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Original Research Article

Effects of Indole-3-carbinol on Inflammatory and Prooncogenic Mediators in Solid Ehrlich Carcinoma Induced Mice

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Abstract

Indole-3-carbinol (I3C) and its metabolites were proven to suppress the proliferation of various cancer cell lines by targeting a wide spectrum of signaling pathways that regulate hormonal homeostasis, cell cycle progression, and cell proliferation. Moreover, I3C inhibited tumorigenesis in mammary glands, liver, lung, and gastrointestinal tract in different animal models. These preclinical findings demonstrate the value of I3C in cancer prevention and therapy, which has led to its trial in cervical dysplasia, breast cancer, leukemia and recurrent respiratory papillomatosis. This study aimed to investigate the effects of different doses of I3C on inflammatory mediators, IL-6 and TNF-α levels and prooncogenic mediator, SphK1 activity, in tumor tissue in Solid Ehrlich Carcinoma (SEC) Tumor Model. Eighty BALB/c male mice were used in this study. Except for mice in the control group, each mouse was implanted subcutaneously with 0.2 ml of the ascites fluid containing $1x10^6$ Ehrlich carcinoma cells (ECCs) into the thigh of the hind limb. Mice were divided into four groups (20 mice per group) as follow: Control group, Solid Ehrlich carcinoma (SEC) control group, Indole-3-carbinol (I3C 1000 ppm) group, in which mice were put on a diet containing 1000 ppm I3C starting seven days before and continued for 42 days after tumor inoculation, Indole-3-carbinol (I3C 2000 ppm) group, in which mice were put on a diet containing 2000 ppm I3C starting seven days before and continued for 42 days after tumor inoculation. Administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in tumor volume and increased survival rate. Subcutaneous implantation of ECCs resulted in significant increase in tumor tissue SphK1 activity, TNF-α and IL-6 levels and administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in all of these oncogenic and inflammatory mediators.
Keywords: Indole-3-carbinol, Inflammatory mediators, Prooncogenic mediators, interleukins.

INTRODUCTION

Dietary and epidemiological studies have provided a correlation between eating cruciferous vegetables and lowering of cancer occurrence. Considerable evidence attributes this chemopreventive effect to the antitumor activity of a common phytochemical, indole-3-carbinol (I3C), and its metabolites [1]. Indole-3-carbinol or its metabolites were proven to have potent suppression effects on various cancer cell lines by affecting on a wide spectrum of signaling pathways that regulate hormonal homeostasis, cell proliferation and cell cycle progression [2, 3]. Moreover, I3C inhibited tumorigenesis in liver, gastrointestinal tract, mammary glands, and lung, in different animal models. These preclinical findings indicated the value of I3C in cancer prevention and cancer therapy, which has led to its trial in cervical dysplasia, leukemia, breast cancer, and recurrent respiratory papillomatosis [4, 5].

Recent studies have reported that I3C and its metabolites have potent anticancer effects. The mechanisms of these effects were attributed to its ability to alter estrogen metabolism and other cellular effects. Estrogen receptors are present on the surface of every type of tissue in the bodies of the men and women and are associated with several hormone-dependent cancers [6].

I3C was reported to induce cell cycle arrest at the G1 phase in human reproductive cancer cells. This leads to prevention and treatment of cancer, as the G1 phase occurs early in the cell cycle, and, for most cells, is the major period of cell cycle during its lifespan. The G1 phase is marked by synthesis of various enzymes

that are required in the next "S" phase, including those needed for DNA replication [7].

I3C can shift estrogen metabolism towards less estrogenic metabolites. Systemic lupus erythematosus is associated with estrogen. In a study using mice bred to develop lupus, the group fed with I3C diet lived longer and had fewer signs of disease [8]. Another study of lupus attributed the mechanism for this improvement to the sequential block of the development of B and T cells of these mice. This resulted in a fall in autoantibody production, thought to be a crucial component of lupus pathogenesis. In addition, I3C of the disease prone mice led to a normalization of their T cell function [9].

Recent studies showed that I3C has beneficial effects on lipid metabolism that could be of great value for prevention of cardiotoxicity [10]. Moreover, other studies reported that I3C might prevent cardiac remodeling via activation of AMP kinase enzyme leading to improvement of the myocardial functions and modulation of the expression of the genes that are responsible for the production of the hypertrophic and fibrotic markers with regeneration of the damaged myocardial tissues which significantly decreases the activity of the cardiac enzymes such as lactate dehydrogenase and creatine phosphokinase [11, 12].

Sphingolipids play a critical role in the cell biological functions. Among them, ceramide and sphingosine induce apoptosis and inhibit cell proliferation while sphingosine 1-phosphate (S1P) inhibits apoptosis and promotes cell survival and proliferation. The balance between ceramide/sphingosine and S1P forms the so-called "sphingolipid-rheostat", which decides the cell fate [13]. Sphingosine kinases are a group of enzymes that catalyze the phosphorylation of sphingosine to S1P [14]. There are two isoforms of sphingosine kinases known as SphK1 and SphK2 [15]. SphK1 was proven to increase the production of the proinflammatory cytokines such as TNF-α and IL-6 through activation of STAT3 and NF-κB which may participate in proliferation and growth of cancer cells [16]. Moreover, S1P which is produced by the action of SphK1 was proven to inhibit apoptosis, possibly through its effect on Phosphatidylinositol 3-Kinase/Akt pathway [17]. Inhibition of SphK1 activity might represent an effective targeted therapy for cancer. Nagahashi *et al.*, [18] reported that specific inhibitor of SphK1 can suppress tumor-induced angiogenesis and lymphangiogenesis and can induce apoptosis. Paugh *et al.*, [19] suggested that a selective sphingosine kinase 1 inhibitor may integrate multiple molecular therapeutic targets in human leukemia. The most prominent mechanisms of this effect were enhanced apoptosis and cleavage of Bcl-2 with alteration of the Akt pathway. Also, Xu *et al.*, [20] reported that concurrent targeting of Akt and SphK1 may inhibit growth of tumor cells, possibly through affection of ceramide production, and subsequent cell death and apoptosis.

The inflammatory changes play an important role, in particular tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NFkB) signaling pathways. The study of Ohta *et al.*, [21] assessed the effects of cisplatin treatment on ovarian cancer cells on nuclear factor kappa B activation and concluded that cisplatin enhanced its phosphorylation significantly, mediated by the PI3/Akt signaling cascade. This finding is consistent with the coexpression of NFkB transcription factors p65 and p50 in ovarian cancer patients who received a chemotherapy regimen that included cisplatin [22].

NFkB signaling is a converging point for controlling downstream signaling cascades that include, interleukin 1 (IL-1), IL-6, IL-8, TNF-α and transcription of other inflammatory genes [23]. The important cytokine in angiogenesis regulation, cell proliferation, and invasion is IL-6. Receptor system of IL-6 involves STAT-3- and extracellular signal regulated kinase (ERK)-mediated pathways. STAT-3 plays multiple roles in cell proliferation and survival and through activation of *bcl-2*, *c-myc*, and *cyclin-D* and persistent activation of STAT-3 is involved in tumorigenesis in a variety of leukemias [24]. Activation of extracellular signal regulated kinase (ERK) induces cell proliferation by phosphorylation of transcription factors such as ELK1 and c-FOS. IL-8 has been implicated in cancer progression, particularly in mediating angiogenesis in various cancer types including pancreatic, colon, non-small-cell, lung carcinoma, and melanoma [25].

Cisplatin treatment leads to increment of TNF-α, and a variety of pharmacological inhibitors attenuate cisplatin nephrotoxicity mediated by TNF-α. Treatment of mouse kidneys with salicylate, leads to attenuation of cisplatin-induced increase in TNF-α mRNA and also reducing TNF-α levels in serum. Rutin treatment on Wistar rats has a beneficial effect on cisplatin's deteriorative effects through inhibition of TNF-α and NFkB pathway-mediated inflammation. Likewise, administration of luteolin in kidneys of mice significantly reduced TNF-α and NFkB, as well as COX-2 expression [26].

OBJECTIVES

This study aimed to investigate the effects of different doses of I3C on inflammatory mediators, IL-6 and TNF-α, and prooncogenic mediator, SphK1 levels in tumor tissue in Solid Ehrlich Carcinoma (SEC) Tumor Model.

MATERIALS AND METHODS

Indole-3 Carbinol

Indole-3-carbinol (I3C) was purchased from Sigma Aldrich Co. and administered daily orally in the

diet at two dose levels; 1000 ppm and 2000 ppm. Mice received a semisynthetic diet (Purina basal diet with 10% corn oil, no. 5755M-S, Purina Mills, Richmond, Indiana, United States). Animals were initially exposed to the powdered diet alone for 3 days before incorporating I3C at 1000 and 2000 ppm concentrations into the diet. Food containers (before and after filling) were weighed every other day. One gram or two grams of I3C were dissolved in 20 ml of acetone and added dropwise to 1 kg of the powdered diet with vigorous mixing that continued over 15 minutes until the acetone odour had completely disappeared to prepare 1000 ppm I3C-containing or 2000 ppm I3C-containing diet respectively. All diets were stored at 4^{oC} in a dark place until the time of use. The diet was freshly prepared approximately every 4 weeks [27].

Solid Ehrlich Carcinoma (SEC) Tumor Model

A model of SEC was used, where 1×10^6 of the Ehrlich carcinoma cells (ECCs) obtained from the Pharmacology and Experimental Oncology unit of the national cancer institute, Cairo University, Egypt were implanted subcutaneously into the right thigh of the hind limb of mice. A palpable solid tumor mass (about 100 mm^3) was developed within 12 days following implantation of ECCs [28].

Animals

BALB/c male mice weighing 20–25 gm, obtained from the animal house of the faculty of medicine, Tanta University were used in this study. Animals were kept in individual metabolic cages at $22^{°C}$, 55% relative humidity and 12/12 hours light-dark cycle through the whole period of the study. All the experiments were conducted according to Helsinki declaration of animal ethics [29]. The protocol of this study was approved by the ethical committee of the faculty of pharmacy, Helwan University.

Experimental Design

Eighty BALB/c male mice were used in this study. Except for mice in the control group, each mouse was implanted subcutaneously with 0.2 ml of the ascites fluid containing 1×10^6 ECCs into the right thigh of the hind limb of mouse. The day of implantation of ECCs was considered as the zero point (day 0) of the experiment. The total period of the experiment was 49 days (7 days before and 42 days after tumor inoculation). Mice were randomly divided into four equal groups (20 mice per each group) as follows:

- **Group 1:** Control group, in which mice received Purina basal diet with 10% corn oil without any additions along the time of experiment.
- **Group 2:** Solid Ehrlich carcinoma (SEC) control group, in which mice received Purina basal diet with 10% corn oil without any additions along the time of experiment and each mouse was implanted subcutaneously with 0.2 ml of the ascites fluid containing 1×10^6 ECCs into the right thigh of the hind limb of mouse.
- **Group 3:** Indole-3-carbinol (I3C 1000 ppm) group, in which mice were put on a diet containing 1000 ppm I3C starting seven days before and continued for 42 days after tumor inoculation [30].
- **Group 4:** Indole-3-carbinol (I3C 2000 ppm) group, in which mice were put on a diet containing 2000 ppm I3C starting seven days before and continued for 42 days after tumor inoculation [31].

On the 40th day after tumor inoculation tumor volume was measured. Two days later (42 days after tumor inoculation), the survival rate was determined. Mice were sacrificed and the tumor tissues were excised for further investigation.

Assessment of the time-course effects of different treatments on tumor volume of SEC

Tumor volume was measured using a Vernier caliper starting on the 15^{th} day post-implantation and thereafter every 5 days till the last record on the 40^{th} day post-implantation prior to scarification of the survived mice. Tumor volume (V) was calculated as V (mm^3) = $(a^2 \times b)^2$ Where: (a) represents length of the small diameter (mm), (b) represents length of the large diameter (mm).

Counting of the survival rate

The number of the survived mice in each group was counted on days 7, 14, 21, 28, 35 and 42 after implantation of Ehrlich carcinoma cells then divided by the total number of animals in each group to get the percentage of the survived mice [32].

Preparation of Tissue Homogenates

The tumor tissues of mice were immediately dissected out and freed from the adjacent tissues, washed with cold saline to remove any excess blood, blotted to dry on filter paper and then weighed. The tumor tissue was divided into two portions, the first portion homogenized in cold saline to give 10 % homogenate using a Branson sonifier (250, VWR Scientific, Danbury, CT, USA). The homogenate was centrifuged at 3000 rpm for 10 minutes at 4 ºC and the supernatant was prepared for further biochemical analysis.

The second portion: was used for histopathological examinations.

Determination of tumor tissue sphingosine kinase-1 (SphK1) activity

Using ELISA kits supplied by MyBioSource, Inc., USA, according to the manufacture's protocol.

Principle

Sphingosine kinase 1 (SphK1) catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate. SphK1 has been implicated in proliferation, survival, migration and regulation of calcium homoeostasis, development and regulation of the cardiovascular and nervous systems, inflammation, immunity, and cancer growth. SphK1 activity assay is prepared by the addition of sphingosine kinase, sphingosine (substrate) in reaction buffer and initiated by the addition of ATP. The reaction is then stopped by adding the ATP detector after the chosen reaction time. The luminescent signal is inversely correlated with the kinase activity.

Determination of tumor and cardiac tissue tumor necrosis factor-alpha (TNF-α)

Using mouse TNF-α ELISA kits supplied by RayBiotech, Inc. according to the instructions of the manufacturer.

Principle

TNF-α ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of TNF- α in serum, plasma and tissue homogenate supernatants. This assay employs an antibody specific for mouse TNF-α coated on a 96-well plate. Standards and samples are pipette into the wells and TNF-α present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Mouse TNF-α antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNF-α bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Determination of tumor tissue interleukin-6 (IL-6) level

Using mouse IL-6 ELISA kits supplied by Sigma Chemical Co. according to the instructions of the manufacturer.

Principle

Mouse IL-6 ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of IL-6 in biological samples, such as serum, plasma, cell culture supernatants, urine, and/or cell and tissue lysates. This assay employs a specific capture antibody coated on a 96 well plate. Standards and samples are pipetted into the wells and IL-6 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and a biotinylated detection antibody specific for IL-6 is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Histopathological Examinations

The tumor sections were prepared and stained with hematoxylin and eosin (H&E) and examined under light microscope.

Statistical Analysis

For statistical analysis, the Statistical Package for the Social Sciences (SPSS) version 16.0 was used. Parameters were shown with mean ± Standard error of mean (SEM). Multiple comparisons were performed using one way analysis of variance (ANOVA) and nonparametric followed by Tukey-Kramer test for post hoc analysis, as appropriate. Unpaired t-test and Mann-Whitney test were used to compare between two different treatment groups. The overall and pairwise comparisons of survival rate were analyzed by applying Breslow test (generalized Wilcoxon) using the Kaplan–Meier method. Differences between the means of the different groups were considered significant at a level of p-value < 0.05.

RESULTS

Administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in tumor volume at the 40th day post-implantation by 25 % and 30 % respectively compared to the SEC control group (Table-1). The results in Table-2 showed that subcutaneous implantation of ECCs resulted in significant decrease in the survival rate at the 42th day post-implantation by 50% compared to the control group. In the same time, administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant increase in the survival rate by 20 % and 40 % respectively as compared to the SEC control group. However, these values were still significantly less than the control group.

Table-3 showed that subcutaneous implantation of ECCs resulted in significant increase in tumor tissue SphK1 activity by 80.9 % compared to the control group. In the same time, administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in tumor tissue SphK1 activity by 18.6 % and 28.4 % respectively as compared to SEC control group. However, these values were still significantly higher than the control group. Subcutaneous implantation of ECCs resulted in significant increase in tumor tissue TNF-α level by 5.1 folds compared to the control group and administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in tumor tissue TNF-α level by 21.9 % and 30.4 % respectively as compared to SEC control group. However, these values were still significantly higher than the control group.

Subcutaneous implantation of ECCs resulted in significant increase in tumor tissue IL-6 level by 5.8 folds compared to the control group and administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in tumor tissue IL-6 level by 20.9 % and 34.4 % respectively as compared to SEC control group. However, these values were still significantly higher than the control group.

Histopathological examinations for tumor tissues showed that subcutaneous implantation of ECC into the right thigh of the hind limb of mice resulted in development of Ehrlich solid tumor (At day 42 post-implantation) showing sheets of small, higher chromatophilic tumor cells of variable shape representing cell proliferation regions surrounding areas of necrosis and differentiated cells (Figure-1) and administration of I3C to mice resulted in improvement of the histopathological picture in a dose-dependent manner manifested as sheets of malignant cells with focal necrosis and apoptosis (Figures 2 & 3).

Table-1: Tumor volume as affected by SEC or I3C treatment

Animal groups	15th day	20th day	25th day	30th day	35th day	40th day
Control	0	0	0	0	0	0
SEC	10% *	22% *	38% *	58% *	82% *	100% *
I3C 1000 ppm	10% *	20% *	35% *	50% *#	70% *#	75% *#
I3C 2000 ppm	10% *	18% *	30% *#	42% *#	68% *#	70%*#

* Significantly different from the control group (P< 0.05)
# Significantly different from SEC control group (P< 0.05)

Table-2: Survival rate as affected by SEC or I3C treatment

Groups	Survival rate (%)	Survival duration (days; % confidence interval (CI), lower bound to upper bound)
Control	100 %	(42.0 ± 0.0 days; 95% CI, 42.0–42.0)
SEC	50 % *	(36.4 ±2.1 days; 95% CI, 33.11–42.07)
I3C 1000	60% *#	(37.8 ±2.1 days; 95% CI, 33.11–42.56)
I3C 2000	70% *%	(39.9 ± 0.72 days; 95% CI, 38.01–42.21)

* Significantly different from the control group (P< 0.05)
# Significantly different from SEC control group (P< 0.05)

Table-3: Tumor tissue SphK1, TNF-α and IL-6 as affected by SEC or I3C treatment

Animal groups	Tumor tissue SphK1 activity (pmol/min/mg protein)	Tumor tissue TNF-α level (pg/g tissue)	Tumor tissue IL-6 level (pg/g tissue)
Control	40.23±2.01	216.01±6.75	208.01±6.3
SEC	72.8±3.4*	1320.5±22.5*	1421.5±20.5*
I3C 1000 ppm	59.26±2.43*#	1031.1±24.6 *#	1124.2±12.5 *#
I3C 2000 ppm	52.11±2.12 *#	918.65±9.76 *#	932.6±10.1 *#

* Significantly different from the control group (P< 0.05)
# Significantly different from SEC control group (P< 0.05)

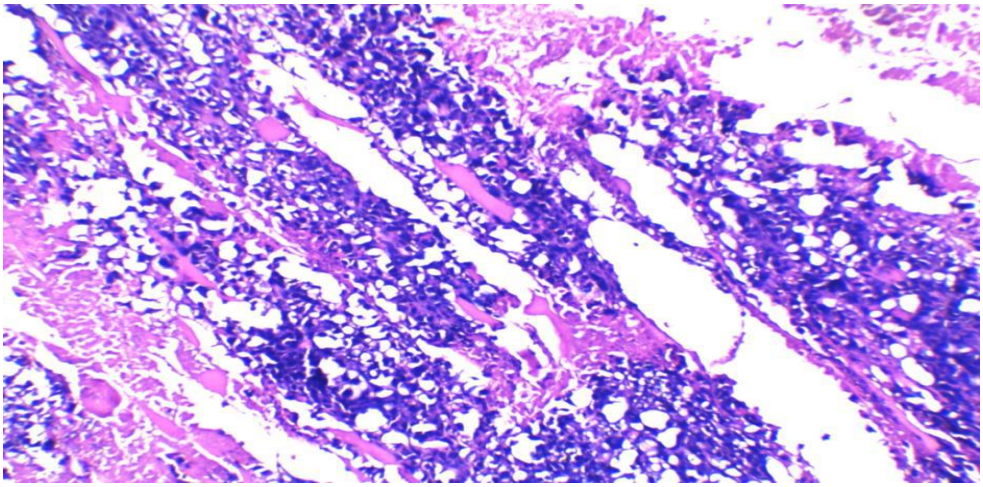

Fig-1: A photomicrograph of Ehrlich solid tumor showing sheets of small, higher chromatophilic tumor cells of variable shape representing cell proliferation regions surrounding areas of necrosis (H&E X 250)

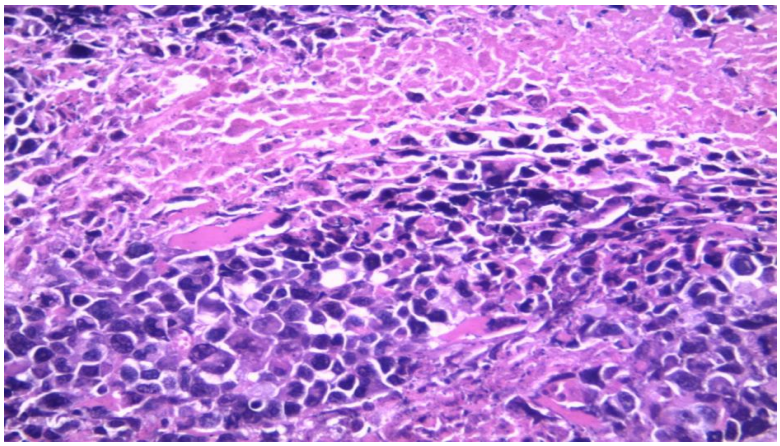

Fig-2: A photomicrograph of SEC sections from mice that received I3C 1000 ppm showing sheets of malignant cells with focal necrosis (H&E X 250)

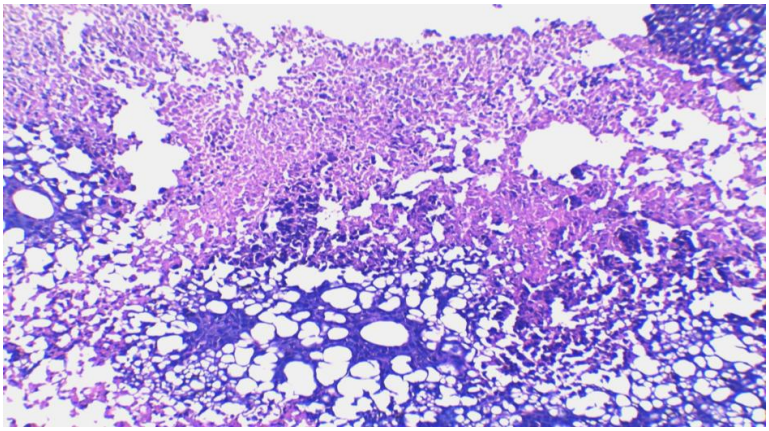

Fig-3: A photomicrograph of SEC sections from mice that received I3C 2000 ppm showing sheets of malignant cells with areas of necrosis (H&E X 250)

DISCUSSION

In the present study, implantation of ECCs resulted in significant increase in tumor tissue TNF-α and IL-6 compared to the control group which was in the same line with other studies which reported that proinflammatory cytokines such as TNF-α and IL-6 have an important role in cancer development [33]. TNF-α and IL-6 are perhaps the best characterised protumourigenic cytokines that were initially suspected to be involved in cancer due to their ability to activate the oncogenic transcription factors such as nuclear factor kappa B (NF-kB), activator protein 1 (AP-1) and signal transducers and activators of transcription 3 (STAT3) in epithelial cells [34].

Sphingosine kinase-1 (SphK1) is an enzyme responsible for phosphorylation of sphingosine to produce sphingosine-1-phosphate (S1P) which is a key regulator of cell growth, differentiation and migration [15, 35]. SphK1 was reported to increase the production of TNF-α and IL-6 through activation of STAT3 and NF-κB which may participate in the pathogenesis of cancer. Moreover, S1P is a mediator of calcium influx during neutrophil activation by IL-6 leading to production of superoxide toxic radicals that lead to further destruction of various body tissues [36]. Many evidences demonstrate that SphK1 is over-expressed in many tumor types and that inhibitors of SphK1 may sensitize tumors to chemotherapy [37]. The results of the present study showed that subcutaneous implantation of ECCs resulted in significant increase in tissue SphK1 activity which in turn increased the production of TNF-α and IL-6 by tumor tissues compared to the control group. Overexpression of SphK1 was found to enhance the expression of Bcl-2 and suppress the expression of Bim in endothelial cells [38] which were in agreement with the histopathological results of our study.

Indole-3-carbinol (I3C) is present in cruciferous vegetables and is considered as a promising chemopreventive agent in experimental studies [39]. In the present study, I3C resulted in significant improvement in tumor tissue antioxidant parameters, survival rate and caspase 3 activity with significant decrease in tumor volume, tumor tissue SphK1 activity, tumor tissue TNF-α and IL-6 and alleviated the histopathological and immunohistochemical changes in a dose-dependent manner compared to SEC group. These results were in agreement with Chen *et al.*, [4]

and Wang *et al.*, [3] who reported that the antitumor effect of I3C was attributed to its antioxidant, anti-inflammatory and apoptosis inducing properties. Chang *et al.*, [10] reported that I3C had anti-inflammatory effects by inhibiting SphK1 activity which in turn inhibits nuclear factor-κB and decreases IL-6 expression.

CONCLUSION

It can be concluded that administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in tumor volume and increased survival rate. Subcutaneous implantation of ECCs resulted in significant increase in tumor tissue SphK1 activity, TNF-α and IL-6 levels and administration of I3C (1000 and 2000 ppm) to ECCs-bearing mice resulted in significant decrease in all of these oncogenic and inflammatory mediators.

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