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Original Article

Potential antitumor activity of nonsteroidal anti-inflammatory drugs against Ehrlich ascites carcinoma in experimental animals

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ABSTRACT

Objectives: Although there is evidence that nonsteroidal anti-inflammatory drugs (NSAID) (e.g., celecoxib [Cxb]) can reduce the occurrence of cancer, the precise mechanism remains under study. The current study aimed to investigate the possible antitumor activity of a selective cyclooxygenase-2 inhibitor on solid tumors, its effect on antioxidant status, and ability to prevent angiogenesis.

Materials and Methods: Solid carcinomas were induced in female Swiss albino mice. Fifty adult female mice were randomly selected and categorized into five groups. The effects of Cxb on hepatic oxidative parameters and the serum level of vascular endothelial growth factors (VEGF) were investigated in parallel to liver histopathological examinations. Biochemical measurements of hepatic malondialdehyde, superoxide dismutase (SOD) activity, hepatic catalase (CAT) activity, and reduced glutathione (GSH) were estimated in liver homogenates prepared from mice in each study group.

Results: The induction of solid tumors in female albino mice was associated with a significant elevation in hepatic lipid peroxidation, whereas the activity of antioxidant enzyme NSAID and CAT was significantly decreased. The level of reduced GSH was decreased. Serum levels of VEGF were significantly increased in tumor-bearing mice compared with normal control mice. These changes were ameliorated when mice were treated with Cxb either before or after the induction of tumors. Antioxidant enzymes were significantly increased, and the serum level of VEGF was significantly reduced compared with the levels in tumor-bearing mice.

Conclusion: Cxb exerts antitumor activity through antioxidative and antiangiogenic activities.

Keywords: Antioxidant status, antitumor, Ehrlich ascites carcinoma, MCF-7, nonsteroidal anti-inflammatory drug

Introduction

Cancer is a multistep process. The phenotype of neoplasia is a result of the accumulation of several genetic modifications. Many genetic variations are required before a regular cell becomes fully neoplastic. These genetic variations involve tumor suppressors, oncogenes, and potentially senescence genes.[1] The main aim of cancer management is to stop, reduce, or reverse the processes of cancer as early as possible.[2]

Cyclooxygenases (COX) are a family of isozyme synthases responsible for the formation of thromboxane and prostaglandins (PGs) from free arachidonic acid.[3] The overexpression of COX-2 was recently shown in numerous solid cancers, including hepatocellular carcinoma, to play a key role in tumorigenesis.[4,5] Tumors regularly synthesize excessive amounts of PGs, which vary in both type and amount, leading to different histological properties of the tumor.[6] Mainly, COX-2 activation results in the synthesis of PGE_2. It has a critical role in the modulation of several aspects of pathophysiology.[7] Many researchers have stated that tumor necrosis factor (TNF-α),[8] interleukins (e.g., IL-1),[9] and growth factors (e.g., platelet-derived growth factor) are inflammatory cytokines that act as positive regulators of COX-2 expression.[10]

Reactive oxygen species (ROS) free radicals or oxidants (e.g., H_2O_2 or O_2) synthesized as a result of environmental

factors or cellular metabolism that destroy proteins and nucleic acids, altering their functions.[11] A high concentration of ROS generates oxidative stress "oxidants," which induce destructive pathways and damage structures in different cells.[12] Oxidative stress indicates an imbalance between the oxidants and the ability of the cell to eliminate them. Thus, the cell structure has its own mechanisms to counteract oxidative stress-based antioxidant production.[13]

Mainly, cellular antioxidants are responsible for maintaining the redox balance in the cell based on the scavenging reactive species causing oxidative stress and cellular damages. Superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT), the classical antioxidant enzymes, are considered important scavengers of free radicals.[14] Oxidants activate the peroxidation of unsaturated fatty acids. Thus, they decrease the activities of enzymatic antioxidants in breast cancers.[15] The peroxidation of unsaturated fatty acids results in the formation of malondialdehyde (MDA) within a cell. Therefore, an increase in oxidants leads to abnormal MDA levels.[16]

Celecoxib (Cxb) is a nonsteroidal anti-inflammatory drug (NSAID). It is used in chemotherapy for cancer inhibition, due to its inhibition of COX-2.[4] The effects of Cxb have been shown in different cells and cell lines (e.g., colon cancer cells and breast cancer).[17,18] Selective COX-2 inhibitors are powerful free radical scavengers because they may inhibit pain and the inflammatory processes in oncogenesis.[19,20] Previous studies have reported that Cxb did not affect cell division, apoptosis, or the epithelial structure of the gut of treated mice.[21] Vascular endothelial growth factors (VEGFs) are activators of tumor growth and metastasis through the mediation of angiogenesis. The overexpression of VEGF is a predictor of poor prognosis in patients with cancer.[22]

At present, the precise mechanisms discussing the antiproliferative effects of Cxb are still under study.[23,24] The main objective of the current study was to investigate the possible biochemical impact of the antitumor activity of a selective COX-2 inhibitor on solid tumors, its effect on antioxidant status, and ability to prevent angiogenesis.

Materials and Methods

Drug, reagents, and cell lines

The selective COX-2 inhibitor (Cxb) was purchased from a local pharmacy and dissolved in dimethyl sulfoxide (DMSO)/saline (70:30) to a final concentration of 40 mM.[25] Other chemicals were purchased from Sigma Company (CA, USA). The human breast cancer cell line (MCF-7) was obtained from the ATCC (VA, Manassas, USA). The Ehrlich ascites carcinoma (EAC) cell line was obtained from the National Cancer Institute, Cairo University.

Animals and experiment design

Fifty Swiss albino adult female mice with a body weight of 20±2 g were selected randomly. They were housed under standard conditions (temperature: 25°C±2°C and 12 h dark/light cycle) in accordance with institutional and national official guidelines. All experimental animals were fed the typical standard nutrition with water *ad libitum* in accordance with the policy of the Ethical Committee Guidelines of the Dentistry College in Qassim University.[26] Ethical consent was achieved from the Committee of Research Ethics of Dentistry College in Qassim University. The experimental mice were randomly divided into five groups, each containing 10 mice: Group I, the healthy control group (no treatment); Group II, the sham group (animals were injected with a 70:30 mixture of DMSO/saline); Group III, the tumor control group (mice were subcutaneously injected with 1×10^6 EAC subcutaneously into the right thigh); Group IV, the Cxb pre-treatment group (mice were injected with 75 mg Cxb daily for 10 days before tumor induction); and Group V, the Cxb post-treatment group (mice were treated with 75 mg/kg Cxb for 10 days after tumor induction. After all treatments, the mice were sacrificed; after sacrifice, the liver and solid tumors were excised and washed in 0.9% saline.

Estimation of solid tumor size

Tumor size was measured based on the method of Geran *et al*.[27] The experimental solid tumors were ellipsoid, with two short axes and one long axis and shortaxis were measured by using a Vernier caliper, and the tumor size was calculated from the following formula: Size = [Length (cm)×Width^2 (cm)]/2.

Biochemical measurements of liver oxidative parameters

Liver homogenate was prepared from the excised livers. Tissue portion samples were collected from a recognized part of the liver, accurately weighed, and homogenized in 10 volumes of 20 mM Tris HCl buffer, pH 7.4. Aliquots of homogenate were prepared and stored at −20°C.

All liver oxidative parameters were assessed in the prepared liver homogenates. The levels of peroxidized fatty acid were assessed by measurement of MDA, based on the method of Ohkawa *et al*.[28] SOD activity was measured based on the procedure of Nishikimi *et al*.[29] CAT activity was measured according to the method of Bock *et al*.[30] GSH levels were measured based on the method of Ellman.[31]

Measurement of serum VEGF level

The level of VEGF was assessed using enzyme-linked immunosorbent assay (ELISA, R&D Systems Inc., Minneapolis, USA), which was based on commercially available matched paired antibodies, as described by Botelho *et al*.[32] The optical density of the solution at 450 nm was measured to quantify the developed color intensity.

Histopathological examination

The livers and solid tumors were separated, fixed in 10% formalin solution, and dehydrated by a series of alcohol dilutions. After xylene treatment, the specimens were processed within the paraffin block at 60°C. A 4µm section was cut and stained with hematoxylin and eosin (H&E). Changes were examined using a light microscope and photographed using a digital camera (Nikon, Japan).[33]

Statistical analysis

The collected data are stated as the mean±standard deviation for each group under study. Comparisons between groups were calculated by one-way analysis of variance using SPSS program (version 22). The intergroup comparisons were performed with the *post hoc* test, with significance accepted for $P < 0.05$.

Results

The mortality rate of the animals in the study groups is shown in Table 1. There was no difference in mortality rate among groups.

The effect of Cxb on the size of the tumors induced by subcutaneous injection of EAC cells is shown in Figure 1. The data are presented as the mean ± standard error of 10 mice.

Table 1: The mortality rate of animals in the study groups

Groups	Number of tested	Number survived	Mortality rate (%)
I	10	9	10
II	10	9	10
III	10	9	10
IV	10	9	10
V	10	9	10

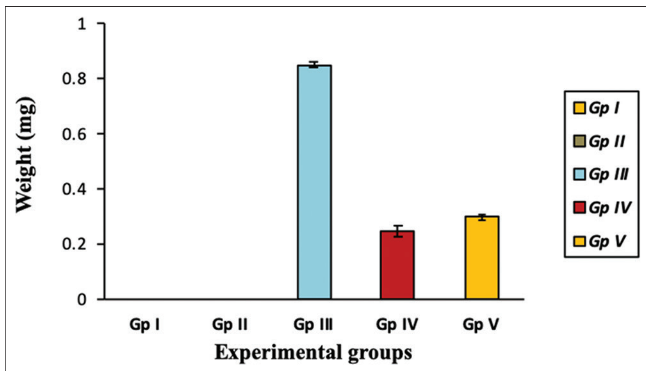

Figure 1: Effect of cyclooxygenase-2 inhibitor on tumor size in the experimental groups of female Swiss albino mice. Group I: The healthy control group; Group II: The sham group; Group III: The tumor control tumor group of animals; Group IV: The celecoxib (Cxb) pre-treatment group; and Group V: The post-treatment group. Figure 1 shows the marked cytotoxic effects of Cxb in both the Cxb pre-treatment group (Group IV) and the Cxb post-treatment group (Group V)

There was a significant difference between tumor control, Cxb pre-treatment, and Cxb post-treatment groups ($P < 0.05$).

The effect of Cxb on oxidative liver status in the experimental mice is shown in Table 2. The administration of Cxb either before or after tumor induction led to a very highly significant decrease in hepatic lipid peroxidation (LPO), as shown by MDA levels, compared with the tumor control group (Group III). In addition, the data in Table 2 illustrate a significant reduction in the antioxidant enzyme activities of SOD, CAT, and GSH in the liver homogenates of solid tumor-bearing mice compared with mice in Group I. In contrast, pre-treatment or post-treatment with 75 mg/kg Cxb (Groups IV and V, respectively) led to significant amelioration of the hepatic oxidant status compared with the tumor control group (Group III).

The levels of VEGF in the serum of mice in the experimental groups are shown in Figure 2. Serum VEGF levels in tumor-bearing mice (Group III; 192.4 ± 31.3 pg/mL) were significantly higher than those in the healthy control mice (Group I; 132.7 ± 19.4 pg/mL). In contrast, serum levels of VEGF in animals pre-treated or post-treatment with Cxb (Groups IV and V; 146.3 ± 18.9 and 137.7 ± 21.3 pg/mL, respectively) were significantly lower than in mice in the tumor control group (Group III).

Representative images of H and E-stained tumors from mice in different experimental groups are shown in Figure 3. In Figure 3a, the solid tumor of EACs shows undifferentiated epithelial cells with the alveolar arrangement as evidence of the tumor. The cells were moderate to small in size. The distinction between cytoplasm and the nucleus was frequently obscured. The intercellular space was wide, and occasional nuclei were apoptotic. Images of the tumor from animals that were treated with Cxb before tumor

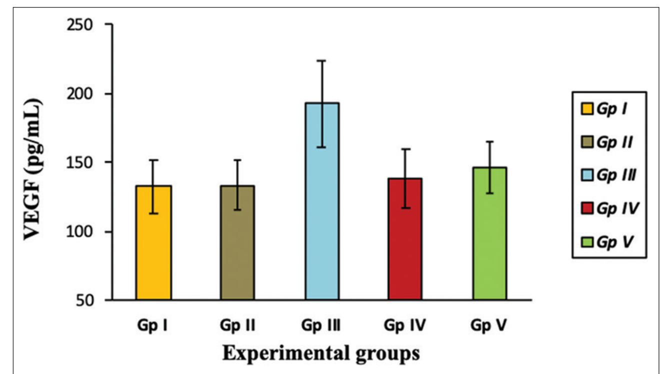

Figure 2: Serum vascular endothelial growth factor (VEGF) levels in the experimental groups. Group I: The healthy control group; Group II: The sham group; Group III: The tumor control tumor group; Group IV: The celecoxib (Cxb) pre-treatment group; and Group V: The post-treatment group. Figure 2 shows the marked cytotoxic effects of Cxb in both the Cxb pre-treatment group (Group IV) and the Cxb post-treatment group (Group V). Serum VEGF levels in the tumor control group (Group III) were higher than in the healthy control group (Group I). Serum levels of VEGF in animals either pre-treated or post-treated with Cxb (Groups IV and V, respectively) were unchanged from the healthy control group (Group I)

Table 2: Effect of celecoxib on the hepatic oxidative status

Oxidative parameters	Group I	Gro] up II	Group III	Group IV	Group V
Malondialdehyde	228±3.1	217±26.8[a]	405.5±31.3	250.5±4.4[a]	249±5.6[a]
Superoxide dismutase	137.5±13.4	134.4±18.6[a]	100±18.9	140.6±18.6[a]	140.9±29.3[a]
Catalase	6.95±0.9	6.4±1.0	4.8±1.2	8.3±1.2[a]	7.8±1.4[a]
Glutathione	0.054±0.01	0.04±0.01	0.037±0.01	0.04±0.01	0.04±0.01

[a]$P<0.05$: Statistically significant

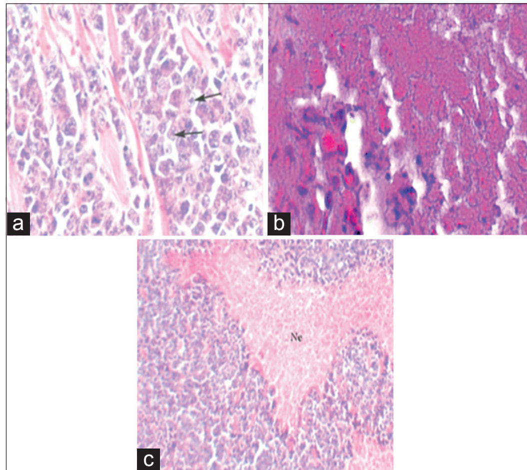

Figure 3: Histopathological examination of solid tumor sections stained by hematoxylin and eosin (×200). Image (a): The tumor control group (Group I); Image (b): The celecoxib (Cxb) pre-treatment group (Group IV); and Image (c): The Cxb post-treatment group (Group IV). The arrows in (a) show infiltration of the tumor section of Ehrlich ascites carcinoma, with a high grade of anaplasia and pleomorphism, and an increase in the nucleocytoplasmic ratio, hyperchromasia, and vesicular nuclei. The arrows in (b) and (c) show the massive area of coagulative necrosis (Ne) with viable tumor cells

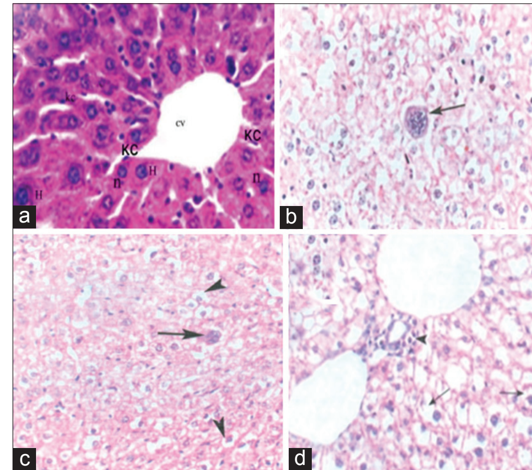

Figure 4: Histopathological examination of the cross-section of hematoxylin and eosin-stained liver sections (×400). Image (a): The liver section from the tumor control group (Group I); Image (b): The liver section from the tumor control group (Group III); Image (c): The liver section of Ehrlich ascites carcinoma from the tumor control group (Group III); and Image (d): Representative section from the liver section of mice in Group V. Image (b) shows mild degenerative changes with a feathery cytoplasm (head of the arrow) and dilated blood sinusoids. The focus shows the malignant tumor spread (arrow). Image (c) shows a solitary giant malignant cell (arrow) between the hepatocytes. This malignant cell showed a high degree of anaplasia, including increased nucleocytoplasmic ratio, hyperchromasia, vesicular nuclei, and prominent multiple nucleoli. Image (d) shows moderate diffuse degenerative changes; feathery cytoplasm (arrows), and mild portal perivascular inflammatory infiltrates (head of the arrow)

induction are shown in Figure 3b. There were small isolated islands of shrunken cells with pyknotic nuclei. The intercellular space was increased due to the shrinkage of cells, which is a pathological characteristic of apoptosis. The treatment of mice with 75 mg/kg Cxb daily for 10 days after tumor induction led to large connected islands of solid tumors with a massive area of coagulative necrosis (Ne) with viable tumor cells on the right side that was interfered by amorphous eosinophilic granules. A variety of pyknotic nuclei were observed [Figure 3c].

The H and E-stained liver sections from mice in the experimental groups are shown in Figure 4. The livers from solid tumor-bearing mice showed infiltration of the tumor section of EAC, with a high grade of anaplasia and pleomorphism, and an increase in nucleocytoplasmic ratio, hyperchromasia, and vesicular nuclei [Figure 4a]. The treatment of solid tumor-bearing mice with Cxb resulted in a massive area of coagulative necrosis with viable tumor cells on the right side.

Discussion

High levels of ROS are responsible for lipid and protein damage and thus, they participate in the development of many human diseases, including cancer.[15] Previous studies have suggested that the anticarcinogenic properties of NSAIDs reduce the permeability of gastrointestinal for carcinogens and their metabolites. NSAIDs are scavengers of ROS involved in both the promotion and initiation of cancer. Thus, the antitumor activity of NSAIDs can be attributed, at least in part, to the inhibition of tumor angiogenesis.[10,12,23]

The oral administration of Cxb (75 mg/kg B.W.) for 10 consecutive days, either before or after tumor induction, resulted in a significant reduction in tumor growth, as evidenced by a reduction in tumor weight of 97% ± 2.6% and 42% ± 3.4%, respectively, compared with the untreated tumor-bearing controls. The results were in agreement with Kansal *et al.*,[34] who reported that Cxb reduced cancer cell proliferation, by 78.7% ± 6.3% in the MCF-7 human breast cancer cell line, and 70.9% ± 7.1% in the MDA-MB-231 human

breast cancer cell line. Other studies showed that pre-treatment with Cxb combined with fish oils resulted in better cancer chemoprevention in rats than 7,12-dimethylbenz(α)anthracene. This treatment resulted in normal histological features, increasing DNA fragmentation, and reducing oxidative stress.[35]

ROS induce procarcinogens, stimulate LPO, deactivate the enzymatic system, and induce cellular changes in the antioxidant defensive systems. The increasing incidence of both oxidative stress and LPO is involved in the carcinogenesis process.[36] The current research shows a significant increase in hepatic LPO products in the tumor-bearing mice (Group III) compared with the healthy control mice (Group I). These data were in agreement with Kamaraj *et al*., who reported a significant increase in MDA level and a decrease in the activities of enzymatic antioxidants in different grades of breast cancer compared with the healthy controls.[37]

Another study demonstrated that both MDA and GSH could be applied to monitor oxidative stress in oral cancer due to the formation of free radicals. An elevated level of MDA led to cancer progression in of squamous cell carcinoma in the oral cavity. The elevation of LPO products induced by ROS is involved in the pathogenesis of malignancy.[38] Another study reported that the elevation of LPO in adenomas and carcinoma of thyroid tissues might be due to the higher production of ROS and the incomplete scavenging of lipid peroxides in carcinoma tissue.[39]

The current study illustrated that the use of selective Cxb for the treatment of tumor-bearing mice resulted in a significant decrease in the level for LPO compared with tumor Group I. These data are comparable with those in the Kirkova study that showed a significant decrease in LPO in patients with cancer after treatment with a COX-2 inhibitor. These findings suggest that the antioxidative activity of Cxb occurs through free radical removal.[40]

Antioxidants are the primary line of defense against ROS. Both SOD and CAT are key antioxidant enzymes that protect against ROS.[38,41] Several studies have reported that the decreased SOD and CAT activities in different cancers may be related to the increased LPO levels.[42,43] The present data demonstrate a significant decrease in hepatic CAT and SOD activities in tumor-bearing mice compared to the healthy control group [Table 2]. The current study agrees with prior studies, in which SOD activity was decreased in the cancerous tissue of the liver compared with the normal tissues. This demonstrated a decrease in CAT and SOD levels in EAC tumor-bearing mice, because of upregulation in response to oxidative stress within tumor cells.[42,43] This may be due to mitochondrial damage or dysfunction arising from oxidative stress that leads to a decrease in SOD activity in the liver. The decreased CAT activity in Group III may be utilized in the removal of H_2O_2, which is converted into water and oxygen.[44,45] In addition, the depletion was also related to the effect of carcinogenesis leading to a defective antioxidant status.

The treatment of tumor-bearing mice with Cxb (75 mg/kg B.W) for 10 consecutive days either before or after tumor induction resulted in a significant increase in the activities of both CAT and SOD in liver homogenates compared with mice in Group III. These results were in agreement with the findings of many authors who reported the increase in CAT and SOD activities in the liver homogenate of tumor-bearing mice treated with Cxb.[44,45]

The electrophilic moieties responsible for the initiation of cancer are scavenged by GSH and its relative enzymes.[42] The current results demonstrate that hepatic GSH level [Table 2] was significantly lower in tumor-bearing mice group compared with the control group. The present data agreed with Kamaraj *et al*. and Weydert and Cullen, who reported a significant decrease in SOD and CAT activities, as well as non-enzymatic antioxidants, including GSH in lung cancer-bearing animals.[35,45]

However, the level of GSH measured in the liver homogenate was significantly higher in tumor-bearing mice after treatment with Cxb for 10 consecutive days either before or after tumor induction compared with those of the tumor-bearing mice group.

The serum of tumor-bearing mice had a significantly higher VEGF level than the healthy controls. Endothelial cell proliferation, migration, and permeability were enhanced and generated by the increase in VEGF.[46] Thus, cells were activated for further angiogenic growth of the tumor.[47] Therefore, this indicated metastasis of the tumor, which was confirmed by an increase in VEGF levels. The tumor-bearing model should be established for further researchers to study the effect of VEGF. There was a positive proportional correlation between tumor sizes and serum levels of VEGF in breast cancer.[48]

The current results are supported by the histopathological findings that elucidated the effect of the anti-COX drug. The histopathological observations of the tumors that were treated or protected with selective Cxb showed distinct areas of coagulative necrosis in solid tumor-bearing mice compared with the normal architecture observed in the EAC control group, as shown in Figure 3. These suggested that necrosis may be the primary cause of anti-COX drug-induced cell death.

Conclusions

Cxb is a chemopreventive agent in carcinogenesis. Cox-2 inhibitors are considered as effective free radical scavengers. In addition, they can act as a complementary drug in cancer chemotherapy. Thus, COX-2 inhibitors inhibit EAC cell cycle progression.

The study indicates the effects of the intrinsic pharmacologic selectivity of Cxb on COX enzymes. Future experiments are required to evaluate the toxicity of Cxb on gastrointestinal and kidney cells arising from long-term use compared with NSAIDs. These conclusions support and promote further additional human investigations to explore this potential and their clinical efficacy.

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