

Therapeutic use of Fisetin and Fisetin Loaded on Mesoporous Carbon Nanoparticle (MCN) in Thioglycollate-induced Peritonitis

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Abstract

Background: The pathophysiology of aseptic peritonitis involves inflammation of the serosal membrane that lines the abdominal cavity and the organs contained therein. The etiology of peritonitis is complicated and is involved in various processes, of which, the most important one is the inflammatory reaction. During the pathological process of peritonitis, NF- κ B plays an activating role in the inflammatory reaction, which might be a potential therapeutic target in the therapy of certain inflammatory diseases. We studied the anti-inflammatory and pro-regenerative actions of Fisetin, a flavonol found in many plants, in a mouse model of thioglycollate-induced peritonitis, as well as the actions of fisetin administered with a nanoparticle such as mesoporous carbon nanoparticle (MCN). BALB/c mice were used in this study.

Results: We found cell recruitment in the blood increased with the administration of thioglycollate (TG) after 24 h, 48 h, 72 h and 96 h, showing that it has induced inflammation. Cell recruitment was successfully inhibited by fisetin, and with MCN+fisetin. In the peritoneal fluid, total cell recruitment was increased, which was successfully inhibited with fisetin and MCN+fisetin treatment. TG treatment significantly reduced cell proliferation in the blood, PF and BM, within 24 h, till 96 h. Interestingly, cell proliferation increased with fisetin treatment, and with MCN+fisetin. The clonogenic potential of the tissues decreased significantly within 24 h, with administration of TG. Both fisetin treatment and MCN+fisetin treatment restored the clonogenic potential of the tissues. There was a decrease in Th2 cytokines with TG treatment, in blood after 48 h, and both fisetin and MCN+fisetin increased the cytokine content.

Conclusion: In conclusion, we found that fisetin had a promising therapeutic effect on the peritonitis.

Keywords: Fisetin; Inflammation; Aseptic peritonitis; Mesoporous carbon nanoparticles; Inflammation; Anti-oxidant; Phenolic compounds; Scavenger activities; Reactive oxygen nitrogen intermediates

Abbreviations: TG: Thioglycollate; F: Only Fisetin; MF: Fisetin loaded on MCN; TG24, TG48, TG72, TG96: Treatment with only TG; sacrifice after 24 h, 48 h, 72 h, 96 h. TG24F, TG48F, TG72F, TG96F: Treatment with TG, followed by Fisetin; sacrifice after 24 h, 48 h, 72 h, 96 h; TG24MF, TG48MF, TG72MF, TG96MF: Treatment with TG, followed by MF; sacrifice after 24 h, 48 h, 72 h, 96 h; PB: Peripheral Blood sample; PF: Peritoneal Fluid sample; BM: Bone Marrow sample; TC: Total cell count; DC: Differential Cell Count; NO: Nitric Oxide; MTS: [3-(4, 5- dimethyl thiazol-2-yl)-5-(3-carboxy methoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; PMS: Phenazine Methosulfate; CFU-c: Colony Forming Units in Culture; MPK: Milligram per Kilogram of Body Weight

Symbols Used:

#: Denotes significance in samples with respect to control.

*: Denotes significance in samples with respect to samples treated with only TG.

*: Denotes significance in samples with respect to samples treated with TG-F.

Background

Peritonitis is the inflammation of the peritoneum, which was the thin tissue that lines the inner wall of the abdomen, and covers most of the abdominal organs. Infected peritonitis is caused by perforation of part of the gastrointestinal (GI) tract, by disruption of the peritoneum or by systemic infections. Local intra-abdominal focus of inflammation caused by the microorganisms can promote the synthesis and secretion

of massive inflammatory cytokines, which would destroy the endothelial junctions and provide access for bacteria into the systemic circulation leading to lethal bacteremia [1,2]. A more severe inflammatory response process usually indicates a much higher mortality. The oxidative stress induced by the direct effect of bacteria and indirect effect of inflammation also contributes to the severity of peritonitis [3]. The microorganisms and their components can immediately activate the transcription factors—nuclear factor- κ B (NF- κ B). NF- κ B can initiate gene expression of cytokines, adhesion molecules, chemokines, and cytotoxic enzymes, which are considered to be directly responsible for the organ injury and death [4-8]. Non-infected peritonitis may be caused by leakage of sterile body fluids into the peritoneum, or by sterile abdominal surgery, which may inadvertently leave behind foreign bodies. In normal conditions, the peritoneum appears grayish and glistening; it becomes dull 2-4 hours after the onset of peritonitis, initially with scarce serous or slightly turbid fluid. Later on, the exudate becomes creamy and evidently suppurative. Although high-class antibiotics and advanced intensive care have proven to be

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effective on the treatment, the morbidity and mortality remain kept at a high level [9,10].

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants [11,12]. It is a protective mechanism by the organism to eliminate injurious stimuli and to initiate the healing process. Inflammation is a mechanism of innate immunity [13,14]. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. The process of acute inflammation is initiated by cells already present in all tissues, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells and mast cells. At the onset of an infection, these cells are activated and release inflammatory mediators, which are responsible for the clinical signs of inflammation [15]. Inflammation leads to increased production of reactive species like ROS (reactive oxygen species), NOS (nitric oxide synthase) and their product peroxynitrite (ONO_2^-) by activated macrophages [16]. This increase in oxidative stress leads to decrease in effectiveness of oxidant defenses, that is, reduction in antioxidants.

Due to the various side effects and other complications of modern medicine, the use of traditional medicines and natural products is gaining popularity. Phytochemicals from fruits are being exploited as possible sources of therapeutic agents. Different biological activities of these chemicals, including their anti-oxidant properties and their anti-inflammatory properties have been tested *in vitro*, as well as *in vivo* [17-19]. Nanomaterials, either as nanodrugs or as nano-vehicles, have the advantages of being small devices that are less invasive than normal medicines, that can be targeted to reach a particular site and that can possibly be implanted inside the body; also biochemical reaction times are much shorter. These devices are faster and more sensitive than typical drug delivery [20-22].

Mesoporous Carbon Nanoparticle (MCN) is a type of porous nanomaterial with a size of 100-200 nm. It has several important properties such as high surface area, large pore volume, and a uniform pore size of 3 nm. It is chemically inert, biocompatible and dispersible in water. Pores of MCN can be loaded with a large amount of drug molecules and then the drugs can be released by slow diffusion or other methods. These properties of the MCN make them useful for biomedical application [23-26].

The aim of our study was to test the anti-inflammatory and pro-regenerative actions of Fisetin, a flavonol found in many plants, including strawberries and apples, in a mouse model of thioglycollate-induced peritonitis. It protects against oxidative stress-induced cell death, by up-regulating expression of heme oxygenase 1 (HO^1). We also aimed to test whether the anti-inflammatory and pro-regenerative actions of fisetin were enhanced when it was administered with a nano-vehicle such as mesoporous carbon nanoparticle (MCN).

Materials and Methods

Reagents and materials

Sodium thioglycollate, Fetal Bovine Serum (FBS), RBC Lysis Buffer, Iscove's Modified Dulbecco's Media (IMDM), powdered Methyl Cellulose and Penicillin-Streptomycin were bought from Himedia, India. EDTA, methanol, Sulfanilamide and NED were bought from

Sisco Research Laboratory (SRL), India. DMEM from Gibco, Murine Stem Cell Factor (SCF) from Biovision, and Bovine Serum Albumin (BSA) from Biosera were used. Ortho-phosphoric acid and NaNO_2 were purchased from Merck, India. 1X phosphate buffered saline (PBS) was prepared using 137 mM NaCl (Merck, India), 2.7 mM KCl (Himedia, India), 10 mM Na_2HPO_4 (Qualigens, India), 2 mM KH_2PO_4 (Himedia, India).

24 well plates and 96 well plates were obtained from Nest Biotech Co. Ltd., China. Dispovan syringes were used to obtain blood and peritoneal fluid. Smears for cell counting were prepared using Cytospin (Centurion Scientific C₂ Series) after centrifuging the sample in a cold centrifuge (Vision VS-15000CFN). Smears were observed under a light microscope (Debro DX-200). Absorbance readings were taken in a multiplate reader (Thermo Fisher Multiskan EX). Plates were incubated in a CO_2 incubator (Thermo Fisher), and colonies in CFU assay were observed using Fluid Cell Imaging Station (Life Technologies, India). All cell-culture work was done inside the biosafety cabinet.

Statistical analysis

All data are presented as mean \pm SEM, and only p values less than 0.05 have been considered as statistically significant. Statistical significance has been calculated using t-test in Graph Pad Prism 6.

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.

Ethics statement

Animal ethical approval has been obtained by Institutional Bioethics Committee (dt 12-5-2010-Registration No. 885/ac/05/CPCSEA).

Induction of peritonitis

BALB/c mice (6-8 week old, weighing 25 gm) were divided into 13 groups (n=4): Control, TG24, TG24F, TG24MF, TG48, TG48F, TG48MF, TG72, TG72F, TG72MF, TG96, TG96F, and TG96MF. 400 μl of 3% TG was injected intra-peritoneally (200 μl near each hind leg). The experiment was repeated twice.

Treatment with fisetin and fisetin loaded onto MCN

1 hour after treatment with TG, mice from four groups (TG24F, TG48F, TG72F and TG96F) were given 40 μl of 2 $\mu\text{M}/\text{kg}$ (0.57 MPK) fisetin orally. 1 hour after treatment with TG, mice from the other four groups (TG24MF, TG48MF, TG72MF and TG96MF) were treated with a mixture of fisetin and MCN. Equal amounts of 2 $\mu\text{M}/\text{kg}$ fisetin was mixed with an equal amount of 5.1 mg/ml MCN, mixed well by vortexing, and administered intraperitoneally into the mice. Mice were sacrificed after 24 hours, 48 hours, 72 hours and 96 hours, and tissues were collected.

Collection of samples

Peripheral Blood (PB): Blood was collected by cardiac puncture, with 5 mM EDTA to prevent clotting, in DMEM. For cell counting, blood was collected in RBC Lysis Buffer, kept at room temperature for 5 minutes, then flushed with 1X PBS, and centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the pellet was

dissolved in 1X PBS and stored at 4°C.

Peritoneal Fluid (PF): 2 ml of 1X PBS was slowly injected into the peritoneal cavity, and the cavity was massaged well to wash the cavity extensively. A 5 ml syringe was inserted into the side of the mouse, and the plunger slowly pulled out to retrieve the maximal amount of peritoneal fluid. The PF was collected in DMEM medium.

Bone Marrow (BM): The bone of the hind leg of the mouse was taken into the biosafety cabinet, and flushed with DMEM till the bone turned white.

Total and differential cell count (TC/DC)

Differential white blood cell count is an examination and enumeration of the distribution of leukocytes in a stained blood smear. Increases in any of the normal leukocyte types or the presence of immature leukocytes or erythrocytes in peripheral blood are important diagnostically in a wide variety of inflammatory disorders.

The total number of cells and their viabilities were determined using a hemocytometer. Differential count was taken using a smear prepared in a cytospin. 100 μ l of each sample was loaded into the wells of a cytospin, and centrifuged at 2000 rpm for 3 mins. The slides were removed, air dried and fixed with methanol. The smears are then stained with hematoxylin, counter-stained with Eosin, and observed under a microscope. The TC and the DC were plotted against each sample.

Determination of nitric oxide content (NO Assay)

Activation of immune system is associated with increase in macrophage NO production. Transient nature of NO makes it unsuitable for detection, but it is oxidized to Nitrite (NO_2^-) and Nitrate (NO_3^-) by nitrate reductase. The concentrations of these anions are used as quantitative measure of NO production using the Griess reaction. In this reaction, acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce diazonium ion. This ion couples with NED (N-1-naphthyl ethylene diamine dihydrochloride) to form a coloured product that is measured spectrophotometrically at 540 nm.

The reaction was standardized using different concentrations of NaNO_2 (100 μ M, 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 3.13 μ M, 1.56 μ M and 0 μ M), using the method in Promega User Guide (Product G2930). 50 μ l of cells from each sample (PB, PF and BM) from all the groups (Control, TG24, TG24F, TG24MF, TG48, TG48F, TG48MF, TG72, TG72F, TG72MF, TG96, TG96F, TG96MF) were plated in the wells of a 96-well plate. The cells were incubated for 24 hours, in a CO_2 incubator at 5% CO_2 , 37°C. Sulfanilamide solution was prepared by dissolving 1% Sulfanilamide in 5% ortho-phosphoric acid. 0.1% NED solution was prepared in distilled water. 50 μ l of sulfanilamide solution was added to each well, and incubated at room temperature for 5 minutes, in dark. 50 μ l of NED solution was then added, and incubated at room temperature for 5 minutes, in dark. Absorbance was measured in a plate reader at 540 nm. Using the standard curve prepared, the absorbance values of the samples were plotted to get the concentrations of NO produced (in μ M). The concentrations of NO were plotted against each sample.

Cell proliferation assay (MTS Assay)

The MTS assay is a colourimetric method for determining the number of viable cells in culture. It uses solutions of a novel tetrazolium compound MTS [3-(4, 5-dimethyl thiazol-2-yl)-5-(3-carboxy methoxy phenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] and

an electron-coupling reagent PMS (Phenazine Methosulfate). MTS is bio-reduced by cells into a formazan product that is soluble in tissue culture medium. Absorbance of formazan is measured at 492 nm. The conversion of MTS to aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. Quantity of formazan product, as measured by the absorbance at 492 nm, is directly proportional to the number of living cells in culture. The assay was performed using the Promega CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay Kit. 100 μ l of cells (PB, PF, and BM) from all the samples were added to the wells of a 96-well plate, and incubated for 1 hour in a CO_2 incubator at 5% CO_2 , 37°C. 20 μ l of MTS/PMS solution was added to each well, and incubated in a CO_2 incubator for 1-4 hours. Absorbance was measured immediately in a plate reader at 492 nm.

The absorbance values for each sample were plotted against time. Taking absorbance of the control as 100 % cell viability, the viabilities of the other samples were calculated. Fold changes in the viability of the samples were calculated.

Determination of clonogenic potential of cells (CFU-c Assay)

CFU-c (Colony Forming Units in culture) assay measures the clonogenic potential of cells. The assay is based on the ability of cells to proliferate and differentiate into colonies in a semi-solid medium, in response to cytokine stimulation. The colonies formed can be enumerated and characterised according to their unique morphology. Clonogenic potential is determined by dividing the number of colonies formed by the number of cells plated.

Number of cells per well taken was 1×10^6 . For PF, 10^5 cells were taken per well. CFU-c media was prepared using IMDM, supplemented with 30% FBS, 10% BSA, 1% Penicillin-Streptomycin and 5 ng/ml murine SCF. Lastly, 1.5% methylcellulose was added into the concoction. 1 ml CFU-c assay media and 500 μ l cell suspension was plated in each 24 well cell culture plate. The plates were kept in CO_2 incubator at 5% CO_2 and 37°C. All Colony types were counted after 7 days using Floid Cell Imaging Station, and pooled to get total CFU-c. A graph of Clonogenic Potential-vs-samples was plotted for each tissue sample.

Cytokine analysis of peripheral blood

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 peripheral blood 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 μ l of wash buffer added to resuspend the bead pellet.

Results

TC of PB

The TC of blood increased 1.94 fold ($p < 0.05$) after 24 h, 2.21 fold

($p < 0.05$) after 48 h, 2.26 fold ($p < 0.05$) after 72 h and 2.51 fold ($p < 0.05$) after 96h, on treatment with TG, as compared to untreated control groups. With fisetin treatment, the TC decreased 1.06 fold after 24 h, 1.14 fold after 48 h, 1.17 fold after 72 h and 1.24 fold ($p < 0.05$) after 96 h, compared to only TG. With MCN+fisetin treatment, the TC decreased 1.24 fold after 24 h, 1.56 fold ($p < 0.05$) after 48 h, and 1.20 fold after 72h, but increased 1.26 fold ($p < 0.05$) after 96 h compared to only TG. Compared to only fisetin, MCN has led to a decrease in TC after 24 h (1.17 fold), after 48 h (1.37 fold) and after 72 h (1.03 fold), but to an increase after 96 h (1.56 fold; $p < 0.05$). This shows that cell recruitment increased in response to the inflammatory stimulus, as TG induces a systemic inflammation. Fisetin inhibits cell recruitment within 24 h, as does MCN+fisetin. Inhibition is better with MCN till 72 h, but there is no inhibition with MCN at 96 h (Table 1 and Figure 1).

TC of PF

The TC of PF increased 1.40 fold after 24 h, 1.47 fold ($p < 0.05$) after 48 h, 1.60 fold after 72 h and 1.77 fold ($p < 0.05$) after 96 h, on treatment with TG, as compared to untreated control groups. With fisetin treatment, the TC decreased 1.02 fold after 24 h, 1.07 fold after 48 h, 1.05 fold after 72 h, and 2.13 fold ($p < 0.05$) after 96 h, compared to only TG. With MCN+fisetin treatment, the TC decreased 1.17 fold after 24 h, 1.40 fold ($p < 0.05$) after 48 h, 1.07 fold after 72h, and 1.36 fold after 96 h, compared to only TG. Compared to only fisetin, MCN has led to

PB	Total Cell Count ($\times 10^6$ / ml) \pm SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	0.53 \pm 0.03			
TG24	1.03 \pm 0.03	(+) 1.94 [#]		
TG24F	0.97 \pm 0.03		(-) 1.06	
TG24MF	0.83 \pm 0.09		(-) 1.24	(-) 1.17
TG48	1.17 \pm 0.09	(+) 2.21 [#]		
TG48F	1.03 \pm 0.09		(-) 1.14	
TG48MF	0.75 \pm 0.05		(-) 1.56* [*]	(-) 1.37
TG72	1.20 \pm 0.12	(+) 2.26 [#]		
TG72F	1.03 \pm 0.03		(-) 1.17	
TG72MF	1.00 \pm 0.06		(-) 1.20	(-) 1.03
TG96	1.33 \pm 0.09	(+) 2.51 [#]		
TG96F	1.07 \pm 0.03		(-) 1.24*	
TG96MF	1.67 \pm 0.09	(+) 1.26* [*]	(+) 1.56*	

(#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; *: $p < 0.05$ versus TG-F)

Table 1: Total cell count of blood samples, taken by hemocytometry. There is a 1.24 fold decrease ($p < 0.05$) with fisetin treatment after 96 h, and a 1.56 fold decrease ($p < 0.05$) with MCN+fisetin treatment after 48 h.

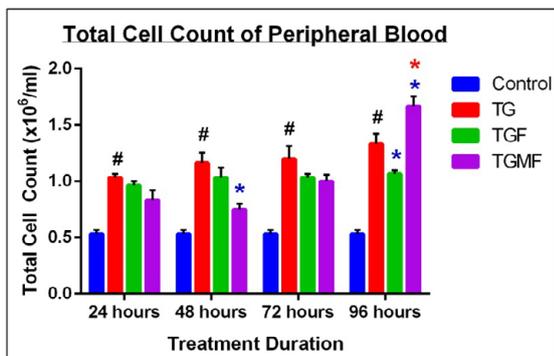


Figure 1: Effect of fisetin and MCN+fisetin on total cell count of peripheral blood (#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; *: $p < 0.05$ versus TG-F).

PB	Total Cell Count ($\times 10^6$ / ml) \pm SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	1.00 \pm 0.06			
TG24	1.40 \pm 0.15	(+) 1.40		
TG24F	1.37 \pm 0.03		(-) 1.02	
TG24MF	1.20 \pm 0.12		(-) 1.17	(-) 1.14
TG48	1.47 \pm 0.03	(+) 1.47 [#]		
TG48F	1.37 \pm 0.03		(-) 1.07	
TG48MF	1.05 \pm 0.05		(-) 1.40*	(-) 1.30*
TG72	1.60 \pm 0.32	(+) 1.60		
TG72F	1.53 \pm 0.03		(-) 1.05	
TG72MF	1.50 \pm 0.18		(-) 1.07	(-) 1.02
TG96	1.77 \pm 0.15	(+) 1.77 [#]		
TG96F	0.83 \pm 0.12		(-) 2.13*	
TG96MF	1.30 \pm 0.12		(-) 1.36	(+) 1.57*

(#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; *: $p < 0.05$ versus TG-F)

Table 2: Total cell count of peritoneal fluid samples, taken by hemocytometry. There is a 2.13 fold ($p < 0.05$) decrease with fisetin treatment after 96 h, and a 1.40 fold ($p < 0.05$) decrease with MCN+fisetin after 48 h.

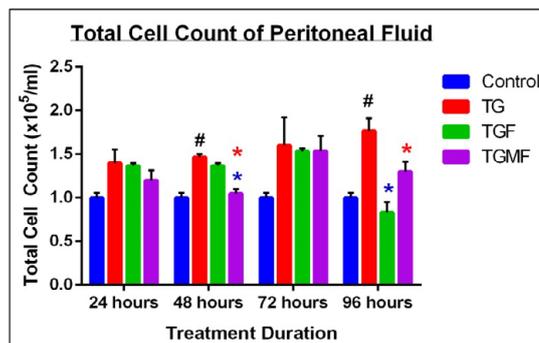


Figure 2: Effect of fisetin and MCN+fisetin on total cell count of peritoneal fluid (#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; *: $p < 0.05$ versus TG-F).

a decrease in TC after 24 h (1.14 fold), 48 h (1.30 fold; $p < 0.05$) and 72 h (1.02 fold), but to an increase after 96 h (1.57 fold; $p < 0.05$). Compared to only fisetin, MCN has led to a decrease in TC after 24 h (1.17 fold), after 48 h (1.37 fold) and after 72 h (1.03 fold), but to an increase after 96 h (1.56 fold; $p < 0.05$). This shows that cell recruitment increases within 24 h in response to the inflammatory stimulus, as TG induces a systemic inflammation, and immune cells localize to the PF. Fisetin successfully inhibits cell recruitment within 24 h, as does MCN+fisetin. Inhibition is better with MCN till 72 h, but there is no inhibition with MCN at 96 h (Table 2 and Figure 2).

DC of neutrophils in PB: The count of polymorphonuclear (PMN) cells in the blood- eosinophils, basophils and neutrophils, increased (data not given) with TG. The numbers of neutrophils in the blood were found to have increased. There is a 1.10 fold increase after 24 h, 1.66 fold ($p < 0.05$) increase after 48 h, 2.39 fold increase after 72 h and a 2.50 fold ($p < 0.05$) increase after 96 h of TG treatment, compared to control. Neutrophil count decreased with fisetin after 72 h (1.12 fold) and 96 h (2.15 fold; $p < 0.05$), and with MCN+fisetin after 48 h (1.17 fold), 72 h (1.02 fold) and after 96 h (1.37 fold). Inhibition of neutrophil infiltration has been enhanced only at 48 h (1.35 fold; $p < 0.05$) (Table 3 and Figure 3).

DC of lymphocytes in PB: The count of mononuclear (MN) cells

Treatment	No. of neutrophils (x10 ⁵ / ml ± SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	3.22 ± 0.16			
TG24	3.53 ± 0.32	(+) 1.10		
TG24F	3.70 ± 0.73		(+) 1.05	
TG24MF	4.78 ± 0.51		(+) 1.35	(+) 1.29
TG48	5.35 ± 0.18	(+) 1.66 [#]		
TG48F	6.20 ± 0.30		(+) 1.16	
TG48MF	4.58 ± 0.34		(-) 1.17	(-) 1.35 [*]
TG72	7.70 ± 1.85	(+) 2.39		
TG72F	6.88 ± 0.25		(-) 1.12	
TG72MF	7.57 ± 1.12		(-) 1.02	(+) 1.10
TG96	8.06 ± 0.72	(+) 2.50 [#]		
TG96F	3.75 ± 0.55		(-) 2.15 [*]	
TG96MF	5.88 ± 0.60		(-) 1.37	(+) 1.57 [*]

([#]: p<0.05 versus Control; ^{*}: p<0.05 versus TG; ^{*}: p<0.05 versus TG-F)

Table 3: Differential count of neutrophils in peripheral blood, seen after HE staining, under light microscope. There is a 2.15 fold (p<0.05) decrease with fisetin after 96 h, and a 1.35 fold (p<0.05) decrease with MCN+fisetin after 48 h.

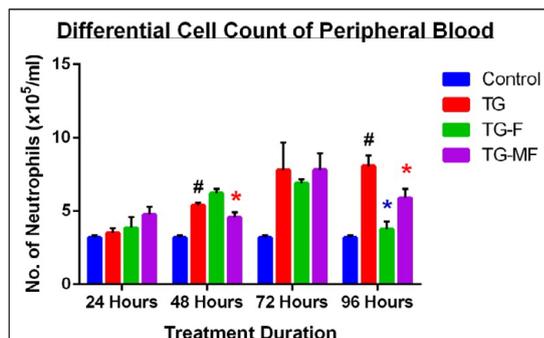


Figure 3: Effect of fisetin and MCN+fisetin on differential count of neutrophils of peripheral blood ([#]: p<0.05 versus Control; ^{*}: p<0.05 versus TG; ^{*}: p<0.05 versus TG-F).

in the blood- lymphocytes and monocytes, increased (data not given) after TG treatment. The numbers of circulating lymphocytes have increased with TG treatment. There is a 1.53 fold increase after 24 h, 1.54 fold (p<0.05) increase after 48 h, 1.57 fold increase after 72 h and a 1.60 fold (p<0.05) increase after 96 h of TG treatment, compared to control. Lymphocyte count decreased with fisetin after 24 h (1.75 fold; p<0.05), 48 h (1.30 fold; p<0.05), 72 h (1.18 fold) and 96h (2.07 fold; p<0.05), and with MCN+fisetin after 24 h (1.65 fold; p<0.05), 48 h (1.70 fold; p<0.05), 72 h (1.18 fold) and after 96 h (1.30 fold; p<0.05). Inhibition of lymphocyte infiltration has been enhanced only at 48 h (1.31 fold; p<0.05) and at 72 h (1.01 fold) (Table 4 and Figure 4).

NO estimation of PB

The NO concentration of peripheral blood shows a 1.03 fold increase after 24 h, a 1.07 fold increase after 48 h, a 1.12 fold increase after 72 h and a 1.23 fold increase after 96 h, with TG, as compared to control. Administration of fisetin alone shows a 1.22 fold decrease after 24 h, a 1.29 fold increase after 48 h, a 1.06 fold increase after 72 h, and a 3.21 fold decrease after 96 h, compared to only TG. With MCN+fisetin, the NO concentration decreased 2.10 fold (p<0.05) after 24 h and 1.18 fold (p<0.05) after 96 h, but increased 1.07 fold after 48 h and 1.44 fold after 72 h, compared to only TG. Compared to fisetin, MCN has reduced the NO concentration 1.72 fold after 24 h and 1.20 fold after 48 h (Table 5 and Figure 5).

Treatment	No. of neutrophils (x10 ⁵ / ml ± SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	4.15 ± 0.56			
TG24	6.35 ± 0.76	(+) 1.53		
TG24F	3.63 ± 0.60		(-) 1.75 [*]	
TG24MF	3.86 ± 0.33		(-) 1.65 [*]	(+) 1.06
TG48	6.40 ± 0.12	(+) 1.54 [#]		
TG48F	4.94 ± 0.06		(-) 1.30 [*]	
TG48MF	3.77 ± 0.28		(-) 1.70 [*]	(-) 1.31 [*]
TG72	6.52 ± 1.09	(+) 1.57		
TG72F	5.54 ± 0.23		(-) 1.18	
TG72MF	5.51 ± 0.58		(-) 1.18	(-) 1.01
TG96	6.64 ± 0.44	(+) 1.60 [#]		
TG96F	3.21 ± 0.53		(-) 2.07 [*]	
TG96MF	5.09 ± 0.33		(-) 1.30 [*]	(+) 1.59 [*]

([#]: p<0.05 versus Control; ^{*}: p<0.05 versus TG; ^{*}: p<0.05 versus TG-F)

Table 4: Differential count of lymphocytes in peripheral blood, seen after HE staining, under light microscope. Lymphocyte count decreased with fisetin, after 24 h (1.75 fold; p<0.05), 48 h (1.30 fold; p<0.05), 72 h and 96 h (2.07 fold; p<0.05), and with MCN+fisetin, after 48 h (1.31 fold; p<0.05) and 72 h (1.01 fold).

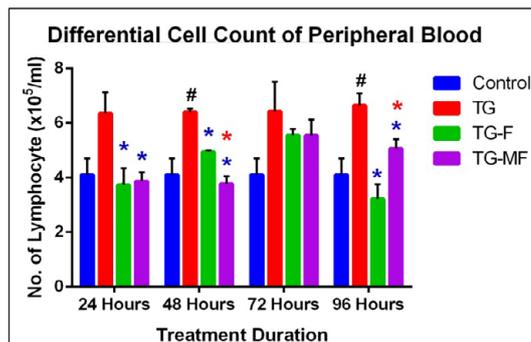


Figure 4: Effect of fisetin and MCN+fisetin on differential count of MN cells of peripheral blood ([#]: p<0.05 versus Control; ^{*}: p<0.05 versus TG; ^{*}: p<0.05 versus TG-F).

PB	Concentration of NO (µM) ± SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	29.59 ± 1.34			
TG24	30.61 ± 1.70	(+) 1.03		
TG24F	25.01 ± 4.39		(-) 1.22	
TG24MF	14.56 ± 2.73		(-) 2.10 [*]	(-) 1.72
TG48	31.76 ± 0.47	(+) 1.07		
TG48F	40.95 ± 22.18		(+) 1.29	
TG48MF	34.10 ± 15.77		(+) 1.07	(-) 1.20
TG72	33.16 ± 2.12	(+) 1.12		
TG72F	35.05 ± 9.09		(+) 1.06	
TG72MF	47.79 ± 6.04		(+) 1.44	(+) 1.36
TG96	36.26 ± 3.15	(+) 1.23		
TG96F	11.29 ± 1.29		(-) 3.21	
TG96MF	30.65 ± 8.90		(-) 1.18 [*]	(+) 2.71

([#]: p<0.05 versus Control; ^{*}: p<0.05 versus TG; ^{*}: p<0.05 versus TG-F)

Table 5: Concentration and fold changes in nitric oxide produced in peripheral blood, assayed by Griess Reagent, at 540 nm. With fisetin, there is a 1.22 fold decrease after 24 h, and a 3.21 fold decrease after 96 h. With MCN+fisetin, there is a 2.10 fold (p<0.05) decrease after 24h and a 1.18 fold (p<0.05) decrease after 96 h.

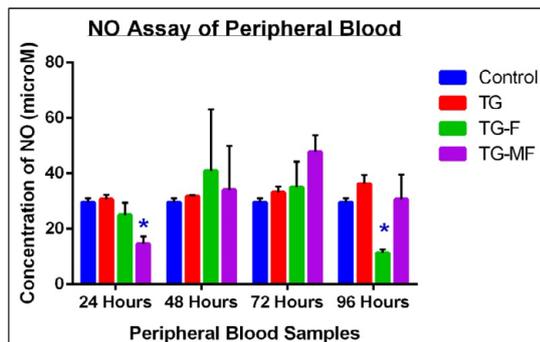


Figure 5: Effect of fisetin and MCN+fisetin on the production of nitric oxide in the blood (#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; **: $p < 0.05$ versus TG-F).

PF	Concentration of NO (μM) \pm SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	5.04 \pm 1.21			
TG24	11.97 \pm 1.56	(+) 2.38#		
TG24F	9.29 \pm 1.38		(-) 1.29	
TG24MF	10.84 \pm 5.20		(-) 1.10	(+) 1.17
TG48	13.03 \pm 2.13	(+) 2.59		
TG48F	10.36 \pm 4.28		(-) 1.26	
TG48MF	8.69 \pm 2.64		(-) 1.50	(-) 1.19
TG72	22.54 \pm 9.29	(+) 4.47#		
TG72F	19.42 \pm 2.31		(-) 1.16	
TG72MF	20.00 \pm 3.31		(-) 1.13	(+) 1.03
TG96	24.67 \pm 1.59	(+) 4.89#		
TG96F	13.10 \pm 4.38		(-) 1.88	
TG96MF	16.60 \pm 5.80		(-) 1.49	(+) 1.27

(#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; **: $p < 0.05$ versus TG-F)

Table 6: Concentration and fold changes in nitric oxide produced in peritoneal fluid, assayed by Griess Reagent, at 540 nm. With fisetin, there is a 1.29 fold decrease after 24 h, a 1.26 fold decrease after 48 h, a 1.16 fold decrease after 72 h and a 1.88 fold decrease after 96 h. With MCN+fisetin, there is a 1.19 fold decrease after 48 h.

NO estimation of PF

The NO concentration of peritoneal fluid shows a 2.38 fold ($p < 0.05$) increase after 24 h, a 2.59 fold increase after 48 h, a 4.47 fold ($p < 0.05$) increase after 72 h and a 4.89 fold ($p < 0.05$) increase after 96 h, with TG, as compared to control. Administration of fisetin alone shows a 1.29 fold decrease after 24 h, a 1.26 fold decrease after 48 h, a 1.16 fold decrease after 72 h, and a 1.88 fold decrease after 96 h. With MCN+fisetin, the NO concentration decreased 1.10 fold after 24 h, 1.50 fold after 48 h, 1.13 fold after 72 h, and 1.49 fold after 96 h. Compared to fisetin, MCN has reduced the NO concentration 1.19 fold only after 48 h (Table 6 and Figure 6).

NO estimation of BM

The concentration of NO in the bone marrow shows a 1.55 fold increase after 24 h, a 1.75 fold increase after 48 h, a 1.95 fold ($p < 0.05$) increase after 72 h and a 2.12 fold ($p < 0.05$) increase after 96 h, with TG, as compared to control. Administration of fisetin alone shows a 1.22 fold increase after 24 h, but there is a 2.16 fold ($p < 0.05$) decrease after 48 h, a 1.36 fold decrease after 72 h, and a 2.29 fold ($p < 0.05$) decrease after 96 h, compared to only TG. With MCN+fisetin, the NO concentration decreased 1.26 fold after 24 h, 2.36 fold after 48 h, 1.23 fold after 72 h

and 2.57 fold ($p < 0.05$) after 96 h, compared to only TG. Compared to fisetin, MCN has reduced the NO concentration after 24 h (1.54 fold), 48 h (1.09 fold) and after 96 h (1.12 fold) (Table 7 and Figure 7).

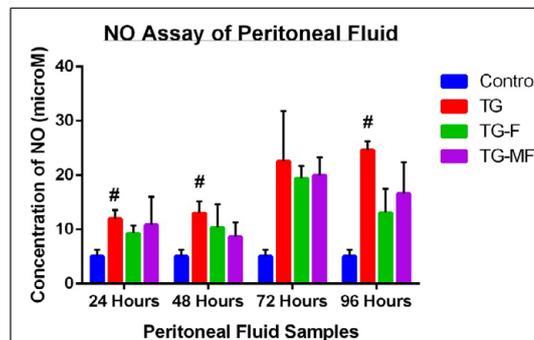


Figure 6: Effect of fisetin and MCN+fisetin on the production of nitric oxide in the peritoneal fluid (#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; **: $p < 0.05$ versus TG-F).

BM	Concentration of NO (μM) \pm SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	9.46 \pm 1.79			
TG24	14.67 \pm 3.84	(+) 1.55		
TG24F	17.96 \pm 1.56		(+) 1.22	
TG24MF	11.65 \pm 3.16		(-) 1.26	(-) 1.54
TG48	16.57 \pm 2.51	(+) 1.75		
TG48F	7.66 \pm 1.55		(-) 2.16*	
TG48MF	7.03 \pm 1.88		(-) 2.36	(-) 1.09
TG72	18.49 \pm 0.63	(+) 1.95#		
TG72F	13.58 \pm 2.01		(-) 1.36	
TG72MF	15.01 \pm 3.96		(-) 1.23	(+) 1.11
TG96	20.05 \pm 1.32	(+) 2.12#		
TG96F	8.74 \pm 1.84		(-) 2.29*	
TG96MF	7.81 \pm 1.11		(-) 2.57*	(-) 1.12

(#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; **: $p < 0.05$ versus TG-F)

Table 7: Concentration and fold changes in nitric oxide produced in bone marrow, assayed by Griess Reagent, at 540 nm. With fisetin, there is a 2.16 fold ($p < 0.05$) decrease after 48 h, a 1.36 fold decrease after 72 h and a 2.29 fold ($p < 0.05$) decrease after 96 h. With MCN+fisetin, there is a 1.26 fold decrease after 24 h, a 2.36 fold decrease after 48 h, a 1.23 fold decrease after 72 h and a 2.57 fold ($p < 0.05$) decrease after 96 h.

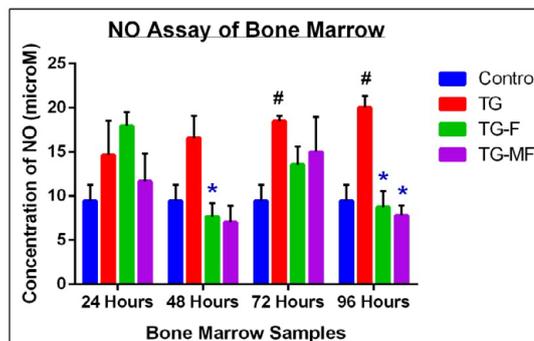


Figure 7: Effect of fisetin and MCN+fisetin on the production of nitric oxide in the bone marrow (#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; **: $p < 0.05$ versus TG-F).

Cell proliferation (MTS) assay of PB

The MTS assay gives an idea about the proliferative potential of the cells. The cell viabilities decreased with TG treatment, as compared to untreated control. There is a 1.23 fold decrease in viability after 24 h, a 1.35 fold decrease after 48 h, a 1.96 fold (p<0.05) decrease after 72 h and a 2.33 fold (p<0.05) decrease after 96 h of infection. Cell viability increased 1.15 fold after 24 h, 1.22 fold after 48 h, 1.26 fold after 72 h and 1.43 fold after 96 h, with fisetin, compared to TG. With MCN+fisetin, viability increased 1.16 fold after 24 h, 1.34 fold after 48 h, 1.24 fold after 72 h and 1.22 fold (p<0.05) after 96 h. Compared to only fisetin, MCN increased the viability of the cells by 1.01 fold after 24 h and by 1.10 fold after 48 h (Table 8 and Figure 8).

Cell proliferation (MTS) assay of PF

The viability of cells in the PF decreased 1.23 fold after 24 h, 1.31 fold after 48 h, 1.64 fold after 72 h and 1.70 fold after 96 h. With fisetin, compared to only TG, cell viability increased 1.15 fold after 24 h, 1.23 fold after 48 h, 1.39 fold after 72 h and 1.42 fold after 96 h. Viability increased 1.16 fold after 24 h, 1.10 fold after 48 h, 1.60 fold after 72 h and 1.35 fold after 96 h, with MCN+fisetin treatment, compared to only TG. MCN increased viability, compared to only fisetin, by 1.01

PB	Absorbance (490 nm) ± SEM	% viability	Fold change, with respect to		
			Control	TG	TG-F
Control	2.321 ± 0.25	100.00			
TG24	1.887 ± 0.45	81.30	(-) 1.23		
TG24F	2.164 ± 0.37	93.23		(+) 1.15	
TG24MF	2.193 ± 0.07	94.48		(+) 1.16	(+) 1.01
TG48	1.725 ± 0.29	74.33	(-) 1.35		
TG48F	2.102 ± 0.11	90.58		(+) 1.22	
TG48MF	2.317 ± 0.06	99.82		(+) 1.34	(+) 1.10
TG72	1.186 ± 0.32	51.11	(-) 1.96 [#]		
TG72F	1.499 ± 0.33	64.61		(+) 1.26	
TG72MF	1.466 ± 0.12	63.19		(+) 1.24	(-) 1.02
TG96	0.995 ± 0.21	42.89	(-) 2.33 [#]		
TG96F	1.419 ± 0.20	61.13		(+) 1.43	
TG96MF	1.215 ± 0.11	52.34		(+) 1.22	(-) 1.17

(#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F)

Table 8: Fold changes in viability of cells in peripheral blood, assayed by MTS reagent, at 492nm. With fisetin, there is a 1.15 fold increase after 24h, a 1.22 fold increase after 48 h, a 1.26 fold increase after 72 h and a 1.43 fold increase after 96 h. With MCN+fisetin, there is a 1.16 fold increase after 24 h, a 1.34 fold increase after 48 h, a 1.24 fold increase after 72 h and a 1.22 fold (p<0.05) increase after 96 h.

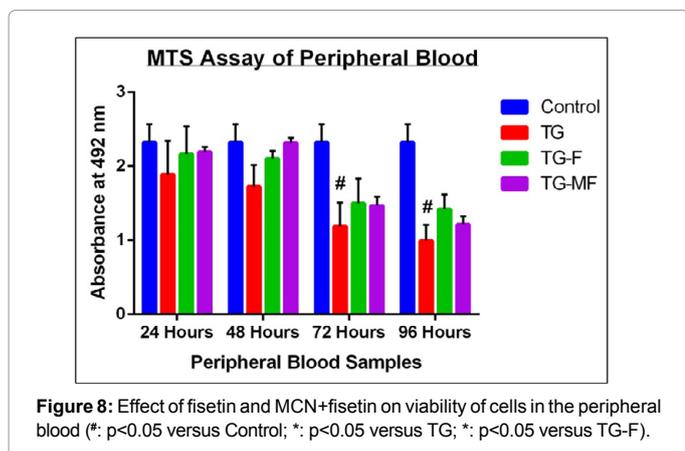


Figure 8: Effect of fisetin and MCN+fisetin on viability of cells in the peripheral blood (#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F).

PF	Absorbance (490 nm) ± SEM	% viability	Fold change, with respect to		
			Control	TG	TG-F
Control	0.945 ± 0.11	100.00			
TG24	0.769 ± 0.08	81.33	(-) 1.23		
TG24F	0.882 ± 0.27	93.29		(+) 1.15	
TG24MF	0.891 ± 0.20	94.31		(+) 1.16	(+) 1.01
TG48	0.722 ± 0.18	76.42	(-) 1.31		
TG48F	0.892 ± 0.05	94.36		(+) 1.23	
TG48MF	0.793 ± 0.21	83.85		(+) 1.10	(-) 1.13
TG72	0.577 ± 0.09	61.06	(-) 1.64		
TG72F	0.804 ± 0.02	85.08		(+) 1.39	
TG72MF	0.921 ± 0.24	97.50		(+) 1.60	(+) 1.15
TG96	0.554 ± 0.17	58.66	(-) 1.70		
TG96F	0.785 ± 0.17	83.11		(+) 1.42	
TG96MF	0.750 ± 0.24	79.33		(+) 1.35	(-) 1.05

(#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F)

Table 9: Fold changes in viability of cells in peritoneal fluid, assayed by MTS reagent, at 492 nm. With fisetin, there is a 1.15 fold increase after 24 h, a 1.23 fold increase after 48 h, a 1.39 fold increase after 72 h and a 1.42 fold increase after 96 h, in the cell viability. With MCN+fisetin, cell viability increased 1.16 fold after 24 h, 1.10 fold after 48 h, 1.60 fold after 72 h and 1.35 fold after 96 h.

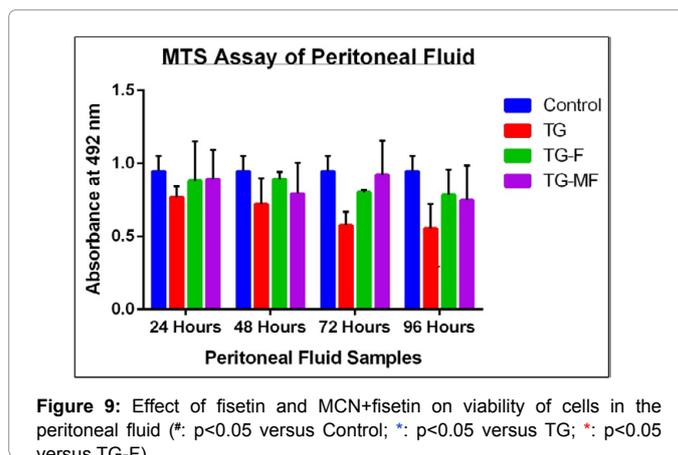


Figure 9: Effect of fisetin and MCN+fisetin on viability of cells in the peritoneal fluid (#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F).

fold after 24 h and by 1.15 fold after 72 h (Table 9 and Figure 9).

Cell proliferation (MTS) assay of BM

The viability of cells in the BM decreased 1.15 fold after 24 h, 1.24 fold after 48 h, 1.37 fold after 72 h and 1.49 fold after 96 h of treatment with TG, compared to control. Viability increased 1.06 fold after 24 h, 1.18 fold after 48 h, 1.10 fold after 72 h and 1.31 fold (p<0.05) after 96 h, with fisetin, compared to only TG. With MCN+fisetin, compared to TG, viability increased 1.08 fold after 24 h, 1.09 fold after 48 h, 1.29 fold after 72 h and 1.22 fold after 96 h. Addition of MCN to fisetin improved viability by 1.02 fold after 24 h, and by 1.17 fold after 72 h (Table 10 and Figure 10).

CFU-c assay of PB

CFU-c assay gives the clonogenic potential of cells, which is the ability of cells to form colonies on a semi- solid matrix. The clonogenic potential of cells in the peripheral blood decreased with TG treatment, as compared to control. There is a 1.63 fold (p<0.05) decrease after 24 h, a 2.82 fold (p<0.05) decrease after 48 h, a 3.65 fold (p<0.05) decrease after 72 h and a 5.17 fold (p<0.05) decrease after 96 h, with TG. It increased 1.26 fold after 24 h, 1.05 fold after 48 h and 1.75 fold after 96 h, with fisetin treatment, but decreased 1.06 fold after 72 h. With

BM	Absorbance (490 nm) ± SEM	% viability	Fold change, with respect to		
			Control	TG	TG-F
Control	1.44 ± 0.51	100.00			
TG24	1.25 ± 0.16	81.33	(-) 1.15		
TG24F	1.33 ± 0.49	93.29		(+) 1.06	
TG24MF	1.35 ± 0.05	94.31		(+) 1.08	(+) 1.02
TG48	1.16 ± 0.22	76.42	(-) 1.24		
TG48F	1.37 ± 0.23	94.36		(+) 1.18	
TG48MF	1.27 ± 0.32	83.85		(+) 1.09	(-) 1.08
TG72	1.05 ± 0.24	61.06	(-) 1.37		
TG72F	1.16 ± 0.23	85.08		(+) 1.10	
TG72MF	1.36 ± 0.07	87.31		(+) 1.29	(+) 1.17
TG96	0.97 ± 0.07	58.66	(-) 1.49		
TG96F	1.27 ± 0.06	83.11		(+) 1.31*	
TG96MF	1.19 ± 0.10	79.33		(+) 1.22	(-) 1.07

(#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F)

Table 10: Fold changes in viability of cells in bone marrow, assayed by MTS reagent, at 492 nm. With fisetin, there is a 1.06 fold increase in cell viability after 24 h, a 1.18 fold increase after 48 h, a 1.10 fold increase after 72 h, and a 1.31 fold (p<0.05) increase after 96 h. With MCN+fisetin, there is a 1.08 fold after 24 h, a 1.09 fold increase after 48 h, a 1.29 fold increase after 72 h and a 1.22 fold increase after 96 h.

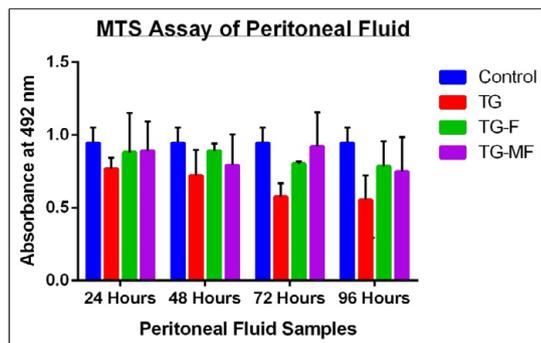


Figure 10: Effect of fisetin and MCN+fisetin on viability of cells in the bone marrow (#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F).

MCN+fisetin, the clonogenic potential increased 1.45 fold after 24 h and 1.65 fold after 72 h. MCN has enhanced the clonogenic potential by 1.15 fold after 24 h and by 1.75 fold after 72 h (Table 11 and Figure 11).

CFU-c assay of PF

The clonogenic potential of cells in PF decreased 1.33 fold after 24 h, 2.00 fold after 48 h, 2.29 fold after 72 h and 3.20 fold after 96h, with TG treatment. Compared to TG, with fisetin treatment, it increased 1.42 fold after 24h, 1.75 fold after 48 h, 1.71 fold after 72 h and 3.20 fold after 96 h. With MCN+fisetin, the clonogenic potential increased 1.33 fold after 24 h, 1.38 fold after 48 h, 2.14 fold after 72 h and 6.80 fold after 96 h. Compared to fisetin, MCN has improved the clonogenic potential by 1.25 fold after 72 h and by 2.13 fold after 96 h (Table 12 and Figures 12-14).

CFU-c assay of BM

The clonogenic potential of cells in the bone marrow decreases with TG treatment, as compared to control. There is a 1.73 fold decrease (p<0.05) after 24 h, a 2.52 fold decrease (p<0.05) after 48 h, a 3.39 fold decrease (p<0.05) after 72 h and a 4.59 fold (p<0.05) decrease after 96 h, with TG. It increases 1.20 fold after 24 h, 1.03 fold after 48 h and 1.53 fold after 96 h, with fisetin, compared to TG. With MCN+fisetin,

PB	Clonogenic Potential (x10 ⁻⁵) ± SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	3.10 ± 0.10			
TG24	1.90 ± 0.10	(-) 1.63#		
TG24F	2.40 ± 0.20		(+) 1.26	
TG24MF	2.75 ± 0.25		(+) 1.45	(+) 1.15
TG48	1.10 ± 0.20	(-) 2.82#		
TG48F	1.15 ± 0.25		(+) 1.05	
TG48MF	0.85 ± 0.05		(-) 1.29	(-) 1.35
TG72	0.85 ± 0.25	(-) 3.65#		
TG72F	0.80 ± 0.10		(-) 1.06	
TG72MF	1.40 ± 0.10		(+) 1.65	(+) 1.75
TG96	0.60 ± 0.10	(-) 5.17#		
TG96F	1.05 ± 0.55		(+) 1.75	
TG96MF	0.40 ± 0.10		(-) 1.50	(-) 2.63*

(#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F)

Table 11: The change in clonogenic potential of cells in the peripheral blood, assessed by CFU-c assay using methyl cellulose. There is an increase in clonogenic potential after 24 h (1.26 fold), after 48 h (1.05 fold) and after 96 h (1.75 fold), with fisetin treatment. With MCN+fisetin, there is an increase in clonogenic potential after 24 h (1.45 fold) and after 72 h (1.65 fold).

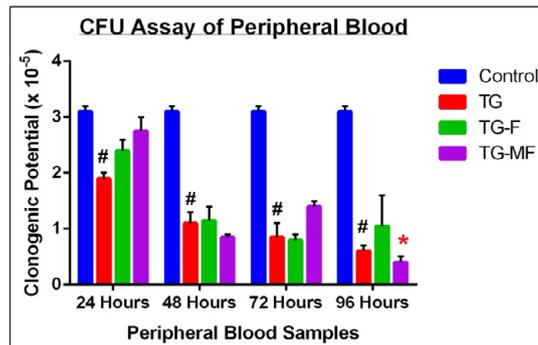
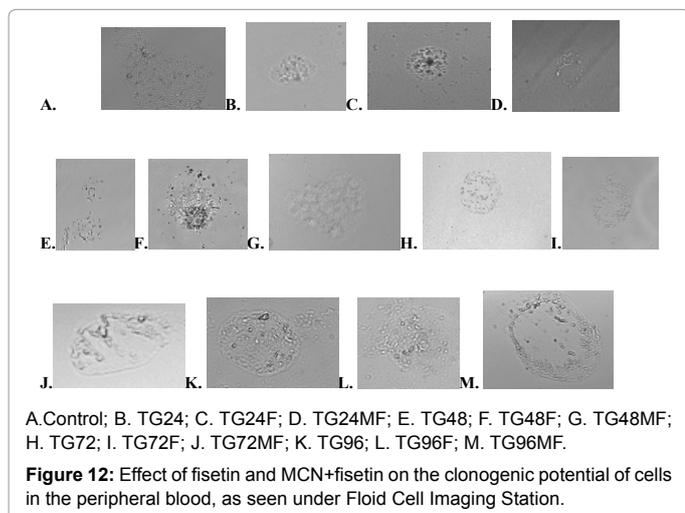


Figure 11: Effect of fisetin and MCN+fisetin on the clonogenic potential of cells in the peripheral blood (#: p<0.05 versus Control; #: p<0.05 versus TG; *: p<0.05 versus TG-F).

PF	Clonogenic Potential (x10 ⁻⁴) ± SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	0.80 ± 0.20			
TG24	0.60 ± 0.10	(-) 1.33		
TG24F	0.85 ± 0.05		(+) 1.42	
TG24MF	0.80 ± 0.40		(+) 1.33	(-) 1.06
TG48	0.40 ± 0.10	(-) 2.00		
TG48F	0.70 ± 0.10		(+) 1.75	
TG48MF	0.55 ± 0.15		(+) 1.38	(-) 1.27
TG72	0.35 ± 0.05	(-) 2.29		
TG72F	0.60 ± 0.10		(+) 1.71	
TG72MF	0.75 ± 0.15		(+) 2.14	(+) 1.25
TG96	0.25 ± 0.05	(-) 3.20		
TG96F	0.80 ± 0.40		(+) 3.20	
TG96MF	1.70 ± 0.60		(+) 6.80	(+) 2.13

(#: p<0.05 versus Control; *: p<0.05 versus TG; *: p<0.05 versus TG-F)

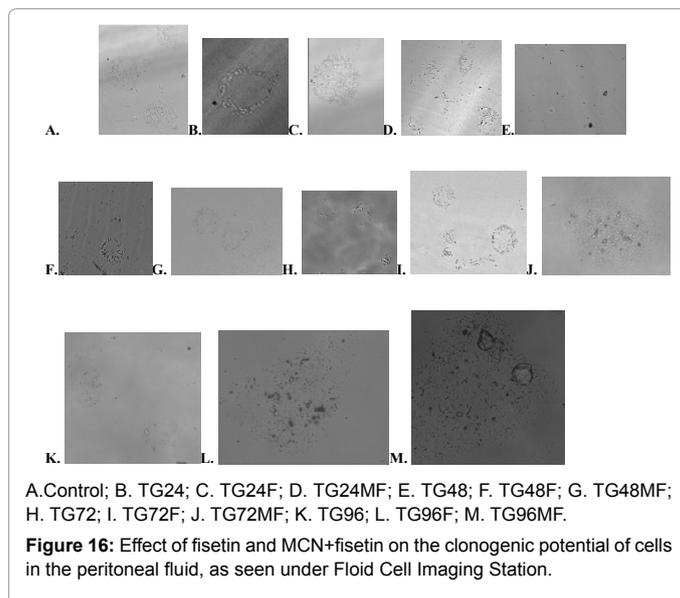
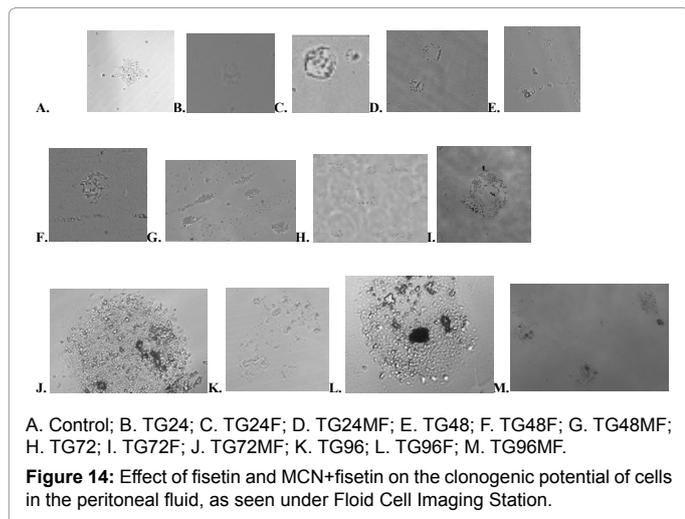
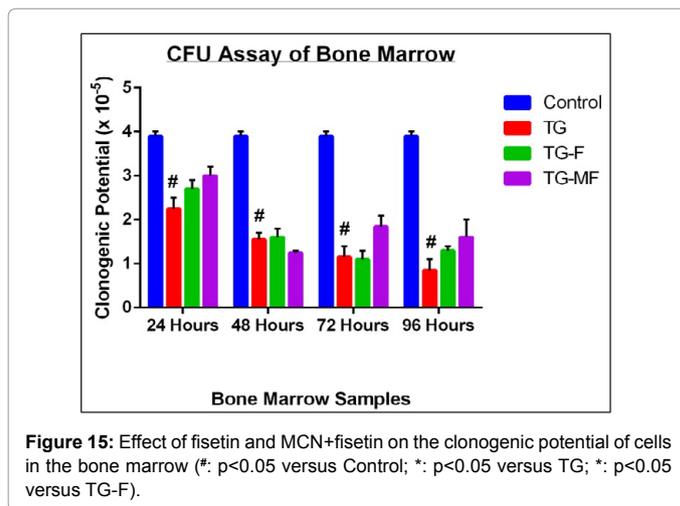
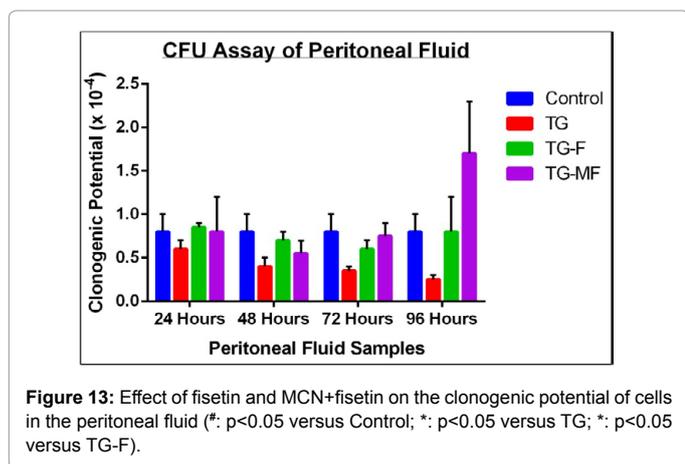
Table 12: The change in clonogenic potential of cells in the peritoneal fluid, assessed by CFU-c assay using methyl cellulose. There is an increase in clonogenic potential after 24 h (1.42 fold), 48 h (1.75 fold), 72 h (1.71 fold) and 96 h (3.20 fold), with fisetin treatment. With MCN+fisetin, the clonogenic potential increased by 1.33 fold after 24 h, 1.38 fold after 48 h, 2.14 fold after 72 h and 6.80 fold after 96 h.



BM	Clonogenic Potential ($\times 10^{-5}$) \pm SEM	Fold change, with respect to		
		Control	TG	TG-F
Control	0.80 \pm 0.20			
TG24	0.60 \pm 0.10	(-) 1.33		
TG24F	0.85 \pm 0.05		(+) 1.42	
TG24MF	0.80 \pm 0.40		(+) 1.33	(-) 1.06
TG48	0.40 \pm 0.10	(-) 2.00		
TG48F	0.70 \pm 0.10		(+) 1.75	
TG48MF	0.55 \pm 0.15		(+) 1.38	(-) 1.27
TG72	0.35 \pm 0.05	(-) 2.29		
TG72F	0.60 \pm 0.10		(+) 1.71	
TG72MF	0.75 \pm 0.15		(+) 2.14	(+) 1.25
TG96	0.25 \pm 0.05	(-) 3.20		
TG96F	0.80 \pm 0.40		(+) 3.20	
TG96MF	1.70 \pm 0.60		(+) 6.80	(+) 2.13

(#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; **: $p < 0.05$ versus TG-F)

Table 13: The change in clonogenic potential of cells in the bone marrow, assessed by CFU-c assay using methyl cellulose. There is an increase in clonogenic potential after 24 h (1.20 fold), 48 h (1.03 fold), and 96 h (1.53 fold), with fisetin treatment. With MCN+fisetin, the clonogenic potential increased by 1.33 fold after 24 h, 1.61 fold after 72 h and 1.88 fold after 96 h.



the clonogenic potential increased 1.33 fold after 24 h, 1.61 fold after 72 h and 1.88 fold after 96 h, but has decreased 1.24 fold after 48 h, compared to fisetin, MCN has increased the clonogenic potential by 1.11 fold after 24 h, 1.68 fold after 72 h and 1.23 fold after 96 h (Table 13, Figures 15 and 16).

Cytokine levels in peripheral blood after 48h of treatment

48 h after challenge with TG, there is an increase in the levels of, IL-4 (1.03 fold), and IFN- γ (1.13 fold), and a decrease in the levels of IL-2 (1.06 fold, $p < 0.05$), IL-5 (1.05 fold) and TNF- α (1.05 fold) compared to control. Compared to TG treated groups, there is an increase in the levels of the cytokines with fisetin treatment {1.13 fold for IL-2, 1.12 fold ($p < 0.05$) for IL-4, 1.10 fold for IL-5, 1.03 fold for IFN- γ , 1.02 fold for TNF- α }, and with MCN+F treatment {1.12 fold for IL-2, 1.27 fold ($p < 0.05$) for IL-4, 1.14 fold for IL-5, 1.07 fold for IFN- γ , 1.03 fold for TNF- α } (Table 14 and Figure 17).

Discussion

The pathophysiology of peritonitis is complicated and is involved in various processes, of which, the most important one is the inflammatory

reaction [27]. During the pathological process of the peritonitis, NF- κ B plays an activating role in the inflammatory reaction [7].

Our study was designed to investigate the potential therapeutic effects of fisetin and fisetin loaded on mesoporous carbon nanoparticle (MCN) on the thioglycollate-induced peritonitis in rodent models. The thioglycollate-induced peritonitis in mice is used as a model to study the potential anti-inflammatory action of investigated test compounds [28]. In this present study, we have induced peritonitis in 6-8 weeks old BALB/c mice using thioglycollate, and then assessed the anti-inflammatory effects of plant flavonoid, fisetin, when administered therapeutically. We have also assessed the anti-inflammatory effects of fisetin, when administered with a nanovehicle, mesoporous carbon nanoparticle. We found that fisetin had a positive therapeutic effect on the peritonitis.

Acute peritonitis differs from other infections because of the broad variety of causes, severity of the infection [29]. Acute peritonitis is one of the most headachy postoperative complications, which was an important cause of death in surgical practice and intensive care units [9]. The most serious consequence of acute peritonitis is sepsis, often leading to an unacceptably high morbidity and mortality [30]. So the research of acute peritonitis is always the hotspot of surgery and critical care medicine. The animal model is one of the most important methods in the scientific research. It can not only provide convenience in deriving a better understanding of the pathophysiology of disease, but also provide important and indispensable tools to explore the therapy of disease. It is the bridge between the fundamental research and clinical application.

The process of peritonitis is mediated by the activation of inducible transcription factors, such as NF- κ B, which play a pivotal role in the immune and inflammatory responses. Previous investigators have found that acute peritonitis and sepsis were associated with the activation of the transcription factor NF- κ B in various organs and tissues [10,31,32] which can regulate the synthesis of TNF- α , IL-6, inducible nitric oxide synthase, cyclooxygenase-2 and many other molecules involved in the inflammatory reaction [18,33].

In this study, we found that, with administration of TG, cell recruitment in the blood increases progressively with time, with maximum recruitment after 96 h. This shows that it has induced inflammation, and the body is synthesizing more immune cells to counter the infection. Cell recruitment is successfully inhibited by fisetin, and with MCN+fisetin. In the peritoneal fluid, total cell recruitment was increased, which was successfully inhibited with fisetin and MCN+fisetin treatments.

Nitric acid is produced by macrophages as a defense against oxidative stress. In case of inflammation, NO content is expected to increase. Our assays have shown that the NO content of the tissues was increased with TG challenge. NO content decreased with fisetin and with MCN+fisetin. However, the addition of MCN to fisetin did not make a significant difference to the NO content.

We found that, TG treatment significantly reduced cell proliferation in the blood, PF and BM. Interestingly, cell proliferation was increased with fisetin treatment, and with MCN+fisetin. In another assay, the clonogenic potential of the tissues decreases significantly within 24 h, with administration of TG. Both fisetin treatment and MCN+fisetin treatment have restored the clonogenic potential of the tissues. Our study demonstrated that, there was a decrease in Th2 cytokines (IL-2, IL-5 and TNF- α) with TG treatment, in blood after 48 h and fisetin and MCN+fisetin was increased the cytokine content. However, in all the

Cytokine	Treatment	Concentration of cytokine (pg/ml) \pm SEM	Fold change, with respect to		
			Control	TG	TG-F
IL-2	Control	19.05 \pm 0.15			
	TG48	18.05 \pm 0.15	(-) 1.06#		
	TG48F	20.40 \pm 0.70		(+) 1.13	
	TG48MF	20.25 \pm 1.15		(+) 1.12	(-) 1.01
IL-4	Control	15.25 \pm 0.05			
	TG48	15.65 \pm 0.35	(+) 1.03		
	TG48F	17.50 \pm 0.20		(+) 1.12*	
	TG48MF	19.85 \pm 0.95		(+) 1.27*	(+) 1.13
IL-5	Control	14.60 \pm 0.20			
	TG48	13.85 \pm 0.55	(-) 1.05		
	TG48F	15.30 \pm 0.30		(+) 1.10	
	TG48MF	15.85 \pm 1.75		(+) 1.14	(+) 1.04
IFN- γ	Control	10.75 \pm 0.45			
	TG48	12.10 \pm 0.30	(+) 1.13		
	TG48F	12.45 \pm 1.65		(+) 1.03	
	TG48MF	12.90 \pm 1.60		(+) 1.07	(+) 1.04
TNF- α	Control	10.80 \pm 1.10			
	TG48	10.30 \pm 0.30	(-) 1.05		
	TG48F	10.55 \pm 1.05		(+) 1.02	
	TG48MF	10.60 \pm 1.40		(+) 1.03	(+) 1.01

(#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; *: $p < 0.05$ versus TG-F)

Table 14: Concentration (in pg/ml) of cytokines IL-2, IL-4, IL-5, IFN- γ and TNF- α , in peripheral blood, after 48 h of treatment, assayed using BD CBA Mouse Th1/Th2 Cytokine Kit. Cytokine analysis shows an increase in the levels of the cytokines with both, fisetin and MCN+fisetin, compared to TG.

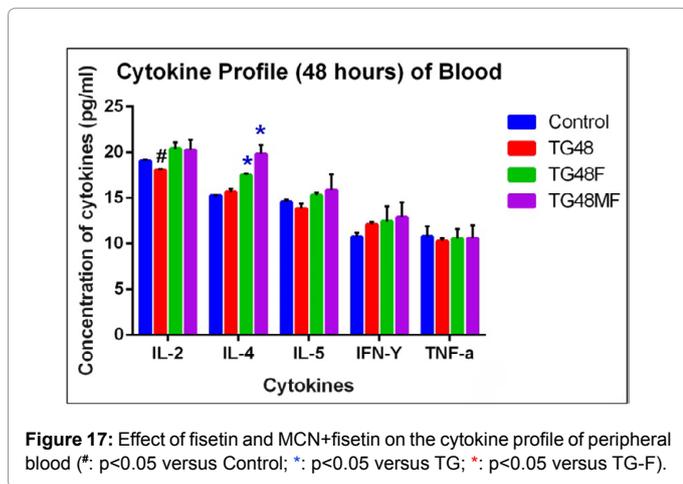


Figure 17: Effect of fisetin and MCN+fisetin on the cytokine profile of peripheral blood (#: $p < 0.05$ versus Control; *: $p < 0.05$ versus TG; *: $p < 0.05$ versus TG-F).

cases, MCN has not had a significant effect. So, the anti-inflammatory and pro-regenerative effects of MCN+fisetin is mainly because of the fisetin.

One of the main results of an inflammatory reaction in a body is the over-production of pro-oxidative agents, like nitric oxide. In response to the increase in pro-oxidative radicals, the body attempts to maintain the oxidative balance, by producing more anti-oxidants, which can scavenge the harmful radicals. This leads to an increase in the proliferation of cells. This has been demonstrated clearly in our experiment, where treatment with TG has led to an increase in NO concentration. Due to the attack caused by TG, the body loses the battle to maintain the oxidative balance, and proliferation is reduced. The cells lose the ability to proliferate and form colonies on semi-solid medium. Fisetin is successful in restoring this balance to some extent. It successfully reduced the NO content, and increased the proliferation. It also increased the clonogenic potential of the cells, showing the cells have regained their ability to form colonies when given a matrix for growth. Fisetin, when loaded on MCN, has a similar effect to fisetin alone, but it does not have any additional effect, which we would want our nano-vehicle to have.

Conclusion

In conclusion, we demonstrated that fisetin and fisetin loaded on mesoporous carbon nanoparticle (MCN) may have anti-inflammatory effects on thioglycollate-induced peritonitis. To our knowledge, this is the first study to date to assess new therapeutic approaches using phytochemicals such as fisetin against peritonitis. Further studies are required to verify the clinical use of fisetin in the treatment of peritonitis, and also to determine the pathways by which it acts. The future research could focus on the combination of fisetin therapy and traditional antibiotics, which might be more efficient than using antibiotics alone. We can also investigate other nano-particles that can be used as vehicles, for better delivery of the drug.

Contribution of Authors

SM performed all experiments, analyzed data, SB gave valuable input to the manuscript, AS and NRJ have prepared the MCN, and ERB initiated the project with her idea, designed the experiments, analyzed all data and wrote the manuscript.

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