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Original Article

A nutrient mixture modulates ovarian ES-2 cancer progression by inhibiting xenograft tumor growth and cellular MMP secretion, migration and invasion

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Abstract: Epithelial ovarian carcinoma, which occurs mainly in post-menopausal women, is the leading cause of death from gynecological malignancy and the fifth most common cancer in the U.S. Since ovarian cancer often remains clinically silent, the majority of patients with ovarian carcinoma have advanced intraperitoneal metastatic disease at diagnosis, resulting in a poor prognosis. Long-term survival of patients with ovarian cancer remains poor, due to metastasis and recurrence. We investigated the effect of a nutrient mixture (EPQ) containing ascorbic acid, lysine, proline, quercetin and green tea extract *in vivo* and *in vitro* on human ovarian cancer ES-2 cell line. In vivo, athymic female nude mice (n=12) were inoculated with 3×10^6 ES-2 cells subcutaneously and randomly divided into two groups: group A was fed a regular diet and group B a regular diet supplemented with 0.5% EPQ. Four weeks later, the mice were sacrificed and their tumors were excised, weighed and processed for histology. Dimensions (length and width) of tumors were measured using a digital caliper, and the tumor burden was calculated using the following formula: 0.5 × length × width. We also tested the effect of EPQ *in vitro* on ES-2 cells, measuring cell proliferation by MTT assay, MMP secretion by zymography, invasion through Matrigel, migration by scratch test and morphology by H&E staining. EPQ inhibited tumor weight and burden of ES-2 tumors by 59.2% ($P<0.0001$) and 59.7% ($P<0.0001$), respectively. *In vitro*, EPQ exhibited 35% ($P<0.0001$) toxicity over the control at 1000 μg/ml concentration. Zymography demonstrated only MMP-2 with and without PMA, which was inhibited by EPQ in a dose dependent fashion, with near total inhibition at 1000 μg/ml. Migration by scratch test and Invasion through Matrigel were inhibited in a dose dependent manner with total block of invasion and migration at 500 μg/ml. These results suggest that EPQ has therapeutic potential in treatment of ovarian cancer.

Keywords: Ovarian cancer ES-2 cell line, nutrients, tumor growth, MMPs, migration, Matrigel invasion

Introduction

Ovarian carcinoma, which occurs mainly in post-menopausal women, ranks fifth in cancer deaths among women; it accounts for more deaths than any other cancer of the female reproductive system [1]. The American Cancer Society estimates that approximately 21,290 women will be diagnosed with ovarian cancer in the United States for 2015 and 14,180 women will die from ovarian cancer [1]. A woman's lifetime risk of getting ovarian cancer is about 1 in 75 and chance of dying from ovarian cancer is about 1 in 100 [1]. Since ovarian cancer often remains clinically silent, the majority of patients with ovarian carcinoma have advanced intraperitoneal metastatic disease at diagnosis, resulting in a poor prognosis. About 85% of ovarian cancer cases are diagnosed at an advanced stage after metastasis has occurred [2]. Mortality rates have only slightly decreased in the last 40 years [2].

Invasion of host tissues is dependent on tumor cell adhesion, cell migration and proteolytic degradation of the ECM by MMPs [3]. MMPs, especially MMP-2 and MMP-9, are prognostic for survival and metastatic potential in ovarian cancer [4-6]. Clinical studies note the association of MMP expression with progression of ovarian cancer [7, 8]. Rath and Pauling proposed that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM degradation, inhibiting MMP activity and strengthening the connective tissue surrounding cancer cells, and thus potentially modulat-

ing tumor growth and expansion [9]. We have developed strategies to inhibit cancer development and its spread using naturally occurring nutrients such as lysine, proline, ascorbic acid and green tea extract. This nutrient mixture has exhibited synergistic anticancer activity *in vivo* and *in vitro* in a number of cancer cell lines through inhibition of cancer cell growth, MMP secretion, invasion, metastasis, and angiogenesis [10].

A previous study on the effect of a nutrient mixture containing ascorbic acid, lysine, proline and green tea extract on ovarian cell line SK-OV-3 demonstrated inhibition of ovarian cancer cell proliferation, MMP-2 secretion, and Matrigel invasion *in vitro* [11]. In this study, our objective was to study the effect of supplementation with a nutrient mixture on xenograft tumor growth of ovarian ES-2 in female nude mice and the effect of the nutrient mixture *in vitro* on cell proliferation, MMP secretion, migration and Matrigel invasion.

Materials and methods

Human ovarian cancer cell line ES-2, along with the culture media DMEM, were obtained from ATCC (American Type Culture Collection, Rockville, MD). Antibiotics, penicillin and fetal bovine serum (FBS), were obtained from Gibco (BRL, Long Island, NY). Twenty-four-well tissue culture plates were obtained from Costar (Cambrdige, MA). Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen Inc.) with 0.1% gelatin in non-reducing conditions. The nutrient mixture (EPQ), prepared by VitaTech (Hayward, CA) was composed of the following ingredients in the relative amounts indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; quercetin from quercetindihydrate, *saphora japonica* 50 mg; selenium 30 μg; copper 2 mg; manganese 1 mg. All other reagents used were of high quality and were obtained from Sigma, unless otherwise indicated.

In vivo studies

Animals: Female nude mice, approximately five weeks of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA and maintained in microisolator cages under pathogen-free conditions on a 12-hour light/12-hour dark schedule for a week. All procedures were performed according to humane and customary care and use of experimental animals and followed a protocol approved by internal institutional animal safety review committee.

Experimental design: After housing for a week, the mice (n=12) were inoculated subcutaneously with 3×10^6 ovarian ES-2 cells in 0.2 ml PBS and 0.1 ml Matrigel (BD Bioscience, Bedford, MA). After injection, the mice were randomly divided into two groups; the Control group of mice was fed regular Purina mouse chow and the EPQ group the regular diet supplemented with 0.5% EPQ (w/w). The regular diet was Laboratory Rodent Diet 5001 from Purina Mills (Gray Summit, MO, USA) LLC/Test Diet. The 0.5% EPQ diet was milled and pressed by Purina Mills, LLC and generated by Vitatech (Tustin, CA, USA). During the study, the mice consumed, on the average, 4 g of their respective diets per day. Thus, the supplemented mice received approximately 20 mg of EPQ per day. After four weeks, the mice were sacrificed and their tumors were excised and processed for histology. Dimensions (length and width) of tumors were measured using a digital caliper, and the tumor burden was calculated using the following formula: 0.5 × length × width. Mean weight of mice at initiation of study and termination of study did not differ significantly between the groups.

Histology: Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4-5 microns. Sections were deparaffinized through xylene and graduated alcohol series to water and stained with hematoxylin and eosin (H&E) for evaluation using a standard light microscope.

In vitro studies

Cell culture: Human ovarian cancer ES-2 cells were grown in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL) in 24-well tissue culture plates. Cells were incubated with 1 mL of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with EPQ, dissolved in media and tested at 0, 50, 100,

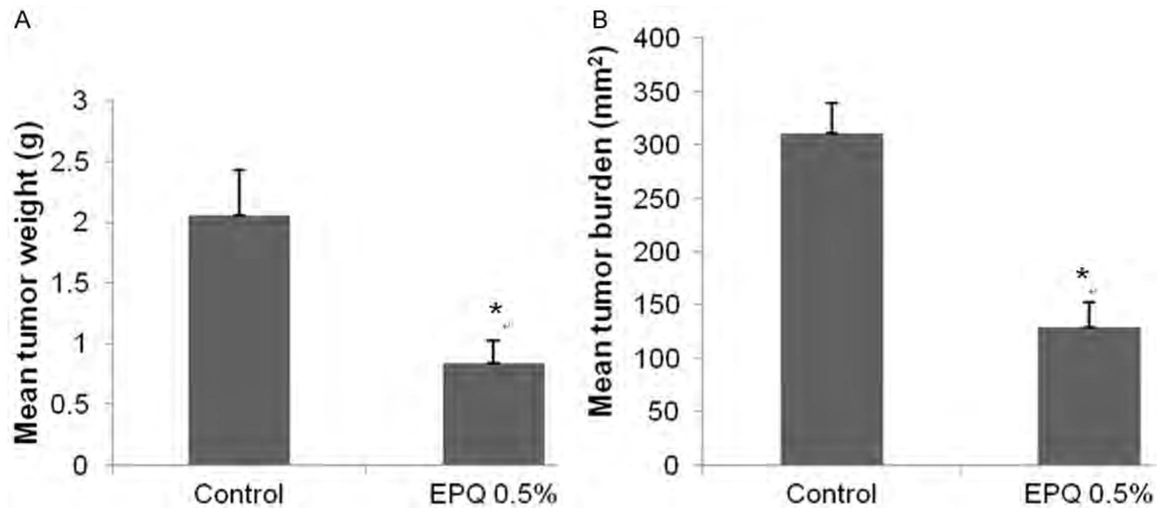

Figure 1. A. Effect of 0.5% EPQ dietary supplementation on mean tumor weight of ES-2 xenografts in female nude mice injected with 3×10^6 ES-2 cells. (*Indicates significance of P<0.0001 with respect to control). B. Effect of 0.5% EPQ dietary supplementation on tumor burden of ES-2 xenografts in female nude mice. (*Indicates significance of P<0.0001 with respect to control).

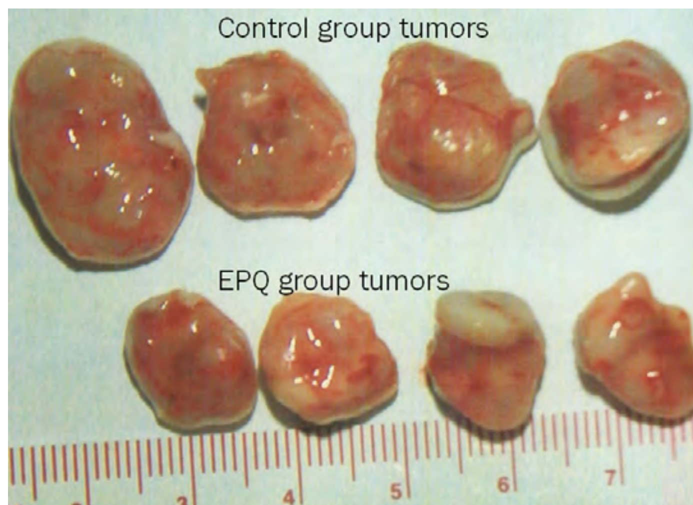

Figure 2. Gross photographs of representative tumors from groups.

250, 500, and 1000 μg/ml in triplicate at each dose. Phorbol 12-myristate 13-acetate (PMA), 100 ng/ml was added to cells to induce MMP-9 secretion. The plates were then returned to the incubator.

MTT assay: Cell viability was evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and 500 μL of MTT (Sigma #M-2128) 0.5 mg/ml in media was added to each well. After MTT addition (0.5 mg/ml) the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered 100%.

Gelatinase zymography: Gelatinase zymography was performed in 10% Novex Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20 μl) were mixed with sample buffer and loaded for SDS-PAGE with Tris glycine SDS buffer, as suggested by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following elec-

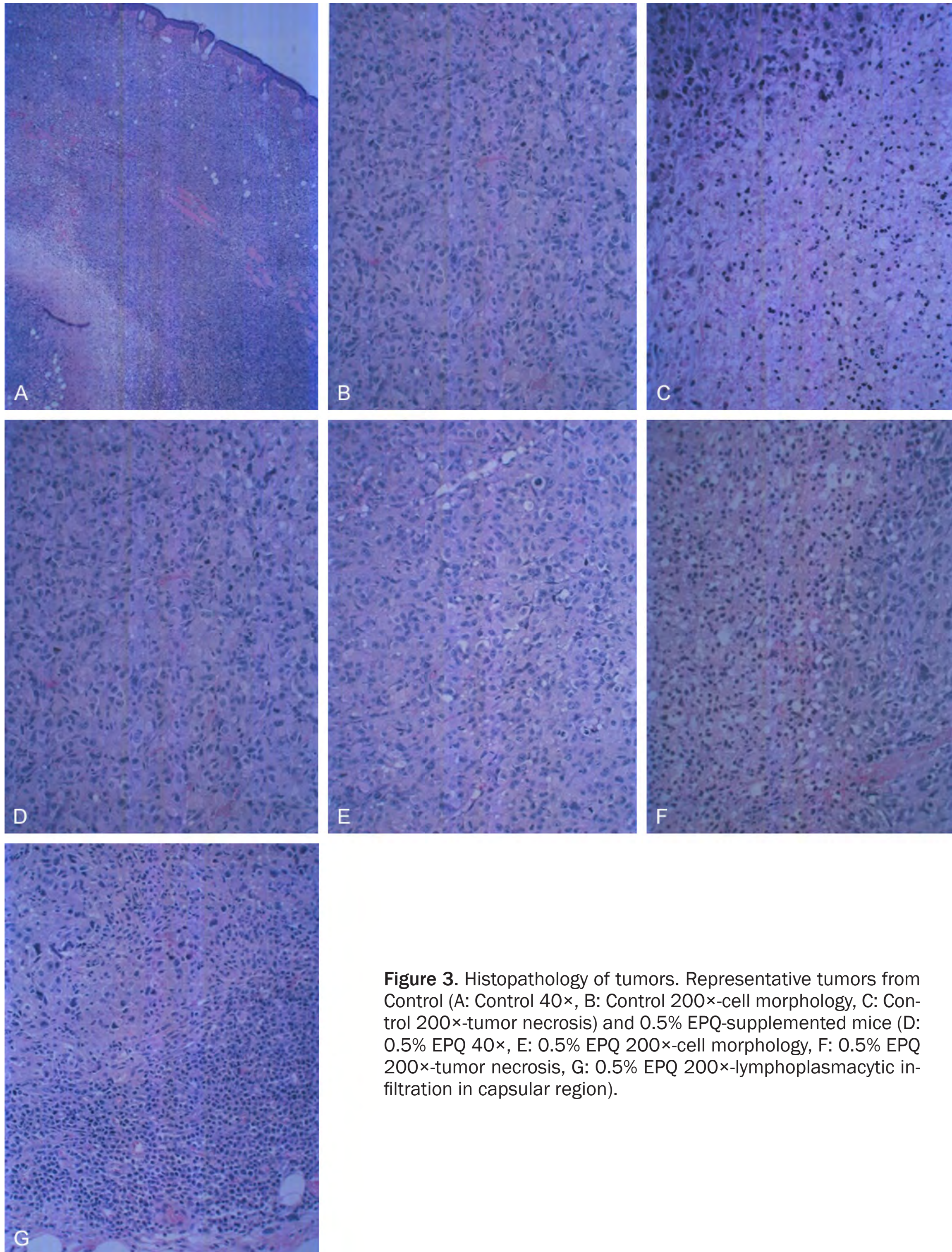

Figure 3. Histopathology of tumors. Representative tumors from Control (A: Control 40×, B: Control 200×-cell morphology, C: Control 200×-tumor necrosis) and 0.5% EPQ-supplemented mice (D: 0.5% EPQ 40×, E: 0.5% EPQ 200×-cell morphology, F: 0.5% EPQ 200×-tumor necrosis, G: 0.5% EPQ 200×-lymphoplasmacytic infiltration in capsular region).

trophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel, producing clear bands

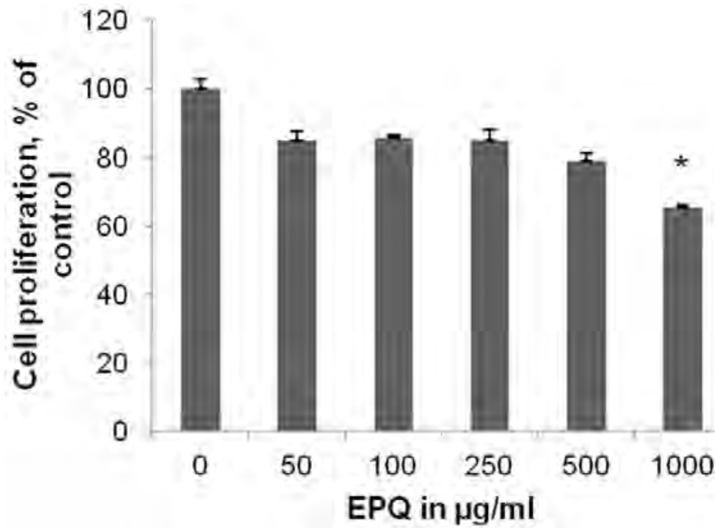

Figure 4. Effect of EPQ on viability of ES-2 cells: MTT 24 h. (*Indicates significance of at least (P<0.0001) with respect to control).

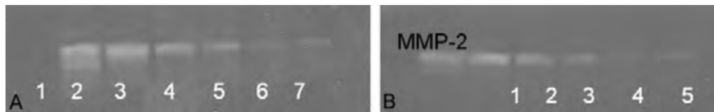

Figure 5. Effect of EPQ on untreated (A) ES-2 cell MMP-2 and MMP-9 secretion and on PMA (100 ng/ml)-treated (B) ES-2 cell MMP-2 and MMP-9 secretion. Legend: 1-Control, 2-6EPQ50, 100, 250, 500, 1000 μg/mL.

against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Matrigel Invasion: Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, ES-2 cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

Cell migration: scratch test: To study cell migration, a 2-mm wide single uninterrupted scratch was made from the top to bottom of culture plates of ES-2 cells grown to confluence. Culture plates were washed with PBS and incubated with EPQ in medium and tested at 0, 50, 100, 250, 500 and 1000 μg/mL, in triplicate at each dose for 24 h. Cells were washed with PBS, fixed and stained with H&E and photomicrographs were taken.

Morphology: H&E: Morphology of cells cultured for 24 h in test concentrations of EPQ were evaluated by H&E staining and observed and photographed by microscopy.

Statistical analysis

The results were expressed as means ± SD, as indicated in the results, for the groups. Data was analyzed by independent sample “t” test using MedCalc software (Mariakerke, Belgium). A threshold of P<0.05 was defined as statistically significant.

Results

In vivo

Tumor growth and burden: NM strongly inhibited the growth of ES-2 xenografts in female nude mice. Mean tumor weight was inhibited by 59.3% (P<0.0001) with EPQ 0.5% dietary supplementation, as shown in **Figure 1A** and tumor burden was inhibited by 58.7% (P<0.0001), as shown in **Figure 1B**.

Histology: Histologically, the tumors from both groups were irregularly round, skin subcutaneous masses, consistent with clear cell ovarian carcinoma. Tumors from Control and EPQ-supplemented mice were similar morphologically, but the tumors from supplemented mice were significantly smaller in size. (See gross photographs of tumors from groups in **Figure 2**). Areas of tumor necrosis ranged from 50-80% of tumor mass in the Control group compared to 20-70% in the EPQ group. Necrosis was not associated with neutrophilic infiltration and the capsular region

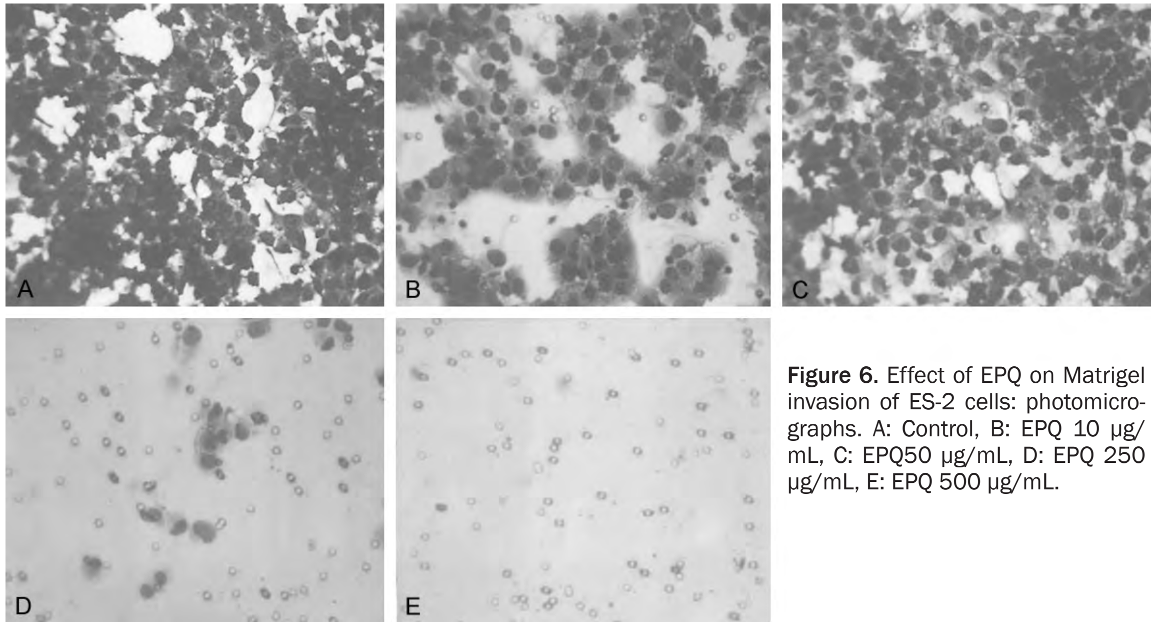

Figure 6. Effect of EPQ on Matrigel invasion of ES-2 cells: photomicrographs. A: Control, B: EPQ 10 μg/mL, C: EPQ50 μg/mL, D: EPQ 250 μg/mL, E: EPQ 500 μg/mL.

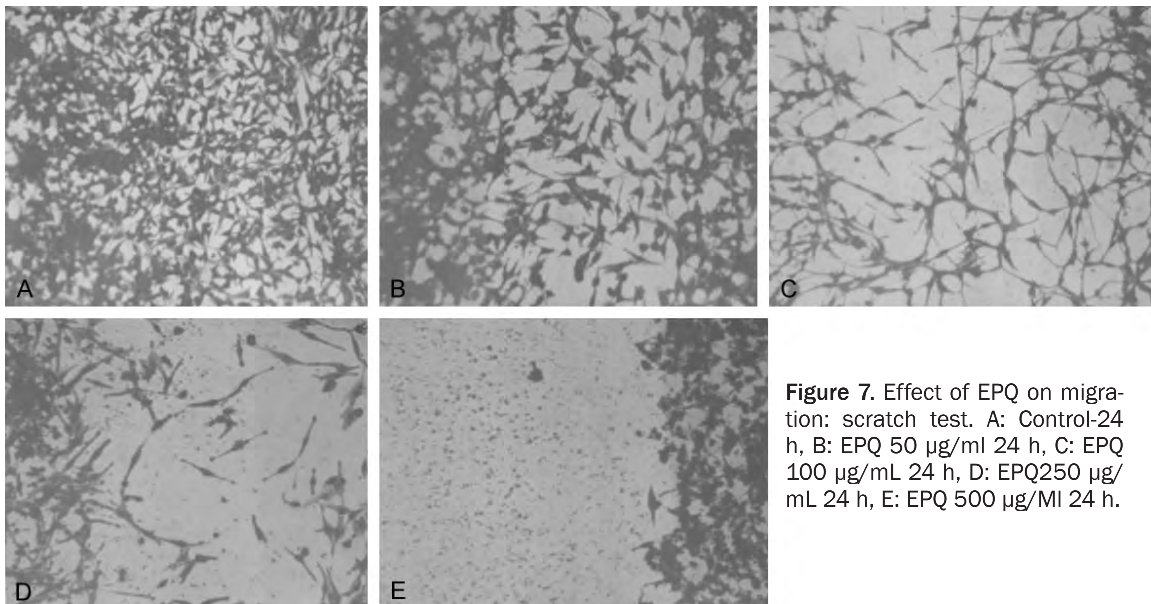

Figure 7. Effect of EPQ on migration: scratch test. A: Control-24 h, B: EPQ 50 μg/ml 24 h, C: EPQ 100 μg/mL 24 h, D: EPQ250 μg/mL 24 h, E: EPQ 500 μg/Ml 24 h.

of some tumors was infiltrated with lymphoplasmacytic cells. See **Figure 3**.

In vitro

Cell proliferation: EPQ exhibited dose-dependent inhibition of ovarian ES-2 cell proliferation *in vitro* with 35% (P<0.0001) decrease at 1000 μg/mL EPQ, compared to the Control, as shown in **Figure 4**.

Gelatinase zymography: Zymography only demonstrated MMP-2 secretion by normal and PMA-treated ES-2 cells. EPQ inhibited MMP-2 secretion in a dose-dependent fashion with near total inhibition at 500 and 1000 μg/mL, as shown in **Figure 5A** and **5B**.

Matrigel invasion: EPQ significantly inhibited ES-2 cell invasion through Matrigel in a dose-dependent manner, with total block at 500 μg/mL, as shown in **Figure 6**.

Cell migration: scratch test: EPQ reduced cell migration in a dose-dependent manner, with complete block of ES-2 cells at 500 μg/mL.

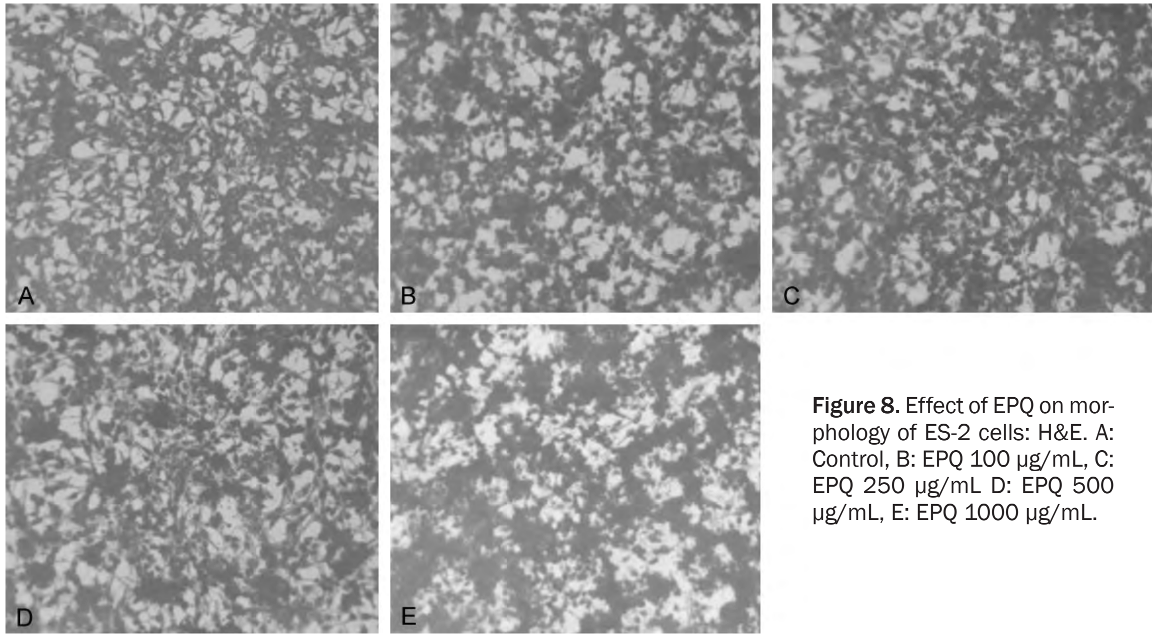

Figure 8. Effect of EPQ on morphology of ES-2 cells: H&E. A: Control, B: EPQ 100 µg/mL, C: EPQ 250 µg/mL D: EPQ 500 µg/mL, E: EPQ 1000 µg/mL.

Photomicrographs of the results for the scratch test for ES-2 cells are shown in **Figure 7**.

Morphology: H&E staining: H&E staining showed no morphological changes below EPQ 1000 µg/ml, as shown in **Figure 8**.

Discussion

The results of the *in vivo* study of human ovarian ES-2 xenografts in immune impaired (athymic) female nude mice demonstrated significant suppression of ovarian cancer ES-2 tumor growth (59.3% inhibition of mean tumor weight and 58.7% inhibition of mean tumor burden) with EPQ0.5% dietary supplementation. Results from the cellular proliferation study support the *in vivo* findings, as EPQ showed increased toxicity in ES-2 cells in a dose-dependent manner, with 35% inhibition of cell growth in cells exposed to 1000 µg/mL EPQ.

Tumor cell invasion is dependent on cell adhesion, cell migration, and proteolytic degradation of the ECM by MMPs [3]. As mentioned earlier, literature documents that MMPs, especially MMP-2 and MMP-9 are prognostic for survival and metastatic potential in ovarian cancer [4-6]. Xu et al. reported significant correlations between activated MMP-2 and invasiveness, metastasis and disease progression in epithelial ovarian carcinomas [6].

The results from our *in vitro* study of ovarian cancer ES-2 cells demonstrated potent, significant suppression of invasive parameters by the micronutrient mixture. EPQ inhibited MMP-2 secretion with near total block at 500 µg/ml and 100% inhibition of invasion of cells through Matrigel at 500 µg/ml. Migration of cells using scratch test showed total block at EPQ 500 µg/ml.

Use of natural health products to prevent, inhibit and reverse carcinogenesis is gaining increasing importance, since scientific evidence shows that high consumption of fruits and vegetables are associated with reduced risk of various cancers [12, 13]. EPQ was formulated by defining critical physiological targets in cancer progression and metastasis. Optimal ECM structure depends upon adequate supplies of ascorbic acid and the amino acids lysine and proline to ensure proper synthesis and hydroxylation of collagen fibers. In addition, lysine contributes to ECM stability as a natural inhibitor of plasmin-induced proteolysis [9, 14]. Manganese and copper are also essential for collagen formation. There is considerable documentation of the potency of green tea extract in modulating cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression [15-21]. N-acetyl cysteine and selenium have demonstrated inhibition of tumor cell MMP-9 and invasive activities, as well as migration of endothelial cells through ECM [22-24]. Ascorbic acid demonstrates cytotoxic and antimetastatic actions on malignant cell lines [25-29] and cancer patients have been found to

have low levels of ascorbic acid [30, 31]. Low levels of arginine, a precursor of nitric oxide (NO), can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis [32]. Cancer preventive effects of quercetin include induction of cell cycle arrest, apoptosis and antioxidant functions [33]. Induction of apoptosis by quercetin in cancer cells during different cell cycle stages without affecting normal cells has been documented in various cancers *in vivo* and *in vitro* [33].

Conclusion

Current treatment methods for ovarian cancer are generally ineffective and have not improved in 40 years. Thus, there is a need for development of effective therapeutic agents for these cancers with minimal toxicity. Our studies demonstrated that the mixture of the non-toxic components of EPQ significantly inhibited the growth and tumor burden of ovarian cancer cell line ES-2 *in vivo*. In addition, invasive parameters, such as ES-2 cell line MMP-2 secretion, migration and invasion were significantly inhibited by EPQ *in vitro*. These findings suggest potential of EPQ in treatment of ovarian cancer.

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Disclosure of conflict of interest

None.

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