DHA induces apoptosis of human malignant breast cancer tissues by the TLR-4/PPAR-α pathways

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Abstract. Docosahexaenoic acid (DHA) oil is an important polyunsaturated fatty acid for the human body. Evidence has demonstrated that DHA is beneficial for inhibiting mammary carcinogenesis. However, the mechanisms of DHA mediating apoptosis induction have not been fully elucidated. Thus, in the present study, the activity levels of total-superoxide dismutase (t-SOD), catalase (CAT), glutathione-peroxidase (GSH-PX) and the concentration of malondialdehyde (MDA) were determined in DHA oil-treated human malignant breast tissues. The results revealed that compared with control, DHA significantly increased the main antioxidant enzymes levels, including t-SOD, CAT, and GSH-PX, but decreased the MDA concentration in the DHA oil treated breast cancer tissues. Furthermore, DHA significantly increased the ratio of cyclic (c)AMP/cGMP, and promoted the expression of Toll-like receptor 4 (TLR-4) and peroxisome proliferator activated receptor (PPAR)-α, thus DHA induced breast cancer cell apoptosis. We hypothesized that the levels of TLR-4 and PPAR-α are involved in the antitumorogenesis properties of DHA in breast cancer. The results of the present study hold significance for the further clinical development of DHA oil in breast cancer treatment.

Introduction

Docosahexaenoic acid (DHA; 22:6 n-3) is an important member of the family of omega-3 polyunsaturated fatty acids and numerous people have added DHA oil to their daily diet (1,2). Currently, breast cancer (BC) is one of the most common types of cancer among women worldwide (3). Dietary unsaturated fatty acids, particularly DHA, are considered to serve an important role in reducing the risk of developing BC (4). Certain evidence has demonstrated that DHA oil is beneficial for inhibiting mammary gland carcinogenesis (5-10). The mechanisms of DHA oil on the inhibition of tumor proliferation and the promotion of apoptosis are complex (5-10). Among the numerous key factors, oxidative stress serves an important role in the initiation, promotion, progression and apoptosis of BC by interfering with the intracellular signal transduction pathways, and inducing DNA damage (11). The downregulation of antioxidant enzyme (AOE) expression levels or their activities have been revealed to be associated with numerous types of cancer, including breast, prostate, bladder and hepatic cancer, and multiple myeloma (12-18). Nevertheless, other studies have reported no significant changes, higher expression or higher activity levels of AOE in certain cancer types. For example, decreased catalase (CAT), with unchanged glutathione-peroxidase (GPH-PX) and increased levels of superoxide dismutase (SOD) levels were reported in the A549 lung cancer cell line, and lung cancer tissues (19). Therefore, maintaining the appropriate activity levels of AOE may be essential in preventing the development of specific cancer types (20). There are specific AOE with essential roles in protecting cells from oxidative stress, including SOD, CAT and GPH-PX. The regulation and increases in AOE activity are associated with cancer (12). To elucidate the anticancer mechanism of DHA in BC, antioxidant activities, including that of SOD, CAT and GPH-PX were analyzed using various assays.

The anticancer mechanisms of DHA are complex (7). Glucose uptake, glycolytic metabolism, lactate production and total glucose oxidation have been demonstrated to be significantly decreased in response to DHA supplementation, thereby decreasing oxidative metabolism, and enhancing metabolic injury (7). Furthermore, the metabolic changes in DHA led to intracellular cyclic (c)AMP and cGMP levels decreasing by 50% in MDA-MB-231, and BT-474 cancer cell lines, which mediated the phosphorylation of AMP-activated protein kinase at Thr172, a metabolic stress marker (7). It effectively provides rationale for enhancement oncurent cancer prevention models and therapies by combining with dietary sources, including DHA oil (7).

In the present study, the tumor suppressor ratio, the activities of SOD, GSH-PX and CAT, the expression of malondialdehyde (MDA), the expression of cAMP/cGMP, and the
expression of Toll-like receptor 4 (TLR-4) and peroxisome proliferator activated receptor (PPAR)-α factor were detected in human malignant BC tissues following treatment with DHA oil. The results aided in explaining the apoptosis mechanism of DHA on human malignant BC. The current study has clinical significance for the further development of DHA oil use in BC.

Materials and methods

Samples and reagents. DHA oil (21) (Bohai algae DHA oil, content ~40%) was provided by the Food Science and Engineering Laboratory of Jinzhou Medical University (Jinzhou, China). Human malignant BC tissue samples were provided by the First Affiliated Hospital of Jinzhou Medical University. The approval of removed tissues for research purposes was obtained from the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Written informed consent was obtained from each patient.

Dexamethasone (1 gram) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-TLR4 antibody produced in rabbit (100 µg) was purchased from Sigma-Aldrich (Merck KGaA). Anti-PPARα rabbit antibody (100 µg) was purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG H&L (1 mg) was purchased from Abcam. Trypsin, Hanks, RPMI-1640 medium and Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The 10% fetal bovine serum and 10% normal goat serum and ammonium molybdate was added to generate a pale yellow compound, 5-dithio‑bis2‑nitrobenzoic acid dithiobis‑nitrobenzoic acid (shipped with dithiobis‑nitrobenzoic acid to produce a yellow compound, 5-dithio‑bis2‑nitrobenzoic acid dithiobis‑nitrobenzoic acid ( Maximum absorption peak at 405 nm). CAT activities were expressed as mU/mg protein (22). SOD activities were determined (at 550 nm) using the Xanthine oxidase method and expressed as nU/mg protein (23). CAT was measured by the reaction of CAT scavenging H₂O₂, and ammonium molybdate was added to generate a pale yellow complex (maximum absorption peak at 405 nm). CAT activities were expressed as nU/mg protein (23). GSH-PX was reacted with dithiobis-nitrobenzoic acid to produce a yellow compound, 5-dithio‑bis2‑nitrobenzoic acid dithiobis‑nitrobenzoic acid anion (maximum absorption peak at 420 nm). The concentrations of GSH-PX were expressed as nU/mg protein (24).

Effect of DHA on the antioxidant activities of BC tissues

Detection of the activities of antioxidant enzymes. The BC tissues were centrifuged at 1,500 x g for 15 min at 4˚C to remove the debris following homogenizing with normal saline solution (0.9% sodium chloride). The supernatant was transferred into new tubes for the evaluation of the SOD, CAT and GSH-PX activities. The measurements were performed following the manufacturer’s protocol of an assay kit (SOD: cat. no. A001-1; CAT: cat. no. A007-2; GSH-PX: cat. no. A005; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China).

SOD activities were determined (at 550 nm) using the Xanthine oxidase method and expressed as nU/mg protein (22). CAT was measured by the reaction of CAT scavenging H₂O₂, and ammonium molybdate was added to generate a pale yellow complex (maximum absorption peak at 405 nm). CAT activities were expressed as nU/mg protein (23). GSH-PX was reacted with dithiobis-nitrobenzoic acid to produce a yellow compound, 5-dithio‑bis2‑nitrobenzoic acid dithiobis‑nitrobenzoic acid anion (maximum absorption peak at 420 nm). The concentrations of GSH-PX were expressed as nU/mg protein (24).

Detection of MDA content. Fatty-acid peroxidation MDA content in the homogenate (0.1 ml) was measured using the thiobarbituric acid (TBA) method according to the manufacturer’s protocol (cat. no. A003-1; Nanjing KeyGen Biotech Co., Ltd.). MDA and TBA condensate to produce a red product (maximum absorption peak at 532 nm). Thus, the MDA content was calculated by measuring the 532 nm absorbance and expressed as nmol/mg protein (23,24).

cAMP and cGMP assays. The cAMP and cGMP content of BC tissues were determined following the manufacturer’s protocol (cAMP: cat. no. 80204; cGMP: cat. no. 80104; Neweast Biotech Company, Wuhan, China) by ELISA. The cAMP and cGMP extracts were diluted (1:10) with sample diluent. Optical densities were measured at 450 nm (25,26).

Immunohistochemistry. The expression of TLR-4 factor was measured by immunohistochemistry. Briefly, 4-µm thick sections were deparaffinized and the endogenous peroxidase activity was blocked for 10 min with 3% hydrogen peroxide at 37°C. After being cultured with or without DHA for 24 h, the BC tissues were embedded in paraffin to observe the effect of DHA oil on the morphology of BC. Five random sections were obtained from each sample. For research on the signal transduction pathway of DHA, BC tissues were cultured for 24 h in RPMI-1640 medium with or without DHA oil, and then the total protein was extracted from the BC tissues. The total protein content was used to detect the effect of DHA on BC antioxidant activities. Each sample was measured three times in parallel.

Histological observation. For hematoxylin‑eosin (H.E.) staining, sections were rinsed in double distilled water and were incubated with hematoxylin solution stain for 5 min at 25˚C. After 5 min, sections were washed with running tap water, sequentially followed by differentiation for 30 sec in 1% acid-alcohol (hydrochloric acid and ethanol) at 25˚C and then washed for 1 min again with running tap water. Subsequently, all sections were stained with eosin for 30 sec and then dehydrated with different concentrations alcohol (70, 80, 90 and 100%) for 2 min each. Sections were covered with xylene-based mounting medium, after two changes of xylene for 5 min each time at 25˚C. The effect of DHA on the H.E. BC sections was observed using an inverted microscope (OLYMPUS, IX73+DP73, 12V 100W halogen lamp). Analysis of the tissue areas occupied was performed using Image Pro 5.0 Plus software (Media Cybernetics, Inc., Rockville, MD, USA). Suppressor ratio (%)=(DHA treatment group entity tissue area-the control group entity tissue area)/control group entity tissue area x100%.
25°C. Subsequently, sections were treated for 30 min with 10% normal goat serum in Tris-buffered saline (pH=7.6) at 37°C. Then, sections were incubated with monoclonal anti-TLR4 rabbit antibody (1:200; cat. no. PRS3141-100UG) overnight at 4°C. After washing three times with PBS, they were incubated with the HRP-sheep anti-rabbit IgG H&L secondary antibody (1:500; cat. no. ab6721) at room temperature for 1 h, followed by incubation with the color reagent 3,3'-diaminobenzidine for 3 min at 25°C (26). Analysis of the average gray density value (GDV) of TLR-4 was performed using Image Pro 5.0 Plus software.

### Western blotting

All BC tissues were stored within liquid nitrogen prior to protein extraction. Briefly, tissues were

| Figure | Group (DHA oil concentration, µg/ml) | α=0.05 | α=0.01 |
|--------|-------------------------------------|--------|--------|
| Fig. 1E | 0                                   | a      | A      |
|         | 100                                 | ab     | AB     |
|         | 150                                 | bc     | B      |
|         | 200                                 | c      | B      |
| Fig. 1F | 100                                 | b      | A      |
|         | 150                                 | ab     | A      |
|         | 200                                 | a      | A      |
| Fig. 2A | 0                                   | d      | D      |
|         | 100                                 | c      | C      |
|         | 150                                 | b      | B      |
|         | 200                                 | a      | A      |
| Fig. 2B | 0                                   | d      | D      |
|         | 100                                 | c      | C      |
|         | 150                                 | b      | B      |
|         | 200                                 | a      | A      |
| Fig. 2C | 0                                   | d      | D      |
|         | 100                                 | c      | C      |
|         | 150                                 | b      | B      |
|         | 200                                 | a      | A      |
| Fig. 2D | 0                                   | a      | A      |
|         | 100                                 | b      | B      |
|         | 150                                 | c      | C      |
|         | 200                                 | d      | D      |
| Fig. 2E | 0                                   | d      | D      |
|         | 100                                 | c      | C      |
|         | 150                                 | b      | B      |
|         | 200                                 | a      | A      |
| Fig. 2F | 0                                   | d      | D      |
|         | 100                                 | c      | C      |
|         | 150                                 | b      | B      |
|         | 200                                 | a      | A      |
| Fig. 3F | 0                                   | b      | B      |
|         | 100                                 | a      | A      |
|         | 150                                 | a      | A      |
|         | 200                                 | b      | B      |
| Fig. 3G | 0                                   | b      | B      |
|         | 100                                 | b      | B      |
|         | 150                                 | b      | B      |
|         | 200                                 | a      | A      |
homogenized with 0.5 ml ice-cold lysis buffer (pH 7.5, 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 20 μg/ml aprotinin, 2 mM sodium orthovandate and 1 mM PMSF). The homogenates were centrifuged at 10,000 x g at 4°C for 20 min and the supernatant was removed. The protein concentration was determined using the bicinchoninic acid method with bovine serum albumin as the standard. Samples (30 μg/ml protein per lane) were boiled for 5 min, separated using 15% SDS-PAGE and transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Subsequently, membranes were blocked with 5% bovine serum albumin at room temperature for 1.5 h and incubated with the anti-PPARα rabbit antibody (1:1,000; cat. no. ab8934) at 4°C for 12 h. The membrane was then washed three times with TBS Tween 20 buffer and incubated with the secondary HRP-conjugated sheep anti-rabbit IgG H&L antibody (1:200; cat. no. ab6721) at room temperature for 1 h. The GDV of specific bands was measured with Quantity One version 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (27).

Statistical analysis. Each experiment was performed in parallel three times. All data are expressed as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance and was analyzed further by Tukey’s honest significant difference test (28). Different lowercase letters in the same column represent significant differences at P<0.05 and different capital letters represent significant differences at P≤0.01. All data analyses were conducted using SPSS 19.0 software (IBM Corp., Armonk, NY, USA).

Results

Effects of DHA on BC morphology and the suppressor ratio of DHA in BC. From the results of paraffin sections (H.E. staining), as DHA oil concentration increased, the morphology of BC cells became more irregular. For example, apoptosis of BC cells was more evident compared with the control group (Fig. 1A). The surrounding of tissues appeared to be shedding, phagocytized and the cell connections had disappeared (Fig. 1B). There were more vesicles observed within cells (Fig. 1C). The overall volume of BC cells had shrunk, whereas the cytoplasmic density of BC cells had increased. Chromatin agglutination occurred, and the nuclear membrane and nucleoli were broken or absent (Fig. 1D).

In Fig. 1E, the area sizes of human malignant breast tissues in the 150 μg/ml DHA group were significantly reduced compared with the control (0 μg/ml DHA group). The suppressor ratio of DHA in the 200 μg/ml DHA group was significantly higher compared with the ratio of the 100 μg/ml DHA group (Fig. 1F).

Effect of DHA on the activities of AOE s. The activities of t-SOD, CAT and GSH-PX in the BC tissues increased in a DHA dose-dependent manner. Compared with the control group (500.33±23.20 nmol/mg protein), the activities of SOD were significantly enhanced by 22.4% in the 100 μg/ml DHA group (612.37±14.98 nmol/mg protein; P<0.001) and reached the maximum at 79.24% in the 200 μg/ml DHA group (896.78±38.87 nmol/mg protein; P<0.001; Fig. 2A).

Compared with the control group (1.59±0.06 mU/mg protein), the activities of CAT was enhanced by 14.44% the 100 μg/ml DHA group (1.82±0.05 mU/mg protein; P<0.001) and reached the maximum at 38.30% in the 200 μg/ml DHA group (2.20±0.14 mU/mg protein; P<0.001; Fig. 2B).

Compared with the control group (104.91±3.51 mU/mg protein), the activity of GSH-PX was enhanced by 57.75% in the 100 μg/ml DHA group (144.51±3.95 mU/mg protein; P<0.001) and reached the maximum at 100.17% in the 200 μg/ml DHA group (210.00±6.01 mU/mg protein; P<0.001; Fig. 2C).

Effect of DHA on the concentration of MDA in the BC tissues. In Fig. 2D, the MDA concentration of BC tissues was significantly reduced compared with the control. Compared with the control group (122.49±5.15 nmol/mg protein), the mean MDA concentration in the 100 μg/ml DHA group (102.31±8.49 nmol/mg protein) was significantly decreased by 16.48% (P<0.001) and in the 200 μg/ml DHA group was decreased by 52.22% (63.96±5.24 nmol/mg protein; P<0.001).

DHA serves as a death receptor via the upregulation of cancer-specific cAMP/cGMP. Intracellular cAMP and cGMP are the secondary messengers involved in the generation of BC. The levels of cAMP and cGMP of the BC tissues with DHA treatment were significantly higher compared with the control groups (Fig. 2E and F).

Compared with the control group (0.50±0.062 nmol/mg), the mean concentration of cAMP in the 100 μg/ml DHA group (0.73±0.043 nmol/mg; P<0.001) significantly increased by 44.78% and reached the maximum at 246.77% in the 200 μg/ml DHA group (1.74±0.056 nmol/mg; P<0.001). Compared with the 100 and 150 μg/ml DHA group (0.73±0.043 and 1.10±0.053 nmol/mg, respectively), the mean concentration of cAMP in the 200 μg/ml DHA group was significantly increased by 139.52, and 59.13%, respectively (Fig. 2E).

Compared with the control group (54.50±1.42 m nmol/mg), the mean concentration of cGMP in the 100 μg/ml DHA group (62.01±1.41 nmol/mg; P<0.001) significantly increased by 13.79% and reached the maximum at 79.64% in the 200 μg/ml DHA group (97.89±1.78 nmol/mg; P<0.001). Compared with the 100 and 150 μg/ml DHA group (62.01±1.41 and 74.32±1.27 nmol/mg, respectively), the mean concentration of cGMP in the 200 μg/ml DHA group significantly increased by 57.87, and 31.72% respectively (Fig. 2F).

Compared with the control group, the 200 μg/ml DHA group revealed significantly higher concentrations of cAMP/cGMP with a 2-fold increase (Fig. 2G).

Effect of DHA on the expression and localization of TLR-4 factor by immunohistochemistry. The immunohistochemical staining of the control group (Fig. 3) was brown in the cell membrane and cytoplasm. The GDV of TLR-4 mainly existed in the cell membrane and cytoplasm. The GDV of TLR-4 showed that staining of the control group (Fig. 3) was brown in the cell membrane and cytoplasm. When DHA was added at 100 μg/ml (Fig. 3B), TLR-4 expression reached the maximum (Fig. 3F).
however, the expression of TLR-4 in the 200 µg/ml (Fig. 3D) DHA group was similar to that of the control group (P>0.05, Fig. 3F). The expression of TLR-4 in the 150 µg/ml (Fig. 3C) DHA group was similar to the 100 µg/ml DHA group (Fig. 3B; P>0.05, Fig. 3F).

**Effect of DHA on the expression of PPAR-α by western blotting.** The western blotting result (Fig. 3E) and the GDV (Fig. 3G) of the control group bands revealed that PPAR-α protein expressed in breast cancer tissue. The results revealed that PPAR-α participated in the generation of BC in Fig. 3E and G. The expression of PPAR-α in BC tissues increased with DHA and reached the maximum in the 200 µg/ml DHA group (Fig. 3E; P<0.01; Fig. 3G). The expression of PPAR-α in the control group, 100 µg/ml DHA group and 150 µg/ml DHA group are similar (Fig. 3E; P>0.05).
Figure 2. Effect of DHA on the activity of antioxidant enzymes and secondary messengers. (A) SOD, (B) GSH-PX and (C) CAT activities, and (D) MDA concentration in the BCTs incubated for 24 h with increasing concentrations of DHA. The results are expressed as the mean ± standard deviation of four independent experiments, two samples per experiment performed in triplicate. The homogeneity of variance comparisons were analyzed using SPSS 19.0 software. Statistical analysis was analyzed by Tukey's honest significant difference test following one-way analysis of variance. Different lowercase letters in the same column represent significant differences at P≤0.05 and different capital letters represent significant differences at P≤0.01, but not significantly different (P>0.05) for the same letter (Table I). DHA serves as a death receptor via cancer-specific cAMP/cGMP upregulation. Effects of DHA on (E) cAMP and (F) cGMP levels in the BCTs following 24 h. (G) Effects of DHA on the ratio of cAMP/cGMP levels in the BCTs following 24 h. Different lowercase and uppercase letters indicate the significance of multiple comparisons tests of the results. Different lowercase letters in the same column represent significance differences at P≤0.05 and different capital letters represent significant differences at P≤0.01 (Table I). SOD, super oxide dismutase; DHA, docosahexaenoic acid; CAT, catalase; GSH-PX, glutathione-peroxidase; MDA, malondialdehyde; cAMP, cyclic AMP; cGMP, cyclic GMP; BCTs, breast cancer tissues.
Figure 3. Effect of DHA on the signaling molecules in breast cancer. Immunohistochemical results of TLR-4 in breast cancer: (A) Control, (B) 100 µg/ml DHA group, (C) 150 µg/ml DHA group and (D) 200 µg/ml DHA group. Magnification, x400. (E) Western blotting results of PPAR-α. The GDV of (F) TLR-4- and (G) PPAR-α-positive expression areas in different groups. The results are expressed as the mean ± standard deviation. Different lowercase and uppercase letters indicate the significance of multiple comparisons tests of the results. Different lowercase letters in the same column represent significance differences at P≤0.05 and different capital letters represent significant differences at P≤0.01 (Table I). DHA, docosahexaenoic acid; PPAR, peroxisome proliferator activated receptor; GDV, gray density value; TLR-4, Toll-like receptor 4.
Discussion

The results of the H.E. staining in the present study demonstrated that DHA oil induced the apoptosis of BC cells. The suppressor ratio of DHA treatment revealed that DHA significantly inhibited the growth and induced the apoptosis of BC cells.

Oxidative stress is the most important cause of cell damage, leading to the occurrence and development of cardiovascular disease. AOE s protect cells from free radicals and oxidative stress, contributing to the prevention of cancer. Numerous studies have reported that the upregulated expression or higher activity of AOE s may be used as effective strategies for cancer prevention and therapy. For example, MRN-100, as an adjuvant therapy was demonstrated to be effective in the treatment of esophageal/gastric carcinoma, exerting an antioxidant effect in the stomach and blood tissues by increasing the levels of GSH-PX, SOD, CAT, GSH-PX, and the total antioxidant capacity (29). *Ganoderma lucidum* significantly enhanced the levels of SOD, CAT and GPH-PX in the plasma, liver, and mammary tissues, thus being an effective chemopreventive agent against BC (30). Tangeretin increased the levels of AOE s, including SOD, CAT, GST and GSH-PX significantly, indicating to be effective, and efficient for the treatment of BC (31).

SOD is a class of AOE s that catalyzes the dismutation of superoxide radicals into H$_2$O$_2$ and O$_2$. Subsequently, CAT and GSH-PX catalyze H$_2$O$_2$ decomposition to H$_2$O and O$_2$ (20). H$_2$O$_2$ is not only a reactive oxygen species, but also a major signaling molecule (32). Multiple studies have indicated that mitochondrial H$_2$O$_2$ is a direct and effective apoptosis inducer (32,33). The present study demonstrated that DHA was able to simultaneously upregulate the expression levels and activities of SOD, CAT, and GSH-PX in BC tissues. Therefore, DHA may inhibit the proliferation of BC cells and the associated oxidative stress mechanism.

Oxidative stress produces fatty-acid peroxidation whose metabolites possess high toxicities and mutagenic properties (34). The main fatty-acid peroxidation is MDA (34). The results in the current study revealed that DHA significantly decreased the MDA concentration of BC tissues.

The cyclic nucleotides cAMP and cGMP have been recognized as important signaling molecules within cells. Under normal physiological conditions, cyclic nucleotides regulate a myriad of biological processes. In addition, altered cyclic nucleotide signaling has been observed in a number of pathophysiological conditions, including cancer. Several studies have demonstrated that activation of cyclic nucleotide signaling leads to the inhibition of proliferation and activation of apoptosis in numerous types of cancer cells, such as bladder (35,36), breast (37-41), colon (42-44), hepatoma (45), leukemia (46,47), lung (36,48), lymphoma (49,50), ovarian (36,51), pituitary (52), prostate (36) and skin cancer (53). In the present study, the results demonstrated that DHA produced significant increase in the ratio of cAMP/cGMP levels (P<0.001), suggesting that DHA inhibits the proliferation and induces apoptosis of BC cells by increasing the ratio of cAMP/cGMP.

Increasing evidence suggests an association between chronic inflammation and cancer development (54). Recent evidence suggests that inflammation and oxidative stress play pivotal roles in the development of clinical conditions like cancers (55) and metabolic syndrome (56). Nonetheless, the underlying molecular signaling pathways associating inflammation, oxidative stress and BC cell death are not well defined.

TLR-4 is an important member of the Toll-like receptor family that are exogenous or endogenous ligands, and activate the nuclear factor (NF)-κB signal transduction pathway and the transcription of the early inflammatory cytokine genes. Ahmed et al (57) demonstrated that lipopolysaccharide, the TLR-4 agonist, enhances 4T1 tumor growth and migration, by increasing the rate of angiogenesis, vascular invasiveness, and tumor invasion. This effect is more evident in TLR-4⁻/⁻ mice, suggesting that TLR-4 on host immune cells may serve an essential role in inhibiting BC genesis and tumor metastasis (58). TLR4 exerts both a defensive role at the host level and a negative role at the cancer cell level in this murine metastatic breast tumor model (58). Other data suggested that the TLR-4 agonist may induce pro- or anti-tumorogenic effects (57-60). The results in the present study revealed that TLR-4 was highly expressed in the BC tissue of the 100 µg/ml DHA treatment group. In the BC tissue, TLR-4 was localized in the cell membrane and cytoplasm, similar to that observed by Yang et al (61). The expression of TLR-4 in the cytoplasm may be due to the presence of BC with lymph node metastasis. Thus, this suggests that TLR-4 participates in the generation of BC and DHA upregulated TLR-4 to induce the apoptosis of BC. The expression of TLR-4 was significantly reduced when DHA was at 200 µg/ml, this may be due to the fact that different concentrations of DHA use different signaling pathways in breast cancer. The low concentrations DHA (100 µg/ml) stimulated the expression of TLR-4 to induce the apoptosis of BC. However, high concentrations DHA (200 µg/ml) decreased the expression of TLR-4 signaling molecules to promote the BC tissue apoptosis thoroughly.

![Figure 4. Apoptosis mechanism of DHA. DHA induced the levels of SOD, CAT, and GSH-PX and decreased the concentration of MDA in the BC tissues. DHA increased the ratio of cAMP/cGMP levels and promoted the expression of TLR-4 and PPAR-α, in order to induce the apoptosis of BC cells. DHA, docosahexaenoic acid; PPAR, peroxisome proliferator activated receptor; TLR-4, Toll-like receptor 4; CAT, catalase; MDA, malondialdehyde; cAMP, cyclic AMP; cGMP, cyclic GMP.](image-url)
PPAR-α, a nuclear transcription receptor, belongs to the PPAR family, and regulates the expression of numerous genes and proteins (62). Activation of PPAR-α has been reported to serve an important role in glucose homeostasis, fatty acid oxidation, lipid metabolism and the inflammatory process (63). The activation of PPAR-α was demonstrated to block the transcription of NF-κB and activator protein-1 signaling pathways (62-64). Previously, PPAR-α-specific agonists have been reported to inhibit the proliferation of various cancer cells in cultured cell lines and in engraft nude mouse models (65-70). Yessoufou et al (71) revealed that TLR-4 mutant mice exhibited significantly higher PPAR-α expression levels following a methionine/choline-deficient diet, while levels in wild types did not change. This suggests that high expression levels of TLR-4 increase the expression of PPAR-α. However, little is known regarding the molecular and cellular mechanisms of PPAR-α-mediated growth inhibition of cancer cells. In the present study, it was demonstrated that DHA promoted the expression of PPAR-α and we hypothesized that DHA regulates the glycolipid metabolic pathways in BC tissues, thus leading to the apoptosis of BC cells.

In conclusion, DHA induced the activities of SOD, CAT and GSH-PX, and decreased the concentration of MDA in the BC tissues. Furthermore, DHA significantly increased the ratio of cAMP/cGMP levels, and promoted the expression of TLR-4 and PPAR-α, in order to induce the apoptosis of BC cells (Fig. 4). DHA may be used as a dietary treatment or for prevention of BC in the future.

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