Docosahexaenoic Acid Reduces Cyto-Genotoxicity of Cytosine Arabinoside in Normal Mouse Bone Marrow

Muthana Ibrahim Maleek¹*

¹Department of Biology, College of Science, University of Wassit, Kut, Wassit, Iraq.

Author’s contribution

The only author performed the whole research work. Author MIM wrote the first draft of the paper. Author MIM read and approved the final manuscript.

ABSTRACT

Aims: This paper evaluates the use of percentage of micronucleus (MN), polychromatic erythrocytes (PCEs), chromosomal aberration (CA) and mitotic index (MI) frequencies in mice bone marrow smears as a method for assessing the ability of docosahexaenoic acid (DHA, is an omega-3 fatty acid) to reduce cyto-genotoxicity damage of cytosine arabinoside (ara-C). Ara-C is widely prescribed antineoplastic drug, especially for the treatment of acute myeloid leukemia. It is a pyrimidine analog, in which the ribose sugar of cytidine is replaced by arabinose moiety.

Methodology: Positive control group of mice was only given intraperitoneal dose of ara-C of 75 mg/kg (every 12 h for 5 days); this dose was selected in accordance with its human therapeutic values. Negative control group of mice group only received 0.1 ml sterile distilled water every 12 hours for 5 days. Three treatment groups of mice were given same dose of ara-C in addition to three different doses of DHA (125, 250 and 500 mg/kg of mice). Experimental data were analyzed using (Mann–Whitney U-test) to compare values of positive and negative controls. However, Kruskal–Wallis test followed by Dunn’s multiple comparisons test were used to compare values of treatments with positive control. All values were accepted at $p = 0.05$.

Results: When 75 mg/kg ara-C was applied, positive control group showed a significant increase in MN and CA, a high decrease in PCE, and a significant decrease in MI. When DHA was used with ara-C, the picture is changed, particularly at a medium dose of DHA of 250 mg/kg where a decrease MN and CA and an increase in PCEs in addition to an
1. INTRODUCTION

Cytarabine, or cytosine arabinoside, is also known as Arabinofuranosyl Cytidine (ara-C) [1], is a chemotherapy agent mainly used in the treatment of acute myeloid leukemia, acute lymphocytic leukemia and lymphomas. However, this drug is not very selective in this setting and causes bone marrow suppression and other severe side effects. Accordingly, this drug is mainly used for the chemotherapy of hematologic cancers [2,3]. It kills cancer cells by interfering with DNA synthesis. It is called cytosine arabinoside because it combines a cytosine base with an arabinose sugar. Normally cytosine combines with a different sugar, deoxyribose, to form deoxycytidine, a component of DNA. Ara-C is structurally similar to human cytosine deoxyribose (deoxycytidine) but when incorporated into human DNA instead it leads to cell death. This mechanism is used to kill cancer cells. Ara-C is the first of a series of cancer drugs that altered the sugar component of nucleosides [4]. It is an S-phase specific cytotoxic drug and has been shown to induce erythroid differentiation in K562, a human erythroleukaemic cell line that has stem cell potential [5]. The stem cells of the hematopoietic system contains a high population of cells in mitotic phase [6,7]. Because ara-C is not selective; it, therefore, kills cells that are actively dividing whether it is neoplastic or normal cells and causing bone marrow suppression, which is a major side effect of the drug [8].

Docosahexaenoic acid (DHA) is an omega-3 fatty acid. It can be synthesized from alpha-linolenic acid or obtained directly from food such as milk, fish oil and other supplements [9]. It is a carboxylic acid with a 22-carbon chain and six double bonds; the first double bond is located at the third carbon from the omega end. DHA trivial name is cervonic acid and its shorthand name is 22:6(n-3) in the nomenclature of fatty acids [10]. DHA possesses moderate anticancer activities against a variety of cancers and shows synergy in pre-clinical models when used in combination with certain drugs and nutritional compounds [11,12]. It can enhance ara-C and doxorubicin toxicity in leukemia cells and ara-C toxicity in transformed rat fibroblasts in vitro [13,14]. In vivo studies showed that DHA had beneficial effects on both bone marrow and gastrointestinal tract of tumor-bearing, ara-C treated rats [15]. Other findings also demonstrated that a low dose of dietary DHA supplementation could prolong the life span and limit the occurrence of toxicity in L1210 leukemic mice [16].

The present study was planned to assess the genomic instability in normal mice treated with single dose of ara-C and multiple doses of DHA. The scoring of MN, CA, mitotic activity (%PCEs) and MI related changes were undertaken as markers of reducing cyto-genotoxicity of ara-C by DHA.
2. MATERIALS AND METHODS

2.1 Mice

Eight to ten week old albino normal (non-cancers) Swiss mice belong to the strain *Mus musculus* were kindly supplied by the Biotechnology Research Center, Al-Nahrain University. Animals weighed 24-26 grams. They were kept at room temperature (23-25ºC) and 12/12 hour light/dark cycle. Mice are fed with a standard mice pellet diet and had free access to water.

2.2 Drugs and Chemicals

Ara-C, DHA and all chemicals used in the present study were purchased from Sigma–Aldrich USA. According to sigma protocol, ara-C was dissolved in sterile distilled water to give a concentration of 50 mg/ml. The solution was clear and colorless and was administered immediately after preparation.

2.3 Experimental Design

2.3.1 Doses treatment

Animals were randomly divided into 5 groups consisting of 5 mice each. All animal groups were treated by intraperitonial injections.

- **Group (1):** Negative control group received 0.1 ml sterile distilled water every 12 hours for 5 days.

- **Group (2):** Positive control group received ara-C of 75 mg/kg [17], which was given every 12 hours for 5 days [18,19].

All groups listed below received ara-C (75mg/kg) and varying concentration of DHA every 12 hours for 5 days [20,21].

- **Group (3):** Received 125 mg DHA /kg
- **Group (4):** Received 250 mg DHA /kg.
- **Group (5):** Received 500 mg DHA /kg.

Mice of all groups were sacrificed after 12 hours of last treatment and femurs were dissected out, and bone marrow samples were taken for determinations cytogenetic analysis to determine the value of MN, CA and MI parameters.

2.4 MN Test and Mitotic Activity % PCE

Groups of five mice were sacrificed and bone marrow cells were collected in tubes containing fetal calf serum, centrifuged at 1100 rpm for 10 min, and the pellet was carefully resuspended in, as little supernatant as possible, before slide preparation. Two smears of bone marrow were prepared from each mouse. After air drying, the smears were coded and stained by May-Gruenwald/Giemsa [22]. From each animal, 1000 polychromatic
erythrocytes (PCE) and 1000 normochromatic erythrocytes (NCE) were examined for micronucleated erythrocytes (MNE) (Fig. 1) under 1000× magnification using a Nikon microscope (Düsseldorf, Germany). In addition, the number of PCE per 1000 NCE per animal was recorded to evaluate bone marrow suppression. Mitotic activity was calculated as % PCE = [PCE/(PCE + NCE)] x100 [23].

![Image](image_url)

**Fig. 1.** Polychromatic erythrocytes with micronuclei induced by ara-C. (1000X)

2.5 Chromosomal Aberration Analysis

2.5.1 Experimental protocol

Animal treatment was the same as in the MN assay except at 90 min before scarification all mice were intraperitonially injected with 4 mg/kg colchicines to arrest cells at metaphase stage [24].

2.5.2 Procedure

The slides for chromosome analysis were prepared and stained as previously described [22]. After both femurs were dissected and bone marrow was flushed from the femoral cavity with fetal calf serum, cells were dispersed by gentle pipetting and collected by centrifugation at 1100 rpm. The harvested bone marrow cells were incubated in 10 ml of 0.075 M KCl for 20 min at 37°C. At the end of the incubation period, Carnoy’s fixative (cold glacial acetic acid-methanol, 1:3, v/v) was added to each tube and then centrifuged for 10 min at 1100 rpm. The supernatant was discarded and 10 ml of fresh fixative was gently pipetted onto the cells without disturbing the pellet. Two to three changes in fixative were required before the preparation of slides. Finally the cells were suspended in small volume of fixative and burst opened (dropping from one foot high) on a clean slide to release chromosomes. The slides were air dried and stained with 5% Giemsa and coded before observation.

2.5.3 Scoring

All slides were scored under 1000x magnification. One-hundred well-spread metaphase plates per mouse (500 metaphases for each group) were scored for both structural and
numerical aberrations (polyploidy) in bone marrow cells. Cells were classified according to the most severe damage which had occurred and were placed in only one of five categories: cells with gaps only, cells with breaks, acentric fragments, centric rings and polyploidy (as shown in Fig. 2). Gaps were defined as achromatic lesions in one or both chromatids not exceeding the width of a chromatid, and breaks as discontinuities greater than the width of a chromatid, irrespective of whether or not the distal fragment was dislocated. Cells with gaps were not included in the percentage of total aberrations [23].

Fig. 2. Chromosomal aberrations induced by ara-C; (a): break; (b): ring; and (c): fragments. (1000X)

2.6 Metaphase Mitotic Activity Analysis Induces MI

From the same slides CA of 1000 cells from each animal were taken into consideration for the mitotic index study. The mitotic activity of bone marrow was evaluated by calculating the number of dividing cells in a population of 1000 cells [23].

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\text{Mitotic Index} = \frac{\text{Number of Dividing Cells}}{\text{Total Number of Cells}} \times 100
\]
2.7 Statistical Analysis

Experimental data were analyzed using (Mann–Whitney U-test) to compare values of positive and negative controls. However, Kruskal–Wallis test followed by Dunn’s multiple comparisons test were used to compare values of treatments with positive control. All values were accepted at $p = 0.05$.

3. RESULTS

3.1 DHA Decrease MNE Arrest by Ara-C Treatment in Normal Mouse

The results of the conventional MN test are presented in Table 1. The frequency of MNE in the positive control (75 mg ara-C/kg alone) was significantly higher when compared to the solvent (H$_2$O) negative control group. When DHA at concentrations 125, 250 and 500 mg/kg were used in combination with ara-C at 75 mg/kg, treatments exhibited lower significant difference in the frequency of MNE compared to the positive control. It may be worth noting that statistical significance varied a little for the dose 250 mg/kg relative to doses of 125 and 500 mg/kg (Table 1).

Table1. Percentages of micronucleated erythrocytes (%MNE) and mitotic activity (% PCE) in bone marrow of mice after treatment with the ara-C alone and ara-C with several doses of DHA (Mean ± SD)

| Chemicals                        | % MNE   | % PCE   |
|----------------------------------|---------|---------|
| Negative control (H$_2$O)        | 0.30 ± 0.01 | 48.2 ± 1.5 |
| Positive control ara-C (75 mg/kg) | 1.67 ± 0.05 | 42.1 ± 2.0 |
| ara-c and 125 mg DHA/kg          | 1.22 ± 0.03 | 45.4 ± 1.6 |
| ara-c and 250 mg DHA/kg          | 0.96 ± 0.01 | 46.8 ± 1.1 |
| ara-c and 500 mg DHA/kg          | 1.38 ± 0.04 | 43.1 ± 2.1 |

*Positive control vs. negative control; † DHA vs. Positive control
All values were accepted at $p = 0.05$.

3.2 DHA Effects on Ara-C Cytotoxicity-induced Mitotic Activity

Mitotic activity at interphase, expressed as % PCE, is also presented in Table 1. The % PCE in positive control decreased from 48.2% to 42.1% relative to the negative control. Increased % PCE in DHA (125 and 250 mg/kg) plus ara-C (75 mg/kg) treated animals was significant, while increasing DHA concentration to 500 mg/kg were not significantly different (Table 1).

3.3 Effect of DHA on Mice Treated with Ara-C Induced Chromosomal Aberrations

Values of chromosomal aberrations are presented in Table 2. Animals treated with the positive control dose (75 mg ara-C/kg) showed a high frequency of total structural and numerical chromosomal aberrations in mice bone marrow cells compared to negative control.
Table 2. Percentages of different types of chromosomal aberrations (CA) and mitotic index (MI) in bone marrow of mice after treatment with the ara-C alone and ara-C with several doses of DHA (Mean ± SD)

| Chemicals                              | Types of chromosomal aberrations (%) | Total CA (%) | MI (%) |
|----------------------------------------|---------------------------------------|--------------|--------|
|                                        | Gaps*                               | Breaks       | Fragments | Rings   | Polyplody |           |         |
| Negative control (H_{2}O only)         | 0.2 ± 0.017                         | 0.6 ± 0.022  | 0.4 ± 0.019 | 0.15± 0.003 | 0.2 ± 0.017 | 1.35 ± 0.06 | 3.4 ± 0.11 |
| Positive control (75 mg ara-C/kg)#      | 1.7 ± 0.023                         | 13.2 ± 0.76  | 1.7 ± 0.053 | 0.7 ± 0.03  | 0.4 ± 0.014 | 16.0 ± 0.21 | 2.0 ± 0.07 |
| ara-C and 125 mg DHA/kg †               | 0.8 ± 0.018                         | 12.3 ± 0.92  | 1.6 ± 0.015 | 0.6 ± 0.016 | 0.3 ± 0.013 | 14.8 ± 0.53 | 2.2 ± 0.08 |
| ara-C and 250 mg DHA/kg †               | 0.2 ± 0.012                         | 8.3 ± 0.58   | 0.2 ± 0.016 | 0.34 ± 0.01 | 0.2 ± 0.011 | 9.04 ± 0.21 | 2.6 ± 0.07 |
| ara-C and 500 mg DHA/kg †               | 0.6 ± 0.014                         | 11.2 ± 0.81  | 0.4 ± 0.017 | 0.31 ± 0.01 | 0.2 ± 0.018 | 12.11 ± 0.42| 2.4 ± 0.08 |

* Positive control vs. negative control;
† DHA vs. Positive control
* Cells with gaps were not included in the total chromosomal aberrations [23].

All values were accepted at p = 0.05.
When similar doses of DHA were given together with ara-C, a decreased chromosomal aberrations, especially at 250 mg/kg, and to a lower degree at doses (125 and 500 mg/kg) was observed. The two major types of aberrations detected in the present study were gaps and breaks and to a lesser degree the rest of aberrations. Calculated total CA (%) and MI (\%) are given in Table 2.

3.4 Effects of DHA on ara-C Cytotoxicity-Induced MI

Mitotic index data recorded in bone marrow cells at metaphase stage are also presented in Tables 2. Drastic inhibition in the mitotic activity of bone marrow cells was recorded in positive control. Enhancements in the MI values was observed in DHA treated groups but were not significant.

4. DISCUSSION

Bone marrow of positive control mice group treated with ara-C (75 mg/kg) alone showed highly significant increase in % MNE and % CA and highly significant decrease in % PCE and % MI (Tables 1 and 2). This effect is the result of ara-C nature, as it kills dividing cells by interfering with DNA synthesis [4]. This would produce cytotoxicity in all mitotic tissues especially in bone marrow. To reduce this cytotoxicity, mice groups were treated with three doses of DHA (125, 250 and 500 mg/kg) in combination with ara-C. Using DHA reduced significantly % MNE and enhanced % PCE especially in medium dose of DHA (250 mg/kg) (Table 1) compared to ara-C alone. Apparently, the protecting effect of DHA is dose-dependent and this is consistent with the previously held views [20,25]. This dose of DHA may have a role in maintaining the structural components of cell membranes. When DHA is incorporated into phospholipids, it affects cell membrane properties such as fluidity, flexibility, permeability and the activity of membrane bound enzymes [26]. At medium dose of DHA (Table 1) the significant reduction of cyto-Genotoxicity of normal bone marrow cells was inferred to a highly significant decline of % MNE and a large increase in % PCE when compared to the positive control data. This justification based on the fact that in the MNE assay, comparison of the PCE/NCE ratio given by % PCE in bone marrow of treated animals relative to that seen in control animals indicated cytotoxicity [27-29]. When erythroblast proliferation is depressed, the % PCE will decrease. In contrast, when erythroblast proliferation is increased to repopulate the PCEs pool, the % PCE is increased. The frequency of MNE reflects the level of damage induced by agents that produce chromosome breakage and/or chromosome loss [27,28]. To further demonstrate this fact CA and MI were tested. At medium dose of DHA we noticed (Table 2) a highly significant decline in CA and a reasonable increase in MI compared to positive control. Chromosomal aberration was used as an indicator to evaluate the enhancement of bone marrow proliferation produced by DHA in normal cells treated with ara-C. Chromosomal aberrations are generated due to a lesion in DNA which leads to discontinuities of the DNA double helix. Experimental analyses have shown that DNA double strand breaks are the principal lesions in the process of chromosomal aberrations formation [30]. Double strand breaks lead directly to structural chromosomal aberrations; all other primary lesions require transformation to double strand breaks by DNA replication and/or repair process [31]. In addition to stable aberrations, chromosome breaks give rise to acentric fragments (i.e. broken chromatin pieces with no centromere), dicentric chromosomes, ring chromosomes, and various other asymmetrical rearrangements that are unstable, in that they usually bring about the death of the cell through loss of vital genetic material [32]. Accordingly, we chose to study these chromosomal aberrations and score the MI. Because the mitotic index is an important
prognostic factor predicting both overall survival and response to chemotherapy in most types of cancer [33], we decided to use it to test its validity in the present investigation. To discuss the results obtained more accurately and objectively and to find explanations for each of the foregoing effects, it is imperative that we look at the following considerations. Tissue such as bone marrow is particularly sensitive to chemotherapeutic drugs because of the high turnover rates of their cells and reliance on nucleoside salvage for survival and proliferation. Ara-C is one of the nucleoside drugs used in cancer chemotherapy, so we noticed (Tables 1 and 2) that a significantly higher increase in chromosomal damage in bone marrow cells when treated with ara-C (positive control). This is in line with previously reported findings [34], in that the reasons for this may be due to that its pyrimidine analogs comprise a diverse and interesting class of drugs that have the capacity of inhibiting biosynthesis of pyrimidine nucleotides, or mimicking the natural metabolites to such an extent that they interfere with vital cellular functions, particularly, in the synthesis and function of DNA [35]. It has also been suggested by a number of investigations [36] that what causes the CA and the emergence of MN is resulting from the impact of ara-C on topoisomerase I and II. This effect leads to gene amplification in addition to breaks in DNA strand and fragmentation in chromosomes. It also inhibits ligation of DNA strand breaks [37]. Both types of topoisomerase are proficient in relaxing supercoiled DNA [38]. It is well documented that the precise mechanism of cell death caused by ara-C is fragmentation of DNA and apoptosis [39]. Therefore, ara-C is mostly S-phase dependent in its actions. Whatever may be the mechanism(s) involved, ara-C induced non-random distribution of chromosomal aberrations (CA) in human lymphocyte chromosomes [40] and micronuclei (MN) in mouse bone marrow [41,42]. However, when mice were treated with a combination of ara-C and DHA, a significant decrease in chromosomal damage was observed, especially when a medium dose of DHA was used. These findings are in agreement with the previously reported findings [15], where it was concluded that dietary DHA enhanced bone marrow cellularity in fibrosarcoma-bearing rats on ara-C treatment. The peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors comprises three distinct gene products, PPARα, β/δ, and γ, that differ in ligand specificity, tissue distribution, and developmental expression [43-45]. In vitro studies have shown that omega-3 PUFAs inhibit cell proliferation and induce apoptosis in cancer cells through various pathways but one of which involves PPARs activation. The differential activation of PPARs and PPARs-regulated genes by specific dietary fatty acids may be central to their distinct roles in cancer [46-48]. Most known target genes of PPARγ regulate lipid metabolism and transport with few cancer-related genes having been confirmed as induced by PPARγ. PPARγ does induce G0/G1 switch gene 2 whose product causes growth arrest in 3T3-L1 cells [49,50]. Accordingly, added DHA activates PPAR, which leads to increasing numbers of cells in G0/G1.

Cell cycle analysis revealed that DHA plus ara-C induced a decrease of proliferation and an increase in the number of cells in the G0 resting phase [43]. This DHA action reduces chromosomal aberrations, which in turn diminishes micronucleus cell formation. These aberrations are usually more prevailed in highly dividing cells such as cancer cells. DHA increases the number of cells at G0 phase, which offers cells a reasonably significant protection against the cytotoxic effect of ara-C. Basically, ara-C affects the process of transcription and DNA replication at S phase. The increase in cell number at G0 phase insignificantly increases mitotic index (MI), as show in Table 2. It could be concluded, however, that DHA produces its therapeutic effect in a synergistic manner. This is achieved through the improvement of the effect of ara-C due to reducing chromosomal aberrations and eventually minimizing the rate of genetic mutations in normal cells. Moreover, it does not increase the rate of cell division so that the drug serves the purpose of treatment. Since the
rate of normal cell proliferation is less than that of cancer cells, therefore, taking DHA with ara-C would offer good protection for the slowly dividing normal cells.

4. CONCLUSION

DHA (250 mg/kg) is able to reduce cyto-genotoxicity of ara-C. This would lead to protecting the normal proliferating cells in bone marrow from the damaging effect of ara-C, thereby improves therapy by ara-C.

CONSENT

Not applicable.

ETHICAL APPROVAL

Author hereby declares that “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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