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Original Article

Fish oil regulates cell proliferation, protect DNA damages and decrease HER-2/neu and c-Myc protein expression in rat mammary carcinogenesis

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SUMMARY

Background & aims: The aim of this study is to assess the effect of dietary fish oil (MaxEPA) on DNA-strand breaks, cell proliferation and anti-apoptotic protein expressions in rat mammary carcinogenesis.
Methods: Eighty-one female Sprague-Dawley rats were divided into two parts, one for DNA-strand breaks study and the other for immunohistochemical study. Female Sprague-Dawley rats were treated with 7,12-dimethylbenz(α)anthracene (DMBA) (0.5 mg/0.2 ml corn oil/100 g body weight) by a tail vein injection. Rats were fed either fish oil or corn oil (0.5 ml/day/rat) by oral gavage.
Results: Fish oil-treated group showed significant protection against generation of single-strand breaks (SSBs) (56.1%, $P < 0.05$) but increased effect (72.3%, $P < 0.05$) was found in the corn oil-treated group when compared to DMBA control group. Furthermore, fish oil-treated group exhibited substantial decrease in Ki-67 ($P < 0.05$), HER-2/neu ($P < 0.05$) and c-Myc ($P < 0.05$) immunolabelling indices when compared to carcinogen counterpart. However, corn oil treatment resulted in significant increase in the above parameters.
Conclusions: The above data support the role of n-3 PUFA as a preventive agent for DNA damages and a potential to inhibit mammary carcinogenesis.

1. Introduction

Omega-3 and omega-6 Polyunsaturated Fatty Acids (PUFAs) are essential Fatty Acids (FAs). Mammals can neither synthesize them de novo nor interconvert them. Many vegetable oils contain high levels of omega-6 PUFAs, whereas fish oil is a rich source of omega-3 PUFAs, mainly eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). Studies of PUFAs of the n-3 series, especially EPA and DHA, indicate that these fatty acids may prevent the development of carcinogen-induced tumors and metastatic disease in experimental tumor models.[1,2] In contrast, PUFAs of the n-6 series appear to enhance tumor development and metastases.[3] Furthermore epidemiological findings of an inverse relation between dietary n-3 PUFA intake and incidence of prostate, breast and colon cancer,[4–6] led us to evaluate the potential benefit of n-3 PUFA in the prevention of rat mammary carcinogenesis. The susceptibility of the rat mammary gland to develop neoplasm has made it a valuable animal model for the mammary carcinogenesis and experimental therapeutic studies. DMBA [7,12-dimethylbenz(a)anthracene]-induced mammary tumors closely mimic human breast cancer: although DMBA is an experimental carcinogen rather than an etiologic factor for human breast cancer, it nevertheless produces mammary tumors that are comparable with human breast tumors in terms of their long relative latency, histotypes, and endocrine responsiveness.[7] DNA double-strand breaks (DSBs) is a particularly hazardous lesion which induces chromosomal aberrations and disruption of genomic integrity.[8] Her-2/neu protein is a growth factor receptor. It over expresses in tumor leading to increased metastatic potential with low responsiveness of chemotherapy regimen.[9] The transforming members of the Myc family (c-Myc, N-Myc, and L-Myc) show deregulated expression in a broad spectrum of cancers, including carcinomas of the lung, breast, and prostate as well as leukemias and lymphomas.[10] c-Myc may increase genomic instability and enhance tumorigenesis. Rapid tumor cell proliferation is a critical feature for tumor aggressiveness and as such we are interested in the cell proliferations studies with Ki-67 as a marker.[11] To examine the effect of fish oil and corn oil on proliferating cells, an immunohistochemical study was undertaken using Ki-67, which is expressed

Abbreviations: DHA, Docosahexaenoic acid; DMBA, 7,12- dimethylbenz(a)anthracene; DSBs, Double-strand breaks; DS-DNA, Double-stranded DNA; EPA, Eicosapentaenoic acid; FAs, Fatty Acids; LI, Labeling index; PUFAs, Polyunsaturated Fatty Acids; SSBs, Single-strand breaks; SS-DNA, Single-stranded DNA.

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in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle.[12] Proliferation Indices (PI) were calculated on the basis of immunostaining of mammary tissue for Ki-67. Accordingly this study explored the effects of fish oil and corn oil on DNA-strand breaks, proliferation index and expressions of Her-2/neu and c-Myc protein in rat mammary carcinogenesis.

2. Materials and methods

2.1. Experimental animals and housing

81 inbred virgin female Sprague-Dawley rats (aged 3 weeks, 60–70 g body weights) were purchased from Indian Institute of Chemical Biology (IICB), [Kolkata, India]. Then the animals were acclimatized for 2 weeks. During the acclimatization and experimental period, the animals were housed in Tarson Cages in standard laboratory conditions [humidity 55 ± 5%, lighting (12-h light/12-h dark cycle) and temperature 23 ± 2 °C]. The recommendations of Jadavpur University's "Institutional Animal Ethics Committee" ["Committee for the Purpose of Control and Supervision of Experiment on Animals" (CPCSEA Regn. No. 0367/01/C/CPCSEA) India] for the care and use of laboratory animals were strictly followed throughout the study.

2.2. Chemicals

All the reagents and chemicals unless otherwise mentioned were obtained from Sigma Chemicals Co. (St Louis, MO, USA), E. Merck (Frankfurter Straβe, Darmstadt, Germany) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), B D Pharmingen, (San Diego, CA, USA).

2.3. Diet and treatment of animals

The entire experiment consisted of two parts:

I) Analysis of DNA-strand breaks in the mammary tissue
II) Immunohistochemical analysis in the mammary tissue.

2.3.1. Analysis of DNA-strand breaks in the mammary tissue

36 animals provided with AIN-76 based diet (Table 1) and de-mineralized drinking water ad libitum, were divided in four treatment groups of 9 rats each. Groups C, FO and CO rats were received a single, tail vein injection of 0.5 mg DMBA/0.2 ml corn oil/100 g body weight whereas group O rats were the normal control that received only single tail vein injection of 0.2 ml corn oil/100 g body weight at 11th weeks of age. Group FO rats received only fish oil by oral gavage[13] at a daily dose of 0.5 ml of Maxepa (Merck) [a commercially available preparation of concentrated fish oil rich in omega-3 fatty acids, {a gelatin capsule (1 ml) contains 180 mg EPA and 120 mg DHA}] starting at 5th weeks and continued upto 11th weeks till the administration of DMBA. Group CO rats however received only corn oil (Sigma) by oral gavage at a daily dose of 0.5 ml corn oil under the same experimental condition. For estimation of DNA-strand breaks, rats were killed after 18–20 h of DMBA injection. All the treatments were withdrawn prior to DMBA administration.

Table 1
Diet composition.

Ingredient	Amount
Mixed Fat	5%
Casein (g)	17.10
DL-Methionine (g)	0.30
Dextrine (g)	45.50
Sucrose (g)	22.70
AIN-salt mixture (g)	3.50
AIN-vitamins (g)	1.00
Cellulose (g)	5.00

The calculated energy value (metabolizable energy) for the diet is 3.87 K cal/g.

2.3.1.1. Isolation of DNA. DNA was isolated from the frozen mammary gland of rats of the 4 groups (i.e., O, C, FO, CO) by a modification of the procedure of Gupta[14] with enzymatic RNA digestion before proteinase K treatment of the tissue homogenate. DNA concentration and its purity were estimated spectrophotometrically[15] and then the solution was stored at −20 °C.

2.3.1.2. Assay of DNA unwinding. The DNA unwinding assay was performed according to the procedure of Sarkar et al., 1997.[16]

2.3.1.3. Estimation of single-strand breaks (SSBs). Estimation of SSBs per DNA fragment was done from the procedure used by Basak 1996.[17]

2.3.2. Immunohistochemical analysis in the mammary tissue

2.3.2.1. Diet and treatment of animals. A total number of 45 rats, 3 weeks of age were divided in three groups of 15 rats each.

Groups were classified as follows:

Group C : DMBA-administered animals served as the carcinogen control.
Group FO : DMBA + fish oil (Maxepa)
Group CO : DMBA + corn oil

At 7th weeks of age all the animals of group C, FO and CO were received a single, intra venous tail vein DMBA injection. Fish oil and corn oil treatment by oral gavage at a daily dose of 0.5 ml were started at 5th weeks in respective groups and continued upto 25th weeks (30 weeks of animal age). All these treatments were withdrawn a week before the animals were sacrificed.

2.3.2.2. Cell proliferation assay by immunostaining with Ki-67. To examine the effect of fish oil and corn oil on proliferating cells, an immunohistochemical study was undertaken using Ki-67, which is expressed in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle.[12] Proliferation Indices (PI) were calculated on the basis of immunostaining of mammary tissue for Ki-67. Immunohistochemical detection of Ki-67 proteins in cold acetone fixed, paraffin-embedded mammary sections was performed by the avidin-biotin-immunoperoxidase-complex method.[18] Briefly 5 μm thin sections on lysine-coated slides were deparaffinized and rehydrated. For immunolabeling of Ki-67, antigen retrievals were facilitated by heating the sections in citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 in 0.1 M Tris–NaCl (pH-7.6) for 30 min. After incubation in 5% normal goat serum, sections were then separately incubated overnight at 4 °C with the respective primary antibodies, mouse MAb anti-rat Ki-67 (clone MIB-5) at dilutions of 1:5000, respectively in 1% BSA. Sections were then incubated with a biotinylated secondary antibody goat anti-rabbit IgG (Sigma) for 30 min at 37 °C with 1:100 dilution. This was followed by incubation with streptavidin peroxidase (1:100) for 1 h and subsequent chromagen development with AEC–H_2O_2 solution (3-amino 9 ethyl carbazole-10 mg, *N*, *N* dimethyl formamide-2.5 ml, 0.1 N acetate buffer-47.5 ml, 3% H_2O_2-0.5 ml.) (Sigma). The sections were then counterstained with Mayers Hematoxylin, dehydrated and mounted and served as positive control. Negative controls were prepared following all the above-mentioned steps omitting the primary