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Research Article

Chemoprevention of Colon Cancer by Combination of Atorvastatin and Fish Oil

Rani I[1], Kumar S[1,2*], Renuka[1], Sharma S[1], Bhargva P[1], Taneja N[1] and Agnihotri N[1*]

[1]Department of Biochemistry, South Campus Punjab University, Chandigarh, India

[2]Block-J, Pharmacology and Toxicology Lab, CSIR-IHBT, Palampur, Himachal Pradesh, India

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***Corresponding to:**
Navneet Agnihotri, Department of Biochemistry, BMS Block-II, Sector-25, South Campus, Punjab University, Chandigarh-160014, India, Tel: 91-172-2534131, Fax: 91-172-2541022, E-mail: agnihotri.navneet@gmail.com

1. Abstract

Cholesterol accumulation in growing tumor cells has recently been implicated in apoptosis evasion and drug resistance. Various reports have suggested the potential of statins (3-hydroxy-3-methylglutaryl-coenzyme Areductase inhibitors) as chemo preventive agents. Side effects associated with statins have encouraged the combination approach to enhance the efficacy of statins at low doses. In this study, a combination regimen of fish oil (FO) and atorvastatin is used in order to reduce the dosage of atorvastatin and evaluate the efficacy of this regimen. Pre-treatment with atorvastatin and/or FO resulted in a significantdecline in number of irregular crypts and increased apoptosis.Though we did not observesignificant effect of this combinational approach on cyclin D1 expression and cell cycle progression, however, there was a significant inhibition of Ras expression and translocation.A significant reduction in the nuclear translocation of NF–κB,a transcription factor that promotes cancer cell survival and metastasis was also observed on treatment with combination regimen. Furthermore, expression of VEGF, a downstream target of NF–κBwas also diminished by the combined regimen. To conclude a combination therapy of atorvastatin with fish oil may be a better approach to prevent cancer and may help in reduction of the optimal dose of stations in clinical use.

2. Keywords: Statins; Fish oil; PUFAs; Apoptosis and Colorectal Cancer

3. Abbreviations: Fish oil (FO); Colorectal cancer (CRC); Atorvastatin (Ator), NN-dimethyl hydrazine dihydrochloride (DMH), Polyunsaturated fatty acids (PUFAs), Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), Ethylenediamine tetra acetic acid (EDTA), Hank's balanced salt solution (HBSS), Dithiothreitol (DTT), bovine serum albumin (BSA), Paraformaldehyde (PFA), Fluo-3-penta-acetoxymethylester (Fluo-3AM), statistical package for social sciences (SPSS)

4. Introduction

Chemotherapy and chemoprevention are the two main strategies which has an impact on the incidence and outcome of cancer by regulating its initiation or progression. Potential limitations and side effects associated with current chemotherapeutic treatments has switched the interest of scientific community to chemoprevention and even WHO has declared cancer chemoprevention as "a high priority area". Chemoprevention includes the use of natural or pharmacological agents; individually or in combination with other dietary components to suppress or reverse carcinogenesis at its early stages. Long chain n-3 PUFAs (EPA and DHA) have been proven to possess major

inhibitory role in carcinogenesis [1,2]. A major source of EPA and DHA is fish oil which has well-established antitumorigenic properties [3,4]. Over the past few years increasing attention has been given to the study of the metabolic processes involved in lipid and cholesterol biosynthesis and their regulation in context with cancer. Consequently much attention has been directed toward the potential of statins as chemo preventive agents in the carcinogenic process [5-8] though the enthusiasm is dampened by certain controversial reports on its use [9]. Cholesterol plays a vital role in maintaining mammalian cell structure and function where it serves to modulate membrane fluidity and permeability. It is evident now that alteration in cholesterol metabolism plays an important role in tumor development and is therefore an attractive target for cancer treatment [10]. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), the major rate-limiting enzyme in cholesterol synthesis that catalyzes the conversion of HMG –CoA to mevalonic acid. Inhibition of mevalonate synthesis also abolishes the production of isoprene units, farnesyl and geranylgeranyl pyrophosphate (GGPP), crucial moieties for activation of cell signalling pathways, regulating cell differentiation and proliferation [11]. Previous studies have established the anti-proliferative, anti-inflammatory, antioxidant and pro –apoptotic properties of statins, and therefore, cancer prevention [12,13]. Much attention has focused on the association between statins and colorectal cancer raising the prospect that these compounds could form the basis of future chemo preventive strategies [14,15]. As fish oil has been shown to decrease the detrimental effects of chemotherapeutic regimen [16], we decided to use fish oil as an adjuvant with a low dose of atorvastatin in the present study and evaluate its chemo preventive efficacy. It has also been documented that n-3 PUFAs have the ability not only to modulate the endogenous synthesis of cholesterol but also its displacement in cell membrane [17,18], which makes the rationale behind this study even more promising.

Excessive and uncontrolled cell proliferation as well as evasion from apoptosis together provides the fundamental platform for neo plastic progression [19]. Genetic aberrations in the regulatory circuits which govern transition through the G1 phase of the cell cycle particularly cyclin D1 have been reported to occur frequently in human cancers [20]. Another regulatory protein involved in cell proliferation is Ras oncoprotein which is also activated and trans located to the membrane by addition of prenyl moieties, intermediates in cholesterol biosynthetic pathway [21]. Cyclin D1 is also a downstream target of Ras protein.

Deregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death and can lead to cancer [22,23]. The major regulators of apoptotic pathway are Bcl-2 family proteins which have both pro and anti –apoptotic members. Over expression of anti-apoptotic Bcl-2 probably occurs in more than half of all cancers and suppresses the initiation of apoptosis in response to a number of stimuli including cytotoxic anticancer drugs [24]. In addition to suppressed apoptosis and increased cell proliferation, chronic inflammation has recently been added to the list of hallmarks of cancer withNF–κB being the major player in the process [25]. NF–κB coordinates the transcription of multiple genes which subsequently regulate apoptosis, angiogenesis and metastasis [26]. In the present study efficacy of combination regimen of atorvastatin and fish oil was assessed by evaluation of the abovementioned key players involved in different aspects of cancer progression.

5. Materials and Methods

5.1. Chemicals

N,N-Dimethyl hydrazine dihydrochloride (DMH) was obtained from Sigma Chemical Company (St. Louis USA). Bovine serum albumin (BSA) and dithiothreitol (DTT) were acquired from Himedia laboratories (Mumbai, India). M30 cytoDEATH antibody was purchased from Boehringer Ingelheim (Mannheim Germany). Monoclonal antibodies against Bax, Bcl-2, pan-Ras, cyclin D1 and vascular endothelial growth factor (VEGF) were procured from Santa Cruz (California USA). Monoclonal antibody against NF-κB (p65 subunit) was obtained from BD biosciences (Maryland, USA). FITC conjugated anti mouse IgG_1 secondary antibody were bought from Bangalore Genei (Bangalore, India). Fish oil under the brand name Maxepa containing 180m/ml EPA and 120 mg/ml DHA was purchased from Merck Limited (Goa, India). Atorvastatin under brand name Lipvas10 was bought from Cipla (Mumbai, India). All other chemicals

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used in this study were of analytical grade.

5.2. Animal Model

Female Wistar rats weighing 100 -120g were obtained from the Central animal house, Panjab University, Chandigarh. The experimental protocols were approved by the Institutional Ethics Committee (IACE/346-356) and conducted according to Indian National Science Academy guidelines for the use and care of experimental models. Animals were acclimatized for a week before inclusion in the study. The animals (2 per cage) were housed in polypropylene cages and fed with standard pellet diet (Ashirwad Industries, Punjab, India) and water *ad libitum*.

5.3. Experimental Design

The animals (N=45) were equally divided into the following experimental groups:

5.3.1. Control Group: The animals were given a weekly intraperitoneal (i.p.) injection of Ethylenediamine tetraacetic acid (1mM EDTA, pH 6.5) i.e. vehicle of DMH for a period of four weeks.

5.3.2. DMH Group: The animals were given DMH (i.p., 20 mg/kg b.w.) once a week for a period of four weeks [27].

5.3.3. Atorvastatin+DMH Group: Atorvastatin was given orally (20mg/kgb.w. dissolved in normal saline) to animals prior to DMH injection [28].

5.3.4. Fish oil+DMH Group: The animals were supplemented with 0.5 ml fish oil [29].

5.3.5. Atorvastatin+Fishoil+DMH Group: The animals of this group were given a combination of atorvastatin (20mg/kg b.w.) and fish oil (0.5 ml).

The treatment (atorvastatin and/or Fish oil) was started at daily dosage one week prior to DMH injection and continued for a period of twelve weeks on every alternate day. Due care was taken during the oral administration of the therapeutic regimen and there was no mortality observed during the procedure. After 12 weeks the animals were sacrificed by cervical dislocation.

5.4. Measurement of Tumor Biomarkers

5.4.1. Histopathological Examination: The colon was removed and flushed with cold PBS and distal end (2-3 cm) was fixed in 10% formalin. Sections were prepared and stained with haematoxylin and eosin. The slides were photographed and evaluated by a trained and qualified histopathologist.

5.4.2. Isolation of Colonocytes: The colonocytes were isolated by the method of Sanders et al. [30]. The entire colon was removed and flushed with normal saline. Lumen was exposed by cutting the colon longitudinally and then kept in warm Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) containing 30 mM EDTA, 5 mM DTT and 0.1% BSA. After incubation with intermittent shaking, the mucosal side was gently scraped to remove intact crypts and surface cells from lamina propria. The isolated cells were then centrifuged at 600 ×g and re suspended in HBSS containing Ca^{2+} and Mg^{2+} and 0.1% BSA. The cell viability was done by trypan blue exclusion method using a hemocytometer (Marienfeld, Germany). The cell viability was found to be in range of 80-90%.

5.5. Measurement of Cell Proliferation Index

Cell proliferation index was measured by cell cycle analysis and measurement of Cyclin D1.

5.5.1. Cell Cycle Analysis: The percentages of cells in G1, S and G2/M phases of cell cycle were determined by using Cycle test plus DNA reagent kit (BD-Pharmingen) as per manufactures protocol by using flowcytometric method.

5.5.2. Cyclin D1 Expression

Cylin D1 expression was assessed in isolated colonocytes using flowcytometer. Briefly, cells were fixed in 70% ice cold ethanol and washed in PBS twice. The cells were permeabilised in saponin buffer (0.1% saponin and 2% BSA) for 30 min at 4°C and incubated with antibodies specific for Cyclin D1, Bcl-2 and Bax for 1 h min at room temperature. Subsequently the colonocytes were treated with corresponding secondary antibodies for 45 min at room temperature and washed with PBS. The acquisition from each sample was conducted on FACS Canto (BD Biosciences) and the collected data were analyzed using the BD FACS Diva software. The corresponding controls for all the different antibodies were also run simultaneously. The results were expressed as the net percentage population of cells = percentage population of cells with Ab - percentage population of cells only.

5.6. Apoptotic Index Analysis

5.6.1. M30 CytoDEATH Method: The percentage

of apoptosis in isolated colonocytes of all groups was estimated by M30 cytoDEATH monoclonal antibody, Boehringer (Boehringer, Mannheim, Germany) using flow cytometric method. Briefly, approximate $2x10^6$ ice cold 70% ice cold ethanol fixed cells were washed once with PBS. After incubationwith M30 cytoDEATH antibody for 60 min at room temperature, the cells were rinsed twice in PBS. For visualization, FITC conjugated Fab_2 fragments of rabbit anti-mouse Ig G_1 (Dako A/S, Glostrup, Denmark) antibody was added in a 1:10 dilution. After incubation for 45 min at room temperature samples were rinsed twice in PBS and the cells were finally re suspended in 0.5 ml cold PBS. The samples were allowed tost and for 15 min on ice in the dark before flow cytometric analysis. In the negative control the primary antibody was omitted.

5.6.2. Hoechst (H33342) and Propidium Staining Method: The isolated colonocytes were suspended with Hoechst dye (10 µg/ml) for 30 min at 37°C. After incubation the cells were washed in PBS twice and counterstained with propidium iodide (1 µg/ml) for 10 min at room temperature. The cells were then washed with PBS and observed using fluorescent microscope (Nikon Eclipse 80i). The images were acquired using Northern Eclipse imaging Elements-D (NIS-D) software. The photomicrographs of cells stained with Hoechst and PI were merged. In the merged photomicrographs the cells were counted and classified on the basis of their morphological and staining characteristics as: live (blue fluorescence cells with normal nuclei) early apoptotic cells (light pink fluorescence dead cells with normal nuclei) late apoptotic cells (blue-violet and dark pink fluorescence dead cells with apoptotic nuclei) and necrotic (red fluorescence). The cells were counted in five random fields in each slide.

5.6.3. Measurement of Bcl-2 Family Members: The isolated colonocytes were fixed by adding 70% ice cold ethanol and washed in PBS twice. For labeling Bcl-2 and Bax, the cells were permeabilised in saponin buffer (0.1% saponin and 2% BSA) for 30 min at 4°C and incubated with antibodies specific for Bcl-2 and Bax for 1 h at room temperature. Furthermore, colonocytes were treated with corresponding secondary antibodies for 1 h at room temperature and washed with PBS. The acquisition from each sample was conducted on FACS Canto (BD Biosciences) and the collected data were analyzed using BD FACS Diva software. The corresponding controls for all the different antibodies were also run simultaneously. The results were expressed as the net percentage population of cells = percentage population of cells with Ab - percentage population of cells only.

5.7. Measurement of Ras and NF –κB Localization

The translocation of membranous Ras protein to cytosol and cytosolic NF –κB to nucleus was studied in isolated colonocytes by immune fluorescence. Briefly, the cells were fixed and air dried on glass slides and allowed to adhere at room temperature. The cells were permeabilized by saponin buffer (containing 0.4% saponin and 2% BSA) and incubated with monoclonal antibody against Ras and NF –κB for 2 hour at 37°C. After rinsing twice in cold PBS, the cells were incubated with FITC conjugated anti mouse IgG1 for 2 hour at 37°C in humid chamber. The cells were counterstained with PI and images were acquired using a fluorescent microscope (Nikon eclipse 80i). Green colour indicates the expression of Ras or NF –κB (p65 subunit) in the cell membrane and cytoplasm respectively and red color represents nuclear staining with PI. In the merged photomicrographs, yellow color indicates the translocation of Ras from cytoplasm to cell membrane and in case of NF –κB (p65 subunit) from cytoplasm to nucleus.

5.7.1. Measurement of Vascular Endothelial Growth Factor (VEGF): The expression of VEGF in colon tissue sections was analyzed by immune histochemical staining technique. Formalin fixed 2-3µm thick sections were mounted on poly-L-lysine coated slides. The sections were deparaffinised, rehydrated and endogenous peroxidase activity was quenched by incubating the tissue sections with 3% hydrogen peroxide for 20 min at 4 °C. After blocking with 6% BSA, antigen retrieval was done with citrate buffer (pH 6.0) in microwave for 5 minutes. The slides were allowed to cool and the sections were incubated with the monoclonal antibody (1:100) against VEGF for overnight at 4°C. After rinsing twice in PBS, the tissue sections were incubated with HRP conjugated anti mouse secondary antibody for 2 h at 37 °C. The slides were then visualized using DAB and H_2O_2 followed by counterstaining with hematoxylin dye. The photomicrographs were acquired and analyzed using the Nikon Eclipse 80i microscope (Japan) and Northern Eclipse Imaging Elements-D (NIS-D) software.

5.7.2. Statistical Analysis: The results are expressed as Mean± Standard Deviation (S.D). Differences between groups were assessed by one way ANOVA using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (L.S.D.) test. A value of $p < 0.05$ was considered to indicate a significant difference between groups.

6. Results

6.1. Effect of Atorvastatin and/or Fish Oil on Histopathological Characteristics of Colon

On histopathological examination, normal colonic mucosa and crypts were observed in the control group (Figure 1a). Significant infiltration of inflammatory cells and malignant transformation was observed on DMH treatment (Figure 1b). However in Atorvastatin + DMH, fish oil+ DMH and Atorvastatin+ fish oil+DMH groups, the inflammation level was markedly decreased as compared to DMH treated group. The effect was pronounced with combination regimens (**Figure 1**).

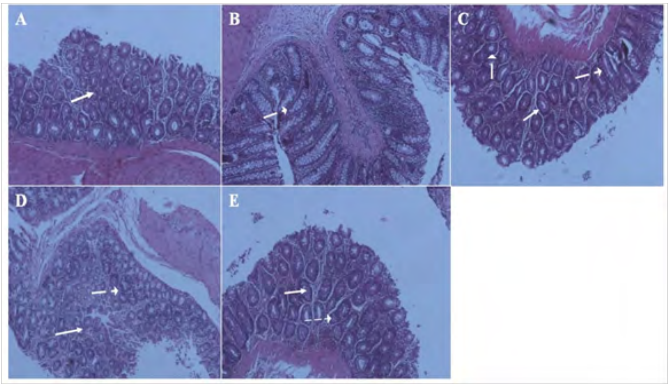

Figure 1: Histopathological analysis of colon sections of different groups. A. Control groupshowing normal crypt architecture with no sign of inflammation.B.photomicrograph representing DMH treated animals with irregular crypt architecture and inflammation rich areas. C. Atorvastatin + DMH groupD Fish oil + DMH groupE Atorvastatin + Fish oil + DMH group.

6.2. Effect of Atorvastatin and/or Fish Oil on Cell Proliferation Index

The results are depicted in (**Figure 2a**, **b**). There was a significant increase in the percentage of cells at S and G2/M phase with concomitant decrease in G1 phase in DMH treated group as compared to control group. However treatment with atorvastatin and/or fish oil led to significant increase in cell population in G1 phase and decrease in S and G2/M phase in comparison to DMH treated group. There was no significant difference among different therapeutic regimens.

6.3. Effect of Atorvastatin and/or Fish Oil on Cyclin D1 Levels

There was a significant increase in the Cyclin D1 levels in DMH treated group as compared to control group (**Figure 3a**, **b**). On the other hand, a significant decline in Cyclin D1 levels was observed in atorvastatin and/or fish oil group with respect to DMH treated group.

6.4. Effect of Atorvastatin and/or Fish Oil on Ras Expression

The immune fluorescence results revealed that DMH treatment led an increase in the number of cells showing the expression of Ras in comparison to control group (**Fig.4B**). However treatment with atorvastatin and/or fish oil significantly decreased the expression and membrane localization of Ras as compared to DMH treated group (**Figure 4a**). But the decrease was found to be more effective with combination group as compared to the other groups (**Figure 4b**).

6.5. Effect of Atorvastatin and Fish Oil on Apoptotic Index

The results are represented in (**Figure 5a** and **6a**). Both Hoechst-PI staining (**Figure 5b**) and M30 CytoDEATH method (**Figure 6b**) have shown that there was a decrease in apoptotic index in DMH treated group as compared to control group. On the other hand treatment with atorvastatin+DMH and fish oil+DMH significantly increased the apoptotic index as compared to DMH treated group. However combined effect of atorvastatin and fish oil in carcinogen treated animals significantly increased the apoptotic index in comparison to the other treated animals.

The results demonstrated that there was a significant increase in Bcl-2 levels in DMH treated animals as compared to control group (**Figure 7A** and **B**). However treatment with atorvastatin and/or fish oil in DMH treated animals led to a significant decrease in Bcl-2 and increase in Bax levels as compared to DMH treated group. But the effect was more pronounced in the combination regimen of atorvastatin and fish oil.

6.6. Effect of Atorvastatin and/or Fish Oil on Expression and Localization of NF–κB

The immunofluorescence results revealed that there was a significant increase in the cytoplasmic expression and

nuclear localization of NF –κB in DMH treated group in comparison to control group (Figure 8a, b). On the other hand the expression and nuclear localization of NF -κB was significantly decreased in atorvastatin+DMH, FO+DMH and atorvastatin+FO+DMH treated group as compared to DMH treated group (**Fig. 8C**, **D**, **E**).

6.7. Effect of Atorvastatin and/or Fish Oil on VEGF Expression

The results are depicted in **Fig. 9**. The colonic mucosa of control animals depicted a weak expression of VEGF (**Figure 9A**). On treatment with DMH an increase in the expression of VEGF was observed (**Figure 9B**). The treatment with either atorvastatin or fish oil led to a marked reduction in VEGF expression (**Figure 9C, D**). In the animals treated with combination therapy, there was a mild expression of VEGF in colonic mucosa (**Figure 9E**).

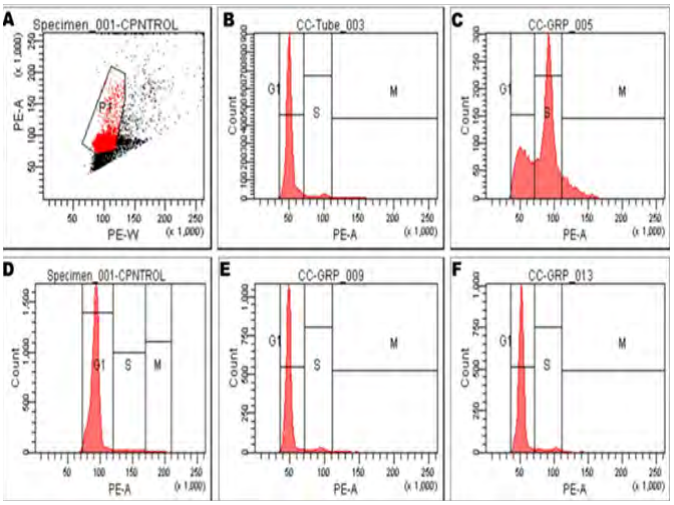

Figure 2(a): Representative images of flow cytometric analysis of propidium iodide stained cells for cell cycle distribution. A. Gated cells.B. Control group.C. DMH groupD.Atorvastatin+DMH group. E. Fish oil+DMH group.F. Atorvastatin+Fishoil+DMH group.

Groups	G1 Phase	S Phase	G2/M Phase
Control	92.8±4.2	5.0±2.2	1.9±3.2
DMH	66.3±4.6[a]	28.4±5.4[a]	4.4±8.2
Ator + DMH	89.1±9.2[b]	9.0±9.0[b]	1.4±0.6[c]
Fish oil + DMH	93.0±1.2[b]	5.7±2.0[b]	1.0±0.8[c]
Ator + Fish oil + DMH	92.7±2.2[b]	6.4±2.0[b]	0.7±2.4[c]

Figure 2(b): Tabulated data of cell cycle distribution in different groups. The results are expressed as mean+S.D. of six animals from each group. ap<0.01 versus control group, bp<0.01 and cp<0.05 versus DMH treated group.

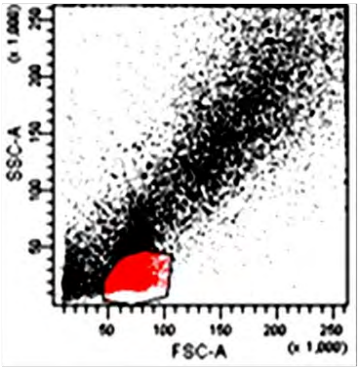

Figure 3(a): Effect of atorvastatin and/or fish oil on Cyclin D1 levels.

Groups	% Cyclin D1 positive cells
Control	4.83.8±1.05
DMH	25.02±6.29[a]
Ator + DMH	9.97±9.3.44[b,c]
Fish oil + DMH	8.06±2.63[c]
Ator + Fish oil + DMH	6.35±1.98[c]

Figure 3(b): The results are expressed as mean±S.D. of 5 animals from each group. ap<0.001 versus control, bp<0.05 versus control, cp<0.001 versus DMH treated group.

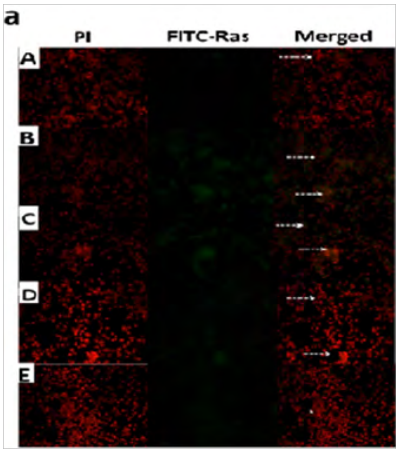

Figure 4(a): Photomicrographs (40X) showing the localization of Ras protein by immunofluorescence staining in the isolated colonocytes. A. Control group B. DMH group C. Atorvastatin+DMH group D. Fish oil+DMH group E. Atorvastatin+Fishoil+DMH group. Green color represents the expression of Ras on membrane; red color represents PI staining.

Groups	% of live cells	% of early apoptotic cells	% of late apoptotic /necrotic cells
Control	80.1±3.6	8.3±1.8	10.0±2.8
DMH	88.2±3.2[b]	4.5±1.4	6.3± 1.4[e]
Atorvastatin+DMH	55.7±4.0[a,d]	28±0.8[a,d]	15.3±2.0[b,d]
Fish oil+DMH	43.2±8.0[a,d,e]	33.7±3.6[a,d,g]	21.7±3.2[a,d,f]
Atorvastatin+Fishoil+DMH	37.6±10.0[a,d,e,i]	42.5±4.6[a,d,e,h]	19.8±2.0[a,d,g]

Figure 4(b): Representative table of Ras expression in isolated colonocytes of different groups.
The results are expressed as mean±S.D. of 4 observations in each group. ap<0.001 versus control, bp<0.01 versus control, cp<0.05 versus control, dp<0.001 versus DMH group, ep<0.01 versus DMH group, fp<0.01 versus atorvastatin+DMH, gp<0.05 versus atorvastatin+DMH treated group.

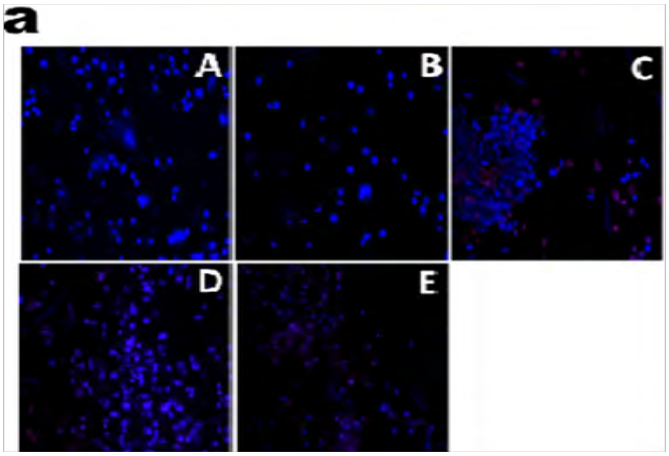

Figure 5(a): Representative images showing morphological alterations in isolated colonocytes by Hoechst-PI staining using immunofluorescence method. A. Control group B. DMH group C.Atorvastatin+DMH group D.Fish oil+DMH group E.Atorvastatin+Fishoil+DMHgroup.

Groups	% of live cells	% of early apoptotic cells	% of late apoptotic /necrotic cells
Control	80.1±3.6	8.3±1.8	10.0±2.8
DMH	88.2±3.2[b]	4.5±1.4	6.3± 1.4[e]
Atorvastatin+DMH	55.7±4.0[a,d]	28±0.8[a,d]	15.3±2.0[b,d]
Fish oil+DMH	43.2±8.0[a,d,e]	33.7±3.6[a,d,g]	21.7±3.2[a,d,f]
Atorvastatin+Fishoil+DMH	37.6±10.0[a,d,e,i]	42.5±4.6[a,d,e,h]	19.8±2.0[a,d,g]

Figure 5(b): Representative table ofapoptotic index in isolated colonocytes from different groups.
The results are expressed as mean±S.D. of four animals in each group. ap<0.001, bp<0.01, cp<0.05 versus control group, dp<0.001 versus DMH treated group, ep<0.001, fp<0.01, gp<0.05 versus atorvastatin+DMH treated group and hp<0.01, ip<0.05 versus fish oil+DMH treated group.

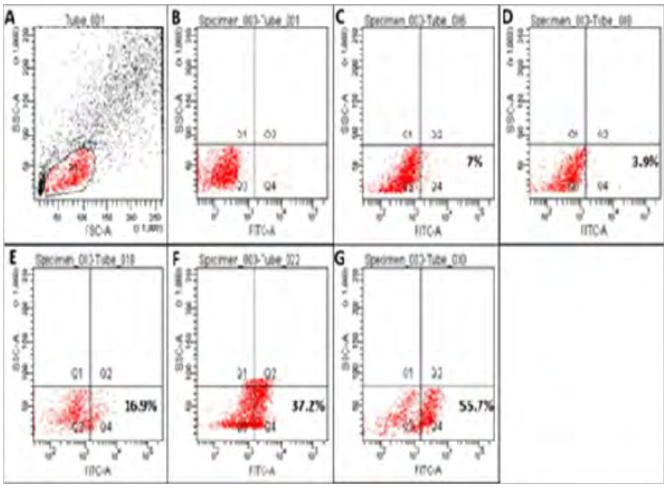

Figure 6(a): Representative images showing percentage of apoptotic cells in isolated colonocytes by M30 cytoDEATH levels using flow cytometry. A. Gated cells. B.Cells without antibody C.Control group D.DMH group E.Atorvastatin+DMH group F.Fish oil+DMH group H. Atorvastatin+Fishoil+DMH group.Q1 quadrant represents the percentage of dead/necrotic cells; Q2and Q4 quadrant represents the percentage ofapoptotic cells; Q3 quadrant represents the percentage of live cells.

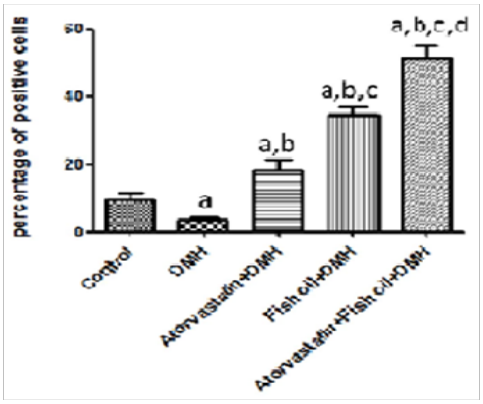

Figure 6(b): Graphical representation ofM30 cytoDEATH levels in isolated colonocytes from different groups.
The results are expressed as mean±S.D. of 5 observations in each group. ap<0.001 versus control, bp<0.001 versus DMH treated group, cp<0.001 versus atorvastatin+DMH treated group and dp<0.001 versus fish oil+DMH treated group.

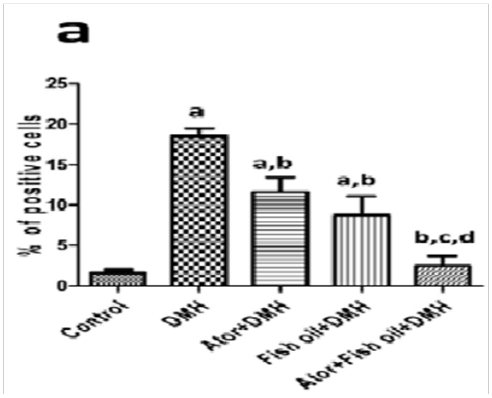

Figure 7(a): The results are expressed as Mean±SD of 4 animals in each group. ap<0.001 w.r.t. control, bp<0.001 w.r.t. DMH, cp<0.001 w.r.t. DMH atorvastatin+DMH treated group and dp<0.001 w.r.t. Fish oil+DMH treated group.

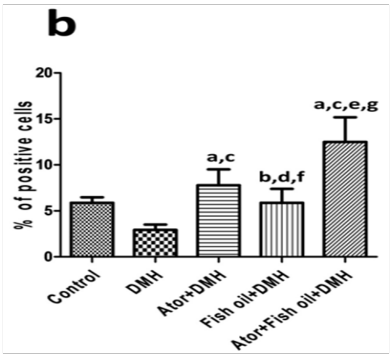

Figure 7(b): The results are expressed as Mean±SD of 4 animals in each group. ap<0.001, bp<0.01 w.r.t. control, cp<0.001, dp<0.01 w.r.t. DMH treated group, ep<0.001, fp<0.05 w.r.t atorvastatin+DMH treated group and gp<0.001 w.r.t. Fish oil+DMH treated group.

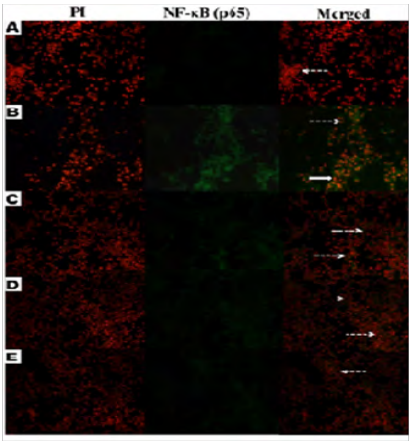

Figure 8(a): Representative microscopic images (40X) showing immunofluorescence staining of NF-κB protein in the colonocytes. A. Control group B. DMH group C. Atorvastatin+DMH group D. Fish oil+DMH group E. Atorvastatin+Fishoil+DMH group. Red color represents PI staining; green color represents the expression of NF-κB in cytoplasm whereas orange color represents the localization of NF-κB protein from cytoplasm to nucleus.
Represents Cytoplasmic expression
Denotes nuclear localization of NF-κB.

Groups	NF-κB(-ve)	cytoplasmic expression	Nuclear expression
Control	86.8±1.7	11.9±9.6	1.27±2.2
DMH	6.7±2.3^{a}	61.6±4.4^{a}	31.54±3.5^{a}
Ator + DMH	39.1±11.6^{a,e}	46.8±12.3^{b}	16.49±3.6^{a,d}
Fish oil + DMH	52.7±6.2^{b,d}	39.8±21.9^{b,f}	7.37±5.6^{e,d,g}
Ator + Fish oil + DMH	71.9±7.1^{d,g}	25.0±6.9^{d,h}	3.01±1.1^{d,g}

Figure 8(b): Representative table of NF-κB localization in isolated colonocytes.
The results are expressed as mean+S.D. of 5 animals in each group. ap<0.001, bp<0.01, cp<0.05 versus control, dp<0.001, ep<0.01, fp<0.05 versus DMH treated group, gp<0.001 versus atorvastatin+DMH treated group and hp<0.05 versus atorvastatin+DMH treated group.

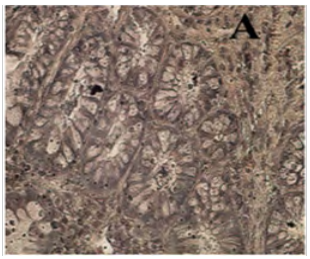

Figure 9(A): Effect of atorvastatin and/or fish oil on VEGF expression using immunohistochemical staining in the colon sections. Control group showing weak expression of VEGF

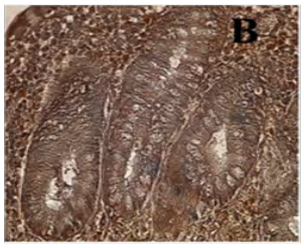

Figure 9(B): DMH treated group showing significant increased VEGF expression (Brown color)

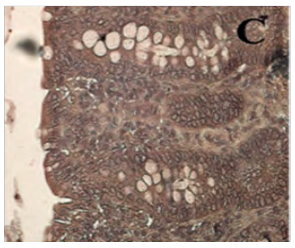

Figure 9(C): Atorvastatin+DMH group

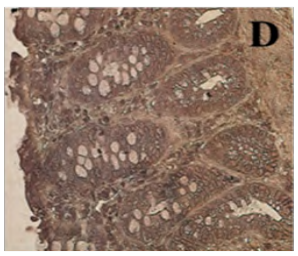

Figure 9(D): Fish oil+DMH E. Atorvastatin+ Fish oil+DMH group

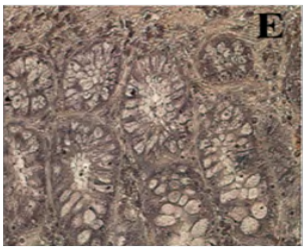

Figure 9(E): Atorvastatin+ Fish oil+DMH group

7. Discussion

Colorectal cancer is one of the leading causes of cancer mortality worldwide and requires urgent intervention anddesign of novel strategiesto reduceits incidence [31]. Though statins were introduced in clinical practice for hypercholesterolemia induced cardiovascular associated diseases [32], their use as cancer chemo preventive agents was mooted as it led to a decrease in incidence of

colorectal cancer [33] and in cancer associated mortality of 13 different cancer types [34]. In general statins are well tolerated but has many clinically relevant side effects which impact muscle, blood glucose levels and liver function [35]. There is increasing interest to use a combination of low doses of chemo preventive agents that may differ in their mechanism of action in order to increase their efficacy and minimise the toxicity. In the present study, we have utilised a similar approach by combining atorvastatinwith fish oil in DMH induced colon cancer. DMH is a highly specific carcinogen that results in the development of dysplastic aberrant crypts, adenoma and adenocarcinoma, the classical features of colon cancer [36]. In this study, DMH treatment led to formation of we observed preneoplastic lesions such as distortion in crypt architecture and inflammation rich areas. Treatment with fish oil alone and atorvastatin alone did not have much impact on the crypt architecture. However, the combination of fish oil and atorvastatin vastlydiminished the colonic inflammation and improved the colon architecture, suggesting the chemo preventive potential of the combination approach. In addition, the effect of the carcinogen is also highlighted by the observation of a significant accumulation of the cell population in S and G2/M phase indicating an increase in cell proliferation [37]. We also observed a higher expression of cyclin D1 in this group. This corroborates our finding on cell cycle analysis as cyclin D1 is responsible for driving the cell cycle progression from G1 to S phase. Atorvaststin and/or fish oil pretreatment resulted in a significant decrease in number of cells in S phase with a corresponding decrease in cyclin D1. Previous studies have also documented inhibition of cell proliferation and associated downregulation of cyclin D1 in the presence of statins or n-3 PUFAs [38-40]. However, there was no additive/synergistic effect of combination regimen on either of these parameters.

Constitutive activation of Ras is considered as a major reason for uncontrolled cell proliferation [40]. A decrease in the translocation and activation of Ras in colon carcinogenesis can act as a determinant to evaluate the effectiveness of tumor inhibition [41]. A significant increase in the trans localization of Ras to plasma membrane in DMH treated animals is suggestive of its activation and its role in regulation of tumor progression. An increase in Ras expression in carcinogenic animals has also been documented previously [42]. On the other hand, supplementation with atorvastatin and/or fish oil+DMH led to a significant decrease in the membrane localization of Ras as compared to DMH treated group but the effect was more pronounced with combination regimen. Inhibition of mevalonate pathway affects the synthesis of isoprenyl moieties i.e. farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) which isoprenylate proteins and assists in their proper localisation within the cell including Ras [43]. Statins may abrogate the process of activation of ras by inhibiting cholesterol biosynthetic pathway [44,45]. n-3 PUFAs have also been reported to inhibit Ras-dependent signalling by decreasing farnesyl protein transferase activity [46,47]. Therefore, the present observations are in complete agreement with the previous studies [47].

Resistance in apoptosis by disruption in apoptotic signaling pathways and their key regulators is one of the common traits that tumor cells acquire during malignant transformation. In the present study on DMH treatment a significant increase in Bcl-2 levels and a corresponding decrease in Bax/Bcl-2 ratio was observed. These results can be corroborated with the reduced apoptosis as shown by decrease in M30 cytoDEATH levels and an increase in live cells by dual staining with Hoechst33342 and propidium iodide. On the other hand treatment with atorvastatin and/or fish oil led to a significant increase in apoptosis with a concomitant decrease in Bcl-2 and increase in Bax levels. Studies have shown that atorvastatin or n-3 PUFAs perturb the dynamic balance between pro- and anti-apoptotic proteins of Bcl-2 family via the diminution of Bcl-2 expression levels [48]. It is postulated that fish oil alters fatty acid composition in the mitochondrial membrane which modulates the interaction between different Bcl-2 members. Down regulation of Bcl-2 and subsequently induction of intrinsic apoptotic pathway by statins have also been observed by other studies [49-51]. It was shown that depletion of GGPP causes improper localisation of proteins which compromises their function and triggers the cell to commit suicide. This is especially interesting since the down regulation of the Bcl-2 protein expression has been proposed as promising treatment strategy for colon cancer. The results suggest that the combination regimen of atorvastatin and fish oil may have greater efficacy than either of the agents alone. A recent study

using lovastatin-DHA conjugate in breast cancer cells has also reported higher apoptotic potential of this regimen in comparison to lovastatin and DHA alone [52]. The increase in apoptotic potential of the combined regimen may be due to the different pathways affected by the two treatment regimens. NF-κB belongs to a family of inducible transcription factors that regulates a wide array of genes involved in immune and inflammatory responses, cell death and survival, cell proliferation and tumor promoting angiogenesis [53]. In the present study there is a significant increase in both expression and nuclear localization of NF-κB in DMH treated group. Constitutive activation of NF-κB has not only been implicated in development but also in increased metastatic potential of various cancers and has also been reported to be elevated in animal models of colon cancer [54]. Furthermore, even a higher expression of VEGF, a downstream target of NF-κB, was observed in DMH treated animals. These results substantiate our hypothesis that angiogenic switch is turned on to favor vascular growth for tumor expansion [55]. In atorvastatin+DMH and fish oil+DMH treated group a significant reduction in both expression and nuclear localization of p65 subunit of NF-κB and VEGF expression was observed. The reduction in NF-κB p65 subunit together with an increase in the NF-κB inhibiting protein IκB by statins has also been reported earlier [56–58]. Statins also regulate the nuclear localisation of NF-κB via inhibition of PI3K/Akt signalling as shown in HUVEC cells [59]. Previous studies have also suggested that EPA/ DHA and their bioactive metabolites are capable of suppressing NF-κB [60-62]. There was further reduction in the expression and nuclear localisation of NF-κB in the animals treated with combination of both atorvastatin and fish oil which may be related to the regulation of diverse pathways by both the agents. A significant decrease in both NF-κB and VEGF suggest the potentiation of the anti-inflammatory and thereby, angiogenic switch, by the combined regimen. Our data indicate that use of statins with n-3PUFAs can be an interesting and more efficient strategy for colorectal cancer prevention. The pharmacologically relevant dose of atorvastatin is 10-80 mg. daily [63]. In the present case we have used the dosage on the lower end of spectrum. Though we did not find any significant effect of combination regimen on cell growth but an increase in apoptotic index, anti-inflammatory and anti angiogenic effect does point to the increased efficacy of the combined regimen. However, the interesting revelations of this study warrants further investigation in clinical and preclinical studies to fully exploit the therapeutic potential of this combination regimen.

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