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Research Paper

The Biochemical Changes Associated with Phytic Acid on Induced Breast Proliferative Lesions In Rats

Preliminary Findings

Mahmoud R. Hussein[1,*]
Mohamad A. Abd EL-Aziz[2]
Nagwa S. Ahmad[3,5]
Faten Omran[4,5]
Mohamad Abdulhameed[6]

[1]Pathology Department; Faculty of Medicine, Assuit University; Assuit, Egypt

[2]Biochemistry Department; School of Pharmacy; AL-Azhar University; Assuit, Egypt

[3]Biochemistry Department; [4]Pharmacology Department; [5]Faculty of Medicine; [6]Faculty of Agriculture; South Valley University; Souhag, Egypt

*Correspondence to: Mahmoud R. Hussein; Department of Pathology; Faculty of Medicine; Assuit University; Assuit, Egypt; Tel.: +093.583.166; Fax: +088.033.272727; Email: mrh17@swissinfo.org

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KEY WORDS

breast, cancer, phytic acid

ABSTRACT

Background: Phytic acid is an anti-neoplastic agent. We hypothesize that during mammary tumorigenesis, the administration of phytic acid is associated with biochemical changes including enhancement of apoptosis and inhibition of oxidative stress.

Materials and methods: An animal model formed of 25 rats was established. The animals were divided into three groups: (1) a control group which received the same phytic acid treatment in the right route and amount; (2) a carcinogen group which received a carcinogenic substance DMBA that can induce proliferative changes in the mammary gland; (3) treated group which received phytic acid, 60 days after the intake of DMBA. The animals were sacrificed, serum and tissue were evaluated for markers of tumorigenicity (serum total sialic acid, TSA); apoptotic changes (tissue caspase-3 activity and % DNA Fragmentation) and oxidative stress (tissue level of nitric oxide, NO).

Results: Following DMBA administration, benign proliferative breast changes occurred in all animals. However, these changes disappeared following phytic acid treatment. As compared to the control group, the development of these proliferative changes in DMBA group was associated with statistically significantly ($p < 0.05$) increased levels of TSA and NO and decreased apoptotic activity. When compared to DMBA group, the disappearance of the proliferative changes in phytic acid -treated group was associated with statistically significantly ($p < 0.05$) decreased levels of TSA and NO and increased apoptotic activity.

Conclusions: Administration of phytic acid reversed the proliferative effects of DMBA, suggesting its protective role.

INTRODUCTION

Mammary tumorigenesis is associated with alterations in both apoptotic and oxidative stress mechanisms.[1] Oxidative stress can cause DNA damage and induces mutations of tumor suppressor genes. Apoptosis is mediated by several molecules such as caspases-3 ; a cysteine protease.[2,3] Phytic acid (PA, Inositol hexaphosphate, IP6), a naturally occurring phosphorylated carbohydrate, is present in almost all plants (wheat germ and wheat bran) and mammalian cells.[4] Inositol can reduce cell proliferation and therefore has a antineoplastic activity. Also, it promotes differentiation of malignant cells with their reversion to normal phenotype.[4,5] Following its administration, IP6 is taken inside the cells and dephosphorylated to lower inositol phosphate. The latter interferes with signal transduction pathways and therefore induces cell cycle arrest5. Although the antitumor effect of phytic acid was examined in breast cancer, our understanding of the biochemical and morphological alterations associated with this effect is still incomplete. In this investigation, we hypothesized that during mammary tumorigenesis, the administration of phytic acid is associated with biochemical changes including enhancement of apoptosis and inhibition of oxidative stress. To test our hypothesis, we carried out this investigation.

MATERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee of South Valley University, School of Medicine, Sohag, Egypt.

Rats and maintenance. Six-week old, female Sprague-Dawley rats were obtained from Assuit University Animal Facility, Faculty of Medicine, Assuit University, Assuit, Egypt. They were housed in Animal facility, with room temperature maintained at 27°C, relative

humidity of 50–70% and an airflow rate of 15 exchange /hour. Also, a time controlled system provided 07.00–21.00 h light and 21.00–07.00 h dark cycles. All rats were given ad libitum access to Taklad rodent chow diet and water from sanitized bottle fitted with stopper and sipper tubes.[6]

Chemicals. The chemicals were purchased from Sigma Chemical Company (DMBA) and Pharco (Phytic acid and brans). Inc., Egypt. The DMBA [7,12-di-methyl benz (a) anthracene] was used to induce cellular transformation in the mammary gland (one-gram was dissolved in 25% dimethyl sulphoxide, DMSO, to make a total of 100-ml solution). Animals were given a single 50 mg/kg body weight intra-gastric dose of DMBA in 1 ml of corn oil administered by oral intubation. The drinking water of female Sprague -Dawwely rats was supplemented with 15 mM Inositol (IP6, exogenous Phytic acid). Wheat bran (WB) and Wheat germ (WG) were taken as 50% of the diet (IP-6, endogenous phytic acid, PA).[7]

Carcinogenic substance-DMBA and the induction of proliferative changes in the mammary gland. Several experiments were executed using a total of 25 rats, five animals each in control and DMBA groups. After a 7-day acclimatization period, a randomized block design based on the animal body weights was used to divide the rats into three groups: (1) control group, (2) carcinogen group and (3) PA-treated group which included three subgroups (PA, WG and WB subgroups). The control group of five rats did not receive DMBA but instead, they received same treatment alone (phytic acid, wheat bran and wheat germ) in the right rout and amount. The remaining 20 rats were treated with DMBA for 60 days. At the end of 60 days treatment period, examination of the animals revealed development of palpable (0.5–2 cm) single and multiple masses in the mammary gland of all DMBA treated-animals. Then, these animals were further divided into two groups: (1) one group received nothing (carcinogen group); (2) the other group (PA-treated group) received either exogenous phytic acid (PA-subgroup), or endogenous phytic acid (wheat germ and wheat bran) for one month.

Specimens. All the animals were scarified at the end of the experiments (three months). The blood samples were collected and the serum was separated for biochemical assays. For each animal, the breast tissues samples were obtained from all mammary glands, and divided into two parts: one was freezed at 70°C for biochemical assays, and the other was fixed in formalin for routine histology. A total of 100 tissue specimens and 50 blood samples were examined. All the analyses were performed in triplicates.

Biochemical evaluations. Evaluation of the apoptotic changes. The tissue caspase-3 activity was determined using Aoptarget caspase-3 protease, calorimetric assay kits (Cat. # KH20022, Biosourse Europe, Belgium). The substrate paranitroanillide and the chemicals for DNA fragmentation were obtained from Sigma Co., USA. The caspase-3 proteolytic activity was determined using a modified procedure described by other groups.[8] Briefly, cytosolic extracts were prepared by homogenization of mammary specimens (50 mg) in lysis buffer. Subsequently the homogenates was centrifuged at 13000 x g for 15 min at 4°C. The supernatant was used to determine caspase-3 activity. A 50 μL of reaction buffer was added to 50 μL of each sample (cytosol extract) and then 50 μL of diluting buffer was added. A total of ten μL of DEVD-PNA substrate was added to each tube and incubated at 37°C for two hours. The samples were kept in the dark during incubation. The protein concentrations were assayed by Biuret method. The samples were read at 405 nm in a spectrophotometer (Schemazu type, Japan) using regular cuvette. The substrate DEVD-pNA was composed of the chromophore para-nitroanilide and a synthetic tetra peptide, DEVD (Asp-Glue-Val-Asp).

Determination of tissue % DNA fragmentation was done following other groups.[9] Briefly, tissue samples were lysed by homogenization in 0.2% triton X-100, ten mM Tris, one mM EDTA, pH 8.0, incubated on ice for a few minutes, homogenized again, and centrifuged at 14000 g for 15 minutes. The supernatant, pellet and the homogenates were used for fragmented, intact and total DNA assays, respectively. Perchloric acid was added to the samples to 0.5 M, i.e., one part of the stock 70%(7 M) acid: 13 parts of sample (20 μL perchloric + 260 μL sample), mixed well and left for ten minutes. A total of 300 μL reagent 2, 150 μL sample/standard/or distilled water blank was incubated for 48 hours. OD575nm was measured against reagent blank and samples content of fragmented DNA was calculated from the standard curve. For tissues, the ratio of DNA in supernatant and pellet of the total was calculated.

Evaluation of markers of tumorigenicity (serum total sialic acid) and oxidative stress (tissue nitric oxide). Total sialic acid, in serum, was determined according to the method described by other groups.[10] For determination of TSA, 20 μL serum/standard + 980 μL distilled water were mixed and put on ice for 20 minutes + 1.0 mL resorcinol reagent, mixed and boiled in water bath for 15 minutes, cooled on ice for 10 minutes. Then, two mL butyl acetate/n-butanol were added and vortexed for one minute to extract the chromogen. Centrifugation was done at 3000 rpm for ten minutes. Finally, the OD of supernatant was determined at 580 nm against water blank.

Nitric oxide (NO) levels in the tissues were measured by the evaluation of total nitrate and nitrites using a method described by other groups.[11] Briefly, 260 μL tissue homogenate was mixed with 26 μL $ZnSO_4$ (30%, w/v in H_2O), incubated for 15 minutes at room temperature then centrifuged (10,000 rpm) for 15 minutes. Cadmium was activated twice with 2% HCl (dilute the conc., 37% solution, 54 mL + 946 mL H2O, v/v) for 30 minutes under continuous shaking .The activated cadmium was washed extensively (ten times) with distilled water with shaking. Cadmium was prepared by soaking zinc rods in 20% cadmium sulfate, w/v in H_2O, overnight, then the formed cadmium precipitate was isolated, washed, activated and washed again with H_2O as above and is stored under H_2O). Nitrate-to-Nitrite reduction was done by mixing 200 μL tissue supernatant with 80 μL of a mixture of three parts of NH_4Cl (2.6%, w/v in H_2O), one part of Sodium borate (Borax, 2.1%, w/v in H_2O), activated Cadmium. The mixture was incubated for 30 minutes at room temperature and centrifuged at 10,000 rpm for ten minutes

Morphological analysis. Histological examination of the specimens: Morphological examination of the tissue specimens was done following other groups.[6]

Statistical analysis. Statistical analysis was done using ANOVA test (analysis of variance). The results were presented as mean ± standard error of mean (SEM). p value <0.05 was considered statistically significant.

RESULTS

Morphological changes following the administration of DMBA in carcinogen-DMBA and phytic acid treated groups. Examination of the animals (60 days from time of DMBA administration) revealed development of palpable variable sized (0.5–2 cm) single or multiple masses involving mammary gland tissues of all DMBA treated-animals. One month later, further examination of animals in DMBA group revealed an increase in the size of these masses

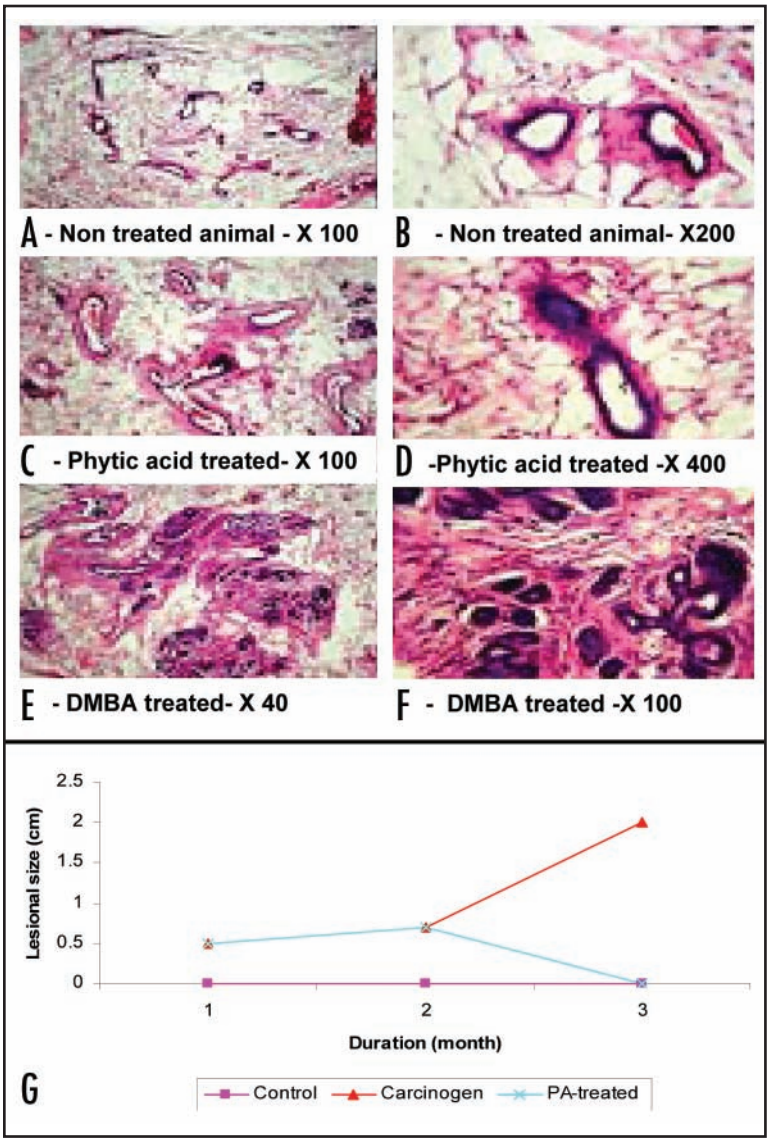

Figure 1. Morphological changes in DMBA, phytic acid and control groups. (A and B). Histological picture of the mammary glands in the control (untreated) animals. The breast is formed of well-circumscribed lobules. The latter is composed of small ducts and loose fibroconnective tissue stroma; (C and D) Histological changes of the mammary gland in the phytic acid-treated animals. The beast tissues show comparable histological features to those encountered in the non-treated group; (E and F) Histological changes of the mammary gland in DMBA (Carcinogen)-treated animals. The breast features the presence of some proliferative changes including adenosis (increased number of the glands), epitheliosis (proliferation of the ductal epithelium) and fibrosis. (G) Size of the lesions (mean values, cm) in the control, carcinogen and Phytic acid treated groups.

(2.0–4.0 cm). The lesions were rubbery in consistency and had whitish cut surface. In PA-treated group (animals which received phytic acid for one month after 60-day period of DMBA), initial lesions (0.5–2 cm) completely disappeared in all animals. Histologically, proliferative changes (adenosis and epitheliosis) were seen in DMBA treated group (Fig. 1). These changes were completely absent in all animals of the control and PA-treated groups (Fig. 1).

Biochemical alterations. Deacreased levels of markers of tumorigenicity and oxidative stress in PA-treated group. As compared to the control, there was a statistically significant increase in serum level of TSA and tissue NO levels in DMBA groups ($p < 0.05$). Alternatively, when compared to DMBA group, disappearance of the proliferative changes in PA-treated group was associated with decreased levels of these markers (Table 1).

Increased tissue levels of caspase-3, % DNA fragmentation in PA-treated group. As compared to the control group, the development of proliferative changes in DMBA group was associated with decreased tissue levels of markers of apoptotic activity. Alternatively, disappearance of these proliferative changes in PA-treated groups was associated with increased levels of markers of apoptotic activity. All these differences reached the level of statistical significance ($p < 0.05$) (Table 1).

DISCUSSION

Our study clearly demonstrated two observations. First, administration of DMBA (carcinogen) was associated with development of benign proliferative breast changes, elevation of the levels of markers of tumorigenicity (TSA) and oxidative stress (NO) as well as a decrease in apoptotic activity (caspase-3 and percent DNA fragmentation). In contrast, administration of phytic acid was associated with disappearance of these proliferative changes, a decrease in the levels of markers of tumorigenicity and oxidative stress as well as an enhanced apoptotic activity.

Administration of carcinogen-DMBA was associated with development of benign proliferative breast changes, elevation of levels of markers of tumorigenicity, and oxidative stress as well as a decrease in apoptotic activity. Sialic acid, a family of acetylated derivatives of neuraminic acid is widely distributed in mammals. The increase in serum level of TSA after the intake of DMBA not only agrees with previous reports[12,13] but also suggests its possible diagnostic value in monitoring breast lesions. This increase may be explained by enhanced activity of sialidase enzyme in breast lesions.[14] The decrease in tissue levels of caspase-3 activity and % DNA fragmentation in DMBA-group as compared to control group is in line with previous reports.[15] This reduction may be due to overexpression of caspase-3 inhibitors and survivin in tumor cells or alterations of death receptors and or mitochonderial mediated pathways of apoptosis.[16] The high levels of nitric oxide in DMBA -group agrees with previous reports.[17-20] It is well known that development of neoplastic changes in the breast is associated with generation of oxygen free radicals. The latter can promote tumor growth and metastasis by enhancing invasive, angiogenic, and migratory capacities of tumor cells.[6]

Administration of IP-6 was associated with disappearance of the proliferative changes in the breast, a decrease in the levels of markers of tumorigenicity and oxidative stress as well as an increase in apoptotic activity. The ability of phytic acid to reverse DMBA induced-proliferative changes in rats not only agrees with previous reports in experimental skin, colon and breast carcinogenesis models but also suggest its anti-proliferative properties.[21-24] This antiproliferative effect of IP-6 may be due to its ability to: (1) enhance baseline Natural Killer cell activity with subsequent destruction of the transformed cells;[21] (2) downregulate genes involved in transcription and cell cycle regulation and to upregulate cell cycle inhibitors[25] and (3) induce apoptotic machinery and to inhibits cell survival signaling.[26] The ability of IP-6 to induce apoptotic activity in our series is in accord with previous studies. In this

Table 1 **Analysis of total lipid sialic acid, caspase-3 activity, % DNA fragmentation and nitric oxide levels in the control, DMBA and phytic acid treated groups**

	Control group	Carcinogen group	Phytic acid (PA) treated group			
	Non-treated Animals	DMBA-treated Animals	Phytic acid (PA) (Exogenous PA)	Wheat bran (WB) (Endogenous PA)	Wheat germ (WG) (Endogenous PA)	Phytic acid (PA) (Total PA: PA+ WB+WG)
TSA (Serum)	84.0 {4.2}**	194.5 {8.2}	133.5 {4.1}**	119.5 {7.5}**	150.5 {5.0}**	134.5 {5.2}
DNA fragmentation (Tissue)	25.2 {1.2}*	17.4 {1.3}	32.8 {1.76}**	27.8 {2.6 }**	26.8 {1.8}**	29.1 {2.1}
Caspase-3 (Tissue)	24.5 {1.4}**	13.5 {1.4}	29.0 {1.9}**	25.7 {1.8}**	25.9 {1.8}**	26.9 {1.8}
Nitric oxide (Tissue)	4.8 {0.3}NS	7.7 {1.5}	5.4 {0.7} NS	5.1 {0.7} NS	4.0 {0.3}*	4.8 {0.6}

The animals were divided into three groups: (1) control (untreated) group; (2) DMBA (carcinogen treated) group; and (3) Phytic acid-treated group that received either phytic acid (exogenous phytic acid) and endogenous phytic acid (wheat germ and wheat bran. As compared to the control, there was a statistically significant (**$p < 0.01$; *$p < 0.1$) increase in serum level of TSA and tissue NO levels in the DMBA groups ($p < 0.05$). Also, as compared to the control group, the development of the proliferative changes in DMBA group was associated with decreased tissue levels of markers of apoptotic activity. Alternatively, disappearance of the proliferative changes in the PA-treated groups was associated with increased levels of markers of apoptotic activity. All these differences reached the level of statistical significance ($p < 0.05$). Values represent mean ± SEM (Standard error of a mean). NS for nonsignificant differences).

respect, administration of IP-6 was able to induce a moderate to strong (up to 14-fold over control) apoptotic cell death in adenocarcinoma cell lines of mouse prostate.[28] Moreover, IP-6 was able to inhibits DMBA-induced mouse skin and mammary tumor development.[23,27] The decreased level of NO following IP-6 administration may be explained by its antioxidant activity. In support, intrinsic IP-6 in corn and soy was protective against lipid peroxidation in colon of pigs.[29]

Taken as a whole, our investigation reported the biochemical and morphological changes associated with administration of IP-6. Here we demonstrated that this natural product has antiproliferative activity. Also, it can increase apoptotic activity and decrease level of detrimental oxidative stress. Although antitumor effect of phytic acid was demonstrated recently in multiple models of cancer, including breast, the novelty of this study is in the use of serum sialic acid and changes in tissue nitric oxide levels to monitor changes due to DMBA treatment. Here we report that phytic acid inhibited the tumor development completely throughout the 60-day and further 1-month treatment time period. These findings suggest possible prophylactic and therapeutic implications of this product in mammary carcinogenesis. The clinical ramifications of these findings mandate further investigations.

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