SHORT-TERM TREATMENT OF ELASTASE-LPS INDUCED EXACERBATION MODEL OF EMPHYSEMA IN MICE WITH DOCOSAHEXAENOIC ACID, A PRECURSOR OF ANTI-INFLAMMATORY AUTACOIDS

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ABSTRACT: An essential pathological phenotype of chronic obstructive pulmonary disease is emphysema or lung tissue destruction. The study aims to assess docosahexaenoic acid (DHA), a precursor for specialized pro-resolving mediators, as a treatment of emphysema. Albino mice were challenged with porcine pancreatic elastase on day 1 and bacterial lipopolysaccharide on day 21 to induce emphysema. The pathological condition was then treated with docosahexaenoic acid for 7 consecutive days. The animals were treated with three experimental doses of docosahexaenoic acid (3, 10 and 30 mg/kg b.w.) and results were compared with another 2 groups receiving standard drugs, i.e., corticosteroid and bronchodilators. Histopathology, automated morphometry, bronchoalveolar lavage (BAL) and lung volume measurements were performed. The experiments showed that the 30 mg/kg dose of DHA has treatment effects similar to the standard drugs. The neutrophil is an inflammatory mediator, and the new treatment was able to reduce the neutrophil count in the bronchoalveolar lavage fluid. Lung volumes were reduced suggesting lesser hyperinflation. Histopathology confirms more inferior airway obstruction in the treatment groups. Even though the experimental doses of docosahexaenoic acid were not superior to the effects of corticosteroids but has shown some signs of improvement in exacerbation model of emphysema in mice and has promised to become a therapy for management of COPD in future.

INTRODUCTION: Chronic Obstructive Pulmonary Disease (COPD) is a complex, debilitating lung disease where the patients have difficulty in exhaling. It comprises of a variety of clinical and pathological phenotypes which range from airway inflammation (chronic bronchitis) to lung tissue destruction (emphysema) and airway remodeling. The pathogenesis of COPD involves abnormal, deregulated and aggressive cellular inflammatory responses of the lung to mainly cigarette smoke exposure.

Emphysema is a significant component of COPD which is characterized by alveolar extracellular matrix destruction resulting in airspace enlargement and the pathogenesis proposes involvement of elastase, matrix metalloprotease imbalance, apoptosis and oxidative stress.

It is seen that pulmonary exposure to porcine pancreatic elastase (PPE), which primarily acts on elastin, can predispose acute lung inflammatory responses through neutrophil and macrophage in animals. These inflammatory cells destroy the lung connective tissues by the production of inflammatory cytokines followed by emphysematous changes and poor lung function. Bacterial lipopolysaccharides or cigarette smoke have been used to reproduce symptoms of exacerbations as seen in computed tomography.
The underlying molecular mechanisms of emphysema have been unveiled, but the knowledge has not been executed to develop effective targeted therapies. Specialized pro-resolving mediators (SPMs) are a new array of molecules that have potent anti-inflammatory, resolution-stimulating and tissue-protective actions. These are structurally distinct signaling molecules namely, resolvins, protectins, and maresins, which constitute novel families of autacoids and have shown potential to stimulate cellular events of resolution in pre-clinical models. The SPMs are derived enzymatically from docosahexaenoic acid and other omega-3 fatty acids in a lipoygenase–dependent manner as well as utilizing the Aspirin-triggered cyclooxygenase-2 pathway. This conversion happens naturally in the body as the SPMs are found in inflammatory tissue exudates.

Neutrophils are one of the key players in precipitating emphysema in humans and rodents. The SPMs can limit the neutrophil migration in vitro, reduces tissue inflammation and damage. Therefore, the objective of this research was to test the hypothesis that docosahexaenoic acid, as a precursor of the SPMs, might be able to limit or reverse the inflammatory changes in the progression of pulmonary emphysema when given as a treatment in an elastase-LPS induced mice model.

The morphological changes in the mice lungs are observed and evaluated. The study quantified inflammatory cells in bronchoalveolar lavage fluid, lung volume measurement by water displacement, histopathology and automated morphometry. The outcomes of DHA treatment were compared to that of established treatments with bronchodilators and corticosteroids for the same emphysema model.

**MATERIALS AND METHODS:**

**Chemicals and Reagents:** Porcine pancreatic elastase (PPE) E1250, Type I and lipopolysaccharide (LPS) from Escherichia coli 0111: B4 were purchased from Sigma-Aldrich, India. Ketamine Hydrochloride injection I.P. (Aneket® 250 mg / 5 ml) was also purchased for anesthetizing/immobilizing the animals. Phosphate Buffered Saline (PBS) had been freshly prepared every time needed in the laboratory with analytical grade reagents. The standard (Prednisone 5 mg tablets & Duova rotacaps®), as well as test drugs (OVEGHA® 400mg DHA), were all marketed products.

**Animals:** 6-8-week-old female albino mice, weighing 15-20 g were purchased and housed in a pathogen-free environment with a 12 h light and dark cycle. The animals were kept in suitable cages and provided with water and food ad libitum. All the experiments described herein were approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi and Institutional Animal Ethics Committee (IAEC); Regd. no. 1458/ PO/E/11/CPCSEA. The animals were divided into six groups (n=6) as follows:

- Porcine pancreatic elastase, followed by Lipopolysaccharide exposure- no treatment (Control).
- PPE and LPS exposure followed by short-term treatment with a corticosteroid (Standard drug).
- PPE and LPS exposure followed by short-term treatment with LABA/LAMA bronchodilators (Standard drug).
- PPE and LPS exposure followed by treatment with DHA Dose I (Test drug).
- PPE and LPS exposure followed by treatment with DHA Dose II (Test drug).
- PPE and LPS exposure followed by treatment with DHA Dose III (Test drug).

**Induction of Emphysema:** Oropharyngeal aspiration was the chosen route of administration for PPE and LPS as it has fewer complications and lesser mortality rate during the procedure. Mice were first administered 100mg/kg intraperitoneal dose of Ketamine for immobilization and suspended vertically. Then 0.25 IU of PPE dissolved in 50μl PBS was administered oropharyngeally to each mouse. Then the mice were challenged with 1mg/kg LPS also dissolved in 50 μl of PBS on the 21st day. The animals were sacrificed on the days 22 and 28 respectively for control & treatment groups for analysis of lung morphological changes.
FIG. 1: MATERIALS AND METHODS. I: CHEMICALS (PPE & LPS); II: MOUSE VERTICALLY SUSPENDED JUST BEFORE OROPHARYNGEAL INSTALLATION OF INDUCING AGENT; III: BALF COLLECTED IN EDTA+ PHOSPHATE BUFFERED SALINE

Treatment Plan: Treatment starts on the day of LPS challenge and continues for seven consecutive days Table 1. Animals were sacrificed on the 28th day, and further pathological tests were carried out. Tissue culture grade water was used as a vehicle for every drug that was administered. The toxicity studies of the test drug showed that acute LD$_{50}$ of rats to be higher than 5000 mg/kg of body weight.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Drug</th>
<th>Dose</th>
<th>Route of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Corticosteroid (Prednisolone)</td>
<td>10 mg/kg/day$^{17}$</td>
<td>Oral</td>
</tr>
<tr>
<td>2</td>
<td>Bronchodilators (Tiotropium/Formoterol)</td>
<td>(60 ng / 40 ng)/day$^*$</td>
<td>Intranasal$^{19}$</td>
</tr>
<tr>
<td>3</td>
<td>The docosahexaenoic acid dose I</td>
<td>3 mg/kg/day</td>
<td>Oral$^{18}$</td>
</tr>
<tr>
<td>4</td>
<td>Docosahexaenoic acid dose II</td>
<td>10 mg/kg/day$^{18}$</td>
<td>Oral$^{18}$</td>
</tr>
<tr>
<td>5</td>
<td>Docosahexaenoic acid dose III</td>
<td>30 mg/kg/day$^{18}$</td>
<td>Oral$^{18}$</td>
</tr>
</tbody>
</table>

*Animal dose calculated from the human dose of the marketed product.

Histopathology and Automated Morphometry: Formalin-fixed lung samples were cut parasagittally and embedded in paraffin. Hematoxylin and eosin staining was performed. Images were acquired in a randomized manner as colored JPG files using a microscope, a 10X objective, a camera, and software TS view that can capture high-quality digital images of the slides. Under-inflated areas were avoided. 25-30 images were captured per lung tissue sample. By using Image J NIH software, these randomized images were converted to binary (black and white) images and saved as TIFF files for further analysis.

The white color represented the tissue and black represented airspaces. This portion of tissue recorded by the software corresponds to any respiratory bronchioles, blood vessels, alveolar ducts and pulmonary sacculles/alveoli$^{21}$. The following parameters were calculated by the software.

A. Mean Linear Intercept (MLI): This parameter was calculated from the images using the direct method with the software Image J. Mean linear intercept (Lm) is quantification of mean distance from one airspace wall to another, which includes the wall of respiratory bronchiole, blood vessels, alveolar ducts, and pulmonary sacculles/ alveoli. A uniform grid of horizontal and vertical lines was superimposed on the binary images. Then the lengths of each chord within areas identified by the software as airspace were quantified. The formula for the mean linear intercept$^{22, 23}$ is as follows:

\[ Lm = \Sigma \{2 \times (\text{length of the line/ number of intercepts})\}/ (\text{total number of lines}) \ldots \ldots \ (i) \]

B. Destructive index (DI): Destructive index was calculated when the slides were observed under a 10X objective. This time a point grid was superimposed on the images and the structures falling under these points were either denoted as normal (N) or destroyed (D).
More than two disruptions in contiguous alveoli or isolated islands of lung parenchyma were classified as destroyed. This parameter is an estimate of parenchymal destruction of the lung. Thickened airways without obvious breaks were also denoted as destroyed (D) structures. The DI was calculated from the formula:

\[ DI = \frac{D}{D + N} \times 100 \]  

**Cell Counts from Bronchoalveolar Lavage Fluid (BALF):** Bronchoalveolar lavage was performed on days 21 (for control) and 28 (for treatment groups) by using a 20 G catheter (intravenous) inserted into the trachea. The cannulated tracheas were lavaged with 0.5 ml increments of ice-cold PBS four times (2 ml total). Red blood cells were made to lysis by erythrocyte lysis buffer. The total cells, lymphocytes, and neutrophils were counted on hemocytometer.

**Lung Volume Measurement by Water Displacement:** Lungs were separated after sacrifice, and the lung volume was measured by water displacement. This parameter is considered as a measure for total lung capacity and hyperinflation caused by emphysema.

**Statistical Analysis:** GraphPad Prism software version 7.03 for Windows had been used for statistical analysis. Data are furnished as mean ± s.e.m. Statistical significance was calculated by one-way ANOVA, between treatment groups versus the disease-induced control group, and the minimum statistical significance was accepted at p<0.05 in all cases. Early death after challenging the animals with the inducing agent was an exclusion criterion. In this experiment, only one mouse died out of all the PPE-LPS treated animals.

**RESULTS:**

**Histopathology:** The lung specimens were evaluated at 22nd day of the disease induced control group along with normal lung specimen, and the treated groups were evaluated after 28 days. No pathological changes were seen in the normal lung specimen which was air-exposed, but chronic airway obstruction and emphysematous changes were observed in the PPE-LPS treated group Figs. 2.

Airspace enlargement and destruction of alveolar structures were evident in the PPE-LPS treated lung sample. In the treatment groups, one can observe lesser obstruction in the bronchioles.

The higher dose of DHA (30 mg/kg) and the corticosteroid-treated lungs have the least inflammation Fig. 3.

**TABLE 2: PARAMETERS OF AUTOMATED MORPHOMETRY (MEAN ± SEM**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Group</th>
<th>Mean Linear Intercept (mean ± SEM)</th>
<th>Destructive Index (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPE-LPS induced</td>
<td>0.4657 ± 0.28</td>
<td>9.667 ± 0.33</td>
</tr>
<tr>
<td>2</td>
<td>Corticosteroid treatment*</td>
<td>0.2305 ± 0.20</td>
<td>8.933 ± 0.08</td>
</tr>
<tr>
<td>3</td>
<td>Bronchodilator treatment*</td>
<td>0.2540 ± 0.4</td>
<td>7.567 ± 1.44</td>
</tr>
<tr>
<td>4</td>
<td>DHA Dose I (3 mg/kg)*</td>
<td>0.2965 ± 0.01</td>
<td>7.767 ± 0.76</td>
</tr>
<tr>
<td>5</td>
<td>DHA Dose II (10 mg/kg)*</td>
<td>0.2367 ± 0.03</td>
<td>7.567 ± 0.77</td>
</tr>
<tr>
<td>6</td>
<td>DHA Dose III (30 mg/kg)*</td>
<td>0.2127 ± 0.01</td>
<td>8.167 ± 1.01</td>
</tr>
</tbody>
</table>

*p<0.05 versus PPE-LPS induced group.
Automated Morphometry: The first parameter calculated was the mean linear intercept Fig. 4. After induction of the disease, the Lm values increased which is indicative of lung parenchymal destruction. The results Table 2 showed that corticosteroid treatment along with the DHA doses (30 mg/kg and 10 mg/kg) was able to decrease the MLI significantly at a 95% confidence interval. But neither the bronchodilator treatment \(p=0.0791\) nor the 3 mg/kg dose of DHA \(p=0.2554\) could show similar significant effect. The second parameter was destructive index calculation. None of the treatments were able to decrease the DI significantly after exposure to elastase and LPS. These results suggest that even though the treatments can halt the progression of the inflammation, tissue repair was not observed in this short period, and hence a long-term experiment is needed.

Inflammatory Cell Counts from Bronchoalveolar Lavage Fluid (BALF): The cellular profile of the bronchoalveolar lavage fluid was evaluated after the 7-day treatments and on the 22nd day of the disease-induced control Table 3.

For the normal lung specimen the total cell count was negligible and differential cell count not
performed. The total cell count for the PPE-LPS treated animals was very high. Three treatment groups of corticosteroid, bronchodilator and DHA 30 mg/kg showed a similar and significant reduction in the cell count when compared to the positive control group. But groups of the two lower doses of DHA (10 mg/kg and 3 mg/kg) were unable to produce significant effect Fig. 5.

**TABLE 3: INFLAMMATORY CELL COUNTS (MEAN ± SEM) OF BALF**

<table>
<thead>
<tr>
<th>S no.</th>
<th>Group</th>
<th>Total cell count × 10³</th>
<th>Neutrophil count/ ml</th>
<th>Lymphocyte count/ ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPE-LPS induced</td>
<td>95.7 ± 7.63</td>
<td>7.43 ± 0.47</td>
<td>112.5 ± 4.6</td>
</tr>
<tr>
<td>2</td>
<td>Corticosteroid treatment**</td>
<td>16.4 ± 4.3</td>
<td>0.72 ± 0.72</td>
<td>13.3 ± 4.1</td>
</tr>
<tr>
<td>3</td>
<td>Bronchodilator treatment**</td>
<td>26.7 ± 6.0</td>
<td>1.25 ± 0.13</td>
<td>19.3 ± 4.6</td>
</tr>
<tr>
<td>4</td>
<td>DHA Dose I (3mg/kg)**</td>
<td>72.3 ± 7.2</td>
<td>2.02 ± 0.13</td>
<td>57.5 ± 6.3</td>
</tr>
<tr>
<td>5</td>
<td>DHA Dose II (10mg/kg)**</td>
<td>58.0 ± 6.0</td>
<td>2.30 ± 0.49</td>
<td>50.2 ± 10.2</td>
</tr>
<tr>
<td>6</td>
<td>DHA Dose III (30mg/kg)**</td>
<td>29 ± 4.9</td>
<td>2.92 ± 0.30</td>
<td>25.4 ± 5.2</td>
</tr>
</tbody>
</table>

**p<0.05, versus PPE-LPS induced group.**

In case of differential cell counts, neutrophils rapidly increased in the BALF of the disease induced group which all the treatment group was able to reduce count significantly at 95% confidence interval and the 30 mg/kg DHA dose was near to significant.

Similarly, the increased lymphocyte counts were decreased in a dose-dependent manner when treated with the three experimental doses of DHA. But only 30 mg/kg dose of DHA had been able to match the extent of effect with the standard treatments and was statistically significant Fig. 6.

**FIG. 6: NEUTROPHIL COUNTS AND LYMPHOCYTE COUNTS FROM BALF**

**Lung Volume Measurement:** The increase in lung volume and hyperinflation of lungs occur due to loss of the elasticity. In this experiment, all the three DHA doses and corticosteroid treatments were able to reduce the increased lung volume caused by chronic inflammation Fig. 7. The bronchodilator treatment is not seen to produce any significant effect.

**FIG. 7: LUNG VOLUME MEASUREMENT BY WATER DISPLACEMENT**

**DISCUSSION:** Elastase and LPS induced emphysema is a simple exacerbation model of emphysema developed by Kobayashi *et al.* This study was initially described in male C57BL/6J mice, unlike this present study where we have experimented on female albino mice. The disease is induced in 3 weeks, and neutrophilic inflammation may contribute to the morphometric changes. This is a novel study to show the treatment effect of docosahexaenoic acid in a mice model of emphysema. Three experimental doses of DHA had been chosen which have previously shown anti-inflammatory effects in an arthritis model with rats. This exacerbation model of emphysema has a very low mortality rate. No significant changes in the body weights or appetite or muscle strength (exercise capacity) of the animals were observed throughout the short span of the experiment. This is an original short-term study which has concluded by performing simple procedures.
Initially, the histopathological analysis revealed the pathological changes to be standard features of emphysema, i.e. free flowing tissue, enlarged air space, thickened epithelial wall, and neutrophil accumulation. Later, we observed it marked an increase in mean linear intercept, neutrophil count, total cell count, and lymphocyte count in the bronchoalveolar lavage fluid. All the treatment doses were significantly able to reduce the airspace enlargement (MLI) up to 50%, and DHA 30 mg/kg is the best treatment. The negative index showed that the destroyed structures of the lungs were not repaired; rather airway remodeling was observed which includes thickened epithelium, sub epithelium fibrosis. The short-term treatment does not have the potential to improve and regenerate tissue but to halt the progression. But resolvin D1, a pro resolving factor derived from DHA has shown the possibility of tissue regeneration after 24 weeks of treatment in a smoking model of emphysema.

Hence, long term treatment with DHA might also have a similar possibility. On the other hand, Resolvin D1 had shown limited effectiveness in reducing hyperinflation of the lungs, which DHA as a precursor of resolvins, protectins, and maresins, have been quite successful. The two main pathogenesis of emphysema is decreased elastic recoil and hyperinflation. The decrease in the lung volume showed that hyperinflation was restricted better when treated with the test drug doses rather than the standard treatments. This implies that a complex mechanism of action lies underneath restoring the elasticity and the size of the lungs, involving all the three classes of specialized pro-resolving factors (SPM) - protectins, resolvins, and maresins. The total cell count was reduced effectively by the 30 mg/kg dose of DHA up to the extent of both the standard treatments with corticosteroid and bronchodilators.

The neutrophil count in the BALF was better reduced by the 3 mg/kg and 10 mg/kg doses of DHA and the standard treatments. Eosinophil count did not significantly change during the disease induction and treatment period. In this case, the high dose of DHA lowered the lymphocyte counts to a larger extent, almost equivalent to the corticosteroid. This may implicate an immune-suppressive activity exhibited by the omega-3 fatty acid. Another significant result of this experiment is that the corticosteroid drug has effectively treated PPE-LPS induced emphysema. But in the cases of smoking models of emphysema, corticosteroid insensitivity is observed very often.

At last, the results show that the standard treatments still had better effects than the test drug doses. But the short-term treatment with DHA have also shown good potential in the mouse model of emphysema of becoming an emerging treatment therapy, but still, it needs to have experimented in a smoking model with modeling other co-morbidities of COPD. The doses will require further adjustments as well as the mode of drug delivery. Previously other treatments like vitamin C, angiotensin receptor blockade, stem cells have attenuated cigarette smoke-induced emphysema in animals. Many other studies suggested statins to be an effective treatment. But these molecules have either failed in randomized clinical trials or needs reproducible results in human beings.

Therefore, pro-resolving mediators must have human trials. A comparison between treatments with DHA and more refined molecules (protectins, resolvins, and maresins) is yet to be established. Lastly, the 30mg/kg oral dose of DHA has shown to improve signs of emphysema in a brief period of treatment. It reduced the inflammatory cell counts in BALF and hyperinflation was restricted. These findings may indicate that DHA can be a treatment for emphysematous lung. Further analysis of the mechanism of action must be elucidated.

**CONCLUSION:** Docosahexaenoic acid has shown signs of improving emphysema in a rodent disease model by reducing the extent of inflammation in a short period of time. It can be called a potential candidate for further investigation as a drug for COPD management.

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**CONFLICT OF INTEREST:** Nil
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