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Original article

Embryo-toxicity of docosahexaenoic and eicosapentaenoic acids: *In vivo* and *in silico* investigations using the chick embryo model

Zohreh Salari [a], Hadi Tavakkoli [b,*], Ahmad Khosravi [c,**], Elahe Karamad [a], Ehsan Salarkia [c], Mehdi Ansari [d], Shahriar Dabiri [e], Abbas Mortazaeizdeh [e], Seyedeh Saedeh Mosallanejad [f], Fatemeh Sharifi [c]

[a] Obstetrics and Gynecology Center, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
[b] Department of Clinical Science, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[c] Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran
[d] Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran
[e] Afzalipour School of Medicine & Pathology and Stem Cells Research Center, Kerman University of Medical Sciences, Kerman, Iran
[f] Afzalipour School of Medicine &Biochemistry Department, Kerman University of Medical Sciences, Kerman, Iran

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ABSTRACT

Objective: The objective of the current study was to evaluate the embryo-toxicity of omega-3 fatty acids.
Methods: Firstly, the embryo-toxicity of docosahexaenoic (DHA) and eicosapentaenoic acids (EPA), as well as their interaction with Bcl-2 family members, were predicted using an *in silico* assay. In the next step, the embryonic pathological lesions and amniotic fluid biochemical changes following omega-3 treatment were investigated using a chick embryo model. Finally, the drug's vascular apoptotic effect on the chick's yolk sac membrane (YSM) was assessed.
Results: In silico simulations revealed the embryo-toxicity, tissue-toxicity (respiratory and cardiovascular), and vascular-toxicity (apoptotic activity) of DHA and EPA. There was also an accurate interaction between DHA and EPA with Bax (Binding affinity: -7.6 and -10.6 kcal/mol) and Bcl-2 (Binding affinity: -8.0 and -12.2 kcal/mol), respectively. Moreover, DHA and EPA administrations were related to various adverse consequences, including weight loss and lesions in the respiratory and cardiovascular systems. Histopathological findings consisted of pulmonary edema, airway dilatation, increased interstitial tissue, and hyperemia in the lungs, heart, liver, kidney, and brain. Morphometric evaluation of the YSM vasculature revealed that the vascular apoptotic effect of omega-3was associated with a significant reduction in mean capillary area. In immunohistochemistry assay, increased expression of BAX and low expression of Bcl-2 affirmed apoptosis in YSM vessels.
Conclusion: According to the results of this study, one could confirm that the possible embryo-toxicity of omega-3 was approved by data presented in this research. The obtained results also support the suspicion that alteration of the apoptotic-related proteins in vessels is an essential pathway in embryo-toxicity of omega-3.

1. Introduction

Omega−3 fatty acids are polyunsaturated fatty acids characterized by a double bond at the third carbon atom from the end of the carbon chain. The various types of omega-3 fatty acids, which are involved in human physiology, are α-linolenic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). ALA is found in plant oils, while DHA and EPA are commonly found in fish oil, microalgae, and

Abbreviations: **ALP**, alkaline phosphatase; **ALT**, alanine aminotransferase; **AST**, aspartate aminotransferase; **BAX**, Bcl-2-associated X protein; **BCL-2**, B-cell lymphoma 2; **DHA**, docosahexaenoic acid; **EPA**, eicosapentaenoic acid; **FDA**, food and drug administration; **H&E**, hematoxylin and eosin; **HH**, hamburger and hamilton; **IHC**, immunohistochemistry; **MCA**, mean capillary area; **YSM**, yolk sac membrane.

* Corresponding author at: Department of Clinical Science, School of Veterinary Medicine, Shahid Bahonar University of Kerman, 22 Bahman Boulevard, Pajouhesh Square, Postal Code: 7616914111, Kerman, Iran.
** Corresponding author at: Leishmaniasis Research Center, Kerman University of Medical Science, 22 Bahman Boulevard, Pajouhesh Square, Postal Code: 7616914111, Kerman, Iran.
E-mail addresses: tavakkoli@uk.ac.ir (H. Tavakkoli), a.khosravi@kmu.ac.ir (A. Khosravi).

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phytoplankton (the sources of diet for fish). Omega-3 fatty acids play a crucial role in cellular functions, and they are available as daily supplements. Various therapeutic products containing omega-3 fatty acids and their derivatives have been widely used in a large variety of applications [1–3].

An overwhelming number of reports indicate the beneficial effects of omega-3 supplements in human nutrition [4–7]. However, some studies in the literature focus on the harmful effects following omega-3 treatment. For example, it has been shown that omega-3 over-nutrition or imbalance during pregnancy had an adverse impact on offspring [8]. Rabbani et al. (2001) showed that sub-chronic consumption of high levels of omega-3 in rats could increase red cell deformity, increased relative liver and spleen weights, and reduced serum HDL, iron, and vitamin E concentrations. Also, Llorente et al. (2003) showed adverse effects of the omega-3 supplement by checked breastfeeding women. The adverse effects following omega-3 exposure were also reported by other experiments [9–11].

Embryo-toxicity of drugs has always been a major concern. Consumption of some medications during pregnancy is associated with an increased risk of growth retardation, gestational malformations, histopathological lesions, and changes in the amniotic fluid's biochemical parameters. It is well approved that the omega-3 would cross the placenta into the fetus circulation [12–16]. Moreover, the drug is classified into group C of the United States Food and Drug Administration (FDA) drugs. Not enough investigation has been conducted to identify the adverse effects of medications during pregnancy [17]. In this respect, it may cause embryo-toxicity and pathological lesions on the fetus during embryo growth.

Although an increasing production and consumption of omega-3 compounds is expected in some areas of the globe, little has been published about the embryo-toxicity and lesions of this drug on the fetus and vascular plexus. Also, the exact mechanisms by which omega-3 affects vascular development are not yet fully defined. One of the most critical tools influencing vascular development is apoptosis [18–21]. Accordingly, we supposed that omega-3 would affect apoptotic-regulator proteins and induce vascular-toxicity. Until now, this possible vascular apoptotic effect of omega-3 has not been evaluated. Consequently, the present study aimed to respond to the following questions:

(i) Do omega-3 fatty acids cause embryo-toxicity?
(ii) What is the primary embryonic lesion following omega-3 treatment?
(iii) Do omega-3 fatty acids alter the biochemical parameters of the amniotic fluid?
(iv) Does omega-3 alter the expression of apoptotic-related proteins in vessels?
(v) How is the interaction mode between omega-3 and apoptotic-regulator proteins?

To answer these questions, *in silico* analysis was performed to predict the embryo-toxicity and apoptotic activity of omega-3. Accordingly, the interactions between DHA and EPA with BAX and Bcl-2 proteins were simulated. In the next step, a chick embryo model was treated with omega-3 fatty acids. The monitoring of the embryos was done to assess embryo-toxicity and lesions following treatment. Computerized analysis of the vasculature on the chick's yolk sac membrane (YSM was done in order to reveal the vascular apoptotic effect of DHA and EPA. The results were also joined with IHC data to affirm the effect of the omega-3 on the apoptotic-regulator proteins (BAX and Bcl-2), which may be associated with drug toxicity. We believe that the acquired results improve our understanding of omega-3's adverse effects.

2. Materials and methods

2.1. Materials

Fertilized chicken eggs (Cobb 500, with the average egg-weight of 56.3 ± 0.4 g) were purchased from Mahan Company (Mahan breeder Co., Kerman, Iran). In that broiler company, the broiler breeders were maintained under optimal conditions of breeding. Omega-3 fatty acids were obtained from Zahravi (Zahravi Pharmaceutical Company, Tehran, Iran). Each 300 mg of the drug contains 120 mg DHA and 180 mg EPA. Paraffin was purchased from Merck (Merck, Darmstadt, Germany Cat # 8042-47-5). Various software and servers like SWISS-MODEL (https://swissmodel.expasy.org/,formolecular simulation of BAX and Bcl-2 (*Gallus gallus*) [22], RCSB Protein Data Bank (https://www.rcsb.org/, for obtaining the PDB format of proteins, which were used in Cross and self-dockings) [23], National Center for Biotechnology Information (NCBI, for obtaining the domain sequences of BAX and Bcl-2 proteins) [24], AutoDockVina (for molecular docking) [25], PROCHECK (http://servicesn.mbi.ucla.edu/Verify3D/, for conformational analysis of simulated proteins) [26], ImageJ® 1.48 (National Institutes of Health, Bethesda, Maryland, USA, for morphometric analysis of vascular pattern) [27], Digimizer®4.3.0 (MedCalc Software, Mariakerke, Belgium, for image analysis and improvement) [28] and MATLAB® (Mathworks Matlab R2015a, for morphometric analysis of vascular pattern) [29,30] were used for *in silico* evaluations.

2.2. Toxicity of DHA and EPA usage in silico prediction

To predict the embryo-toxicity aspects of DHA and EPA, tissue-toxicity (pulmonary and cardiovascular) and vascular-toxicity (vascular apoptotic activity) were evaluated. The interactions of DHA and EPA with proteins associated with apoptosis were also accessed via the docking technique.

2.2.1. Toxicity evaluation of DHA and EPA

DHA and EPA's molecular structures were acquired in the MDL file from the PubChem server (https://pubchem.ncbi.nlm.nih.gov/, PubChem CID: 445580 and 446284, respectively). The DHA and EPA structures were uploaded in the PASS online server to access the agents' toxicity and apoptotic activity based on the structural conformation (http://www.pharmaexpert.ru/passonline/). The toxicity and *Pa* (probability to be active) values were recorded [31].

2.2.2. Molecular simulation and docking

We evaluated the possible interaction of DHA and EPA with BAX and Bcl-2 proteins. The domain sequence of BAX and Bcl-2 proteins (*Gallus gallus*, Gene ID: *ACR83547.1* and *NP_990670.2*) were obtained from the National Center for Biotechnology Information (NCBI) GenBank, and appropriate template structures were detected via blasting the NCBI server. In the next step, we determined the closest homologous structures of proteins from the Protein Data Bank and retrieved the suitable homologous structures to serve as templates for BAX and Bcl-2. According to the user template models, the 3D structures of BAX and Bcl-2 (*Gallus gallus*) were generated by SWISS-MODEL (https://swissmodel.expasy.org/). The accuracy of the SWISS-MODEL database has been previously confirmed [32].

Ligands and receptors were prepared for docking by inserting hydrogen and partial charges. A docking study was made using AutoDock Vina to identify DHA and EPA's binding affinity to BAX and Bcl-2 proteins [25]. A grid box was determined with 40 × 46 × 48 points and a resolution of 1 Å to include the complete proteins. After the grid box was centered in the macromolecule, docking was performed. The docked structures were classified based on the binding energies, and the best energy poses were selected.

Cross-dockings were also conducted to clarify the interaction mode of DHA and EPA in a way that BAX and Bcl-2 proteins were connected to

a specific activator or inhibitor. To reach this goal, the structures of 5W5X (known structure of BAX connected with activator) and 5JSN (known structure of Bcl-2 connected with inhibitor) were selected from the Protein Data Bank (Fig. 5A and B). Following the splitting of the ligands from the receptors, the interactions between DHA and EPA with 5W5X and 5JSN were investigated. Finally, we decided to perform a validation stage by self-docking between 5W5X, 5JSN, and their original ligands. This stage was considered as the primary indication of docking accuracy.

2.3. Embryo-toxicity of DHA and EPA using the chick embryo model

The study was conducted according to the suggested European Ethical Guidelines for animals' care in experimental research. It was approved by the Animal Ethics Committee of the Research Council of Kerman University of Medical Sciences, Iran (Ethic number IR.KMU.REC.1395.47).

Chicken eggs were incubated at 37.5 °C and 60 % relative humidity (incubator: Beldechin Company, PLC-DQSH, Rasht, Iran). The eggs were divided into two experimental groups of fifteen eggs. On day 4 of the incubation period, (Hamburger–Hamilton developmental stage 22–24), the eggs of the first group were injected (into the yolk sac) with omega-3 fatty acids at a dosage that was equivalent to the maximum safe daily dosage (the maximum safe daily dosage recommended by the FDA for a 70-kg human is a total of 3,360 mg/day of EPA and HDA) [33]. The next group was considered as a sham control. The embryos were re-incubated for a further period of fourteen days and evaluated on day eighteen of the incubation period. For gross evaluation, the embryonated eggs were kept on ice for 20 min [34]. Following opening the eggshell, the embryos were removed, and the body-weight was measured by a digital scale (Sartorius TE 1535, Germany, with a range up to 150 g reading to ± 0.001 g). To study any pathological abnormalities, the tissues, including the brain, liver, kidney, and heart, were dissected and placed in 10 % neutral buffered formalin. Following standard preparation, serial sections of paraffin-embedded tissues were made and stained with hematoxylin and eosin (H&E) and reticulin.

2.3.1. Effect of DHA and EPA on the biochemical parameters of the amniotic fluid

Cobb 500 fertilized eggs were purchased and incubated. The incubation condition and drug injection were similar to those described in the previous section. The amniotic fluid was collected on day fourteen of the embryonic development [35–37]. After opening the eggshell and shell membranes of the incubated eggs, the allantoic membrane and allantoic fluid were removed to allow the amniotic fluid to be collected. The amniotic fluid (2–2.5 ml) was sampled via an 18-gauge syringe. The samples were centrifuged (10 min at 3000 g), and then the supernatants were taken for biochemical measurement. The activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were evaluated. The levels of urea, creatinine, uric acid, glucose, total protein, phosphorus, sodium, and potassium were also assessed via the ELISA commercial Parsazma kits (Parsazma Company, Tehran, Iran) and analyzed using an automated analyzer (Dirui CS-400, China).

2.4. Effects of DHA and EPA on apoptotic-related proteins in vessels

In this survey, we utilized a chick's YSM model for investigating the

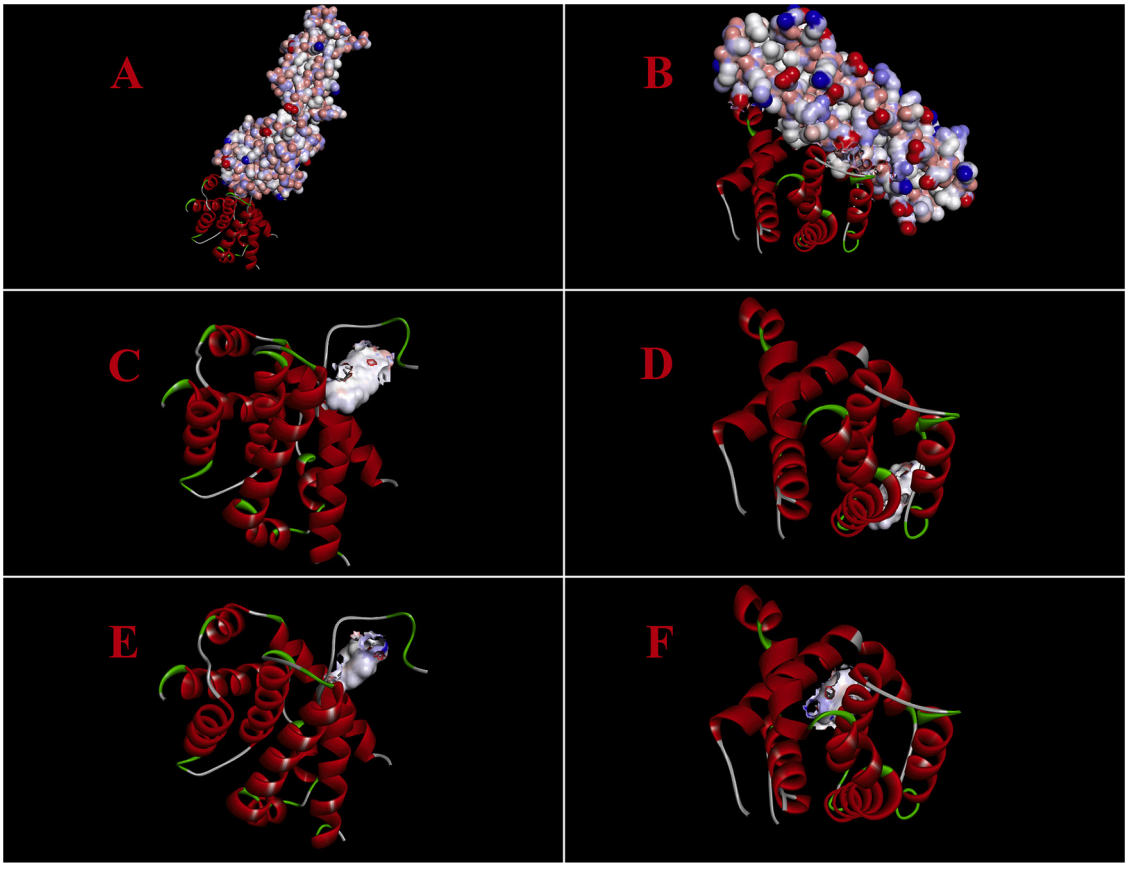

Fig. 5. Cross-docking between omega-3 fatty acids (white/right) and apoptotic-regulator proteins (left). (A) 5W5X; the crystallographic structure of human BAX protein linked with specific activator. (B) 5JSN; the crystallographic structure of human Bcl-2 protein linked with specific inhibitor. (C) Successful cross-docking between DHA and 5W5X. (D) Successful cross-docking between DHA and 5JSN. (E) Successful cross-docking between EPA and 5W5X. (F) Successful cross-docking between EPA and 5JSN.

effects of DHA and EPA on apoptotic-related proteins in vessels. Over recent years, the YSM of the *Gallus gallus* has provided a valuable model for *in vivo* evaluation of the vascular-toxicity of the drugs and compounds [38–40]. Hence, we applied this model to investigate the omega-3 fatty acids' effect on the vessels' apoptotic-related proteins. That effect was evaluated via morphometric analysis of vascular patterns from the chick's YSM. The IHC assay was also performed to evaluate the expressions of Bax and Bcl-2 proteins following drug injection. The details are explained below.

2.4.1. Omega-3 inoculation and image capturing

Embryonated chicken eggs of the breed Cobb 500 were incubated at 37.5 °C and 60 % relative humidity. Treated embryos were assigned to two groups as follows: group 1 (n = 10): phosphate-buffered saline-treated group (control group), group 2 (n = 10): omega-3-treated group, in which embryonated eggs were treated with omega-3 fatty acids. Before drug inoculation, eggs were examined for embryonic growth via candling. After 24 h of incubation, fifty microliters of omega-3 fatty acids (contained 8.3 mg EPA and 5.5 mg DHA) were inoculated on the YSM. After inoculation, the eggs were re-inoculated at two different time points: 24 and 48 h following the first inoculation. On Hamburger–Hamilton developmental stage 25–26 (day 5 of the incubation period), the eggshell was cut with scissors, and a window of 25 × 25 mm was opened to allow image capture. The images (High-resolution: 4000 × 3000 pixels) were taken from the YSM vasculature. After capturing, the YSM was cut with scissors (15 × 15 mm in diameter), and surrounding embryonic tissues were surgically removed. The cut YSM was used as a sample for the immunohistochemistry assay.

2.4.2. Morphometric analysis of vascular pattern

Morphometric analysis of vascular pattern was performed by image processing software such as ImageJ® 1.48 (National Institutes of Health, Bethesda, Maryland, USA) and MATLAB® (Mathworks Matlab R2015a). In the first step, a distinct area (32 mm^2) on the YSM was selected to enhance its contrast (Fig. 1A). The format of the selected area was converted to a binarized picture (Fig. 1B). Five areas without any vessels were extracted, and the percentages of the black pixels were calculated (Fig. 1C). The black pixels indicate the blood (red color) in the original pictures. The mean of all areas calculated in an image is described as the mean capillary area (MCA) [41,42].

2.4.3. Immunohistochemistry evaluation

The tissue sampling method is presented in the "Omega-3 inoculation and image capturing" section. The samples of the chick's YSM were collected and fixed in 10 % buffered formalin. Following tissue preparation, 3 μm thick sections were prepared by a rotary microtome (Slee Germany), and IHC staining was performed for Bax (mouse monoclonal antibody Zytomed_Germany, ID number: 502_17990) and Bcl-2 (mouse monoclonal antibody, American, ID number: PDMO16- lotH147) markers. To visualize Bax and Bcl-2 protein expression, a Mouse/Rabit PolyVue plus TM HRP/DAB Detection System (Cat No: PVP25D) was applied as recommended by the manufacturer. Images were captured by a light microscope (LH100, Olympus, Tokyo, Japan) equipped with a digital camera. The expression levels of Bax and Bcl-2 were determined by counting the dark brown stained cells and calculating the mean in 10 microscopic fields (40×). and determining Bax/Bcl2 ratio [43].

2.5. Statistical analysis

Statistical analysis was performed via SPSS version 20 (SPSS Inc., Chicago, IL, USA). Fisher's exact was utilized to identify the significant differences in lesion occurrence between groups. The T-test was done to determine the significance of differences in the body-weight, biochemical parameters of the amniotic fluid, and MCA. A *p-value* of <0.05 was considered statistically significant.

3. Results

3.1. In silico results

3.1.1. Toxicity of omega-3 in PASS

The structures of DHA and EPA were uploaded in the PASS online server to evaluate their embryo-toxicity, tissue-toxicity (respiratory and cardiovascular),and vascular-toxicity (vascular apoptotic activity). The *Pa* values were predicted by PASS server. The acquired data were filtered via *Pa* (probability to be active) values. This filtering was performed through PASS outputs by keywords such as including: embryotoxic, weight loss, toxic respiration, pulmonary edema, hypertension, toxic vascular and apoptosis We detected high *Pa* values for embryotoxicity (*Pa = 0.516, Pa = 0.530*), weight loss (*Pa = 0.525, Pa = 0.537*), respiratory toxicity (*Pa = 0.966, Pa = 0.969*), pulmonary edema (*Pa = 0.779, Pa = 0.797*), hypertension activity (*Pa = 0.651, Pa = 0.658*), vascular-toxicity (*Pa = 0.746, Pa = 0.764*) and apoptotic activity (*Pa = 0.626, Pa = 0.627*) of DHA and EPA, respectively.

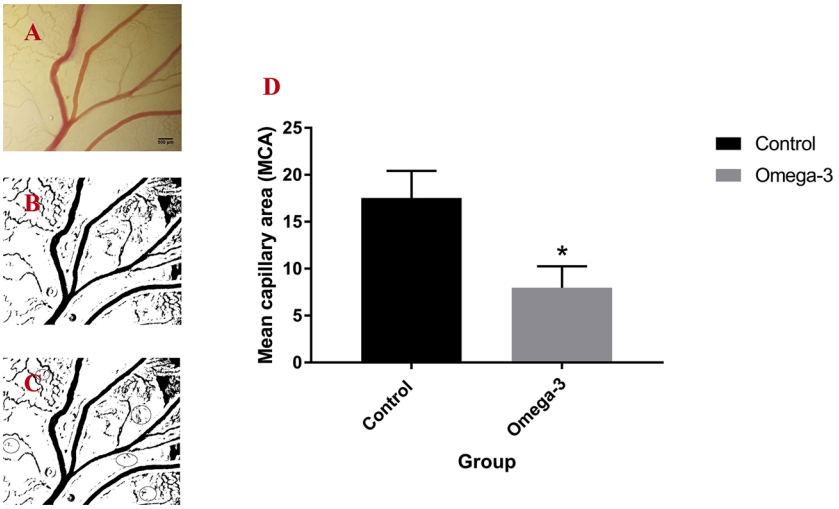

Fig. 1. The mean capillary area (MCA)analysis. (A) A distinct area (32 mm^2) is selected from the image. (B) The format of the selected area is converted to a binarized picture. (C) Five areas (circles) without any vessels were extracted and the percentages of the black pixels were calculated. (D) The MCA is significantly decreased in the omega-3 treated group compared to control (error bars show standard error of the mean; *p<0.03, T-test).

3.1.2. Conformational analysis of simulated proteins

The bioinformatics servers were used to simulate the 3D conformations of the apoptotic-regulator proteins (BAX and Bcl-2, *Gallus gallus*) (Fig. 2A and B). Ramachandran plots analyzed the simulated structures at the PROCHECK server (Fig. 2C and D). Ramachandran data, for Bax and Bcl-2, are described as below, respectively: 87.2 % and 88.1 % of the residues situated in most favored regions, 9.9 % and 9.4 % of the residues located in additional allowed areas, and 2.3 % and 2.5 % of the residues situated in generously allowed regions. The Ramachandran plot is a fundamental tool in the analysis of protein structure. It locates the amino acid residues of the simulated models [44]. A good quality Ramachandran plot has over 85–90 % in the most favored regions. Ramachandran plots of simulated proteins (BAX and Bcl-2, *Gallus gallus*) have 87.2 % and 88.1 %of residues in the most favored regions; therefore, these data exhibited that good quality model were simulated. The PDB format and Ramachandran plots of affected protein (BAX and Bcl-2, *Gallus gallus*). The reproducibility of the results can be check by uploading the structure of the protein structures and analysis of the Ramachandran plots at the PROCHECK server (https://servicesn.mbi.ucla.edu/PROCHECK/).

The molecular characteristics of the simulated Bax included the number of amino acids = 196, the number of atoms = 3085, the number of positively charged residues (Arg + Lys) = 17, the number of negatively charged residues (Asp + Glu) = 19, and weight = 21666.16.

The molecular characteristics of the simulated Bcl-2 including the number of amino acids = 194, the number of atoms = 2954, the number of positively charged residues (Arg + Lys) = 17, the number of negatively charged residues (Asp + Glu) = 22, and weight= 21422.02.

3.1.3. Interactions of DHA and EPA with BAX and Bcl-2

Molecular docking was performed to identify the interactions of DHA and EPA with BAX and Bcl-2 proteins. The docking results have shown that DHA was docked with BAX and Bcl-2 proteins (binding affinity=-7.6 and -8.0 kcal/mol, respectively) (Fig. 3A and D). As presented in the critical model, DHA was bound to the active site of BAX by van der Waals interaction and 2 hydrogen bonds between Lys, Asn, Gly, Leu, Arg, Tyr, Pro, and Val (Fig. 3B and C). When searching for binding residues between DHA and Bcl-2, we detected Leu, Arg, Asp, Glu, His, Leu, Lys, Glu, Asp, Val, Ala, and Pro interactions (Fig. 3E and F). Binding residues were determined using discovery studio visualize software (BIOVIA Discovery studio 2017R2 Client 2017).

The docking results have also confirmed that EPA also could bind to the BAX and Bcl-2 proteins with binding affinities of -10.6 and -12.2 kcal/mol, respectively (Fig. 4A and D). The van der Waals interaction and hydrogen bonds between EPA and BAX are presented in Fig. 4B and C. For EPA, various residues, including Ile, Lys, Ala, Leu, Glu, Gly, Tyr, and Ser were predicted to interact with BAX. The docking results analysis showed that the EPA also interacted with Bcl-2 by the binding

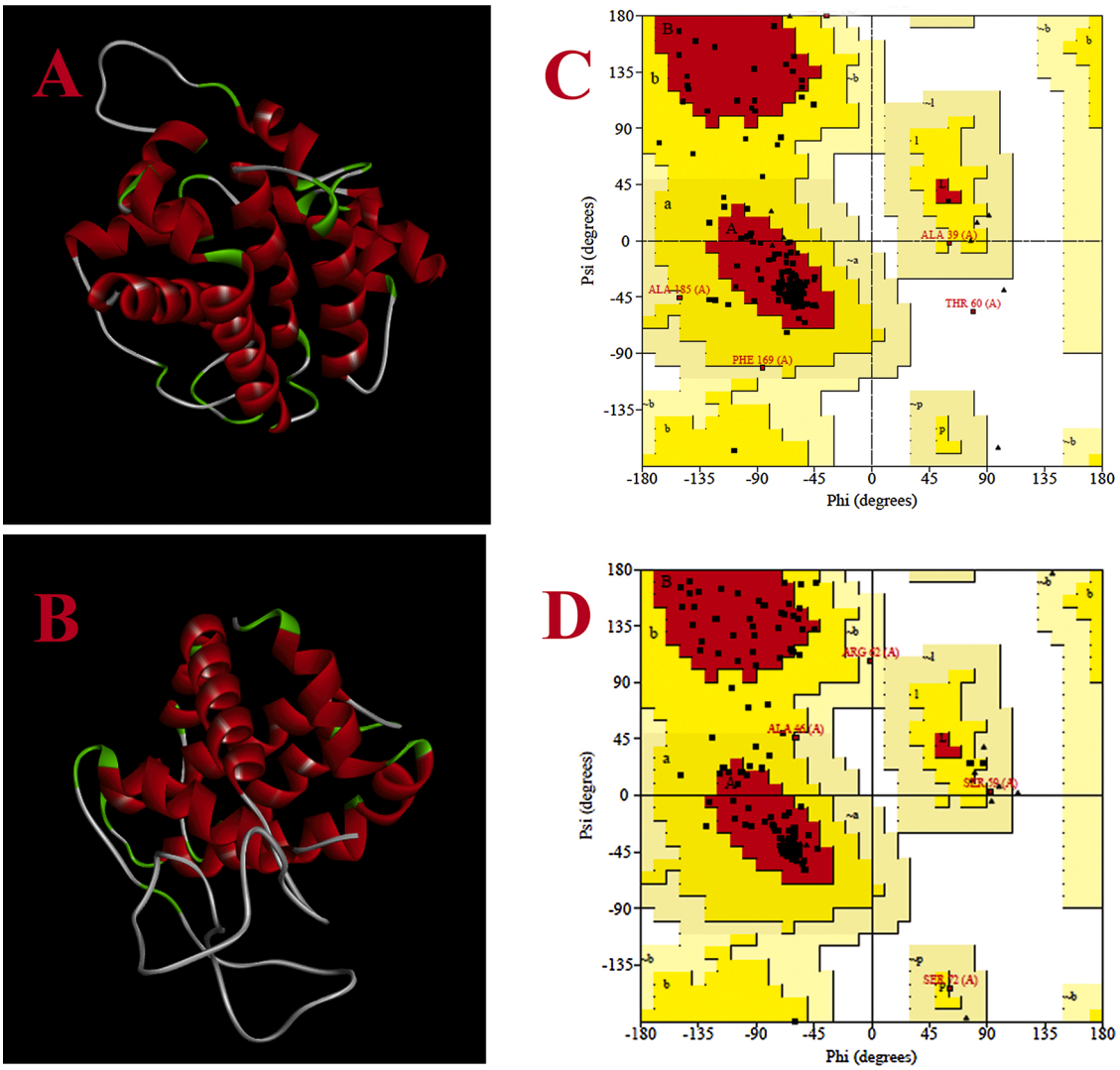

Fig. 2. (A and B)3D structures of simulated BAX and Bcl-2 *Gallus gallus,* respectively. (C and D) Ramachandran plots for the protein model of BAX and Bcl-2.

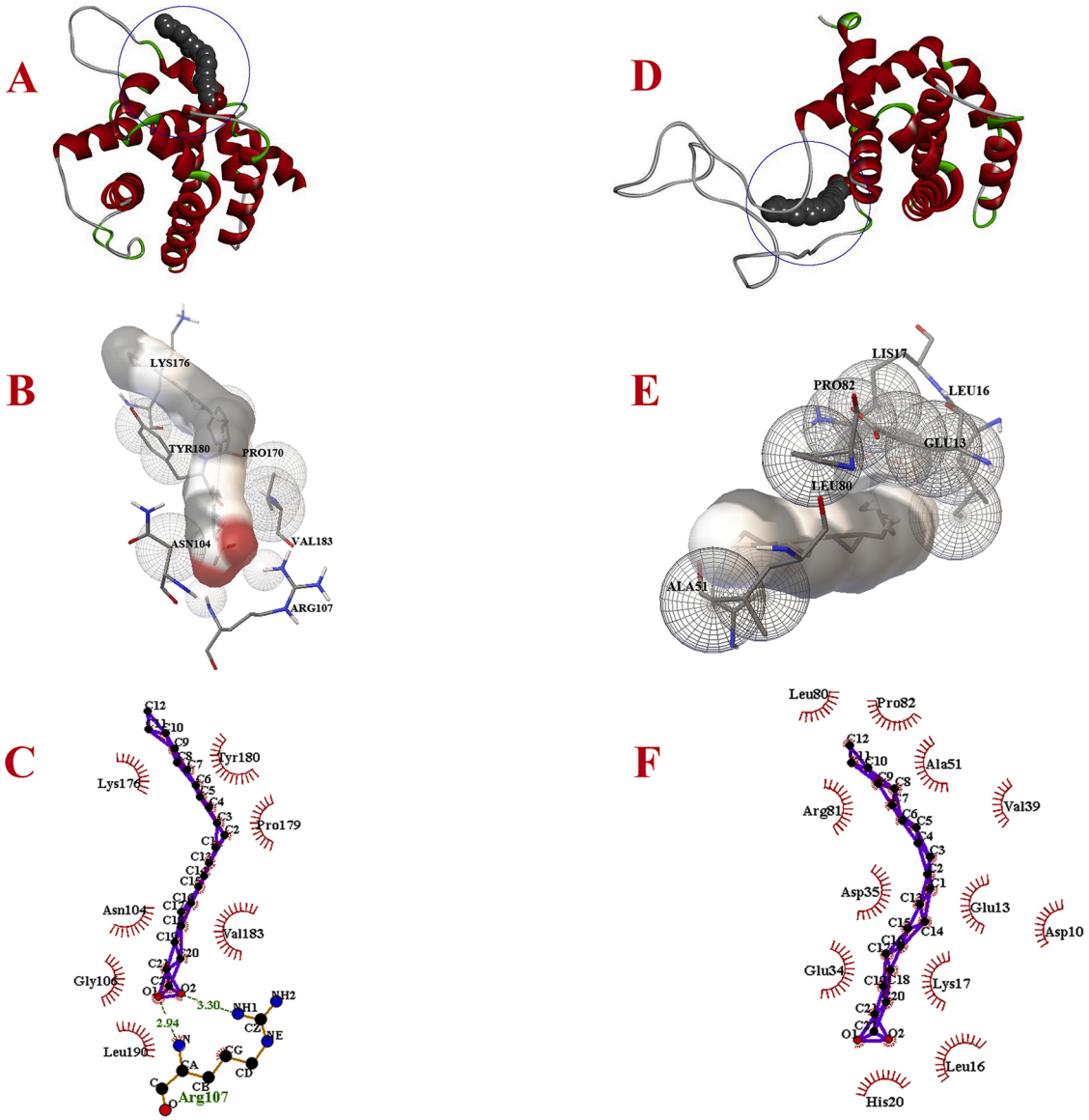

Fig. 3. Best docked conformations of docosahexaenoic acid with *Gallus gallus* BAX (A-C) and Bcl-2 (D-F). (A) The platform of DHA inserted in the BAX binding site (blue circle). (B and C) Van der Waals interactions and hydrogen bonds (green dots) between DHA and amino acid residues of the nearby binding site. (D) The platform of DHA inserted in the Bcl-2 binding sites (blue circle). (E and F) van der Waals interactions between DHA and amino acid residues of the nearby binding sites.

residues including Trp, His, Phe, Val, Val, Tyr, Glu, His, and Glu (Fig. 4E and F).

3.1.4. Cross-docking results

As declared earlier, cross-dockings were done with 5W5X and 5JSN and, instead of their specific ligands, to assess the affinities of DHA and EPA for BAX and Bcl-2 proteins in a way that they are connected to a specific activator or inhibitor. The results are shown in Fig. 5. The cross-docking results showed that after separating the specific ligands from 5W5X and 5JSN, the DHA and the EPA still interacted with them.

Moreover, cross-dockings showed that DHA was docked with the active sites of BAX (5W5X) and Bcl-2 proteins (5JSN) (binding affinity=-7.2 and -7.9 kcal/mol, respectively) (Fig. 5C and D). Cross-docking results also verified that EPA could bind to the BAX (5W5X) and Bcl-2 (5JSN) proteins with binding affinities of -10.3 and -12.4 kcal/mol, respectively (Fig. 5E and F). The 5W5X and 5JSN are crystallographic structures of human BAX and Bcl-2, linked with a specific activator or inhibitor. When searching for binding residues on the surfaces of 5W5X and 5JSN facing toward their specific activator or inhibitor, different residues including SER, GLU, SER, LEU, LYS, ARG, ILE, GLY, ASP, GLU, LEU, ASP, SER, ASN, MET as well as ASP, PHE, SER, ARG, ARG, TYR, ARG, ARG, ASP, PHE, ALA, GLU, MET, SER, SER, GLN, and LEU were predicted, respectively (Fig. 5 A and B). By comparing these results, it is revealed that many of these residues are known to interact with DHA and EPA and are located near the binding sites of 5W5X and 5JSN with DHA and EPA, suggesting that DHA and EPA may alter the activity of 5W5X and 5JSN in a way similar to that of their specific inhibitor or activator.

3.1.5. Self-dockings

We also used a self-dockings assay to prove the accuracy of the results (validation stage). The total numbers of five runs were performed between 5W5X, 5JSN, and their original ligands. The self-dockings results for 5W5X and 5JSN are demonstrated in (Fig. 6 A–D). The results revealed that all runs with 5W5X, 5JSN, and their ligands resulted in correct binding site recognition (Fig. 6 C and D). The applied docking assay was confirmed to be very accurate for predicting the binding sites based on the acquired results.

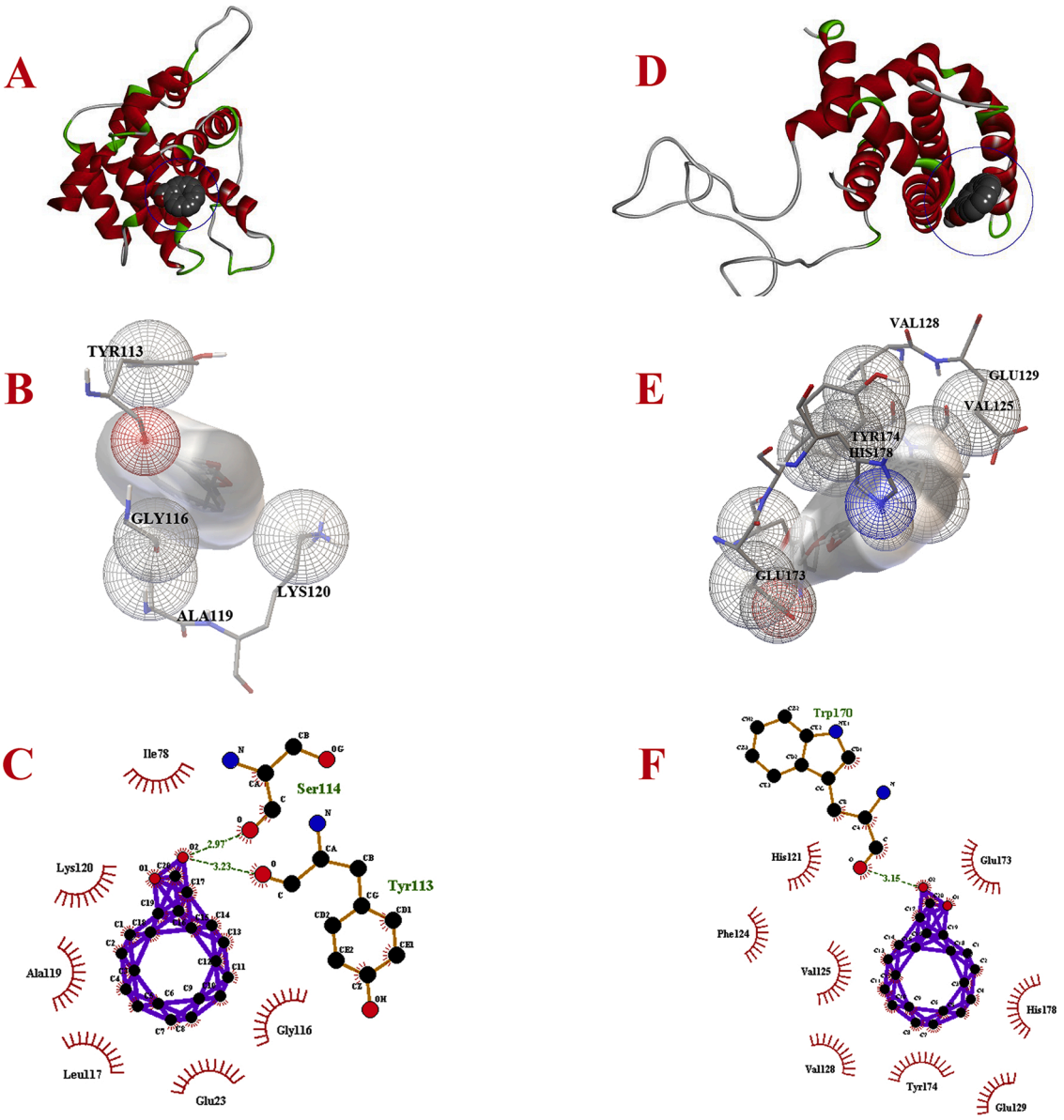

Fig. 4. Best docked conformations of eicosapentaenoic acid with *Gallus gallus* BAX (A-C) and Bcl-2 (D-F). (A) The platform of EPA inserted in the BAX binding site (blue circle). (B and C) Van der Waals interaction and hydrogen bonds (green dots) between EPA and amino acid residues of the nearby binding sites. (D) The platform of EPA inserted in the Bcl-2 binding sites (blue circle). (E and F) van der Waals interactions and hydrogen bonds (green dots) between EPA and amino acid residues of the nearby binding sites.

3.2. Lesions following omega-3 treatment in the chick embryo model

3.2.1. Embryo weight

The weights of the treated embryos were measured on day 18 of the incubation period (Fig. 7). The analysis of acquired results showed that the omega-3 treated embryos were lighter than the control (embryo weight/egg weight: 24.01 ± 2.25 g, $p = 0.001$). The control embryos have a normal weight (embryo weight/egg weight: 38.47 ± 1.46 g).

3.2.2. Histopathological lesions

Histopathological lesions following omega-3 inoculation to the chick embryos were mainly identified in the respiratory and cardiovascular systems. In this regard, the treated embryos' lungs were hyperemic with signs of edema, characterized by fluid accumulation and airway dilatation were observed (Fig. 8A). In some instances, the interstitial tissues of the lung were increased. The embryos' cardiomyocytes were normal, while severe hyperemia and vessel dilatation were noted in the heart (Fig. 8C). The structures of the liver (Fig. 8C), kidney (Fig. 9A), and brain (Fig. 9C) were normal, while severe hyperemia was noted in those organs. In the control embryos, the respiratory and cardiovascular tissues' structures were normal (Figs. 7B, 8 D, F, 9 B and D).

3.2.3. Biochemical analysis of the amniotic fluid Biochemical analysis of the amniotic fluid

Chick embryos were treated with omega-3 fatty acids on the 4th day of embryonic development. The biochemical analysis of the chick's amniotic fluid was done 10 days after the treatment (14th day of the growing period). The biochemical parameters of the treated and control embryos are presented in Table 1 .. Inoculation of omega-3 oil caused alterations in some parameters. The AST levels and total protein levels were increased in the treated embryos compared to the controls ($p < 0.05$).

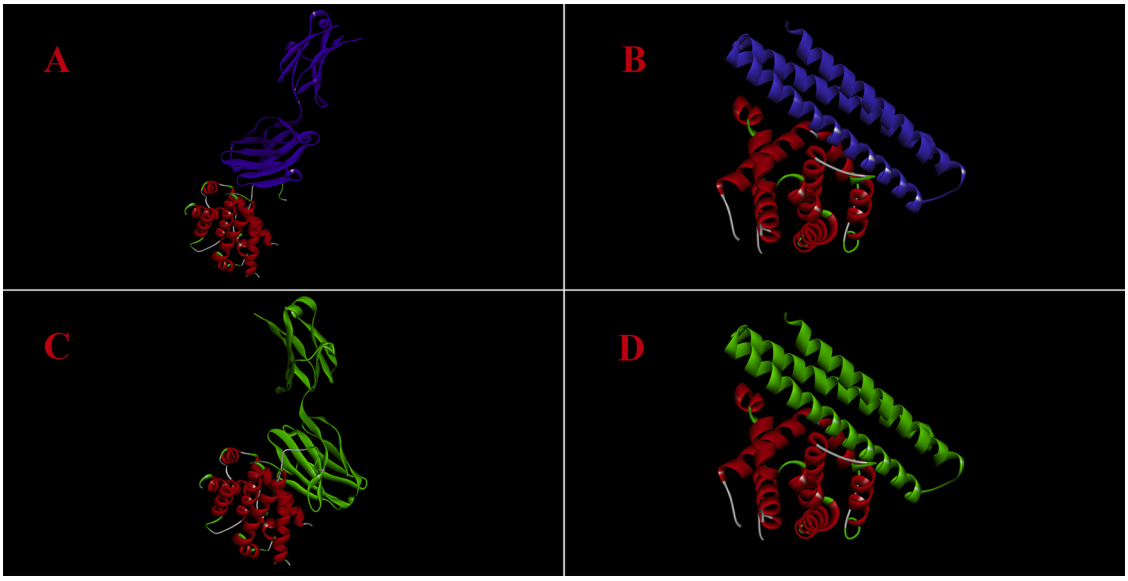

Fig. 6. Validation stage by self-docking assay. (A) 5W5X, the known structure of BAX connected with activator. (B) 5JSN, the known structure of Bcl-2 connected with inhibitor. (C and D) Binding sites predicted by the self-docking (green) are in agreement with the binding sites revealed by the experiment (blue).

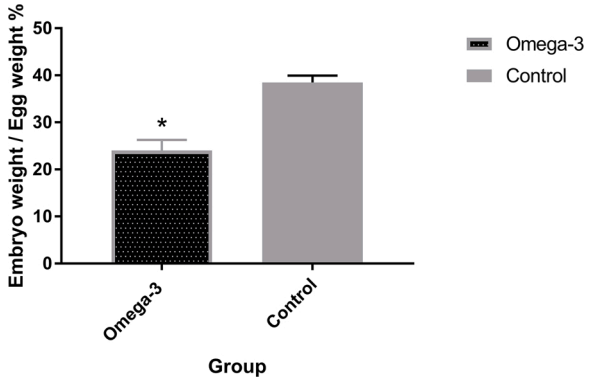

Fig. 7. The weight of the chick embryos following omega-3 treatment. The weight is presented on day 18 of the incubation period comparing control and omega-3-treated embryos. The embryos that received omega-3 were lighter than controls (*p=0.000, T-test).

3.3. Vascular analysis and IHC results

As explained earlier, the chick's YSM model was used to determine the effect of omega-3 fatty acids on apoptotic-related proteins in vessels. The results are presented as follows.

3.3.1. Mean capillary area analysis

The vascular response following omega-3 inoculation on the chick's YSM is presented in Fig. 1D. There was a significant decrease in MCA values from the treated eggs compared to the controls (control group, 29.98 ± 2.72; treated group, 9.12 ± 1.80; $p = 0.001$).

3.3.2. Immunohistochemistry results

The H&E, reticulin staining, and IHC assay were applied to evaluate the omega-3 fatty acids' effect on apoptotic-related proteins in the chick's YSM vasculature. As shown in Fig. 10, increased Bax expression and low expression of Bcl-2 are accrued in omega-3 inoculated groups. The ratio of BAX/Bcl2 was 6/1.

4. Discussion

Consumption of some drugs during pregnancy is associated with an increased risk of lesions in the embryo or surrounded fluid. As pointed out in some literature reviews, increasing the production of omega-3 compounds is expected in some areas of the globe leading to an increase in this drug's consumption in pregnant women [4,13].

Drug toxicity is of great concern during pregnancy. In this respect, we assessed the details of omega-3 fatty acids' embryo-toxicity in the present study. Some mechanisms, which may relate to the embryo-toxicity of drugs, were also investigated.

Determination of the toxicity of drugs needs a preclinical model to be employed. The chick embryo and its extra-embryonic membranes provide appropriate models for this purpose due to good reproducibility of results, cost-benefit, and reduced ethical and legal aspects. The growing repository of genetic, anatomical, and physiological data continues to expand the tractability of the chick embryo for toxicology studies. On the other hand, the United States Food and Drug Administration (FDA) has approved drugs preclinically evaluated with chick embryos [45–50]. Based on those reasons, we used the chick embryo model for our investigation. Various alterations and pathological lesions were seen following omega-3 inoculation. These alterations and lesions are depicted in multiple indications as follows:

Weight loss was a significant indicator of omega-3 outcomes. Measurement of the body-weight at the end of the growing period revealed that it decreased from expected values. Based on this result, the omega-3 fatty acids' administration during the gestational period is suggested, thereby causing a lighter weight in the fetus.

The second indication of the toxicity of omega-3 in the chick embryo model is various microscopic lesions in multiple tissues. The injuries following omega-3 treatment in embryos are not clearly defined. Based on our results, it is concluded that omega-3 fatty acids can mainly affect the respiratory and cardiovascular systems. In our investigation, the primary microscopic lesions were noticed in those organs, at least until further data are provided on injuries in other tissues. Several types of disturbances were observed in the chick's embryo due to respiratory and cardiovascular injuries. These symptoms are described as follows: 1) severe hyperemia, edema, airway dilatation in the lung; 2) hyperemia and vessel dilatation in the heart and 3) severe hyperemia in the liver, kidney, and brain.

Since no gross pathological lesion was observed on the external body

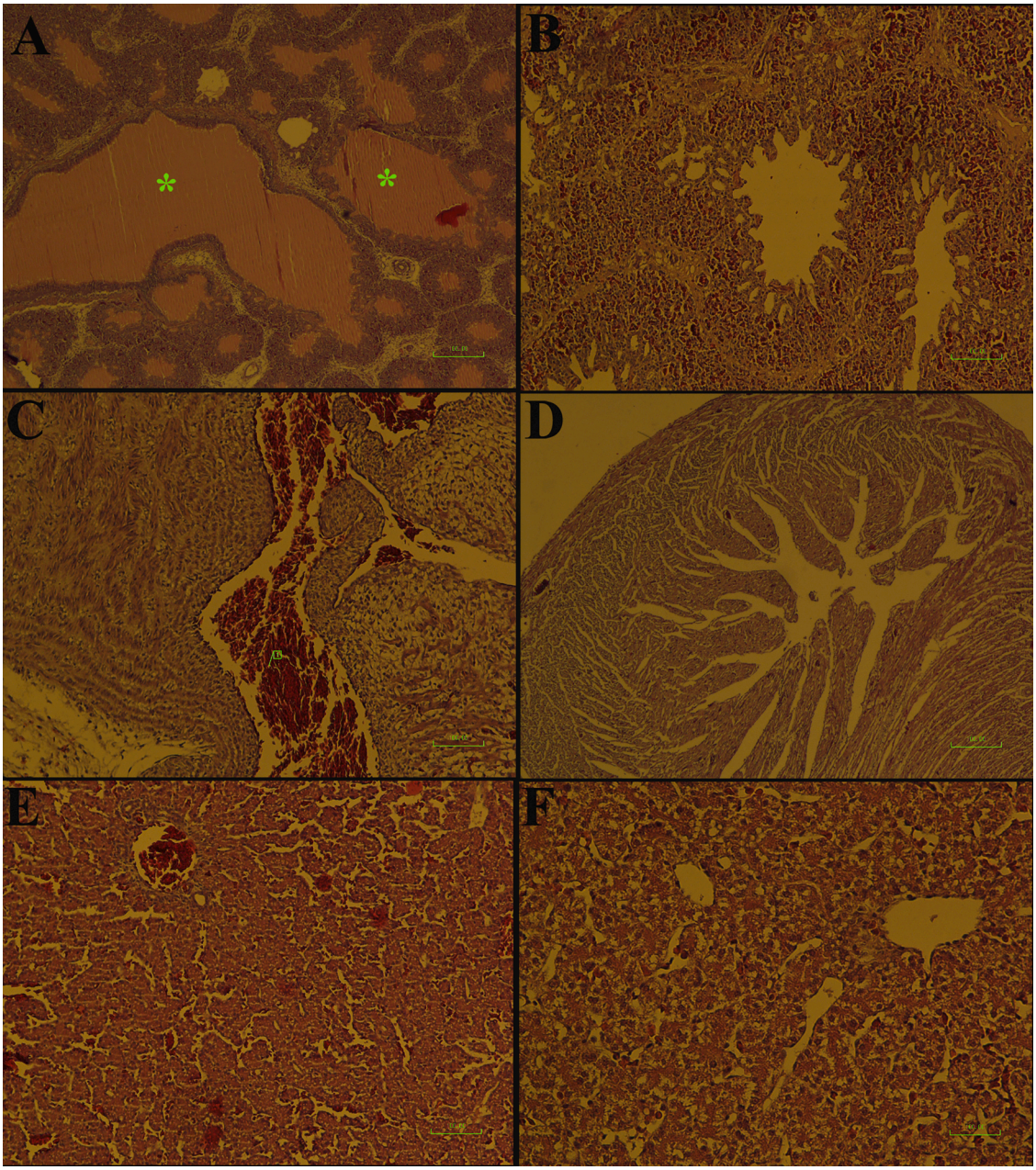

Fig. 8. Histopathological lesions of docosahexaenoic and eicosapentaenoic acids in the chick embryo model with H&E staining. (A) Edema and airway dilatation in lungs (asterisks) (100X). (B) Normal structure of the lung (X100). (C) Severe hyperemia and vessel dilatation in the heart (X200). (D) Normal structure of the heart (X100). (E) Hyperemia in the liver (X100). (F) Normal structure of the liver (X200).

surfaces of chicks on day 18, it seems that administration of omega-3 fatty acids can induce microscopic lesions without the presence of any macroscopic lesion. This concept is also supported by other experiments in which there were no lesions in the external appearance of mice following acute and chronic toxicology of omega-3 fatty acid[10]. In our study, the omega-3 fatty acids were inoculated into the yolk sac of the chicken eggs. It is demonstrated that developmental toxicity following omega-3 consumption depends on the length of treatment [10,51]. This issue prompted us to extend the drug absorption length so that the drug was inoculated into the yolk sac to cover the whole period of embryo organogenesis.

The next indication of the omega-3toxicity was a significant alteration in the chick's amniotic fluid's biochemical parameters, such as AST and total protein. Amniotic fluid is a dynamic fluid surrounding the embryo. Monitoring of its biochemical has been widely applied in clinical diagnosis as an indicator of embryo status. The AST is the intracellular enzyme and is present in the liver, kidney, heart, skeletal muscle, and brain, and its elevation is associated with tissue damage. Various factors influence the total protein. Overall, dehydration, immune stimulation, and edema may elevate its value. In our investigation, an increase in the amniotic fluid above is suggested due to tissue injuries and edema, following omega-3 treatment, and possible transfer of them into the amniotic fluid. Alterations in the biochemical parameters of amniotic fluid due to the administration of some drugs and compounds have been previously reported [35–37]. Our study lends evidence that the alteration in the normal values of the amniotic fluid biochemical can be induced via omega-3 exposure during embryonic development.

Various cellular and molecular pathways may be related to the embryo-toxicity of omega-3. In the current study, details about the vascular apoptotic effect of DHA and EPA were investigated through *in*

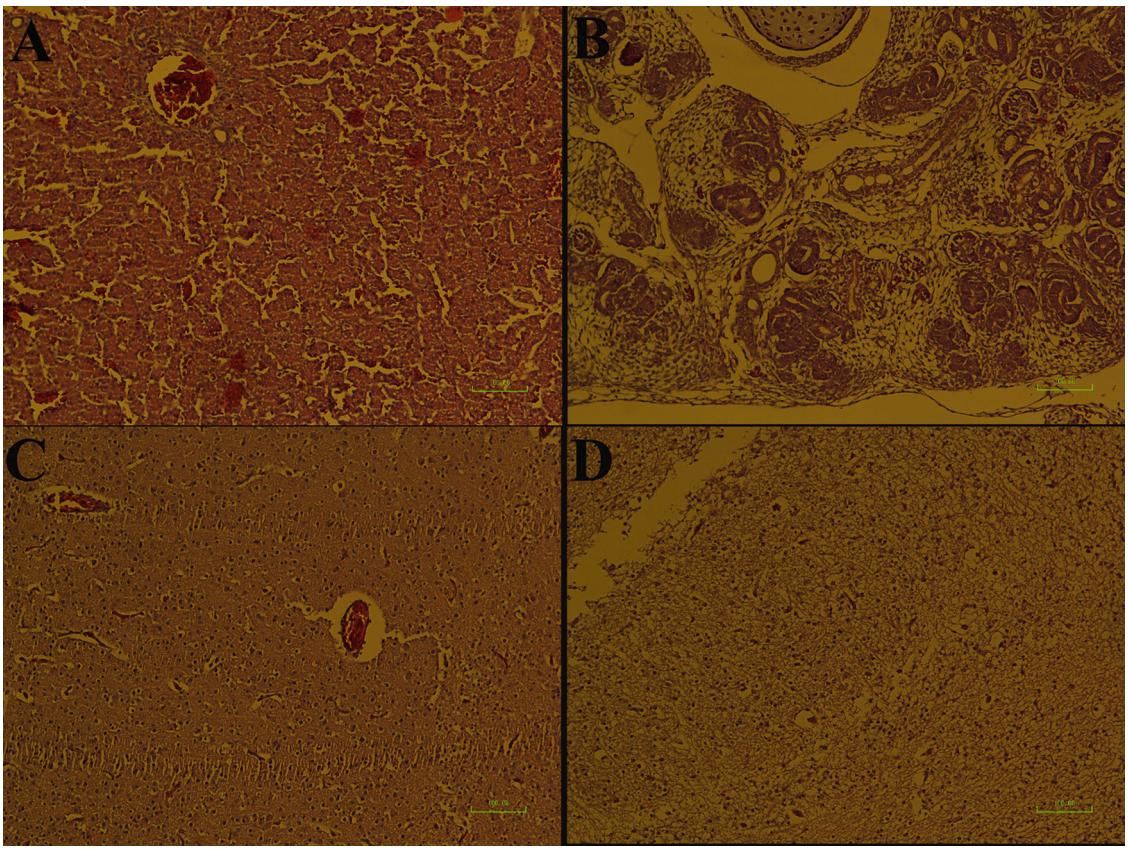

Fig. 9. Histopathological lesions of docosahexaenoic and eicosapentaenoic acids in the chick embryo model with H&E staining. (A) Hyperemia in the kidney liver (X100). (B) Normal structure of the kidney (X100). (C) Hyperemia in the brain (X100). (D) Normal structure of the brain (X200).

Table 1
Biochemical parameters of amniotic fluid in chick embryo following omega-3 fatty acids treatment (data are acquired on the 14th day of the embryonic development).

Biochemical parameters	Control	Omega-3 fatty acids (docosahexaenoic and eicosapentaenoic acids)	
	Level	Level	P-value
ALT (IU/L)	1.80 ± 0.24	2.50 ± 0.29	0.098
ALP (IU/L)	7.00 ± 2.16	5.33 ± 2.97	0.647
AST (IU/L)	**4.66 ± 1.25**	**10.42 ± 2.75**	**0.000**
Urea (mg/dL)	19.50 ± 2.95	20.42 ± 2.83	0.823
Creatinine (mg/dl)	0.77 ± 0.10	0.92 ± 0.12	0.373
Uric acid (mg/dl)	15.53 ± 1.54	16.22 ± 1.14	0.749
Glucose (mg/dL)	69.58 ± 19.73	57.00 ± 11.19	0.570
Total protein (mg/dl)	**1.91 ± 0.39**	**3.77 ± 1.37**	**0.027**
Phosphorus (mg/dl)	17.20 ± 2.82	18.02 ± 3.27	0.855
Sodium (mEq/L)	95.00 ± 6.6	93.70 ± 5.42	0.883
Potassium (mEq/L)	10.00 ± 1.42	9.86 ± 1.26	0.944
Calcium (mg/dl)	6.19 ± 0.99	7.01 ± 1.06	0.582

Values are mean ± SEM, T-test. ALT, alanine aminotransferase, ALP, alkaline phosphatase; AST, aspartate aminotransferase.

silico and *in vivo* investigations. In this regard, we discuss various highlights of the discoveries on the vascular alteration and interaction of DHA and EPA with proteins associated with apoptosis.

In the current study, we utilized a docking technique to identify some details about the vascular apoptotic effect of omega-3. This technique is presently considered an effective approach to clarify the interaction between ligands and receptors [52,53]. It is well known that the Bcl-2 family members are critical targets for apoptotic and anti-apoptotic agents[54,54–56]. Herein, an essential highlight in our research is DHA and EPA's interaction with Bax and Bcl-2 proteins. The docking results have shown that DHA and the EPA were bound to Bax and Bcl-2 proteins' active site by van der Waals and hydrogen interaction. Our results suggest the modulation of Bcl-2 family members via the binding of DHA and EPA. Future experiments should confirm this phenomenon. As mentioned previously, the DHA and EPA structures were also uploaded to the PASS online server to evaluate their toxicity and adverse effects. The acquired data was also confirmed the apoptotic activity of DHA and EPA.

The next highlight of the ligand-protein interaction is the binding affinity between DHA, EPA, and Bcl-2 family proteins. By analyzing the docking data, it is predicted that the affinity of the EPA for Bax and Bcl-2 is higher than DHA because it obtained the lowest-scoring energy (-10.6 and -12.2 kcal/mol) compared to DHA (-7.6 and -8.0 kcal/mol). Therefore, the EPA is predicted to affect BAX and Bcl-2 more than DHA. It can be considered a promising target for designing anti-apoptotic agents to alleviate the devastating outcomes of omega-3 toxicity in embryos.

In our investigation, the chick's YSM vasculature's morphometrical analysis showed that the omega-3 fatty acids negatively affect vascular plexus. The method, which was applied to investigate the vascular apoptotic effect of omega-3, computed the mean capillary area on the acquired images. Up to now, this method has been widely applied in vascular analysis [19,29,41].

The next highlight to be discussed is the significant alteration in Bax and Bcl-2 proteins' expression following omega-3 inoculation. The mechanisms by which omega-3 induces vascular alteration have not been clearly discussed. However, due to *in vivo* results, we propose that alteration in Bcl-2 family members' regular expression is one of the mechanisms related to the apoptotic activity of omega-3 in vessels because the IHC results affirm the changes in the expression of Bax and Bcl-2 in omega-3 exposed embryos.

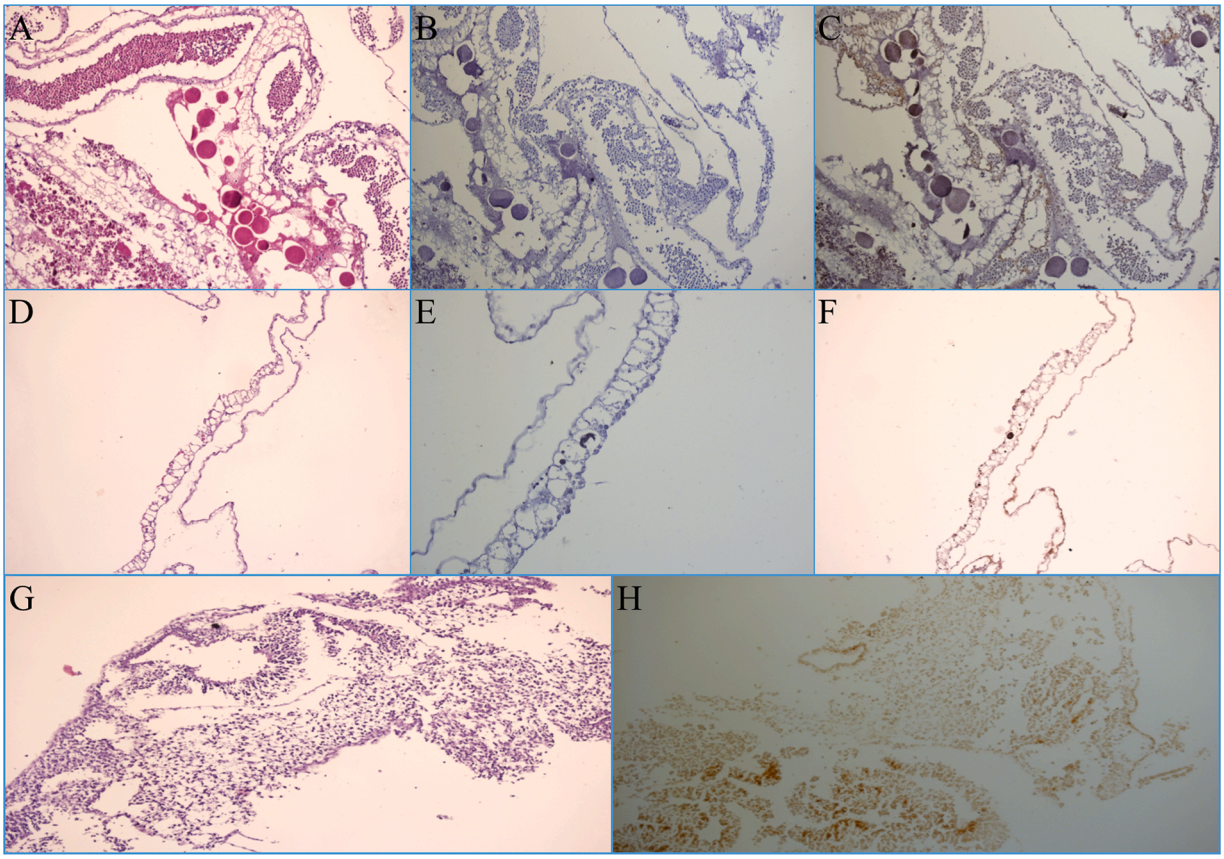

Fig. 10. Control sample: (A) H&E staining shows Normal vascularization (X100) (B, C) BCL-2 and Bax staining are equivalency in accession, confirmed Normal vascularization (X100). Omega Treatment: (D) H&E staining shows decreased vascularization (X40). (E, F) Bcl-2 and Bax staining show weakly staining of Bcl-2 and strong for Bax. This finding confirmed decreased vascularization in treated samples (X40). (G, H): H&E and reticulin staining in treated samples decreased vascularization that reticulin confirms H&E findings (X100).

In the present study, the chick's YSM and *in silico* assays were the two methods applied to evaluate embryo-toxicity and vascular apoptotic effects of docosahexaenoic eicosapentaenoic acids. To date, these methods have been widely used in biological investigations. However, it needs further studies on the human fetus (if ethics committees rule that a protocol is ethical) to support our results.

As far as the authors are aware, this is the first study to target the different aspects of omega-3 toxicity with a chick embryo model. This model offers a promising model to investigate the toxicity of various drugs and agents in the embryo. Our results show that acute administration of omega-3 harms the embryonic growth and biochemical parameters of the amniotic fluid and produced evidence of tissue lesions, which was determined by histopathology. The significant pathological effects of the omega-3 fatty acids occur in respiratory and cardiovascular systems. The acquired data also indicate that omega-3 alters the expression of apoptotic-related proteins in the vessels.. On this matter, it is suggested that apoptotic activity of omega-3 fatty acid in vessels is one of the unique mechanisms in the embryo-toxicity of the drug. Accordingly, the drug consumption must be limited during gestation or only be given when the benefit outweighs the risks. In the current paper, the apoptotic activity of omega-3 was predicted by assessing the altered expressions of BAX and Bcl-2 in vessels. The omega-3 may alter various apoptotic regulator proteins and pathways like bcl-xS, bax-alpha, and bak. These provide meaningful recommendations for future researches.

Declaration of Competing Interest

The authors report no declarations of interest.

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