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IN VIVO AND IN VITRO EFFECT OF CELEBREX ON EHRLICH ASCITES CARCINOMA CELL LINE

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ABSTRACT

Evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandin (PG) synthesis can reduce the incidence of cancers via its antioxidant activity. Celebrex (celecoxib), a cyclooxygenase-2 (COX-2) inhibitor, is a NSAID with known antioxidant and antineoplastic activity. The present study has been carried out to investigate the chemopreventive effects of Celebrex on tumor development incidence and cytotoxicity on Ehrlich ascites carcinoma cell line and bearing mice. Celebrex was given orally in a dose 30 mg/kg. Antioxidant parameters were studied in blood samples and liver tissue, whereas immune evaluations were performed in spleen tissue samples. Results show the ameliorative effect of Celebrex on antioxidants status and immunity system of the treated mice.

Keywords: Celebrex, In vivo, In vitro, Tumor, Cytotoxicity, Ehrlich ascites, Immune System

INTRODUCTION

Cancer is one of the most complex diseases in the world (Devi et al., 2015). It is initiated with a series of genetic and epigenetic alterations causing formation of abnormal cell populations (López-Lázaro, 2018). Inflammation has become a promising target for cancer prevention and treatment. Among various inflammatory factors, cyclooxygenase 2 (COX-2) is the most commonly studied anti-inflammatory/anticancer target (Li et al., 2018). The cyclooxygenases (COX) are enzymes responsible for the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2) as the common precursor molecule to all prostaglandins which plays a vital role in multiple physiologic and pathologic processes and subsequently converted to a number of eicosanoids, including PGD2, PGE2, PGF2a,

PGI2, and thromboxane (Hassanzade et al. 2018).

There are two major isoforms of COX which are COX-1 and COX-2. COX-1 is substantial expressed in most tissues, whereas COX-2 is usually caused by several physiologic stimuli. It has been shown that PGE2 and PGI2 are mainly derived from the COX-2 pathway (Thomas and Morton, 2017). COX-2 is an important inflammation factor where it increased at the inflammation sites and in several types of cancers (Liu et al., 2015 and Tupáa et al., 2019). Therefore, it is a vital therapeutic tool in treatment of cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of analgesic anti-inflammatory compounds acts on COX inhibition (Szabo-Revesz, 2018). The anti-tumor effects of NSAIDs have been documented in a variety of cells and cell lines, including colon, breast cancer cells, and fibroblasts via both COX-dependent and COX-independent mechanisms NSAIDs have also been shown to be powerful radical scavengers (Polychronisa et al., 2019). Celecoxib (Cxb) is one of a medication in NSAIDs class drugs, which has a low water solubility, and variable oral absorption (Van Nguyen et al., 2016). It reduces the prostaglandins synthesis through specific inhibition of the COX_2 enzyme and has been used for the treatment of various chronic musculoskeletal conditions, acute pain, dysmenorrhea, colorectal polyps (Safaeian et al., 2018) and was used in breast cancer remedy and acute promyelocytic leukemia (Uner et al., 2019). It has, therefore, been suggested that many anti-inflammatory drugs might exert part of their action by scavenging oxidants (Muller, and Wurl, 1987). In cancer cells high ROS levels can originate from increased oncogene activity, cyclooxygenases, metabolic activity, mitochondrial dysfunction, increased activity of oxidases, lipoxigenases, peroxisome activity, or through infiltrating immune cells (Movaheda et al., 2019). Experimental, clinical and epidemiological investigations have provided evidence supporting the role of reactive oxygen species (ROS) such as singlet oxygen (O_2), superoxide anions (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) in the etiology of cancer (Ray et al., 2000 and Zhou, 2014). Several studies showed that Cxb was related to suppression of cancer cell proliferation, decrease in cancer incidents and its efficient role in triggering in vitro cancer cell death (Sacchetti, 2013 and Li et al., 2018). Normal defense mechanisms have decisive role in carcinogenesis and tumor spread. In this regard, evidence has been documented, that chronic inflammation plays an important role in the development of certain cancers (Zamarron and Chen, 2011). However, cells of the immune system can also inhibit tumor growth and progression through the

recognition and rejection of malignant cells, a process referred to as immunosurveillance (Bui and Schreiber, 2007). In this respect, immunodeficiency can lead to development of both spontaneous and virally induced cancers, and tumors formations predominatingly create immunosuppressive microenvironments which block productive antitumor immunity (Zamarron and Chen, 2011).

The present study has been carried out to investigate the possible anti-tumor activity of Cxb on the growth of solid Ehrlich Carcinoma, its role in the enhancement of the antioxidant status as a free radical scavenger and its immunosurveillance effect.

MATERIALS AND METHODS

Chemicals:

Celebrex and chemicals for biochemical measurements were purchased from Sigma Chemical Co. (St. Louis, MO 6, USA).

Animals:

Adult female Swiss albino mice weighing 18 to 20 gm were purchased from the animal farm of Vacsera, Helwan, Egypt, and were housed in the same conditions for one week prior to the experiment for acclimatization.

Cell line:

Ehrlich Ascites Carcinoma (EAC) cell line was purchased from the National Cancer Institute, Cairo University, Egypt.

Tumor model:

A model of solid tumor was induced in female Swiss albino mice by injecting of $1x10^6$ EAC cells subcutaneously into the right thigh of the lower limb of the mice.

Experimental protocol:

In vitro cytotoxicity of Cxb on EAC. Two individual sets of *in vivo* experiments were carried out to investigate the time-course effect of the drug treatment on: 1) the volume of solid Ehrlich carcinoma (SEC); and 2) the growth delay of SEC for each experiment, a total of 40 mice were distributed into 5 equal groups (n=8) and injected by oral route with the drug and their control vehicles (0.1 ml), as follows:

Group 1, served as the control group. Group 2, mice were injected with the dissolving solution (DMSO and saline) and served as the solution sham group. Group 3, mice were implanted with EAC for 7 days to form a solid tumor. Group 4, mice were protected with Cxb drug (75mg/25ml) for 10 days before tumor implantation. Group 5, mice were treated with Cxb drug (75mg/25ml) for 10 days after tumor implantation.

In vitro cytotoxicity of Cxb on EAC:

Cell viability was determined by using trypan blue staining and the cytotoxicity values were calculated against control EAC cells (Om Ali et al., 2003) the results are expressed as % cell death.

Estimation of tumor weight:

Tumor weight was estimated according to Geran et al. (1972) method using the following formula:

$$\text{Weight (g)} = \frac{\text{Length (cm)} \times \text{width2 (cm)}}{2}$$

Biochemical analysis:

At the end of the experimental period, animals were sacrificed. A tissue sample from a known portion of the liver was accurately weighed and homogenized (Potter-Elvehjem) in a 10-fold volume of ice-cold (20mM) tris-HCl buffer pH 7.4. The homogenates were taken for the following analyses. Lipid peroxides was estimated by the method of Ohkawa et al. (1982), superoxide dismutase (SOD) Niskimi et al. (1972); catalase (CAT) bock et al. (1980); glutathione s transferase (GST) habig et al. (1974) and reduced glutathione (GSH) prinse and loose (1969).

Molecular analysis:

Protein fractionation was done by using one-dimensional polyacrylamide gel electrophoresis according to the method of (Laemmli, 1970). Then gel was analyzed by using image analyzer software to determine the protein molecular weight.

Immunological analysis:

Isolation of lymphocytes from spleen was done according to the method of Weaver and Cross (1981).

Assessment of lymphocytes viability was done according to the method of Leffel (1990).

Lymphocytes proliferative assay was done according to the method of Colley et al. (1979).

Statistical analysis:

The results were expressed as mean ± standard deviation (SD) for eight animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the Tukey test; significance at P values <0.05**.**

RESULTS

The in vitro study showed that Cxb exhibited a cytotoxic activity on EAC as shown in table 1 and figure 1.

Table 2 and Figure 2; show the decrease in tumor weight in the protected and treated groups as compared with that of tumor in control group.

Table 3, shows the concentration of MDA as a marker of lipid peroxidation in liver homogenate. Data revealed that the administration of Cxb either before or after tumor induction caused a very highly significant decrease in hepatic lipid peroxidation as compared with that of tumor control group.

Table 4, shows the activities of antioxidant enzymes in hepatic tissue of

various experimental groups of animals. Highly significant reductions in the activities of antioxidant enzymes (SOD, CAT, GST and GSH) were observed in the tumor bearing mice. These changes were ameliorated and increase in these enzymes activities occur in both Cxb protected and treated groups.

Table 5 and figure 3, show The SDS-PAGE of liver homogenate and protein molecular weight to each lane.

Table 6 and figure 4, show the splenic total lymphocytes count (10^6/ml) in control and different treated mice groups.

Cxb concentrations	10	20	40
% of cytotoxicity	33.3	60	90.9
SD ±	2.1	1.8	1.7

Table 1: In vitro cytotxicity of Cxb on EAC

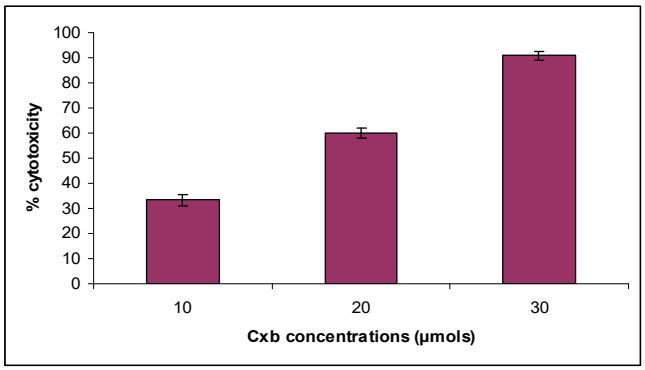

Figure 1: In vitro cytotxicity of Cxb on EAC

Table 2: Tumor weight (G) in tumor group and different treated groups

	DMSO	Tumor	Cxb protected	Cxb treated
mean	0.723	0.936	0.319	0.544
SD±	0.085	0.17	0.077	0.17

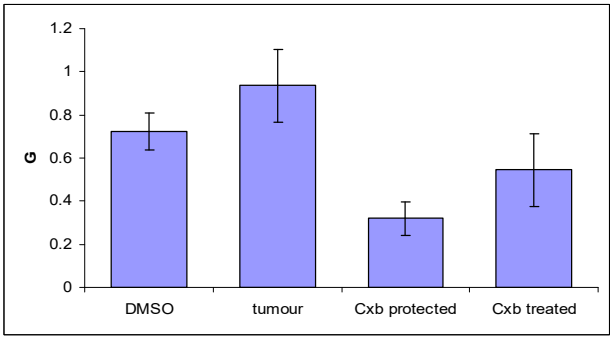

Figure 2: Tumor weight (G) in tumor group and different treated groups

Table 3: Effect of Cxb on the level of lipid peroxidation as MDA in liver homogenate (values are expressed as mean ± S.D. for 8 animals in each group and statistically significant at P < 0.05)

Parameter	Control	DMSO	Tumor	Cxb protected	Cxb treated
MDA	247±4.1	217±26.8[a]	405.5±31.3	250.5±4.4[a]	249±5.6[a]

Table 4: Effect of Cxb on the activities of antioxidant enzymes in liver homogenate (values are expressed as mean ± S.D. for 8 animals in each group and statistically significant at P < 0.05)

Parameter	Control	DMSO	Tumor	Cxb protected	Cxb treated
SOD	137.5±13.4	134.4±18.6[a]	100±18.9	140.6±18.6[a]	140.9±29.3[a]
CAT	6.95±0.9	6.4±1.0	4.8±1.2	8.3±1.2[a]	7.8±1.4[a]
GST	0.5±0.02	0.3±0.03	0.2±0.05	0.3±0.07	0.4±0.1[a]
GSH	0.054±0.01	0.04±0.01	0.037±0.01	0.04±0.01	0.04±0.01

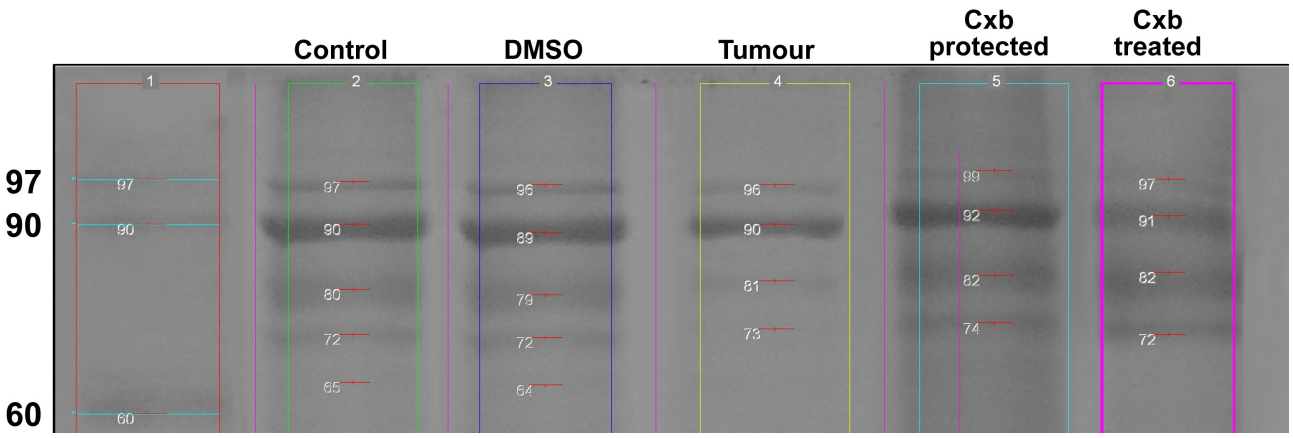

Figure 3: The SDS-PAGE of liver homogenate

Table 5: The protein molecular weight to each lane

Lanes:	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
Bands	(mol.w.)	(mol.w.)	(mol.w.)	(mol.w.)	(mol.w.)	(mol.w.)
1	97	97	96	96	99	97
2	90	90	89	90	92	91
3	60	80	79	81	82	82
4	36	72	72	73	74	72
5	18	65	64	-1	45	
6		53	52		25	
7		45	44		1	
8		34	34			
9		22	21			
10		-1	-2			

Table 6: The splenic total lymphocytes count (10^6/ml) in control and different treated mice groups

	Control	DMSO	Tumor	Cxb protected	Cxb treated
mean	16.07±**0.6**	13.6±**1.1**	2.6±**0.97**[a]	10.3±**1.3**[a]	3.7±**0.75**[a]

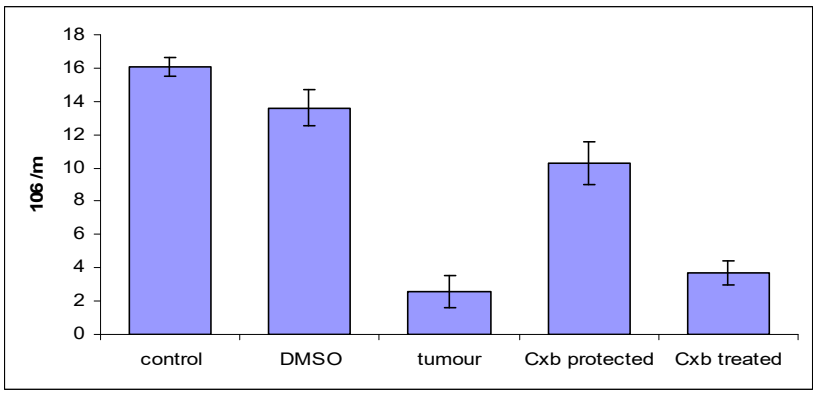

Figure 4: The splenic total lymphocytes count (10^6/ml) in control and different treated mice groups

DISCUSSION

Several studies have been explained the anticarcinogenic properties of Celebrex through scavenging of Reactive oxygen species (ROS) involved in the initiation and promotion of cancer Ralph et al., 2018. The present study demonstrated a remarkable inhibitory effect *in vitro* of Cxb on the Ehrlich ascites cells in a dose-dependent manner. These results confirm other studies of Wu et al. (2003) and Fukada et al. (2007) which indicated the apoptotic and antiproliferative effect of Cxb on cancer cells in a time and dose-dependent manner. This *in vitro* observation was extended in mice to disclose its efficacy *in vivo*. Oral administration of Cxb (30 mg/kg B.W) for 10 constitutive days either before or after implementation of EAC to tumor-bearing mice resulted in a significant reduction in tumor growth evidenced by reduce tumor weight by 97% and 42% respectively as compared with that of untreated tumor-bearing control group. These results run in parallel with that of Harris et al. (2000) who found that Cxb produces striking reductions in the incidence of tumor volume (81%) compared to those of the control group. Also Alshafie et al. (2000) and Kundu and Fulton (2002) showed that Cxb significantly inhibits the induction of mammary tumors by 7, 12-dimethyl benz (α) anthracene in rats. In addition, Cxb supplemented diet (1500 mg/kg diet) significantly decreased the size of the mammary tumours, resulting in an average reduction in tumour volume of approximately 32% which exhibits a chemopreventive impact of Cxb against mammary carcinogenesis. Human studies by Steinbatch et al. (2000) reported that the patients received 400 mg of Cxb twice a day for 6 months had a 28% reduction in the mean number of colorectal polyps and 30.7% reduction in the polyp burden as compared with reductions of 4.5% and 4.9% respectively, in the placebo group. Moreover, Wu et al. (2003) mentioned that Cxb inhibits intestinal tumor multiplicity by up to 71% compared with controls in mouse model and inhibits colorectal tumour burden in the rat azoxymethane model. ROS are implicated in several pathological conditions including tumerogenesis, where it activates procarcinogens, alter the cellular antioxidant defense system and initiate lipid peroxidation (LPO) (Perumal et al., 2011). Malondialdehyde (MDA) is a low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Hence, it is of interest to assess MDA as a marker of oxidative stress and the role played by LPO and the modulation of antioxidants during the progression of cancer (Anbuselvam et al., 2007). In the present study, a significantly increase in hepatic LPO products (table 3) was observed in the tumor-bearing mice group as compared with that of the control group. These results run in parallel

with that of Kamaraj et al. (2007) who reported a significant increase in MDA level and decrease in the activities of enzymatic antioxidants as compared to non-cancerous tissues. Also, Beevi et al. (2004) observed elevated levels of MDA in patients with oral cavity cancer, which could be attributed to free radicals formation. The present study illustrated that treatment of tumor-bearing mice with Cxb resulted in a significant decrease in the level of LPO as compared with that of the tumor control group. These data are compatible with the studies of Weger et al. (2002) and Kirkova et al. (2007) that showed a significant decrease in LPO in cancer patients after treatment with Cxb. Also, protein oxidation determined as protein carbonyl level was significantly decreased after treatment of tumor-bearing mice with Cxb as compared with that of the tumor controls. These findings suggest the antioxdative activity of Cxb via free radical removal. The same result was revealed by (Pejic et al., 2006). Antioxidants are the primary line of defense against ROS. Superoxide dismutase (SOD) and Catalase (CAT) are the key antioxidative enzymes that provide protective defense against ROS (Weydert et al., 2006 and Rajkapoor et al., 2007). Several reports have cited decreased activities of SOD and CAT in various carcinogenic conditions that may be due to the increased LPO (Ramakrishnab et al., 2006; Kamaraj et al., 2007 and Sreedharan et al., 2008). The present data demonstrate significant decrease in hepatic CAT and SOD activities in tumor-bearing mice as compared with that of the control (table 4), which might be due to its utilization in the removal of H_2O_2 that converted into water and oxygen (Anbuselvam et al., 2007). This depletion also might be due to altered antioxidant status caused by carcinogenesis (Mila-Kierzenkowska et al., 2004). These results are in harmony with the previous work of Rajkapoor et al. (2007) who illustrated that SOD activity decreases in cancerous tissue of lung when compared with that of normal tissues. In addition, Hu et al. (2005) demonstrated an increase in SOD activities in cancer tissues because of up-regulation in response to oxidative stress in cancer cells.

Treatment of tumor-bearing mice with Cxb (30 mg/kg B.W) for 10 constitutive days either before or after implementation of EAC resulted in a significant increase in SOD and CAT activities in liver homogenate when compared with those of tumor- bearing mice group. These results are in agreement with the findings of Kirkova et al. (2007) and Sinbel et al., 2014. These authors illustrated an increase in the activities of SOD and CAT in liver homogenate of tumor-bearing animals treated with Cxb.

The glutathione *S*-transferase (GST) family of enzymes comprises a long list of cytosolic, mitochondrial, and microsomal

proteins that are capable of multiple reactions such as catalyzing the conjugation of reduced glutathione (GSH) via the sulfhydryl group, to electrophilic centers on a wide variety of substrates which is useful in the detoxification of endogenous compounds (Hubatsch et al 1998). The present results demonstrate that hepatic GST activity and GSH level (tables 4) were significantly decreased in tumor-bearing mice group when compared with those of control mice group. These results run in parallel with the results of Kamaraj et al. (2007) and Prabhu and Bhat (2007) who reported a significant decrease in the activities of SOD, CAT and GST as well as non enzymatic antioxidants including GSH in lung cancer bearing animals. Also, these findings are in agreement with the study of Nair et al. (2006) who showed a significant decrease in hepatic GST activity in patients with cancer as compared with that of the control group.

However, levels of GST and GSH measured in the liver homogenate were significantly increased in tumor-bearing mice after treatment with Cxb for 10 constitutive days either before or after implementation of EAC when compared with those of tumor-bearing mice group. These results run in parallel with the findings of Beckett and Hayes (1993); Hayes and Pulford (1995) and Van Lieshout et al, (1998) Sinbel et al., 2014, who reported that Cxb exerts its anticarcinogenic mechanism through the enhancement of GST enzyme activity.

Immunotherapy has proven to be a promising way to improve cancer treatment outcomes where Tumor growth associated with the maintenance of an immunosuppressive tumor microenvironment (Pardoll, 2012). Whereas productive reactive oxygen species suppress T-cell activation, the amelioration of oxidative status is vital for cancer prevention (Zhao et al., 2017). The present study showed that the splenic total lymphocytes count in both treated and protected groups of Cxb is higher than that of tumor control group (table 6). These results agree with the study of li et al., (2016) which showed that Cxb enhanced T cell immunity, reduced immunosuppression, inflammation and tumor angiogenesis.

Finally, it can be concluded that Cxb act as a good free radical scavenger and improve immunity status and can be used as a complementary drug in cancer chemotherapy.

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