]

Each value represents the mean of 6 records ± SE. [a]Significant differences versus normal control group. [b]Significant differences versus Ehrlich carcinoma (EC) bearing animals group.

the reported methods of Yoshioka et al. (1979). Reduced glutathione (GSH) were determined according to the method of Beutler et al. (1963). Catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined according to the method of Sinha (1972), Masayasu and Hiroshi (1979) and Gross et al. (1967), respectively.

Histopathological studies

Parts of the excised liver and tumor tissues were fixed in 10% formalin for 48 h, then transferred to 70% ethyl alcohol processed and embedded in paraffin blocks. Sections of 5 to 6 µm thickness were stained with haematoxylin and eosin (H&E) for histopathology examination.

Statistical analyses

All values are presented as mean ± standard error of mean (SEM). All groups were compared by one-way analyses of variance (ANOVA) and post hoc multiple comparisons were done with Duncan test in SPSS/PC software program (version 12.0; SPSS Inc., Chicago, IL, USA) to determine the differences in all parameters. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

EC size monitoring is illustrated as shown in Figure 1. It is clear that the inoculation of 2.5 millions of EC cells in 2 ml physiological saline in the neck region of healthy normal mice produced a tumor with a mean size of 332.8±8.83 mm^3 on the 7th day after tumor inoculation (ATI). EC size exceeds 500 mm^3 on the 10th day ATI. The increase in size of EC proceeds by days reaching 3300±194.7 mm^3 on the 30th days ATI.

Gavages of the experimental animals with ω-3 FATs (0.3 g/kg body weight/day) 7 days before EC tumor cell inoculation, recorded 18.72±1.26 mm^3 EC tumor size on the 7th day ATI and reaching 165±10.73 on the 30th days ATI. The percentage of growth inhibition in the 7th days ATI was 94.375% and the percentage of growth inhibition on the 30th days ATI was 95%

Effect of ω-3 FATs administration to mice on biochemical parameters

Table 1 revealed that subcutaneous injection of EC cells on the back of neck region of female mice produced a significant increase ($P<0.001$) in total leucocytes count (TLC) by 153%, increase serum TNF-α level by 159.3%, and remarked increase in serum CRP level significant in comparison with normal control group values. Treatment of the experimental mice-bearing EC with ω-3 FATs produced a significant decrease in TLC by 29.77%, decrease in serum TNF-α level by 65.58% and great reduction in the CRP level in comparison with EC bearing group.

EC has been found to cause abnormalities in liver function as shown by the significant increase in the activity of serum enzymes. The results demonstrated in Table 2 showed that the EC animal bearing group exhibited a significant increase in serum activity of ALT, AST, ALP, and LDH compared to that of the normal control group. After administration of ω-3 FATs at the dose of 300 mg/kg body weight to EC bearing mice significantly reduced serum activity of liver function enzymes were recorded in comparison with EC control group.

Table 3 showed that EC inoculation; significant increase

Table 3. Effect of ω3 FATs on TBARS levels and antioxidant status of liver tissue in control or Ehrlich carcinoma (EC) bearing mice.

Group	TBARS (umol/g)	GSH (GSH/g)	CAT (umol/min/g)	SOD (ug/mg)
NC	152±17	38.80±3.96	152.33±6.74	5.70±0.46
EC	180±19[a]*	32.83±2.13[a†]	140.17±6.11[a†]	5.06±0.33[a‡]
ω-3	135±18[b‡]	37.93±2.92[b†]	159.00±5.55[b‡]	5.58±0.31[b†]
ω-3+EC	154±14[b]*	36.20±1.77	144.50±4.18[a]*	5.47±0.20[b]*

Each value represents the mean of 6 records ± SE. [a]Significant differences versus normal control group. [b]Significant differences versus Ehrlich carcinoma (EC) bearing animals group.

Table 4. Effect of ω3 FATs on TBARS levels and antioxidant status of EC tumor tissue in mice bearing Ehrlich carcinoma (EC).

Group	TBARS (umol/g)	GSH (GSH/g)	CAT (umol/min/g)	SOD (ug/mg)
EC	99±1.70	18±0.37	92±2.48	4.08±0.16
ω-3+EC	127±1.70[b‡]	17±0.43[b‡]	84±2.80[b‡]	4.65±0.26[b‡]

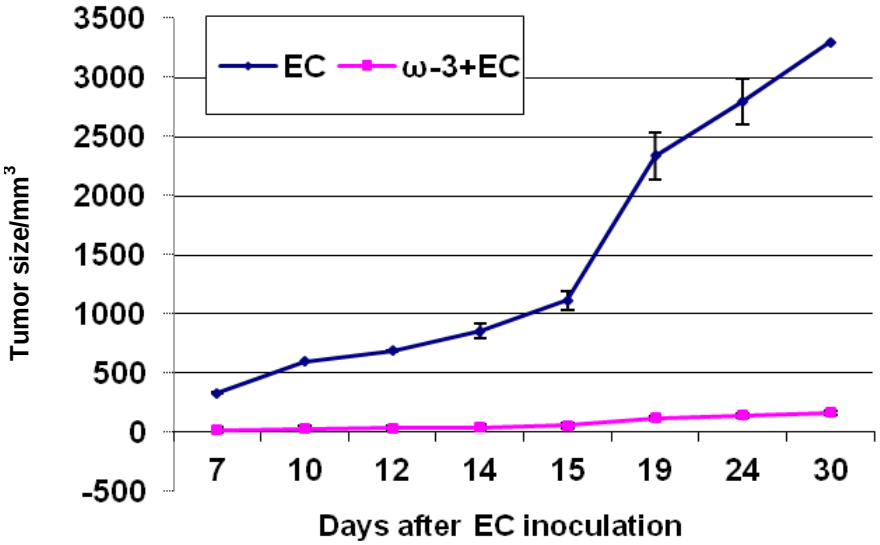

Figure 1. Effect of ω3 FATs on tumor size of Ehrlich carcinoma.

increase (P<0.05) in liver TBARS level by 18.4%, meanwhile, a significant decrease in liver GSH, GSH-Px, CAT and SOD content by 15.37, 14.44, 8 and 11.14%, respectively, were observed in comparison with the normal control values (Table 3). Treatment of animals with ω-3 FATs at the dose of 300 mg/kg, increased hepatic antioxidant enzymes compared to that of EC control group. The treatment of EC-bearing mice with ω3 FATs was more effective in restored and corrected the biochemical parameters level (Table 3).

Treatment of the experimental mice-bearing solid EC with ω3 FATs produce significant increase (P<0.05) in tumor TBARS and SOD content by 28.28 and 13.97%, respectively, against EC-bearing group. Meanwhile, a significant decrease in tumor GSH, GSH-PX and CAT content was observed by 5.68, 17.65 and 8.57%, respectively in comparison with EC-bearing group (Table 4).

Histopathological changes in EC tumor tissues and liver as effected by ω-3 FATs treatment.

EC tumor tissue

Histopathological examination of the EC tumor under

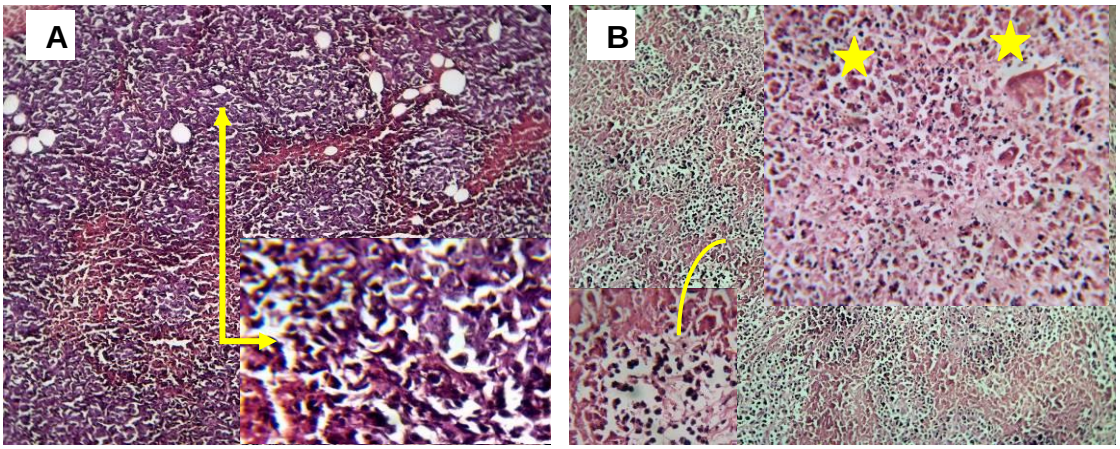

Figure 2. Photomicrograph represents subcutaneous solid Ehrlich carcinoma in mice.
(A): Control solid Ehrlich carcinoma. (B): Solid Ehrlich carcinoma in mice treated by ω3 FATs. (H & E x100-Encircled parts x400).

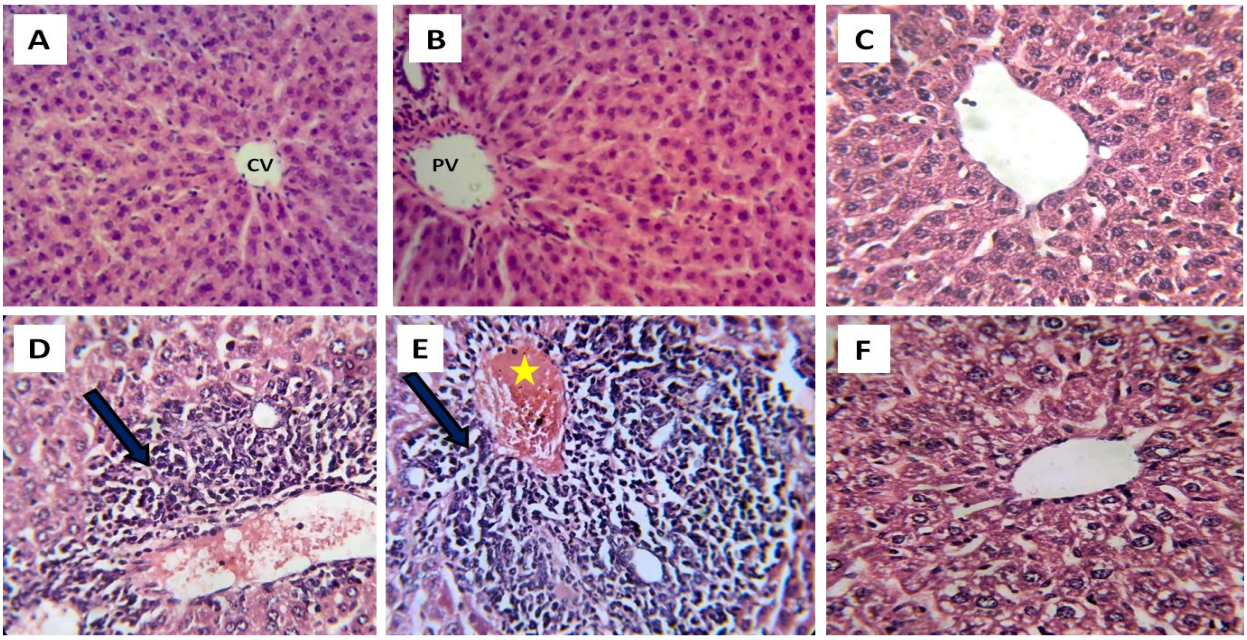

Figure 3. Photographs of sections in liver of mice.
A, B: Control sections showing the normal appearance of hepatocytes and the central vein (CV) in A and portal vein (PV) in B. C: Normal appearance in ω-3 treated group. D, E: Liver sections of mice bearing EC represented accumulation of EC cells (blocked arrow) around a congested portal blood vessel (star) with completely haemolysed RBCs. F: Liver tissue represents great disappearance of metastatic ECs in ω3 FATs treated group. (H&E stain × 400).

Light microscope showed compactness and aggregation of the tumor tissue cells spread subcutaneously within the soft tissues in the neck region. EC tumor showed groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei and binucleation. Several degrees of cellular and nuclear pleomorphism were seen (Figure 2A).

Treatment of female mice bearing EC tumor by ω-3 FATs recorded great destruction of tumor tissue represented by the appearance of dead (arc) and necrotic cells (star) (Figure 2B).

Liver tissue

Normal histological pattern of the control liver of young mice is as shown in Figure 3A and B. Normal central vein (CV) is surrounded by radiating cords of hepatocytes with prominent Kupffer cells. Normal sinusoidal spaces, branches of the hepatic portal veins (PV) and branches of the hepatic arteries and bile ducts could also be noticed. Treatment of female mice with ω-3 FATs represented a normal appearance of liver tissue section (Figure 3C).

The liver sections of mice bearing EC showed

accumulation of EC cells around congested portal blood vessels with completely haemolysed red blood cells (RBCs) in the portal vein (Figure 3D and E). Treatment of the experimental mice-bearing solid EC with ω-3 FATs revealed great disappearance of metastatic EC cells (Figure 3F) from the liver tissue. Some hydropic degeneration in hepatocytes cytoplasm and increase in Kupffer cells were also detected.

DISCUSSION

Two major limitations are known to negatively affect the utilization of anticancer agents for therapy; firstly, the lack of selectivity for tumor cells and the second cytotoxicity of these substances, especially when applied in high doses. Implantation of EC tumor into female Swiss albino mice has been proved to induce oxidative stress provoking oxidative damage, organ dysfunction and metabolic disturbances. Oxidative stress is a main mediator in ROS-induce liver dysfunction and has been implicated in many pathological conditions, including, DNA damage, tumor promotion and cancer. Cytotoxic ROS were identified in tumor cells and are possibly associated with depletion of antioxidant enzymes (Yamamoto et al., 2003). Some polyunsaturated fatty acids (PUFAs) exert a selective cytotoxic or anti-proliferative effect on tumor cells rather than on normal cells (Judé et al., 2006).

The results obtained in the present study showed that, the serum levels of ALT, AST, ALP and LDH increased in mice bearing-EC. The elevation in liver enzymes of mice treated with EC has already been reported in earlier studies and is still consistent with more recent results (Sakr et al., 2011). Most experimental solid tumors have elevated levels of ROS and consequently oxidative stress (Maeda and Akaike, 1998). Oxidative stress has been suggested to represent an important contributory factor to liver injury, and to enhanced morbidity related to liver hypo-function (Siddique et al., 2004). Therefore, accumulation of ROS and GSH depletion in tumor cells could induce unsaturated fatty acid to undertake lipid oxidation, thereby disordering the transport and storage of Ca^{2+} in mitochondria, endoplasmic reticulum and cell membrane, increasing the content of plasmic Ca^{2+}, and ultimately causing death of cell. ALT, AST, ALP and LDH are then released into the blood (Hua and Ya-Wei, 2005; Wallace, 2007).

LDH activity was inhibited in tumor group due to increase in inflammatory cells which caused reduction in protein level (Saad-Hossne et al., 2003) or due to hepatotoxicity (Kalapos et al., 1993) or the other possible reason for elevated levels of LDH may be due to higher glycolysis in cancerous conditions (Al-Jasabi et al., 2013).

Treatment with ω-3 FATs caused improvement in which ameliorating hepatic enzyme levels which related to the free radical scavenging activity, so ω-3 FATs protect liver membrane from free radicals and decrease lipid peroxidation level, as a result of that, LDH was prevented from releasing outside the cell (Schmidt et al., 2005).

The results of the current study indicated that treatment of the experimental mice-bearing EC with ω-3 FATs produced significant increase in tumor TBARS and SOD content associated with a significant decrease in tumor GSH, GSH-PX and CAT content in comparison with EC-bearing group. In the present work, the increase of TBARS level in tumour tissue might be attributed to the deficiency of antioxidant defence mechanisms or probably due to the generation of ROS and altered redox statuses which are common biochemical aspects in tumor cells. ROS can react with the PUFAs of lipid membranes and induce lipid peroxidation. Earlier studies observed increased lipid peroxidation and decreased antioxidant levels in the cancer patients (Manju et al., 2002).

Significant elevation in SOD in tumor group may be correlated to the oxidative stress in response to the continual generation of free radicals by the increase of tumor load (Fahim et al., 2003). The significant decrease in GSH level in tumor tissue may be due to lack of amino acids which is used in the making of GSH (Deepa and Varalakshmi, 2003).

Long chain ω-3 PUFAs, are highly susceptible to lipid peroxidation (LPO), because of their double bonds. Hence, their incorporation into phospholipids of cellular membranes may sensitize cells to ROS and thereby induce oxidative stress in tumour cells (Sawyer and Field, 2010).

The results of the current study revealed that subcutaneous transplantation of EC cells on the back of neck region of experimental mice produced a significant increase in liver TBARS level associated with a significant decrease in liver GSH, GSH-Px, CAT and SOD content in comparison with the normal control values. The results obtained support previous findings that EAC has been suggested to cause the generation of ROS resulting in oxidative stress, alter the antioxidant defense system in tissues and cellular injury, which may be one of the factors in the etiology of cancer (Bansal et al., 2005).

Moreover, the inhibition of antioxidants enzyme activity and a reduction in glutathione level as a result of tumor growth was reported (Gupta et al., 2004). This phenomenon could be attributed to the exhaustion of these antioxidants especially glutathione and glutathione containing enzymes in the detoxification of free radicals and peroxides, generated due to the presence of the EC tumor. The decrease in liver SOD activity in EC bearing mice is probably due to loss of Mn^{2+} containing SOD activity in EAC cells and the loss of mitochondria, leading to a decrease in total SOD activity in the liver (Sun et al., 1999). A small amount of CAT in tumor cells was reported (Sun et al., 1999).

Most experimental solid tumors have elevated levels of ROS (Maeda and Akaike, 1998). Thus, in the present study, a significant expected elevation of free radicals in

the tumor group was observed. It has been suggested that ω-3 FATs may stimulate the production of ROS generating cells, such as activated macrophages and T lymphocytes (Avogadri et al., 2008). The prominent antioxidant activity of ω-3 FATs was extensively documented in different experimental situations; it inhibit hepatocellular carcinoma cell growth through blocking β-catenin and cyclooxygenase-2 (Kyu et al., 2009).

In the present study, the presence of EC tumor in female Swiss albino mice was accompanied by a systemic inflammation as manifested by a significant increases in C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α) and leukocyte counts (leukocytosis) reflecting systemic inflammation in the liver. All of these could come from the tumor C-reactive protein (CRP) is an exquisitely sensitive systemic marker of inflammation and liver tissue damage (Kerner et al., 2005). The significant increase in the level of serum CRP might be attributed to EC-induced oxidative damage and increases in inflammatory activity (Hayashi et al., 2001).

Etiologically, leukocytosis is a primary pathological condition affecting the white blood cells, but it frequently arises as a reaction to infection, chronic inflammation and cancer (Ruka et al., 2001). The increase in total leucocytes count (leukocytosis) observed in the present study could be due to induction of solid tumor in mice which was accompanied by marked cellular, molecular, and biochemical changes. Leukocytosis which observed in the current study might be attributed to the decreased significantly number of lymphocytes, or due to lipid peroxidation (Tibaldi et al., 2008).

Significant increases in proinflammatory cytokines (as TNF-α), in the present study might be attributed to the fact that Ehrlich solid tumors can cause a series of deleterious side-effects, including oxidative stress that may triggers lipid peroxidation which in turn initiates release of malondialdehyde and binds to hepatocyte proteins initiating a potentially harmful immune response and stimulate neutrophil chemotaxis or activates transcriptional factor NFκB which in turn increases the production of proinflammatory cytokines as TNF-α, promoting hepatocyte injury and apoptosis and hepatic stellate cell activation (Duvnjak et al., 2007).

In the present study supplementation of mice with ω-3 FATs has significantly ameliorated serum systemic inflammatory level. This could be attributed to the role of ω-3 FATs in EC tumor regression and minimizing EC-induced oxidative injury as well as to its anti-inflammatory effect (Raso et al., 2002). Clinical studies have reported that fish oil supplementation has beneficial effects in rheumatoid arthritis, inflammatory bowel disease, and among some asthmatics, supporting the idea that the ω-3 FATs are anti-inflammatory and immunomodulatory (Philip, 2001). The first mechanism whereby ω-3 FATs can reduce inflammation is by producing metabolites which exert potent anti-inflammatory effects, including resolvins, docosatrienes and protectins (Bazan, 2007; Dyall, 2010). A second mechanism by which ω-3 FATs can reduce inflammation is by lowering the level of omega-6 (ω-6) PUFAs in the brain that can stimulate pro-inflammatory eicosanoid metabolites including prostaglandins, leukotrienes and thromboxanes (Farooqui et al., 2007).

In the present study, treatment of tumor-bearing mice with ω-3 FATs exerted a marked effect in the retardation of tumor growth as compared to tumor bearing mice group. These observations are consistent with the previous findings by Majumder et al. (2006). The increase of tumor size in EC might be attributed to EC-induced oxidative stress (Dwivedi et al., 2007) or due to activation of activate nuclear factor (NF-κB)-induces tumor growth and metastasis and reduces cytokines-induced apoptosis (Takada et al., 2006).

Experimental studies suggested a beneficial role of ω-3 FATs in health, owing to their antioxidant properties, and their ability to modulate the activity of various enzymes (Depasis et al., 2002). ω-3 FATs, especially the eicosapentanenoic acid (EPA) and docosahexanoic acid (DHA), were shown to exert cancer-protective activity. On the other hand, the selectivity of DHA and other PUFAs) on tumor cells has been a worthwhile goal of research. In this context, *in vitro* cell culture studies have led to a consensus that some PUFAs exert a selective cytotoxic or anti-proliferative effect on tumor cells rather than on normal cells (Judé et al., 2006).

Evidence increasingly suggests that ω-3 FATs, particularly eicosapentaenoic acid (20:5n−3) (EPA) and docosahexaenoic acid (22:6n−3) (DHA) are protective against cancer, and the data is the strongest for breast and colon cancer. These protective effects are mediated by a variety of different mechanisms, including the incorporation of ω-3 FATs into cell membranes, which changes membrane fluidity, may affect the association of proteins within cell membranes (Wang et al., 2000), and/ or may initiate different signal-transduction processes. ω-3 FATs have also been shown to decrease cell proliferation and/or increase apoptosis during the tumorigenesis process and modulating angiogenesis (Szymczak et al., 2008).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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