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The Combination of Natural and Synthetic Agents – A New Pharmacological Approach in Cancer Chemoprevention

L. RIVERÓN-NEGRETE, S. CABALLERO-SALAZAR, M.G. ORDAZ-TELLEZ[1] & F. ABDULLAEV*

Instituto Nacional de Pediatría, Avenida Imán # 1, Colonia, Insurgentes Cuiculco, 04530, México D.F., México;
[1]Facultad de Ciencias, UNAM, México
**e-mail: fikrat@servidor.unam.mx*

Cancer chemoprevention involves intervening in the carcinogenic process with natural or synthetic agents that either block neoplasia development or prevent malignant phenotype progression in humans [1]. Recently, the rationale of combining different chemopreventive agents to target multiple pathways of the carcinogenetic process has received considerable attention as a new pharmacological approach. The process of identifying potential synergistic combinations of chemopreventive agents should be based upon result of *in vitro* studies followed by testing in animal models of carcinogenesis and clinical trial.

Previous studies have demonstrated that natural and synthetic agents such as saffron and sodium selenite, or sodium arsenite alone, inhibited the development of human tumor cells *in vitro* [2-7]. In contrast to saffron extract, the synthetic agents used alone also exhibited cytotoxic effects on normal human cells [2,3,6]. This experimental study was designed to evaluate *in vitro* cytotoxic effects of saffron extract in combination with sodium selenite and sodium arsenite against human tumor cells.

METHODS:

Chemicals. Sodium selenite and sodium arsenite were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco´s modified Eagle's medium (D-MEM) was purchased from Gibco/BRL Products (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone (Road Logan, UT). Culture dishes and flasks were obtained from Costar (Corning, NY). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium selenite (Na_2SeO_3) and sodium arsenite (Na_2AsO_3) were dissolved in distilled water, adjusted to pH 7.4 and added to the culture medium. Final concentrations of sodium selenite and sodium arsenite in cultures are shown in Tables 1 and 2.

Preparation of saffron extract. Stigmata of pure saffron (Mancha, Spain) were purchased from a local market and stored in the dark at 4°C. The concentrated saffron extract was prepared as previously described [3,4]. The final concentrations of saffron extract in the cultures ranged as indicated in Tables 1 and 2.

Cell culture. HeLa (human cervix epitheloid carcinoma), A-204 (human rhabdomyosarcoma) and HepG2 (human hepatocellular carcinoma) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in D-MEM with 10% FBS in a CO_2 water-jacketed incubator (Nuaire, Plymouth, MN) at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air. Incubation with the saffron extract and sodium selenite or sodium arsenite (alone or in combination) were carried out as described previously [2-4,7].

Colony formation assay. Tumor cells (2×10^5) were seeded into 100-mm Petri dishes with 10 ml D-MEM medium. After 2 days, different concentrations of tested agents were added and incubation continued 1 h for sodium selenite and 3 h for saffron. The cells were then trypsinized (0.05 M trypsin), counted and 200 cells were seeded in 35-mm Petri dishes for determination of colony formation. At least 3 replicate colony determinations were carried out for each culture. After 10 days of incubation, the resulting colonies were rinsed with 50 mM phosphate buffer, pH 7.6, containing 150 mM NaCl, fixed with methanol, stained with Giemsa (Sigma) and the number of colonies (with diameter <0.05 mm) per dish were determined as described [2-4].

Statistical analysis. Data were analyzed using Statistical Analysis System software (SAS Institute Inc., Cary, NC 27511, US. Release 6.02). Values were considered significant when $p < 0.05$.

Table 1. Inhibitory effect (%) of saffron extract in combination with sodium selenite on colony formation of tested human tumor cells

Agents	HeLa cells	A-204 cells	HepG-2 cells
10μM of selenite	29 ± 3.6	16 ± 1.9	8 ± 1.0
50μg/ml of saffron	31 ± 4.8	21 ± 3.6	31 ± 5.3
100μg/ml saffron	32 ± 3.1	34 ± 2.8	38 ± 5.4
10μM of selenite + 50μg/ml of saffron	43 ± 4.3	44 ± 6.2	37 ± 2.8
10μM of selenite + 100μg/ml of saffron	48 ± 3.7	47 ± 1.5	50 ± 2.8

Cells (Hela, A-204 and HepG2) were exposed to the indicated concentrations (alone or in combination) of sodium selenite, for 1 h and of saffron extract for 3 h. The number of cells that were able to form colonies was determined as described in Methods. Results are presented as the percentage of inhibition of colony formation (colonies/dish formed by untreated cells accepted as 100%; for HeLa cells = 41; for A-204 cells = 64; for HepG2 cells = 31). Data are mean ± SD of values obtained with four culture dishes per treatment ($p < 0.05$).

RESULTS First, we have examined the effect of different concentrations of saffron extract (from 50 to 400 μg/ml), sodium selenite (from 10 to 200 μM) and sodium arsenite (from 10 to 200 μM) on colony formation of these human malignant cells (HeLa, A-204 and HepG2 cells) *in vitro* and demonstrated that these agents inhibited colony formation of all tested cell lines in a dose-dependent manner.

The treatment of tested tumor cells with saffron extract in combination with sodium selenite synergistically inhib-

ited colony formation, in comparison to the effect of these agents alone at the same concentrations (Table 1). A more effective inhibition (48%, 47% and 50% for HeLa, A-204 and HepG2 cells, respectively) was found when these tumor cells were treated with the combination of saffron (100 μg/ml) and sodium selenite (10 μM).

Table 2 indicates that saffron extract in combination with sodium arsenite also caused a synergistic inhibitory effect on colony formation of tested cells, in comparison to the effect of these agents alone at the same concentrations. A more effective inhibition (42%, 47% and 44% for HeLa, A-204 and HepG2 cells, respectively) was found when these tumor cells were treated with the combination of saffron (100 μg/ml) and sodium arsenite (10 μM).

Thus, our results demonstrate that natural extract of saffron in combination with well-known synthetic anticancer agents such as selenium and arsenic compounds enhanced inhibitory effect of these agents on the development of different human malignant cells *in vitro.*

Table 2. Inhibitory effect (%) of saffron extract in combination with sodium arsenite on colony formation of tested human tumor cells

Agents	HeLa cells	A-204 cells	HepG-2 cells
10 μM of arsenite	28 ± 2.0	17 ± 0.9	17 ± 1.3
50 μg/ml of saffron	24 ± 1.6	23 ± 2.5	21 ± 2.9
100 μg/ml saffron	33 ± 4.3	35 ± 4.7	41 ± 5.2
10 μM of arsenite + 50 μg/ml of saffron	35 ± 3.7*	36 ± 3.1	38 ± 4.9
10 μM of arsenite + 100μg/ml of saffron	42 ± 1.7	47 ± 0.8	44 ± 3.7

Cells (Hela; A-204 and HepG2) were exposed to the indicated concentrations (alone or in combination) of sodium arsenite, for 1 h and of saffron extract for 3 h. The number of cells that were able to form colonies was determined as described in Methods. Results are presented as the percentage of inhibition of colony formation (colonies/dish formed by untreated cells accepted as 100%; for HeLa cells = 41; for A-204 cells = 64; for HepG2 cells = 31). Data are the mean ± SD of values obtained with four culture dishes per treatment ($p < 0.05$).

DISCUSSION: Cancer chemoprevention is a new discipline whose foundation rests upon epidemiological evidence suggesting that dietary or synthetic compounds may be inhibitors of carcinogenesis [9].

This study evaluated the efficacy of naturally occurring saffron extract in combination with synthetic sodium selenite and arsenite on the development of different human malignant cells *in vitro*. It was reported that selenium alone and in combination with ß-carotene and vitamin E was effective in reducing the incidence of malignant tumors *in vivo* [10,11] and in combination with adriamycin or taxol inhibited different cancer cells *in vitro* [12]. It was also shown that arsenic compounds alone and in combination with other agents (all-trans retinoid acid, ascorbic acid or interferon-alpha) inhibited human cancers, particularly acute promyelocytic leukemia [7,13-15]. Inhibition of nucleic acid synthesis in tumor cells by these natural and synthetic agents (alone or in combination) might explain their cytotoxic activities [2-7]. Furthermore, intracellular level of SH compounds in tumor cells can be another important factor in the relative sensitivity of malignant cells to the effect of saffron and sodium selenite or sodium arsenite, while SH compounds play a role in the cytotoxicity of these agents [2,5,6]. Previously, we demonstrated that the pre-treatment of tumor cells with saffron resulted in increased levels of intracellular SH-compounds [6]. It seems that the marked difference in the inhibition of colony formation in tumor cells by saffron in combination with sodium selenite or arsenite may be explained by an increase in the levels of intracellular SH-compounds in tested malignant cells or through modulation of the GSH-redox system [7,14].

In conclusion, the present study supports the increasing evidence that naturally occurring saffron extract alone and in combination with synthetic sodium selenite or sodium arsenite may have an important role in cancer chemoprevention. Additional studies are needed to determine the biologically active ingredients of saffron as well as the most effective approach to treatment and concentrations of these agents and the molecular mechanism(s) of their antitumor and antimutagenic effects. Futhermore, clinical studies to evaluate the safety and effectiveness of these agents as further prospects in cancer chemoprevention, both alone and in combination, are warranted.

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