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Inhibitory effect of polyunsaturated fatty acids on apoptosis induced by *Streptococcus pneumoniae* in alveolar macrophages

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***Background & objectives*: Apoptosis is considered as a major defense mechanism of the body. Multiple pathogens induce macrophage apoptosis as a mode of immune evasion. In earlier studies, n-3 polyunsaturated fatty acids (PUFA) have been reported to be protective against neuronal apoptosis and neuronal degeneration, seen after spinal cord injury. In this study, we tried to evaluate the role of n-3 polyunsaturated fatty acids on the process of macrophage phagocytic activity and apoptosis in mice.**

***Methods*: Mice were divided into three groups (n=60); Group I was fed on sea cod oil; Group II on flaxseed oil supplementation for 9 wk along with standard laboratory chow diet. Group III was fed on standard diet and served as control. After supplementation, phagocytic and apoptotic (morphological staining: acridine orange plus ethidium bromide; H-33342 plus propidium iodide staining and DNA ladder formation) activities of mouse alveolar macrophages were assessed.**

***Results*: Alveolar macrophages (obtained from sea cod oil and flaxseed oil fed group mice) showed significant increase in bacterial uptake as well as intracellular killing ($P< 0.05$) of *Streptococcus pneumoniae.* Significant decrease ($P<0.05$) in apoptotic cells was observed among alveolar macrophages from sea cod and flaxseed oil fed mice whereas maximum apoptosis was observed in control alveolar macrophages on interaction with bacteria *in vitro* which was confirmed by DNA laddering.**

Interpretation & conclusions*: These findings suggest that dietary supplementation with n-3 polyunsaturated fatty acids to mice led to enhanced phagocytic capability of their alveolar macrophages as well as provided protection against apoptosis upon challenge with *S. pneumoniae.

Key words Apoptosis - flaxseed oil - phagocytosis - pneumonia - polyunsaturated fatty acids - sea cod oil - *Streptococcus pneumoniae*

Pneumococcus usually colonizes the nasopharynx of humans asymptomatically, although sometimes it moves to the lungs, brain, and blood. This can lead to diseases associated with high morbidity and mortality such as pneumonia, septicemia, and meningitis[1,2]. Pneumococci are capable of inducing apoptosis in respiratory tree epithelium, endothelium, neuronal cells and alveolar macrophages (AM)[1,3]. AM are the first line defenders in the lungs and play an essential role against infections because of their capability to phagocytose and kill the invading microorganisms. To induce and potentiate inflammatory immune processes, AM release the required cellular mediators, such as tumour necrosis factor-alpha (TNF-α) and eicosanoids[4]. The

critical role of AM in lung defense against infection has been reported on the basis of high frequency of pneumonia in severely neutropenic patients and in cases of lung macrophage dysfunction[4].

Several compounds in or derived from the diet modulate apoptosis in cell cultures *in vitro*. These observations have important implications concerning the mechanism whereby dietary components affect health. Proapoptotic compounds could protect against cancer by enhancing elimination of initiated, precancerous cells and antiapoptotic compounds could promote tumour formation by inhibiting apoptosis in genetically damaged cells[5]. Oxidative stress activates apoptosis, and antioxidants protect against apoptosis *in vitro*; thus, a central role of dietary antioxidants may be to protect against apoptosis[5]. However, little data are available to directly link diet with altered apoptosis as an underlying determinant of disease. Omega-3 polyunsaturated fatty acids are important nutritional elements for humans, and these have the potential to inhibit excessive inflammatory responses; hence are widely recommended against atherosclerosis, coronary heart diseases, arrhythmias and in allergic conditions like asthma[6,7]. In an attempt to understand the role of dietary n-3 polyunsaturated fatty acids (PUFA) on phagocytosis and apoptosis, we studied interaction of alveolar macrophages obtained from the mouse fed on n-3 PUFA with *Streptococcus pneumoniae*, *in vitro*.

Material & Methods

Bacterial strain: A standard strain of *S. pneumoniae* D39 serotype 2 (provided by Dr Dong Kwon Rhee, College of Pharmacy, Sungkyunkwan University, South Korea) was used in this study. Organism was maintained on blood agar plates. The strain was found to be virulent in mice as confirmed by intra-peritoneal inoculation[8]. Experimental pneumonia was developed in mice by intra- tracheal administration as described by Zeng *et al*[9].

Test animals: Both male and female BALB/c mice, 6-8 wk old, healthy weighing 25 ± 5 g were procured from Central Animal House, Panjab University, Chandigarh, India. Animals in groups of eight were randomly housed in propylene cages and had free access to an antibiotic free diet (Hindustan Lever Limited, Mumbai) and water *ad libitum*. The experiments were performed in the Department of Microbiology, Panjab University, Chandigarh, after taking the permission from the Institutional Animal Ethics committee.

Feeding with n-3 polyunsaturated fatty acid (n-3 PUFA) to mice: Mice (n=60) were divided into three groups and consisted of 20 mice each. Group I was fed on standard laboratory chow diet by daily supplementation of 0.5 ml sea cod oil (Seven seas ^{R} Seacod ^{TM}, Universal Medicare, India) providing 900 mg per human body weight per day of n-3 PUFA. Group II was fed with daily supplementation of 0.5 ml flaxseed oil (Flax Oil, Nature's Bounty, USA) providing 2000 mg per human body weight per day of n-3 PUFA administered orally with a feeding catheter (Romsons Ltd., India) for 9 wk along with standard laboratory chow diet. Control mice (Group III) were fed on standard diet along with daily oral intake of 0.5 ml of normal saline.

Phagocytosis: Uptake and intracellular killing of *S. pneumoniae* D39 type 2 by mouse alveolar macrophages was studied as described earlier[10].

Relative uptake value was expressed as percentage of viable bacteria taken up by the macrophages at respective sampling time interval. For intracellular killing, bacterial suspension (10^8 cfu/ml) was mixed with normal mouse serum and kept for 30 min at room temperature. Macrophages (10^6cells/ml) were added to above bacterial suspension, incubated and centrifuged. The cells were lysed by the addition of normal saline containing 0.5 per cent sodium deoxycholate at time intervals of 1, 2 and 3 h. The colony forming units (cfu) were counted after overnight incubation at 37 ^{o}C.

Apoptosis studies: Alveolar macrophages were collected from the lungs of different groups of mice supplemented with dietary n-3 PUFA following the method of Morissette *et al*[11]. Briefly, Broncheoalveolar lavage (BAL) was performed using 1.5 ml of sterile phosphate buffer saline (PBS) containing 0.1 per cent (w/v) EDTA. The lavage cells were centrifuged, washed and suspended in RPMI-1640. The cell density of alveolar macrophages was adjusted after checking the viability with 0.2 per cent (w/v) trypan blue staining. A ratio of prokaryotic to eukaryotic cell 100:1 was achieved[12,13] by incubating macrophages (10^6 cells/ml) with 10^8 cfu/ml of bacteria for 3 h in humidified atmosphere containing 5 per cent CO_2 at 37^{o}C.

Acridine orange plus ethidium bromide staining: For morphological evaluation of alveolar macrophage apoptosis, staining of cells with acridine orange/ethidium bromide (Sigma, USA) was performed by the method of Singhal *et al*[14]. Briefly, at the end of the scheduled incubation, 2 µl of a combined dye of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide were added to 20 µl of the cell suspension (10^6 cells/ml), and 5 µl of the stained cell suspension was rapidly

transferred to a glass slide for immediate analysis using an ultraviolet fluorescence microscope (Olympus BH-2, Japan). Staining with acridine orange plus ethidium bromide combined with fluorescent microscopy was used to distinguish early apoptotic cells from necrotic cells. Ten random fields were counted and percentage of apoptotic cells was recorded by the observer unaware of experimental condition.

H-33342 plus propidium iodide staining: H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence, whereas propidium iodide costain the dead cells. Double staining by these two agents provides the percentage of live, apoptotic and necrosed cells under control and experimental conditions. For this the method of Kapasi *et al*[15] was followed. Briefly, at the end of the scheduled incubation periods, aliquots of methanol containing H-33342 (final concentration, 1 µg/ml) were added and incubated for 10 min at 37°C. Subsequently, cells (without a wash) were placed on ice, and propidium iodide (final concentration, 1 µg/ml) was added to the cell suspension. Cells were incubated with the dyes for 10 min on ice, protected from light, and then examined under ultraviolet light. Percentage of apoptotic cells was recorded in ten random fields by the observer unaware of experimental conditions.

Assessment of apoptosis by DNA laddering: DNA isolation and gel electrophoresis are simple techniques to confirm the occurrence of apoptosis[16,17], as these provide morphologic evidence of DNA fragmentation. To confirm the occurrence of macrophage apoptosis when incubated with *S. pneumoniae*, cells were lysed in DNA lysis buffer [10mM Tris-HCl (pH-8,) 0.1M EDTA, 0.5% SDS and 20 µg/ml RNase]. DNA was extracted and run on a 1.6 per cent (w/v) agarose gel and electrophoresed.

Statistical analysis: Data were analyzed for significant differences by two way ANOVA. Individual group comparisons were made by two tailed Student's t test. Each experiment was done in triplicates.

Results & Discussion

Alveolar macrophages are critical to early defence in the lung and their ability to phagocytose bacteria represents an integral part of the host immune defense against *S. pneumoniae*[18]. Following phagocytosis, macrophages recruit activated inflammatory cells to the site of infection, as well as are involved in the processing and presentation of bacterial antigens, thereby linking innate and adaptive immunity[19]. In the present study, phagocytosis of *S. pneumoniae* with mouse alveolar macrophages obtained from animals supplemented with n-3 PUFA for 9 wk was studied. When compared with control, macrophages showed significant increase in uptake as well as intracellular killing (P<0.05) of *S. pneumoniae* (Fig. 1). Macrophages from sea cod oil fed group showed 30 per cent increase in phagocytic uptake which was slightly higher than the flaxseed oil group (27%). Similarly, increase in intracellular killing of *S. pneumoniae* by alveolar macrophages from sea cod oil as well as flaxseed oil fed groups was 36 and 30 per cent, respectively. Previous studies have observed that macrophages enriched with saturated fatty acids such as myristate or palmitate showed decrease of 28 and 21 per cent, respectively in their ability to phagocytose unopsonized zymosan particles. Those enriched with polyunsaturated fatty acids showed 25-55 per cent enhancement of phagocytic capacity[20]. In another study, short-term enteral feeding with an eicosapentaenoic acid-enriched or eicosapentaenoic with gamma-linolenic acid-enriched diet rapidly modulated the fatty acid composition of alveolar macrophage phospholipids, promoted a shift toward formation of less inflammatory eicosanoids by stimulated macrophages, but did not impair alveolar macrophage bactericidal function relative to responses observed after feeding a linoleic acid diet[21].

Tissue macrophages, including AM of the lung, exhibit low levels of constitutive apoptosis[22]. Therefore,

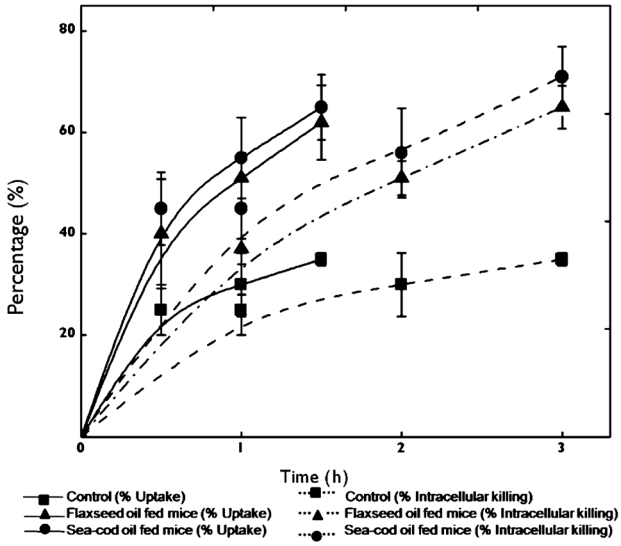

Fig. 1. Uptake (%, solid line) and intracellular killing (%, dotted line) of macrophages obtained from control (square) unsupplemented mice and flaxseed oil (triangle), sea cod oil (circle) supplemented mice infected with *S. pneumoniae*.

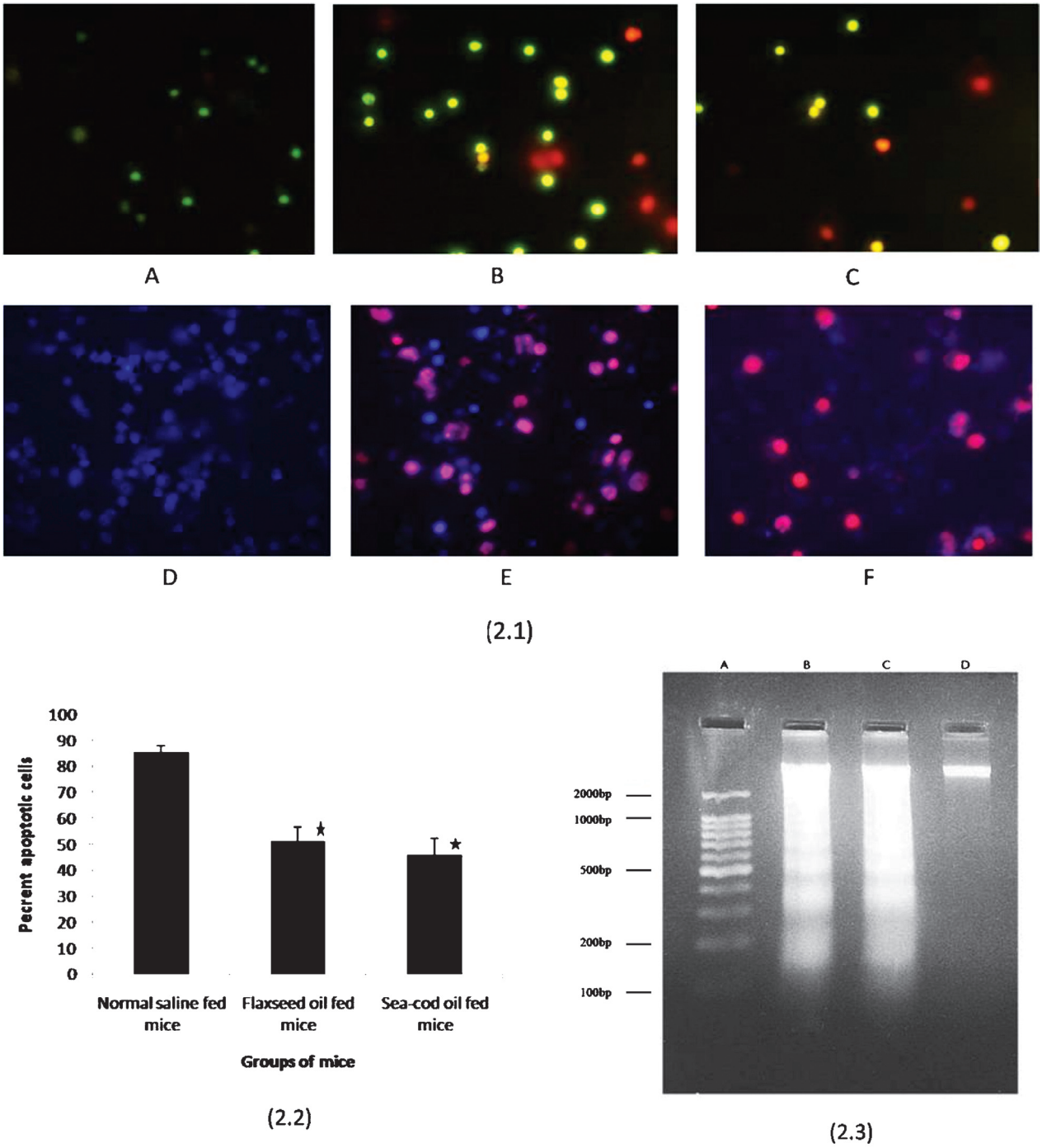

Fig. 2. Representative photographs showing presence of apoptotic cells **(2.1)** and per cent of apoptotic cells **(2.2)** among alveolar macrophage obtained from normal as well as n-3 PUFA fed mice on interaction with *S. pneumoniae in vitro*. Macrophages were stained with acridine orange plus ethidium bromide and Hoechst-33342 plus propidium iodide. (**2.1 A, D:** macrophages from normal mice (negative control); **2.1 B, E:** macrophages from normal mice after interaction with *S. pneumoniae* (positive control); **2.1 C, F:** macrophages from n-3 PUFA supplemented mice after interaction with *S. pneumoniae*). Live cells stain green or blue; necrotic cells stains orange-red or red; apoptotic cells stain greenish yellow or pink depending on the stain used. DNA ladder formation **(2.3)** in alveolar macrophages obtained from normal as well as n-3 PUFA fed mice upon exposure to whole cells of *S. pneumoniae* (Lane A: 100bp DNA ladder marker; Lane B: DNA of alveolar macrophages extracted from flaxseed oil supplemented mice; Lane C: DNA of alveolar macrophages extracted from sea cod oil supplemented mice showing characteristic ladder formation; Lane D: DNA of alveolar macrophages extracted from normal mice (negative control).

induction of apoptosis is a major mechanism by which macrophage function is regulated during infection. Morphologically, apoptosis is characterized by intact plasma membranes and cellular organelles, nuclear condensation, DNA fragmentation and formation of apoptotic bodies[22]. DNA fragmentation can be detected by TUNEL labeling of breaks at 3-OH ends in DNA strands. However, this technique lacks specificity for apoptosis since DNA strand breaks have also been seen in necrotic cell death. Ethidium bromide (EB) intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it is used to identify cells that are in the final stages of apoptosis - such cells have much more permeable membranes. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence[14]. Hoechst 33342 (H-33342) binds preferentially to DNA rich in thymidine and adenine base pairs and is widely used as a marker for DNA condensation that accompanies apoptotic cell death in various cell types. The difference in H-33342 fluorescence emission between normal and apoptotic cells is attributed to a more rapid membrane uptake of H-33342 by cells undergoing apoptosis. This stain is used in conjunction with propidium iodide[15]. The potential of *S. pneumoniae* to induce apoptosis in alveolar macrophages of n-3 PUFA supplemented mice was evaluated. In order to quantify the apoptotic population, the nuclear morphology of cells was observed following acridine orange plus ethidium bromide staining and H-33342 plus propidium iodide staining. The macrophages in the control samples were moderate green (Fig. 2.1 A). The apoptotic cells were greenish yellow in colour whereas the necrotic cells were orange-red (Fig.2.1 B). With H-33342 plus propidium iodide staining viable cells showed moderate blue fluorescence while apoptotic cells showed nuclei with bright pink (Fig. 2.1 D, E). Significant decrease ($P<0.05$) in apoptotic cells was observed among alveolar macrophages from sea cod and flaxseed oil fed mice (Fig. 2.1 C & F) whereas maximum apoptosis was observed in control alveolar macrophages (Fig.2.2) on interaction with bacteria *in vitro* which was confirmed by DNA laddering (Fig. 2.3).

The present study demonstrated that dietary supplementation with n-3 PUFA not only enhanced phagocytic capability of alveolar macrophages but also decreased alveolar macrophage apoptosis by *S. pneumoniae* D39 type 2. In earlier studies, it has been reported that regulation of the inflammatory infiltrate is critical for the successful outcome of pneumonia and alveolar macrophage apoptosis is a feature of pneumococcal infection and this aids disease resolution[2,23]. Previous studies have also found that the degree of apoptosis in the lungs correlates with the severity of injury[2,23]. Thus, modulation of macrophage life span can be an important mechanism for the regulation of macrophage function. The incorporation of n-3 PUFA in immune cell membranes may influence the membrane fluidity, structure and function of several membrane receptors, transporters, enzymes and ionic channels[21,24]. These alterations can in turn indirectly modulate macrophage function as has been reported in earlier studies related to central nervous system associated problems. Wu *et al*[25] have suggested that dietary supplementation with n-3 PUFA may be helpful in spinal cord injury by inhibiting neuronal apoptosis and thus has a potential means to delay the onset of the disease and/or the rate of progression. PUFA can also block apoptosis[26,27] and several studies have documented their neuroprotective effects *in vitro* and *in vivo*[19,28]. In neurodegenerative diseases of retina, docosahexaenoic acid (DHA), the major retinal polyunsaturated fatty acid, prevents photoreceptor apoptosis during early development *in vitro*, and upon oxidative stress[29,30].

In conclusion, the present observations are important as AM are the first line of defence in respiratory tract infections. These observations, therefore, form the basis for future experimentation to study the molecular basis of altered macrophage function on exposure to n-3 PUFA.

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