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Mesoporous Nano-carbon particle Loaded Fisetin has a Positive Therapeutic Effect in a Murine Preclinical Model of Ovalbumin Induced Acute Allergic Asthma

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Abstract

Inflammation is a complex process initiated by several factors ranging from bacterial infection and chemical injury to environmental pollution that causes cell injury or death, release of inflammatory mediators which further trigger the up-regulation of other pro-inflammatory cytokines and chemokines as well as sequestration of specific immunoglobulin molecules. Th2 inflammation is an allergenic reaction by primed T and B cells and during manifestation of acute asthma, this immune reaction takes the form of super-activated macrophages, eosinophils, neutrophils and an array of Th2 skewed inflammasome development. An anti-inflammatory molecule should be able to potently reduce or inhibit one or more of the aforementioned traits. Several organic and inorganic substances have been studied to evaluate their potential as anti-inflammatory molecules, among which nanotechnology has emerged as a promising aid to anti-inflammatory activities. Nanoparticle-incorporated mesoporous carbon particles have a wide range of applications in controlled drug delivery. These nano particles are capable of delivering drugs inside the cell, even in a sub-cellular organelle specific manner. In this study, inflammation is initiated by using different classes of pro-inflammatory stimuli, such as OVA (chicken ovalbumin), LPS (lipopolysaccharide) and TG (thioglycollate), and by applying such nano-concoctions, the anti-inflammatory activities of certain compounds are assessed in in vivo models. The anti- inflammatory action of a novel drug made from extract of strawberry (fisetin) has been evaluated in an in vivo model of acute allergic asthma in Balb/c mice. Also, attempts are being made to narrow the delivery window within the cell, by loading the drug onto the constructed mesoporous carbon nanoparticle. It has been found to maintain cell viability significantly by reducing oxidative and nitrosative stress and ameliorating the overall development of composite asthma phenotype in this murine preclinical model.

Keywords: Inflammation; Anti-oxidant; Phenolic compounds; Scavenger activities; Reactive oxygen; Nitrogen intermediates

Introduction

Inflammation, either acute or chronic, is the body's response to disturbed homeostasis caused by infection, injury or trauma resulting in systemic and local effects [1,2]. Inflammatory response occurs in three distinct phases [1]. The first phase is caused by an increase in vascular permeability resulting in exudation of fluids from the blood into the interstitial space, the second phase involves the infiltration of leukocytes from blood into tissue space and in third phase granuloma formation and tissue repair occurs [1,3]. Infiltration of innate immune system cells, specifically neutrophils and macrophages, characterizes the acute inflammation, while infiltration of T lymphocytes and plasma cells are features of chronic inflammation [2-4]. Monocytes/macrophages play a central role in both, contributing to the final consequence of chronic inflammation which is represented by the loss of tissue function due to fibrosis. At the molecular level, inflammation is regulated by numerous molecules and factors, including cytokines, chemokines, proinflammatory transcription factors and proinflammatory enzymes. Among all these mediators, NF-κB is the central regulator of inflammation [3,5-7].

Inflammation leads to the clinical manifestation of diseases such as asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, obesity and inflammatory bowel disease [8-15]. Asthma is an inflammatory disease of the airways affecting millions of people worldwide. Long-term airway inflammation leads to episodes of airflow obstruction that typically respond well to steroids in mild to moderate asthmatics. Therapeutic goals for treating moderate to severe asthma are to restore normal longterm airway function and reduce the morbidity and associated mortality. The current standard of care for long-term asthma is the use of inhaled glucocorticoids and beta receptor agonists to provide symptomatic relief. There is still a need for effective therapies for treating patients with moderate and severe asthma [9,16-19].

Despite the advances in medical technology and hygiene, the introduction of vaccines and modern medications the prevalence of inflammatory disorders has continued to increase in the last few decades throughout the globe [20]. Although the best approach to investigate various inflammatory processes, and to identify crucial pathways and potential novel targets for drug therapy, is to perform studies in human patients, due to ethical reasons these are not always

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possible necessitating the development of various preclinical animal models and cell lines, both human and non-human, to elucidate the pathophysiology of various inflammatory disorders, and to identify and evaluate novel therapeutic targets for them. Preclinical disease models need to be carefully selected if they are to be predictive of the biology, expected in treating human disease [9]. From the ancient period the role of traditional medicines in the solution of health problems is invaluable on a global level. In traditional medicine medicinal plants continue to provide valuable therapeutic agents. Due to various side effects and complications of the modern medicine, and to address unmet needs of the particular disease, specially complex etio-pathophysiological pathways traditional medicine is gaining importance and is now being studied systematically and using biotechnological tools, to find the scientific basis of their therapeutic actions [5].

Recently, the use of natural dietary substances found in fruits, vegetables, and herbs has received considerable attention as chemopreventive and chemotherapeutic agents worldwide [21-24]. Flavonoids are commonly found in most plants and exert a significant range of biological activities such as antioxidant, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, and antiviral [25-27]. Flavonoids and their polymers comprise one of the largest groups of phytonutrients that afford beneficial health effects. Fisetin is a bioactive flavonol molecule found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber. The highest concentration of fisetin was found in strawberries (160 μg/g) followed by apple (26.9 μg/g) [21].

Nanoparticles (NPs), defined as particles with a diameter less than 100 nm, are being increasingly used as carriers for drug delivery [28]. The diminutive size of nanoparticles used in such applications facilitates their interaction and/or uptake by cells, as well as potentially enabling them to interfere with specific subcellular components, enabling less toxic and more efficient therapeutic actions [29]. Several organic and inorganic substances have been studied to evaluate their potential as anti-inflammatory molecules, among which nanotechnology based knowledge has emerged as a promising aid to anti-inflammatory activities [5,7]. Nanoparticle-incorporated mesoporous carbon particles (MCP) have a wide range of applications in controlled drug delivery. These nano particles are capable of delivering drugs inside the cell, even in a sub-cellular organelle specific manner [5-7].

Phytochemicals from fruits and other edible plant parts have been shown to possess significant antioxidant properties that may be associated with lower incidence and lower mortality rates of degenerative diseases in human. Different biological properties, antioxidant capacities and radical-scavenging activities of various herbal extracts have been widely demonstrated, using in vitro techniques and in vivo models by different groups of researchers [23,26,27,30,31]. The anti-proliferative and anti-inflammatory activities of these herbal extracts have been documented in human oral, breast, colon, cervical, and prostate cancer cell lines as well as preclinical animal models by attenuating some inflammation intermediates, including nitric oxide, NF-κB, and TNFα [6,7].

The aim of this study was to detect potential anti-inflammatory activities of fisetin from a fruit that is traditionally known as an anti-geriatric compound against acute allergic asthma in a murine model. High content of various phenolic and non-phenolic compounds and other uncharacterized moieties may contribute to its use not only as a highly nutritive edible plant part but also position it as a nutraceutical substance and a prophylactic cum therapeutic compound in oxidative inflammatory diseases. In addition to fisetin, mesoporous carbon nanoparticles (MCN) were loaded to provide direction to the drug like compound to see whether it indicated a better drug delivery and therefore enhanced therapeutic power.

Materials and Methods

Animals

BALB/C mice were used in this study. All experiments were performed according to rules laid down by the Institutional and departmental animal ethics committee and the animals housed under specific pathogen-free conditions at the animal housing vivarium of the Department of Zoology, University of Calcutta. In total, the following number of animals were used in each group: WT (Control)=5 per experiment, +OVA=5 per experiment, +OVA+fisetin=3 per experiment, and +OVA+(MCN+fisetin)=2 per experiment. A total of three independent experiments for development and analyses of the OVA model and a total of two independent experiments for drug discovery studies were performed. Data presented are mean ± SEM for all experiments and only p value less than 0.05 have been considered as significant.

Allergen sensitization and challenge: Mice were sensitized and later challenged with OVA (Pierce, Rockford, IL). Mice were immunized with OVA (100 μg) complexed with aluminium sulfate in a 0.2 ml volume, administered by i.p. injection on day 0. On days 8 (250 μg of OVA) and on days 15, 18, and 21 (125 g of OVA), mice were anesthetized briefly with inhalation of isoflurane in a standard anesthesia chamber and given OVA by intratracheal (i.t.) administration. Intratracheal challenges were done. Mice were anesthetized and placed in a supine position on the board. The animal's tongue was extended with lined forceps and 50 μl of OVA (in the required concentration) was placed at the back of its tongue. The control group received normal saline with aluminium sulfate by i.p. route on day 0 and 0.05 ml of 0.9% saline by i.t. route on days 8, 15, 18, and 21.

Development of acute asthma phenotype in murine model

Animals were treated intraperitoneally with 100 μg ovalbumin conjugated with aluminium hydroxide adjuvant and then on day 8 treated with 250 μg ovalbumin intratracheally. On day 15, 18 and 21 they were treated intra tracheally with 125 μg of ovalbumin. For drug treated groups, 5 MPK Fisetin (dissolved in DMSO) was given intratracheally one hour before each intra tracheal challenge, and 2 μM/Kg BW (i.e 50 nM/animal) Fisetin loaded on mesoporous carbon nano particles (dissolved in sterilized autoclaved double distilled water) was given intratracheally one hour before each intra tracheal challenge. Placebo treated groups were given normal saline. All animals were sacrificed 24 hours after final ovalbumin challenge.

BALf

The mouse underwent exsanguination by infra-orbital arterial bleeding and then BAL (0.4 ml three times) of both lungs. Total BAL fluid cells were counted from a 50 μl aliquot and the remaining fluid was centrifuged at 200 g for 10 min at 4°C and the supernatants stored at -70°C for assay of BAL cytokines later. The cell pellets were resuspended in FCS and smears were made on glass slides. The cells, after air drying, were stained with Wright-Giemsa (Biochemical Sciences Inc, Swedesboro, NJ) and their differential count was taken under a light microscope at 40X magnification. Cell number refers to that obtained from lavage of both lungs/mouse.

Lung parenchyma

Lung mincing and digestion was performed after lavage with 100 µ/ml collagenase for 1 hr at 37°C, and filtered through a 60# sieve (Sigma). All numbers mentioned in this paper refer to cells obtained from one lung/mouse.

NO estimation

In culture, the NO released by the macrophages into the medium is converted to several nitrogen derivatives, from which only nitrite is stable, being easily measured by Griess reagent (1.0% sulphanilamide and 0.1% N-(1)-naphthylethylenediamine in 5% phosphoric acid). After incubation, 100 µl of culture medium supernatant was mixed with the same volume of Griess reagent, during 10 min, at room temperature. The nitrite produced was determined by measuring the optical density at 540 nm, in a microplate reader (Thermo Fisher), and extrapolating from a standard curve.

Catalase estimation

Catalase is an antioxidant enzyme that protects against harmful ROS (reactive oxygen species), produced during metabolism. Catalase concentration was measured in this study. 0.5 ml of culture medium supernatant (lung sample) was added to the reaction mixture containing 1 ml of 0.01M phosphate buffer (pH 7.0), 0.5 ml of 0.2M H_2O_2, and 0.4 ml of H_2O. The reaction was stopped by adding 2 ml of acid reagent (dichromate/ acetic acid), made by mixing 5% potassium dichromate with glacial acetic acid, in the ratio of 1:3 by volume. The tubes were heated for 10 minutes, and absorbance was measured at 610 nm using a spectrophotometer (Shimadzu). The concentration of catalase produced was determined from a standard curve.

Ascorbic acid estimation

Ascorbic acid is an antioxidant, free-radical scavenger, which is present in normal conditions to protect against ROS. Inflammation leads to a decrease in the concentration of ascorbic acid, which can be measured colourimetrically. The reaction mixture for quantification of ASA comprised 0.1 ml of the sample, 2.9 ml distilled water, 1 ml of 2% DNPH (Dinitrophenyl hydrazine) and 1-2 drops of thiourea. After incubation for 3 hours at 37°C, the osazone crystals formed were dissolved with 7 ml of 80% sulphuric acid. Absorbance was read after 30 minutes at 540 nm using a spectrophotometer. The concentration of ASA was determined from a standard curve.

Cytokine analysis of BAL fluid

The BD CBA Mouse Th1/Th2 Cytokine Kit (Catalog No. 551287) was used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interferon-γ (IFN- γ) and Tumour Necrosis Factor (TNF) protein levels in BAL fluid samples. Bead array technology was used to simultaneously detect multiple cytokines in samples. Five bead populations with distinct fluorescent intensities are coated with capture antibodies, specific for the above-mentioned proteins. The beads are mixed to form the bead array, and resolved in a red channel of a flow cytometer. After addition of the samples to the sample assay tubes containing the capture beads, the Mouse Th1/Th2 PE Detection Reagent was added to each tube. The tubes were incubated for 1 hour at room temperature, in the dark, and then washed with 1 ml of wash buffer (centrifuge at 200 g for 5 mins). The supernatant is carefully discarded and 300 µl of wash buffer added to resuspend the bead pellet.

Gene expression

Gene expression was analysed by isolating RNA, followed by reverse transcriptase PCR, using gene specific primers for one housekeeping gene (GAPDH), and genes for four inflammatory cytokines (iNOS, IL-9, TNF-α and IL-1β). GAPDH is constitutively expresses at high levels in most cells and tissues. iNOS is the gene for inducible nitric oxide synthase, which catalyzes the production of nitric oxide as an immune defense mechanism. The gene for IL-9, a cytokine produced by CD4+T helper cells, has been identified as a candidate gene for asthma. TNF-α is a cell signaling protein in systemic inflammation, which helps in neutrophil migration. IL-1β is produced by activated macrophages, and is an important mediator of inflammatory responses.

RNA isolation

RNA was isolated using TRIzol reagent (Life Technologies, California). Lung tissue was homogenized in 1 ml TRIzol reagent, incubated at room temperature for 5 mins, 0.2 ml chloroform added and shaken vigorously for 15 seconds. After incubation at room temperature for 2-3 minutes, it was centrifuged at 12000 xg for 15 minutes at 4°C, and the upper aqueous layer removed into a new tube. To it, 0.5 ml of 100% isopropanol was added, incubated at room temperature for 10 minutes, and centrifuged at 12000 xg for 10 minutes at 4°C. The pellet was washed with 1ml of 75% ethanol, vortexed briefly, centrifuged at 7500 xg for 5 minutes at 4°C, and the pellet dried, before resuspending it in RNase-free water. It was incubated at 55°C-60°C for 10-15 minutes, before proceeding to cDNA preparation.

cDNA preparation

The isolated RNA is converted to cDNA using SuperScript III Reverse Transcriptase (Life Technologies, California). 50-250 ng of random hexamer primer was mixed with the isolated RNA, 10 mM dNTP mix and water, and heated to 65°C for 5 minutes, and then incubated on ice for at least 1 minute. To this, 5X buffer, 0.1M DTT and 200 units/µl SuperScript III RT were added, mixed by gentle pipetting, and incubated at 50°C for 30-60 minutes, after which the enzyme is inactivated by heating at 70°C for 15 minutes.

Primers used

The primers for the genes were obtained from Xcelris Genomics, India.

Gene	Primer	
GAPDH	Forward	5'TGTGATGGGTGTGAACCACGA
	Reverse	5'TGCTGTTGAAGTCGCAGGAGAC
iNOS	Forward	5'CCCTTCCGAAGTTTCTGGCAGCAGC
	Reverse	5'GGCTGTCAGAGCCTCGTGGCTTTGG
IL-9	Forward	5'CATCCTTGCCTCTGTTTTGC
	Reverse	5'CGTCCCCAGGAGACTCTTC
TNF-α	Forward	5'CATCTTCTCAAAATTCGAGTGACAA
	Reverse	5'ACTTGGGCAGATTGACCTCAG
IL-1β	Forward	5'CTCTAGAGCACCATGCTACAGAC
	Reverse	5'TGGAATCCAGGGGAAACACTG

PCR amplification

10X PCR buffer, 50 mM $MgCl_2$, 10mM dNTP mix, 10 µM forward primer, 10 µM reverse primer, 5U/µl Taq DNA polymerase, the prepared cDNA and water were mixed to prepare the PCR reaction mixture.

OVA specific IgE in serum

Anti-mouse IgE (R35-118) from BD Biosciences, San Diego, CA were used for measuring OVA specific IgE (in serum previously frozen at -70°C) respectively by standard ELISA procedures.

Lung histology

Lungs of other animals of same group were fixed in 4% paraformaldehyde overnight at 4°C. The tissues were embedded in paraffin and cut into 5 μm sections. A minimum of 15 fields were examined by light microscopy. The intensity of cellular infiltration around pulmonary blood vessels was assessed by Hematoxylin and Eosin staining. Airway mucus was identified by staining with Alcian blue and Periodic Acid Schiff staining.

Fluorescin-activated cell sorter (FACS) analysis

Cells from hemolysed peripheral blood (PB), bone marrow (BM), bronchoalveolar lavage (BAL), lung parenchyma (LP), spleen, mesenteric lymph nodes (MLN), cervical lymph nodes (CLN), axillary lymph nodes (LNX) and inguinal lymph nodes (LNI) were analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA) by using the CELLQuest program. Staining was performed by using antibodies conjugated to fluorescin isothiocyanate (FITC), phycoerythrin (PE), allophucocyanin (APC) , Peridinin Chlorophyll Protein (Per CP-Cy5.5) and Cy-chrome (PE-Cy5 and PE-Cy7). The following BD pharmingen (San Diego, CA) antibodies were used for cell surface staining : APC-conjugated CD45 (30F-11), FITC-conjugated CD3(145-2C11), PE-Cy5 conjugated CD4 (RM4-5),PE-conjugated CD45RC (DNL-1.9), APC-conjugated CD8(53-6.7), PE-Cy5 conjugated B220 (RA3-6B2), FITC-conjugated IgM, PE-conjugated CD19 (ID3), PE-conjugated CD21(7G6), FITC-conjugated CD23 (B3B4), APC-conjugated GR-1(RB6-8C5), and PE-conjugated Mac1(M1/70). PE-Cy5 conjugated F4/80 (Cl:A3-1(F4/80)) was obtained from Serotec Ltd., Oxford, UK. PE-conjugated anti-α4 integrin (PS2) and anti-VCAM-1(M/K-2) was from Southern Biotechnology, Birmingham, Ala. Irrelevant isotype-matched antibodies were used as controls.

Results

Inflammatory recruitment into lung lumen- (Total BALf cell content)

One of the first signs of successful inflammation is the recruitment of inflammatory cells into the tissue site of injury which, in this case, is the lumen of the respiratory tree and the lung. To quantitate this, the lungs were lavaged and the total cells counted using a hemocytometer and reconfirmed using TALI cell counter and viability tested using Trypan blue dye exclusion method. More than 98% of the cells were viable (data not presented) and total cell number in BALfluid increases 3.91 fold significantly than the untreated control groups after ovalbumin challenge whereas in fisetin treated groups, total cell number in bronchoalveolar lavage fluid decreases 2.78 fold than the control groups (Figure 1). In MCN+Fisetin treated groups, total cell number increases 3.60 fold than the control groups. This denotes that, after fisetin treatment, recruitment of cells in bronchoalveolar lavage fluid is 10.85 fold less, and after MCN+Fisetin treatment, cell recruitment is 1.09 fold less, than the only ovalbumin treated groups. Both Fisetin and MCN+fisetin effectively inhibit the cellular recruitment (Table 1).

Cell count at the site of poiesis – the bone marrow

Total cell number in bone marrow (aspirated from the femur) increases 29.5 fold significantly than the untreated control groups after ovalbumin challenge whereas in fisetin treated groups, total cell number in bone marrow decreases 4.29 fold than the control groups (Figure 2). In MCN+Fisetin treated groups, total cell number increases 10.45 fold than the control groups. This indicates a 126.43 fold decrease with Fisetin, and a 2.82 fold decrease with MCN+Fisetin, as compared to only Ova. This shows that the synthesis of cells in the bone marrow, which increases due to the Ova challenge, is reduced due to the treatment (Table 2).

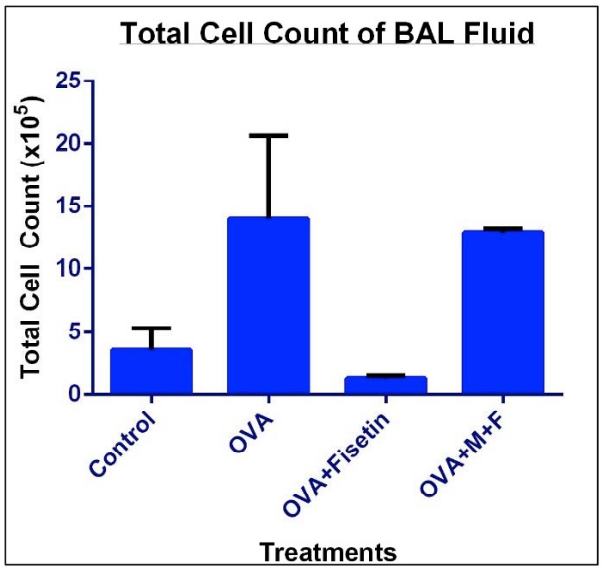

Figure 1: Effect of fisetin and MCN-fisetin on the total cell count of BAL fluid.

Cell count in circulating blood

Total cell count in peripheral circulating blood increases 16.88 fold in the OVA treated compared to control. The count decreases 4.48 fold with fisetin, and increases 3.48 fold with MCN+fisetin, as compared to control. This indicates a 75.59 fold decrease with fisetin, and 4.86 fold decrease with MCN+fisetin, as compared to only Ova (Figure 3 and Table 3).

Spleen

Total cell count in spleen increases 16.34 fold in the OVA treated compared to control. The count decreases 1.84 fold with fisetin, and increases 1.52 fold with MCN+fisetin, as compared to control. This indicates a 30 fold decrease with fisetin, and 10.76 fold decrease with MCN+fisetin, as compared to only Ova. Both fisetin and MCN+fisetin inhibit the cellular recruitment to the spleen to varying degrees but MCN+fisetin inhibits only 1.09 fold and this is not effective as compared to fisetin alone (Figure 4 and Table 4).

Axillary lymph nodes (AXL)

Total cell number in axillary lymph nodes increases 2.06 fold significantly ($p<0.05$) than the untreated control groups after ovalbumin challenge whereas in fisetin treated groups total cell number increases 1.05 fold than the control groups. This denotes that, after fisetin treatment, recruitment of cells in axillary lymph nodes is 1.96 fold less ($p<0.05$) than the only ovalbumin treated groups. Fisetin effectively inhibit the cellular recruitment to the axillary lymph nodes (Figure 5 and Table 5).

	TC (× 10^5)
Control	3.58 ± 1.75
Ova	14.0 ± 6.64
Ova+Fisetin	1.29 ± 0.21
Ova+(MCN+F)	12.9 ± 0.30

Table 1: Total cell count of BAL fluid, taken by hemocytometry and TALI cell counter. There is a 10.85 fold decrease with fisetin treatment, and a 1.09 fold decrease with MCN+fisetin.

	TC (× 10^8)
Control	0.60 ± 0.23
Ova	17.7 ± 8.84
Ova+Fisetin	0.14 ± 0.01
Ova+(MCN+F)	6.27 ± 0.07

Table 2: Total cell count of bone marrow, taken by hemocytometry and TALI cell counter. There is a 126.43 fold decrease with fisetin treatment, and a 2.82 fold decrease with MCN+fisetin.

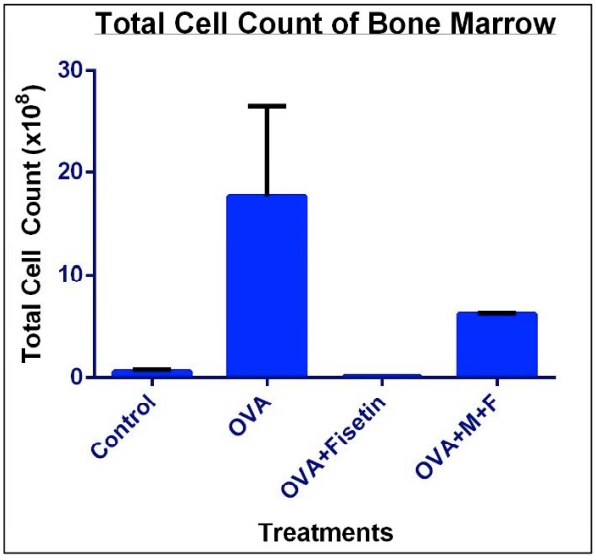

Figure 2: Effect of fisetin and MCN-fisetin on the total cell count of bone marrow.

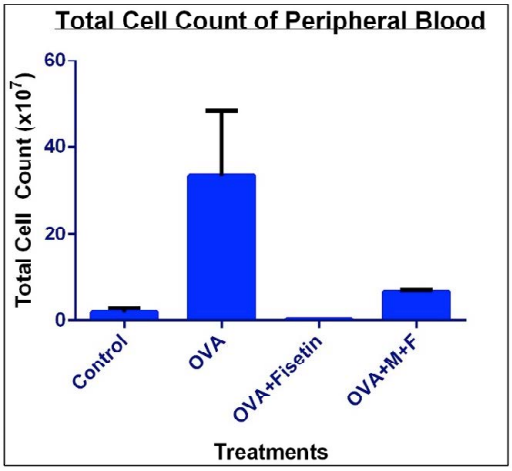

Figure 3: Effect of fisetin and MCN-fisetin on the total cell count of peripheral blood.

Thymus

Total cell number in thymus gland increases 2.74 fold significantly ($p<0.05$) than the untreated control groups after Ovalbumin challenge whereas in fisetin treated groups total cell number increases 1.35 fold than the control groups. This denotes that, after fisetin treatment, recruitment of cells in thymus is 2.03 fold less ($p<0.05$) than the only ovalbumin treated groups. Fisetin effectively inhibit the cellular recruitment to the thymus (Figure 6 and Table 6).

Payer's patch

Total cell number payer's patch increases 2.44 fold significantly ($p<0.05$) than the untreated control groups after ovalbumin challenge whereas in fisetin treated groups cell number decreases 1.26 fold than the control groups. This denotes that, after fisetin treatment, recruitment of cells in payer's patch is 3.08 fold less ($p<0.05$) than the only ovalbumin treated groups. Fisetin effectively inhibit the cellular recruitment to the Payer's patch (Figure 7 and Table 7).

Lung parenchyma

Total cell number in lung parenchyma increases 21.48 fold significantly ($p<0.05$) than the untreated control groups after ovalbumin challenge where as in fisetin treated groups total parenchyma cell types increases 1.43 fold than the control groups. This denotes that, after fisetin treatment, recruitment of cells in lung parenchyma is 15.03 fold less ($p<0.05$) than the only ovalbumin treated groups. Fisetin effectively inhibit the cellular recruitment to the lung parenchyma (Figure 8 and Table 8).

Differential counting (PBL/2ml)

There is a 3.94 fold increase ($p<0.05$) in lymphocyte count, a 7.34 fold increase ($p<0.05$) in eosinophil count, a 5.76 fold increase ($p<0.05$) in monocyte count and a 2.10 fold increase in neutrophil count, in the ova treated groups, compared to control. The counts of all the cell types decrease with fisetin treatment, and with MCN+fisetin treatment, as compared to only Ova. A 2.59 fold ($p<0.05$) decrease in lymphocyte count, a 4.70 fold ($p<0.05$) decrease in eosinophil count, a 2.09 fold ($p<0.05$) decrease in monocyte count, and a 1.21 fold decrease in neutrophil count, is seen with MCN+fisetin, compared to only Ova (Figure 9 and Table 9).

Differential counting (BALfluid/ml)

There is a 9.0 fold increase ($p<0.05$) in neutrophil count, a 52.50 fold increase ($p<0.05$) in eosinophil count, a 10.76 fold increase ($p<0.05$) in macrophage count and a 2.64 fold increase in lymphocyte count, in the ova treated groups, compared to control. The counts of all the cell types decrease with fisetin treatment, and with MCN+fisetin treatment, as compared to only Ova. There is a 1.43 fold decrease in neutrophil count, a 4.84 fold decrease ($p<0.05$) in the eosinophil count, a 1.60 fold decrease in macrophage count and a 2.96 fold decrease in lymphocyte count, after treatment with MCN+fisetin (Figure 10 and Table 10).

NO estimation (nM)

After ovalbumin treatment Nitric Oxide (NO) production in lung homogenate increases 1.66 fold ($p<0.05$) than the placebo treated groups whereas NO production increases 1.35 fold after fisetin treatment, and 1.29 fold after MCN+fisetin treatment, than the control groups. Thus a 1.23 fold decrease ($p<0.05$) after fisetin treatment, and a 1.29 fold decrease ($p<0.05$) after MCN+fisetin treatment, is observed in NO production in lung tissue homogenate (Figure 11 and Table 11).

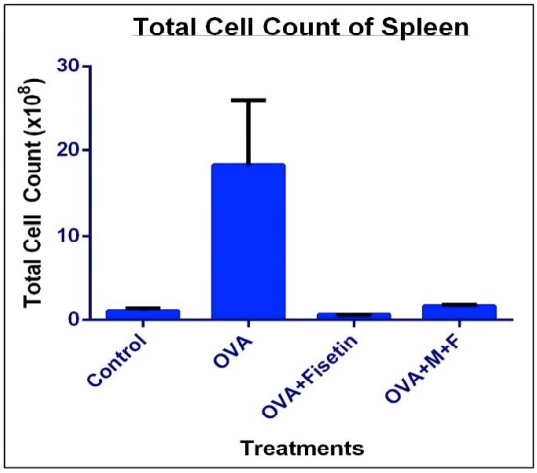

Figure 4: Effect of fiseitn and MCN-fisetin on the total cell count of spleen.

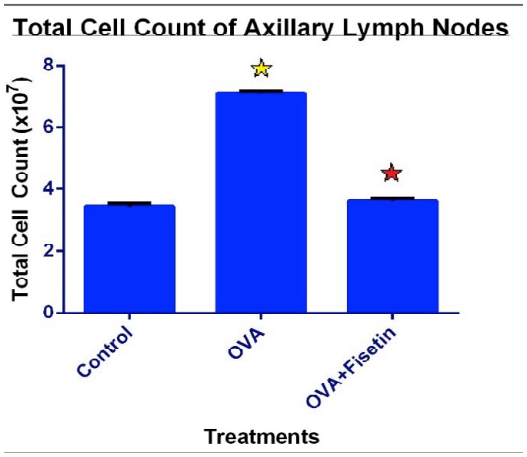

Figure 5: Effect of fisetin on the total cell count of axillary lymoh nodes. (*P<0.05 versus Control; *P<0.05 versus Ova).

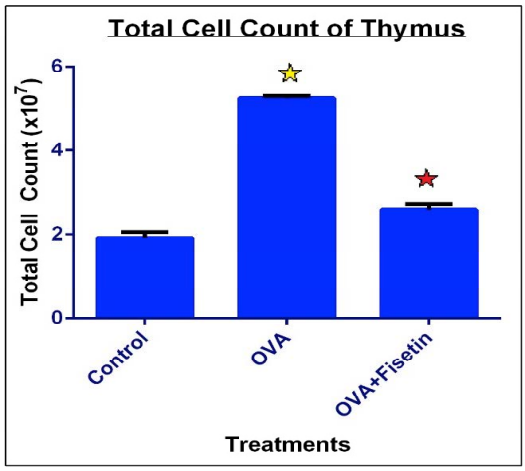

Figure 6: Effect of fisetin on the total cell count in thymus. (*P<0.05 versus Control; *P<0.05 versus Ova).

	TC (× 10^7)
Control	1.97 ± 0.97
Ova	33.26 ± 15.16
Ova+Fisetin	0.44 ± 0.01
Ova+(MCN+F)	6.85 ± 0.35

Table 3: Total cell count of peripheral blood, taken by hemocytometry and TALI cell counter. There is a 75.59 fold decrease with fisetin treatment, and a 4.86 fold decrease with MCN+fisetin.

	TC (× 10^8)
Control	1.12 ± 0.32
Ova	18.30 ± 7.73
Ova+Fisetin	0.61 ± 0.01
Ova+(MCN+F)	1.70 ± 0.16

Table 4: Total cell count of spleen, taken by hemocytometry and TALI cell counter. There is a 30.0 fold decrease with fisetin treatment, and a 10.76 fold decrease with MCN+fisetin.

	TC (× 10^7)
Control	3.45 ± 0.10
Ova	7.11 ± 0.07
Ova+Fisetin	3.62 ± 0.08
Ova+(MCN+F)	-

Table 5: Total cell count of axillary lymph nodes, taken by hemocytometry and TALI cell counter. There is a 1.96 fold decrease (p<0.05) with fisetin treatment.

	TC (× 10^7)
Control	1.92 ± 0.14
Ova	5.26 ± 0.05
Ova+Fisetin	2.59 ± 0.14
Ova+(MCN+F)	-

Table 6: Total cell count in thymus, taken by hemocytometry and TALI cell counter. There is a 2.03 fold decrease (p<0.05) with fisetin treatment.

	TC (× 10^7)
Control	1.11 ± 0.02
Ova	2.71 ± 0.04
Ova+Fisetin	0.88 ± 0.05
Ova+(MCN+F)	-

Table 7: Total cell count of Payer's patch, taken by hemocytometry and TALI cell counter. There is a 3.08 fold decrease (p<0.05) with fisetin treatment.

	TC (× 10^7)
Control	0.21 ± 0.01
Ova	4.51 ± 0.11
Ova+Fisetin	0.30 ± 0.01
Ova+(MCN+F)	-

Table 8: Total cell count of lung parenchyma, taken by hemocytometry and TALI cell counter. There is a 15.03 fold decrease (p<0.05) with fisetin treatment.

	Lymphocyte (× 10^5)	Eosinophil (× 10^5)	Monocyte (× 10^5)	Neutrophil (× 10^5)
Control	0.81 ± 0.52	1.34 ± 0.05	1.27 ± 0.04	1.53 ± 0.63
Ova treated	3.19 ± 0.18	9.83 ± 0.30	7.31 ± 0.33	3.21 ± 0.64
Ova+Fisetin treated	0.35	2.28	1.26	2.60
Ova+(MCN+Fisetin) treated	1.23 ± 0.03	2.03 ± 0.23	3.50 ± 0.30	2.66 ± 0.44

Table 9: Differential cell count of peripheral blood, seen after Wright-Giemsa staining, under light microscope. There is a 2.59 fold decrease (p<0.05) in lymphocytes, a 4.70 fold decrease (p<0.05) in eosinophil count, a 2.09 fold decrease (p<0.05) in monocyte count, and a 1.21 fold in neutrophil count, after treatment with fisetin.

Catalase estimation (pM)

After ovalbumin treatment Catalase expression in lung homogenate increases 1.13 fold than the placebo treated groups whereas after fisetin treatment catalase expression increases 1.31 fold than the control groups. After treatment with MCN+fisetin, the catalase expression decreases 1.40 fold compared to control. This shows a 1.16 fold increase with fisetin treatment, and a 1.58 fold decrease with MCN+fisetin treatment, as compared to Ova (Figure 12 and Table 12).

Ascorbic acid estimation

After ovalbumin treatment ascorbic acid contents in lung homogenate decreases 1.37 fold ($p<0.05$) than the placebo treated groups whereas after fisetin treatment ascorbic acid contents in lung tissue decreases 1.01 fold than the control groups. After MCN+fisetin treatment, the ASA contents decreases 1.16 fold compared to control. Thus a 1.39 fold increase ($p<0.05$) in ascorbic acid contents is observed after fisetin treatment, and a 1.18 fold increase is seen after MCN+fisetin treatment, compared to only Ova (Figure 13 and Table 13).

NO level in BALfluids (nM)

After ovalbumin treatment Nitric Oxide (NO) production in BAL fluid increases 1.41 fold than the placebo treated groups whereas NO production increases 1.49 fold after fisetin treatment, and decreases 1.51 fold after MCN+fisetin treatment, than the control groups. Thus a 1.06 fold increase after fisetin treatment, and a 2.13 fold decrease after MCN+fisetin treatment, is observed in NO production in BAL fluid (Figure 14 and Table 14).

NO level in BM (nM)

After ovalbumin treatment Nitric Oxide (NO) production in bone marrow increases 1.58 fold ($p<0.05$) than the placebo treated groups whereas NO production increases 1.21 fold after fisetin treatment, and decreases 1.64 fold after MCN+fisetin treatment, than the control groups. Thus a 1.31 fold decrease after fisetin treatment, and a 2.59 fold decrease ($p<0.05$) after MCN+fisetin treatment, is observed in NO production in bone marrow (Figure 15 and Table 15).

NO level in spleen (nM)

After ovalbumin treatment Nitric Oxide (NO) production in spleen increases 1.42 fold than the placebo treated groups whereas NO production increases 1.1 fold after fisetin treatment, and decreases 1.55 fold after MCN+fisetin treatment, than the control groups. Thus a 1.29 fold decrease after fisetin treatment, and a 2.20 fold decrease after MCN+fisetin treatment, is observed in NO production in spleen (Figure 16 and Table 16).

Cytokine levels in BAL fluids

After challenge with ova, there is an increase in the levels of IL-2 (1.78 fold), IL-4 (1.74 fold), IL-5 (1.84 fold), IFN-γ (2.86 fold) and TNF-α (2.67 fold; $p<0.05$) compared to control. The levels of all the cytokines decrease (1.03 fold for IL-2, 1.04 fold for IL-4, 1.15 fold for IL-5, 1.11 fold for TNF-α) for fisetin treated group compared to control, except IFN-γ (1.02 fold increase). There is a decrease in the levels of all the cytokines for MCN+F treated group, compared to untreated control (1.02 fold for IL-2, 1.05 fold for IL-4, 1.19 fold for IL-5, 1.05 fold for IFN-γ, 1.07 fold for TNF-α). This shows that, compared to ova treated groups, there is a decrease in the levels of the cytokines with fisetin treatment (1.85 fold for IL-2, 1.81 fold for IL-4, 2.11 fold for IL-5, 2.82 fold for IFN-γ, 2.96 fold for TNF-α), and with MCN+F treatment (1.82 fold for IL-2, 1.83 fold for IL-4, 2.19 fold for IL-5, 3.00 fold for IFN-γ, 1.07 fold for TNF-α) (Figure 17 and Table 17).

Gene expression analysis

Gene expression analysis shows no change in the level of the GAPDH gene, as it is a housekeeping gene that is produced constitutively. iNOS expression increases 4320 fold on treatment with ova. Fisetin is successful in reducing its expression almost to the level of control. IL-9 expression increases 1.77 fold with ova compared to control, and decreases 1.27 fold with fisetin compared to ova. There is a 2.00 fold increase in the expression of TNF-α with ova, and a 1.16 fold decrease with fisetin. IL-1β expression is increased 2280 fold with ova challenge, and a 1.52 fold down regulation after fisetin treatment. All these results show that there is an up-regulation in the production of inflammatory cytokines on challenge with ova, which is successfully reduced by administration of fisetin (Figure 18 and Table 18).

IgE estimation in serum

There is a 90.67 fold increase in the serum IgE concentration after challenge with Ova, and a 26.79 fold increase with fisetin treatment, as compared to control. This shows a 3.38 fold decrease in serum IgE with fisetin, compared to only ova, indicating a successful reduction in inflammation with administration of fisetin (Figure 19 and Table 19).

a) Histological studies (Figure 20)

b) Alcian blue – PAS staining (Figure 21)

c) Masson's trichrome staining (Figure 22)

Discussion and Concluding Remarks

To the best of our knowledge, this is the first report of MCN particle loaded fisetin being used as a therapeutic drug and nanodrug delivery vehicle in preclinical acute allergic asthma.

It has long been considered that the inflammatory response is instrumental to supplying growth factor and cytokine signals that orchestrate the cell and tissue movements necessary for repair [32]. In various experimental animal models and human wounds, it has been demonstrated that the inflammatory response during normal healing is characterized by spatially and temporally changing patterns of various leukocyte subsets [33]. Chronic inflammation culminates in devastating events that can lead ultimately to major organ dysfunction due to abnormalities in tissue architecture after regeneration and replacement by non-functional fibrous tissue. Once this stage is reached, little can be done. Some of the chronic diseases present a fairly simple aetiology of Mendelian inheritance, while others present with a much more complex framework influenced by a number of known and unknown factors. Therapies developed against these illnesses are not foolproof.

Fisetin has recently received some attention for its beneficial effects against several diseases. In the past years, fisetin was a subject of research because of its presence in various human foods and its antiproliferative [30,34-39], apoptotic [35,37,40,41], and antioxidant [42] activities. Several studies indicate that fisetin is a promising novel antioxidant. Fisetin has been reported to inhibit human low-density lipoprotein (LDL) oxidation *in vitro* [43]. It induced quinone oxidoreductase activity in murine hepatoma 1c1c7 cells in a time- and dose-dependent manner, and the induction of activity was associated with increase in mRNA expression. It was found that fisetin prevented LDL from oxidation, in part, through reducing CD36 gene expression in macrophages, a possible effect in ameliorating atherosclerosis [44].

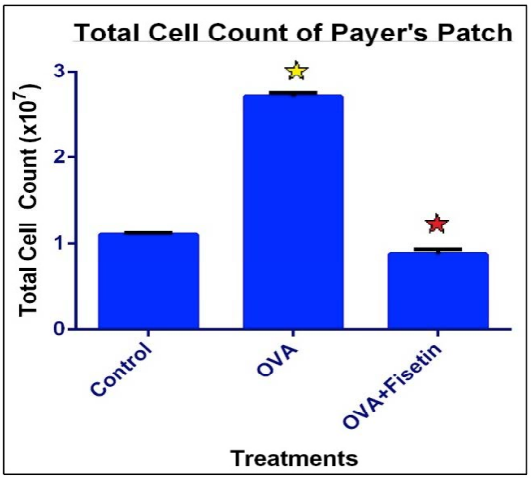

Figure 7: Effect of fisetin on the total cell count of Payer's patch. (*P<0.05 versus Control; *P<0.05 versus Ova).

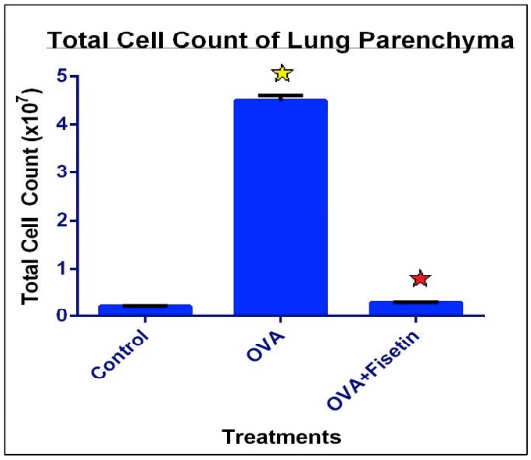

Figure 8: Effect of fisetin on the total cell count of lung parenchyma. (*P<0.05 versus Control; *P<0.05 versus Ova).

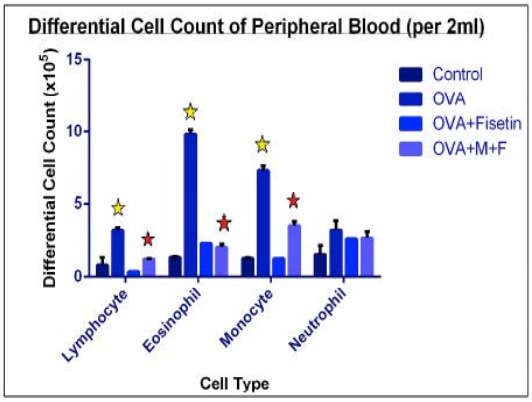

Figure 9: Effect of fisetin and MCN-fisetin on the differential cell count of peripheral blood. (*P<0.05 versus Control; *P<0.05 versus Ova).

	Neutrophil (× 10^4)	Eosinophil (× 10^4)	Macrophage (× 10^4)	Lymphocyte (× 10^4)
Control	0.31 ± 0.02	1.07 ± 0.09	3.95 ± 0.21	0.28 ± 0.1
Ova treated	2.79 ± 0.21	56.20 ± 2.25	42.50 ± 4.88	0.74 ± 0.07
Ova+Fisetin treated	1.56	16.40	28.60	0.20
Ova+(MCN+Fisetin) treated	1.95 ± 0.15	11.60 ± 1.20	26.60 ± 1.60	0.25 ± 0.05

Table 10: Differential cell count of BAL fluid, seen after Wright-Giemsa staining, under light microscope. There is a 1.43 fold decrease in neutrophil count, a 4.84 fold decrease (p<0.05) in the eosinophil count, a 1.60 fold decrease in macrophage count and a 2.96 fold decrease in lymphocyte count, after treatment with fisetin.

	NO Conc. (nM)
Control	5.19 ± 0.20
Ova	8.60 ± 0.08
Ova+Fisetin	6.99 ± 0.12
Ova+(MCN+F)	6.69 ± 0.13

Table 11: Concentration of NO produced in lung tissue. There is a 1.23 fold decrease (p<0.05) with fisetin, and a 1.29 fold decrease (p<0.05) with MCN+fisetin.

	Catalase Conc. (pM)
Control	56.32 ± 7.60
Ova	63.80 ± 8.03
Ova+Fisetin	73.67 ± 0.61
Ova+(MCN+F)	40.29 ± 0.38

Table 12: Concentration of catalase in lung tissue. There is a 1.16 fold increase with fisetin, and a 1.58 fold decrease with MCN+fisetin.

	ASA Conc. (nM)
Control	23.60 ± 1.40
Ova	17.22 ± 0.88
Ova+Fisetin	23.93 ± 0.37
Ova+(MCN+F)	20.30 ± 0.30

Table 13: Concentration of ascorbic acid in lung tissue. There is a 1.39 fold increase (p<0.05) with fisetin and a 1.18 fold increase with MCN+fisetin.

	NO Conc. (nM)
Control	4.47 ± 0.58
Ova	6.32 ± 0.90
Ova+Fisetin	6.67 ± 0.21
Ova+(MCN+F)	2.97 ± 0.10

Table 14: Concentration of NO produced in BAL fluid. There is a 1.06 fold increase with fisetin, and a 2.13 fold decrease with MCN+fisetin.

	NO Conc. (nM)
Control	5.91 ± 0.90
Ova	9.33 ± 0.57
Ova+Fisetin	7.15 ± 0.34
Ova+(MCN+F)	3.60 ± 0.03

Table 15: Concentration of NO produced in bone marrow. There is a 1.31 fold decrease with fisetin, and a 2.59 fold decrease (p<0.05) with MCN+fisetin.

	NO Conc. (nM)
Control	8.50 ± 1.44
Ova	12.07 ± 3.11
Ova+Fisetin	9.33 ± 0.63
Ova+(MCN+F)	5.48 ± 0.07

Table 16: Concentration of NO produced in spleen. There is a 1.29 fold decrease with fisetin, and a 2.20 fold decrease with MCN+fisetin.

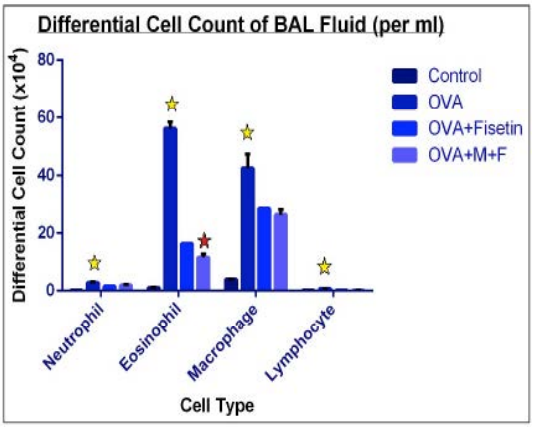

Figure 10: Effect of fisetin and MCN-fisetin on the differential cell count in Bronchoalveolar lavage fluid. (*P<0.05 versus Control; *P<0.05 versus Ova).

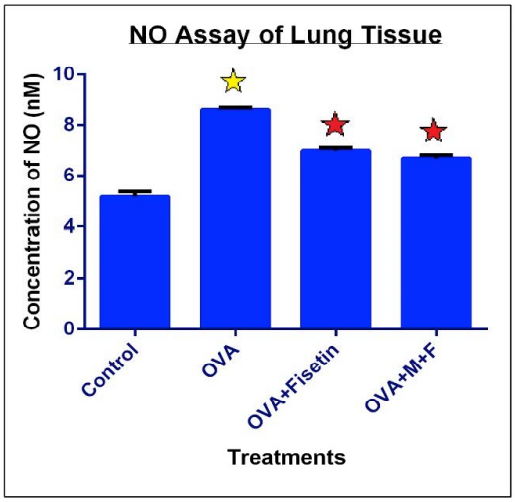

Figure 11: Effect of fisetin and MCN-fisetin on the production of nitric oxide in lung tissue. 0.1 ml of sample was added to 0.1 ml of Griess reagent, incubated and absorbance measured at 540 nm in a microplate reader (Thermo Fisher). Concentration of NO was determined from a standard curve. (*P<0.05 versus Control; *P<0.05 versus Ova).

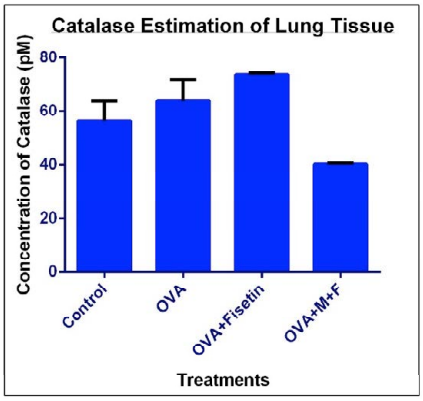

Figure 12: Effect of fisetin and MCN-fisetin on the production of catalase in lung tissue. The sample was added to 0.01M phosphate buffer (pH 7.0), 0.2M hydrogen peroxide and water. The reaction was stopped with dichromate/acetic acid reagent, and absorbance was measured at 610nm in a spectrophotometer. Concentration of catalase was determined from a standard curve.

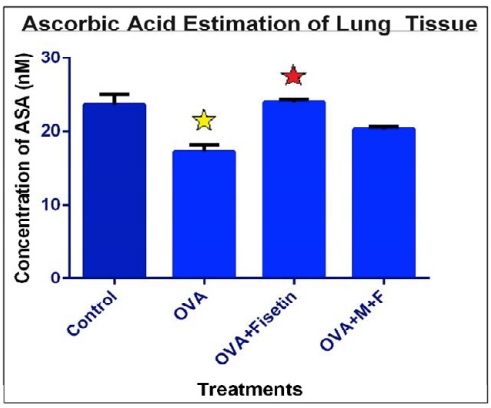

Figure 13: Effect of fisetin and MCN-fisetin on production of ascorbic acid in lung tissue. The sample was incubated with distilled water, DNPH and thiourea, after which, the crystals formed were dissolved in 80% sulphuric acid. Absorbance was read at 540 nm in a spectrophotometer (Shimadzu). Concentration of ASA was determined from a standard curve. (*P<0.05 versus Control; *P<0.05 versus Ova).

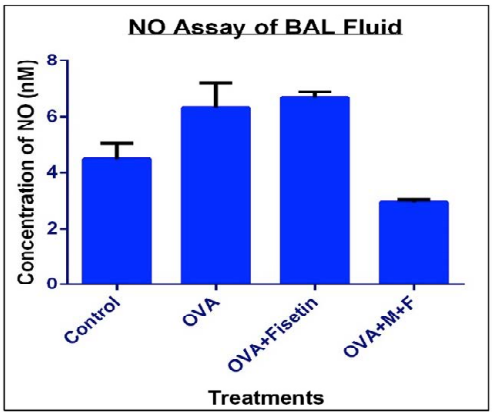

Figure 14: Effect of fisetin and MCN-fisetin on the production of nitric oxide in BAL fluid. 0.1 ml of sample was added to 0.1 ml of Griess reagent, incubated and absorbance measured at 540 nm in a microplate reader (Thermo Fisher). Concentration of NO was determined from a standard curve.

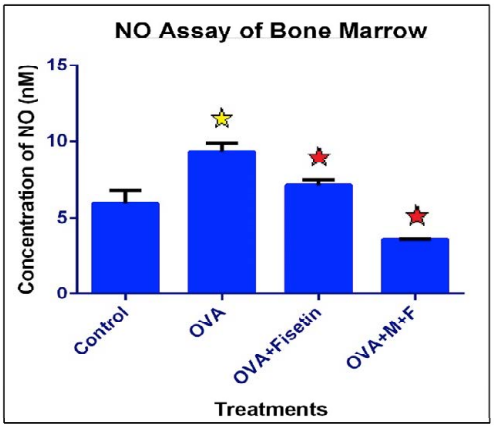

Figure 15: Effect of fisetin and MCN-fisetin on the production of nitric oxide in bone marrow. 0.1 ml of sample was added to 0.1 ml of Griess reagent, incubated and absorbance measured at 540 nm in a microplate reader (Thermo Fisher). Concentration of NO was determined from a standard curve. (*P<0.05 versus Control; *P<0.05 versus Ova).

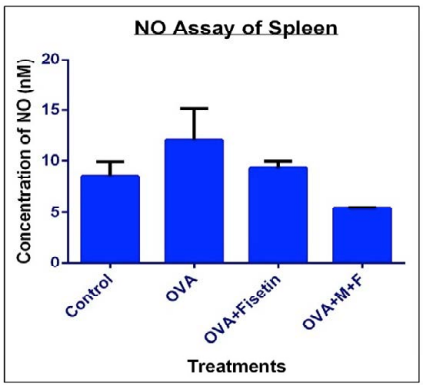

Figure 16: Effect of fisetin and MCN-fisetin on the production of nitric oxide in spleen. 0.1 ml of sample was added to 0.1 ml of Griess reagent, incubated and absorbance measured at 540 nm in a microplate reader (Thermo Fisher). Concentration of NO was determined from a standard curve. (*P<0.05 versus Control; *P<0.05 versus Ova).

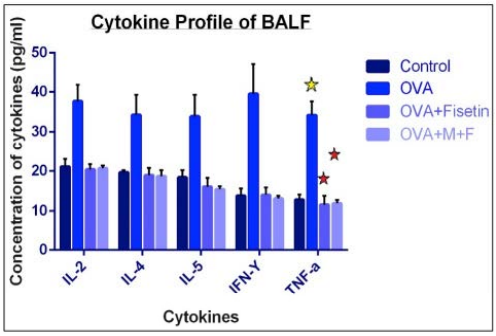

Figure 17: Effect of fisetin and MCN-fisetin on the concentrations of cytokines IL-2, IL-4, IL-5, IFN-γ and TNF-α (in pg/ml), in BAL fluid. This assay was done using the BD CBA Mouse Th1/Th2 Cytokine Kit, in which five bead populations, coated with specific antibodies, are mixed to form a bead array, and resolved in a flow cytometer.

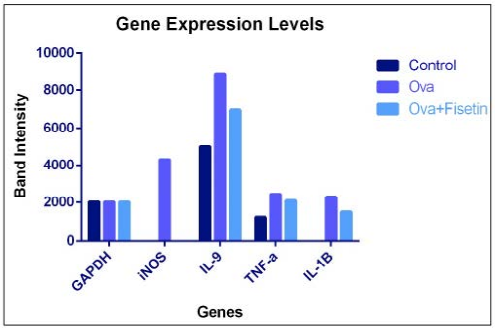

Figure 18a: Effect of fisetin on the level of expression of genes for GAPDH, iNOS, IL-9, TNF-α, and IL-1β. Gene expression was analysed by isolation of RNA, followed by reverse- transcriptase PCR.

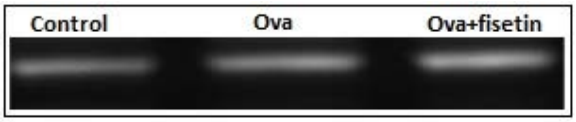

Figure 18b: Bands showing expression of GAPDH. There is no change in the intensity of the bands.

Figure 18c: Bands showing expression of iNOS. Challenge with ova induces expression of iNOS gene (4320 fold).

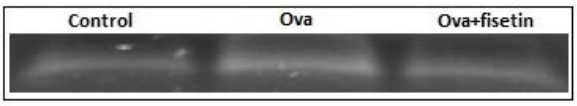

Figure 18d: Bands showing expression of IL-9. Expression is upregulated 1.77 fold with ova, and again reduces 1.27 fold with fisetin.

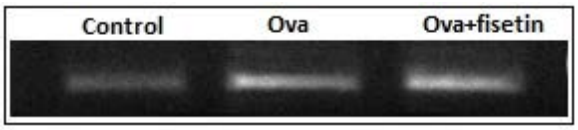

Figure 18e: Bands showing expression of TNF-α. Expression increases 2.0 fold with ova, and decreases 1.16 fold with fisetin.

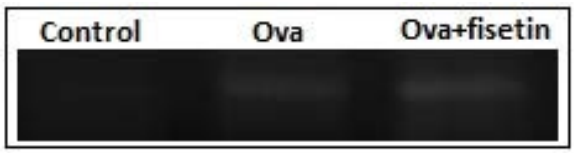

Figure 18f: Bands showing expression of IL-1β. Expression is upregulated 2280 fold with ova, and downregulated 1.52 fold with fisetin.

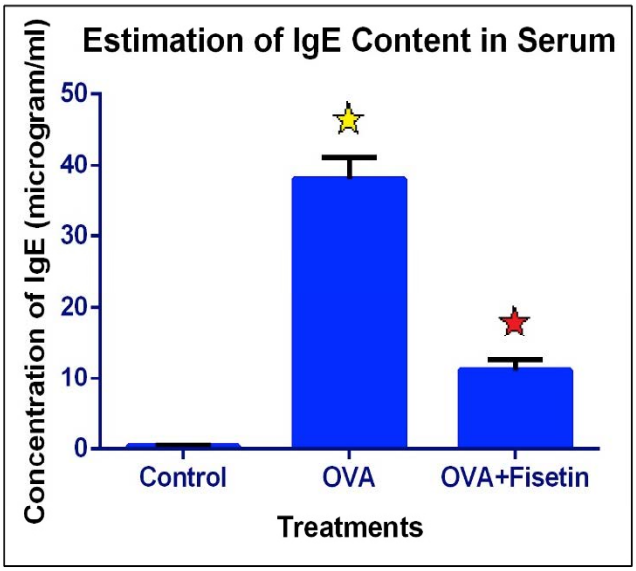

Figure 19: Effect of fisetin on the concentration of serum IgE. IgE content was estimated by standard ELISA. (*P<0.05 versus Control; *P<0.05 versus Ova).

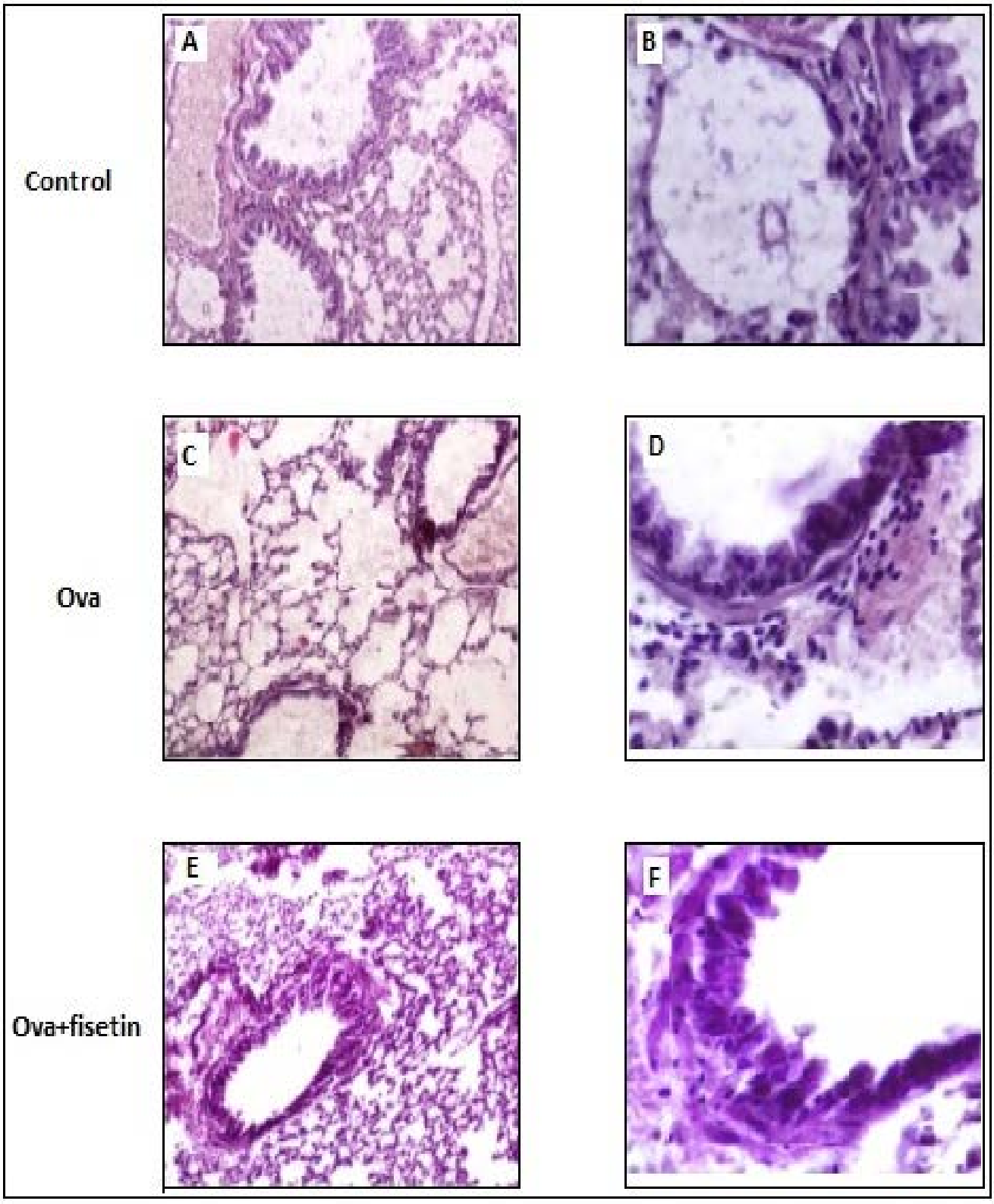

Figure 20: Hematoxylin and Eosin staining of lung sections. Lung tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut into 5 μm sections, stained and observed under light microscope (Olympus). A, C, E: Lung sections observed under 10X magnification; B, D, F: sections observed under 40X magnification.

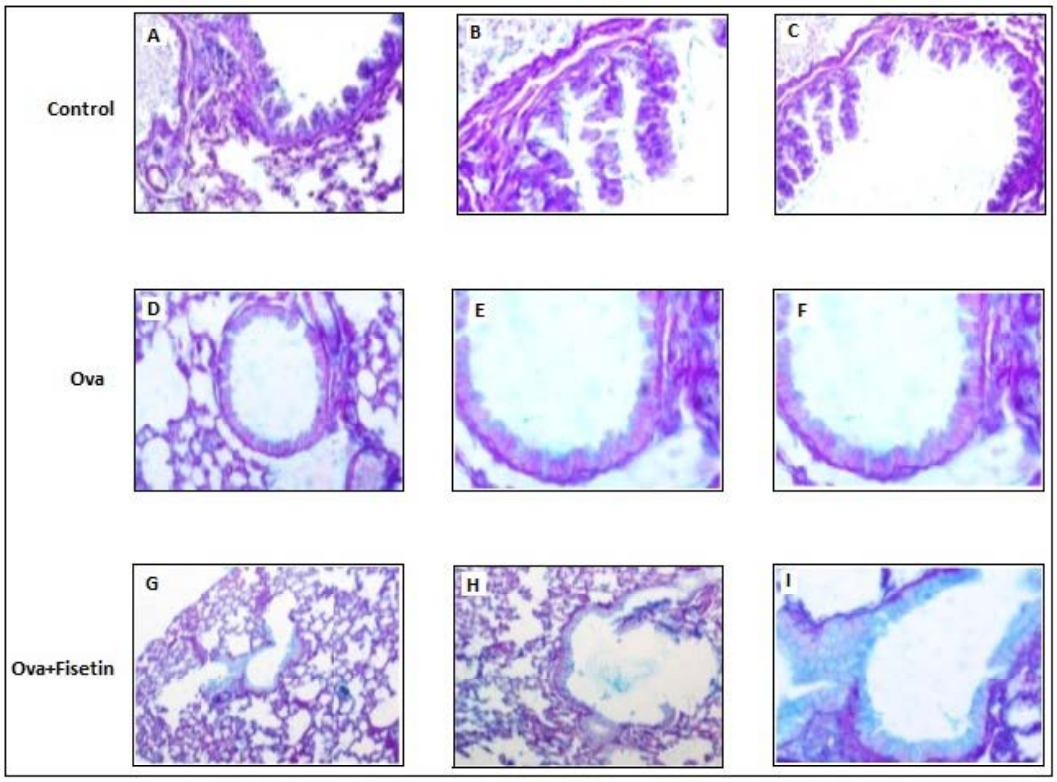

Figure 21: Alcian Blue-PAS staining of lung sections. Lung tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut into 5 µm sections, stained and observed under light microscope (Olympus). A, D, G: Lung sections observed under 10X magnification; B, E, H: sections observed under 40X magnification; C, F, I: Lung sections observed under 100X magnification.

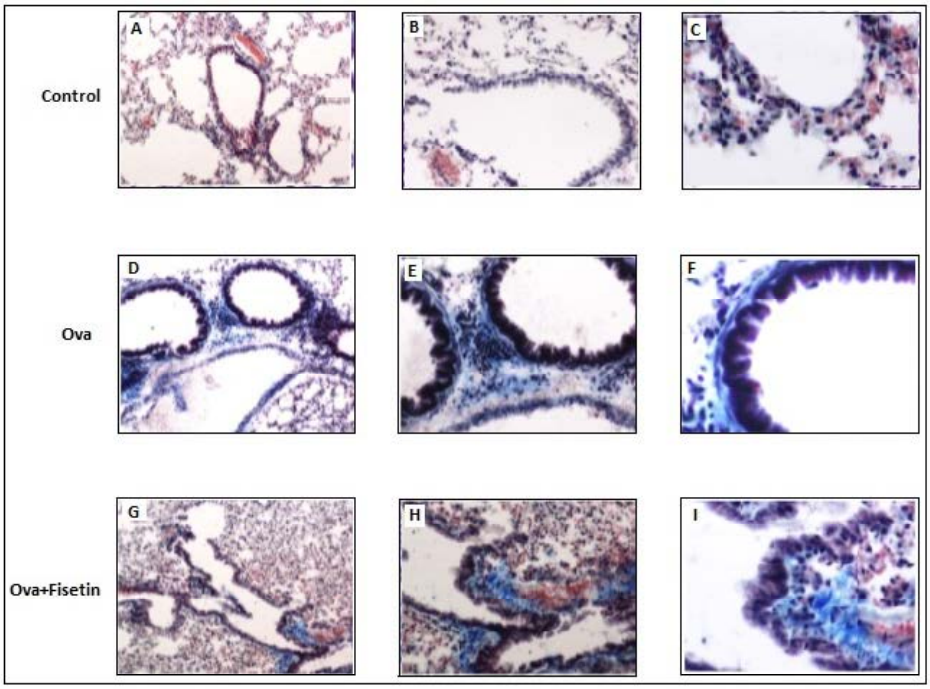

Figure 22: Masson's Trichrome staining of lung sections. Lung tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut into 5 µm sections, stained and observed under light microscope (Olympus). A, D, G: Lung sections observed under 10X magnification; B, E, H: sections observed under 40X magnification; C, F, I: Lung sections observed under 100X magnification.

	Concentration of ctokines (in pg/ml)				
	IL-2	**IL-4**	**IL-5**	**IFN-γ**	**TNF-α**
Control	21.10 ± 1.90	19.65 ± 0.55	18.40 ± 1.80	13.80 ± 1.80	12.80 ± 1.30
OVA	37.65 ± 4.15	34.25 ± 5.05	33.90 ± 5.40	39.50 ± 7.70	34.20 ± 3.40
Ova+Fisetin	20.40 ± 1.30	18.95 ± 1.85	16.05 ± 2.25	14.00 ± 1.90	11.55 ± 2.25
Ova+(MCN+F)	20.70 ± 0.70	18.70 ± 1.50	15.45 ± 0.75	13.15 ± 0.65	11.95 ± 0.85

Table 17: Concentration of cytokines IL-2, IL-4, IL-5, IFN-γ and TNF-α (in pg/ml), in BAL fluid. Cytokine analysis shows a decrease in the levels of the cytokines with both, fisetin and MCN+fisetin, compared to ova.

	Band Intensities				
	GAPDH	**iNOS**	**IL-9**	**TNF-α**	**IL-1β**
Control	2025	0	5014	1215	0
OVA	2025	4320	8856	2432	2280
Ova+Fisetin	2025	0	6976	2100	1496
Ova+(MCN+F)	-	-	-	-	-

Table 18: Expression levels of genes for GAPDH, iNOS, IL-9, TNF-α, and IL-1β. There is an increase in cytokine levels with ova challenge, which is decreased with fisetin. There is no change in level of housekeeping gene, GAPDH.

	IgE Conc. (μg/ml)
Control	0.42 ± 0.09
Ova	38.08 ± 3.08
Ova+Fisetin	11.25 ± 1.42
Ova+(MCN+F)	-

Table 19: Concentration of IgE in serum. There is a 3.38 fold decrease ($p<0.05$) with fisetin.

Nanoparticles (NPs) have promising applications in medicine [45]. We should take advantage of the benefits from the immunomodulating properties of NPs and, on the other hand, avoid the undesirable immune responses in order to minimize the systemic side effects. The factors affecting the immune response are complex, including particle composition, size, surface chemistry, plasma protein binding, and exposure route. Investigation of the relationship between properties of NPs and systemic immune response is crucial for their application in medicine and other areas.

In this study, we demonstrated data from a murine model of preclinical acute allergic asthma, where fisetin was dosed orally before each intra-tracheal instillation. Composite physiological asthma phenotype was developed through periodic intra-tracheal challenge to the mice which were first primed/sensitized with allergen. Repeated challenges with minute quantities of allergen causes the Th2 skewed immune response, a classic marker of clinical asthma.

Human allergic asthma is a chronic inflammatory disease of the airways, characterised by inflammation in the airways, airway hyper-responsiveness (AHR) and obstruction of the airways. Besides these, certain structural changes also take place, which are together termed 'airway remodelling'. Allergen-induced asthma is a Th2 driven inflammatory response, which leads to an increase in Th2 cytokines, elevated levels of serum IgE, and goblet cell metaplasia. Bronchoaveolar lavage fluid and histology studies have shown that there is increases recruitment of inflammatory cells, especially eosinophils. Th2 cytokines that are mainly produced include IL-4, IL-5, IL-6, and IL-9, among others, which are responsible for antibody production, eosinophils activation, inhibition of several macrophage functions, and regulation of B cell class switching to IgE. IL-4 suppresses Th1 and Th17 responses, by upregulation of transcription repressor, IFN-γ and IL-17.

In this study, we have induced acute asthma in Balb/c mice using ovalbumin, and then assessed the anti-inflammatory effects of plant flavonoid, fisetin, alone, and loaded on a nanovehicle, mesoporous carbon nanoparticle (MCN), when administered therapeutically. We demonstrated that fisetin had a postitive therapeutic effect on the asthma, and in most cases, this effect has been enhanced by the addition of MCN. Total cell recruitment increases with the administration of ovalbumin, showing that the allergen has induced inflammation, and the body is synthesizing more immune cells to counter the infection. Cell recruitment is successfully inhibited by fisetin and MCN+fisetin treatment, allthough the inhibition is much less when MCN is added, in comparison with fisetin alone. Eosinophil recruitment increases significantly in the blood and BALF, which is inhibited by the treatment, more with MCN+fisetin, than with fisetin alone.

Nitric acid is produced by macrophages as a defense against oxidative stress. Catalase is produced by cells to break down harmful ROS. Ascorbic acid, an antioxidant, is normally present in the body to protect against ROS. In case of inflammation, both NO and catalase content increases, but ascorbic acid content decreases. Our assays have shown that the NO and catalase content of the lung has increased with ova challenge, and has decreased with fisetin and MCN+fisetin both, with the decrease being more pronounced with MCN. The ASA concentration has decreased with ova, and has increased significantly with treatment.

The cytokine profile showed an increase in the Th2 cytokines, IL-2, IL-4, IL-5 and IFN-γ, along with TNF-α. The levels of the cytokines are reduced with treatment. Gene expression studies corroborate these findings, with an increase in expression in the genes for cytokines IL-9, IL-1β and TNF-α, and also in the inducible gene iNOS. Administration of fisetin leads to a downregulation of these genes. Serum IgE also shows an increase with ova, and a subsequent decrease with fisetin. Histological studies showed that there are structural changes in the lung due to administration of ova. Treatment with fisetin appears to somewhat restore the structure.

Possible reasons for lower efficacy of fisetin loaded on MCN, than fisetin alone:

Nanovehicles are used in the hope that they will increase a drug's efficacy. The main advantage of nanovehicles is that they can carry the drug into the cells, by crossing the cell membranes, after which they disintegrate to release the drug. In our experiments, we have found that fisetin alone has better effect in disease amelioration, than fisetin loaded on MCN. This may be because:

- The fisetin loaded on MCN gets encapsulated by the MCN, and does not get released when it reaches the site of inflammation [46].

- Perhaps the MCN cannot get across the membrane and thus, drug is not effectively released inside the cells [47].

- Interaction between the Nanoparticles and the tissue or cell may have different effect in to the body. Nanoparticles permeate membrane cells and spread along the nerve cells synapses, blood vessels and lymphatic vascular. At the same time, Nanoparticles accumulate in the different cells [48]. Therefore, Nanoparticles of strong permeability provide inflammatory effects, at the same time, it give rise to potential threats on the health.

- Some impurities such as amorphous carbon, contribute increase level of reactive oxygen species (ROS), thus inducing the oxidative stress in cells [49].

- Nano-carrier-drug conjugates for example MCN loaded Fisetin can be phagocytosed by cells. Other issues include low drug loading capacity, low loading efficiency, and poor ability to control the size distribution of carriers [50].

Nanoparticles can be linked to biological molecules that can act as address tags, to direct the nanoparticles to specific sites within the body and specific organelles within the cell [51-53]. Also the nanosized drug delivery systems for herbal drugs can potentially enhance the biological activity [54]. Their interactions with biological systems are relatively unknown [55,56]. We found in around 70% or more cases Fisetin is more effective than F+MCN (mesoporous carbon nanoparticle). The possible reason could be that, the Fisetin when administered with MCN was not able to reach directly to the target site or was not able to release properly. Another reason could be, Fisetin is being partially absorbed by the nanoparticles. It has been reported that, the drug may be able to remain inside the mesoporous carbon nanospheres at physiological pH, but be efficiently released in the acidic environment [57]. There are some reports on toxicity of nanoparticles [57,58]. Key factors in the interaction with living structures include nanoparticle dose, the ability of nanoparticles to spread within the body, as well as their solubility. Some nanoparticles dissolve easily and their effects on living organisms are the same as the effects of the chemical they are made of. However, other nanoparticles do not degrade or dissolve readily. Instead, they may accumulate in biological systems and persist for a long time, which makes such nanoparticles of particular concern [55]. It is vital to perform more rigorous and detailed long-term toxicological studies before using spherical nanocarbon materials in real biomedical applications [56]. The biocompatibility and toxicity of porous carbon nanospheres must also be assessed before they can be widely used as carriers.

Since fisetin exhibits several activities that are potentially beneficial in treating inflammatory diseases, further studies directed toward target identification, and pathway analysis could pave the way for the addition of fisetin to the inflammatory diseases therapy. Additional studies are required to establish the anti-inflammatory properties of fisetin and to understand the precise molecular mechanisms involved in the animal models of inflammatory diseases. More in vivo studies are needed to further establish its role and leading the way for future clinical trials. To translate the anti-inflammatory potential of fisetin to clinical use, well-designed clinical trials along with the development of reliable analytical biomarkers are required. The investigation of the molecular mechanisms associated with the anti-inflammatory effects exhibited by fisetin is essential. Finally, the findings of this study shall have far reaching implications in designing therapeutic molecules in the management and modification of clinical asthma.

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Author Contribution

PP and SM performed experiments and acquired and analyzed data. KM and MJ performed some preliminary experiments. SB wrote a major part of the manuscript. Design and synthesis of MCN was entirely AS and NRJ's forte. The project was ERB's idea and design for execution was also entirely ERB's. Overall, ERB designed the experiments, analyzed the data and wrote the manuscript.

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