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EXPLORING THE MECHANISM OF ANTI-INFLAMMATORY ACTIVITY OF PHYTO-STABILIZED SILVER NANORODS

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In the present study, the anti-inflammatory activity of the phyto-stabilized silver nanorods has been investigated. We have already optimized the synthesis of silver nanorods using germinated fenugreek seed extract and reported its anticancer activity in skin cancer cell lines. Inflammation was induced in skin cell lines using lipopolysaccharides and thereafter the anti-inflammatory activity of silver nanorods was analysed by measuring the protein and mRNA expression of interleukins (IL-1, IL-6) and macrophage chemoattractant protein-1 (MCP-1). Our results revealed that silver nanorods could attenuate LPS-induced inflammation by inhibiting the expression of IL-1, IL-6 and MCP-1. Our results suggest that the phyto-stabilized silver nanorods may be used in the prevention of skin diseases associated with inflammation. The anti-inflammatory activity of silver nanorods might be due to its diminished size, and the phytochemicals of germinated fenugreek seeds capped on the surface of the nanoparticles. As most of the diseases including cancer are associated with inflammation, the silver nanorod is expected to emerge as an effective biomedical cum pharmacological candidate.

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1. Introduction

We have recently reported the supremacy of green synthesis protocol to produce therapeutic silver nanorods. The therapeutic efficacy may be attributed to the plant phytochemicals like polyphenols, which reduce the silver nitrate and functionalize the surface of the nanoparticles to stabilize them and to impart biomedical activities. Recently, we have reported the rapid synthesis of silver nanorods using germinated fenugreek seed extract (GFS-SNR) and predicted the synergistic anticancer activity of the silver nanorods and the surface coated polyphenols [1].

Inflammation is the key pathological changes manifested in many diseases like cancer, arthritis and asthma [2, 3, 4]. Hence, compounds which inhibit inflammation may emerge as a therapeutic tool for many diseases. *In-vitro*, inflammation can be induced using the lipopolysaccharide (LPS), the extracellular antigenic product of the Gram-negative bacteria [5, 6]. Interleukins (IL-1, IL-6) and macrophage chemoattractant protein-1 (MCP-1) are well documented and reliable inflammatory markers [7, 8, 9]. Previous studies have revealed the anti-inflammatory activities of metals such as Titanium, copper and nanoparticles like Gold, Zinc [10, 11, 12, 13]. However, so far there is no report on the anti-inflammatory activity of green-synthesized and phytochemicals-capped silver nanorods. Silver nanoparticles are widely used in cosmetic products like skin lotions and creams, enhancing the possible dermal exposure to nanoparticles [14]. Therefore, for the first time we have investigated the anti-inflammatory activity of silver nanorods synthesized using germinated fenugreek seeds during LPS-induced inflammation in A375 (Human malignant skin melanoma) cell line, and explored the mechanism of action. The extracellular

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concentration and the expression of mRNA of the marker proteins viz., IL-1, IL-6 and macrophage chemoattractant protein-1 (MCP-1) were evaluated. This study might lead to the validation of biomedical activity of the silver nanorods synthesized by us.

2. Experimental

2.1. Synthesis of germinated fenugreek seed extract stabilized silver nanorods (GFS-SNR)

Dry fenugreek seeds were purchased from local market and the extraneous matter were cleaned. The seeds were germinated by soaking them in water for a day, and then incubating under room temperature for two days. The germinated sprouts were then extracted and used for reducing silver nitrate to silver nanorods, which was then characterized as reported earlier [1]. The optimized protocol is briefly described here: 20g of the germinated seeds were heated with 150 ml of Millipore water at 80°. The mixture was then filtered thrice using Whatmann no.1 filter paper. The filtrate was then called germinated fenugreek seed extract (GFS), which was stored at -20° C. Silver nitrate was purchased from Aldrich chemicals (99.9% purity). A 1mM solution of silver nitrate solution was prepared using double distilled water and stirred for 10 minutes. To the solution, GFS added dropwise. The mixture was then refluxed at 80 - 90° degree for 2 hours to obtain colloidal silver which was further air dried and stored. The product was then characterized using called germinated fenugreek seed stabilized silver nanorods (GFS-SNR), which was then characterized using RIGAGU X-ray diffractometer, FE-SEM and Perkin – Elmer Infra-red Spectroscopy.

A375 (Human malignant skin melanoma) cells were procured from PCBS (Pondicherry, India). A375 (Human malignant skin melanoma) cells were maintained in DMEM, 10% Fetal Calf Serum at 37°C in a humidified atmosphere containing 5% CO_2. Lipopolysaccharide (LPS, *Escherichia coli* 0111: B4) was obtained from Sigma-Aldrich. MCP-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biosource International, Inc. (Camarillo, CA, U.S.A.).

2.2. Experimental protocol for cell line studies

A375 (Human malignant skin melanoma) cells were seeded in 6-well plates at a density of $1x10^5$ cells per well. The cell population was cultured to get 70-80% sub confluence and incubated for a day in serum-free medium, permitting organized cell growth. After this, the cells were divided into different aliquots. One aliquot was left as control. Another aliquot was incubated with 100 ng/ml of LPS for 10 hours. A third aliquot was pretreated with the IC_{50} concentration of GFS-SNR is 50µg/ml for 1 h, and then incubated with 100 ng/ml LPS for 10 hours. Francis *et al*., [15] has used nanoparticles to investigate IC_{50} concentrations for biomedical applications. Similarly we have reported the IC_{50} concentration and the therapeutic concentration of GFS-SNR as 50µg/ml.

2.3. Determination of IL-1, IL-6 and MCP-1 proteins

After the incubation time, the supernatant of the cells were subjected to IL-1, IL-6 and MCP-1 assay, along with untreated control cells. The Interleukins IL-1 and IL-6 were quantified using ELISA method. 100 µl of Tris and Nacl buffer were used as Standard diluent buffers, 100 µl of standards and cell hydrosylate (>1:10 dilution) were filled in microtiter wells and thoroughly mixed. The wells were covered and incubated for 2 hours. The liquid from the wells are discarded and washed well for 4 times thoroughly. 100 µl Biotin-conjugated Detection Antibody IL-1 and IL-6 (phosphoELISA™) solution was pipetted into each well except the chromogen blank, the side of the plate was gently tapped to mix the solution. The wells were covered for 1 hour at room temperature. The solution present in the well is discarded and washed well for 4 times thoroughly. 100 µl anti-rabbit IgG-HRP (phosphoELISA™ kits, ABCAM, Merck Germany) solution was pipetted to each well except the chromogen blank. The wells are covered and incubated for 30 minutes at room temperature. Again the solution present in the wells were discarded and washed thoroughly for 4 times. 100µl of Stabilized Chromogen was pipetted to each well. The liquid in the

wells will begin to turn blue. Incubate for 30 minutes at room temperature and in the dark. The readings were read at 410nm. The specificity of this method is 1 pg/ml.

MCP-1 was quantified using a sandwich ELISA kit, as per the manufacturer's instructions, as briefed below: The ELISA plates were coated with anti-MCP-1 primary antibody and incubated for 2 hours at room temperature. The wells were then differently filled with the samples and standards and incubated for 1 hour. The wells were then rinsed with water and then filled with biotinylated goat anti-rat MCP-1 antibody and incubated for 1 hour. The plates were washed again, and then streptavidin-labeled horseradish peroxidase was added and incubated for 10 min. The wells were again washed and filled with tetramethylbenzidine which led to color development. After this, 1 mol/l H_2SO_4 was added and the absorbance was measured at 490 nm. Values were expressed as pg/ml.

2.4. Determination of Expression of mRNA of IL-1, IL-6 and MCP-1

IL-1, IL-6 and MCP-1 mRNA expression were determined using Real-Time RT-PCR. Total mRNA of the cultured A375 cells were isolated using TRIzol and reverse transcribed into cDNA according to previously reported protocol [16]. PCR was performed in a 15 μl PCR mixture volume consisting of 7.5 μl of SYBR green Real-Time PCR master mix (TOYOBO, Osaka, Japan) containing Hotmaster *Taq* DNA polymerase and SYBR solution, 0.3 μl (10 μM) of each forward and reverse primer, 1 μl of cDNA, and 5.9 μl of nuclease-free water. PCR amplification reactions were performed in a Chromo4 Four-color. Real-Time PCR Detection System (Bio-Rad, California, U.S.A.). Thermal cycle conditions were as follows: initial denaturation was at 94 °C for 10 min, followed by 40 cycles of amplification at 94 °C for 15 s and annealing at 60 °C for 1 min, with extension at 60 °C for 1 min. Each sample was analyzed in triplicate. Data were normalized to GAPDH and calculated as the change (*n*-fold) in value of the treatment groups over the control groups according to the 2_DD*Ct* method [17]

The following primers were used:

GAPDH:
5_-CAGGGCTGCTTTTAACTCTGGTAA-3(Forward), 5-GGGTGGAATCATATTGGAACATGT-3_(Reverse);

IL-1:
5_-GAATTGAATGGGTTTGCTAGA-3_ (Forward), 5_-CACTGTGAGGTAAGATGGTGG-3_ (Reverse);

IL-6:
5_-GAATTGAATGGGTTTGCTAGA-3_ (Forward), 5_-CACTGTGAGGTAAGATGGTGG-3_ (Reverse);

MCP-1:
5_CAGCCAGATGCAATCAATGC-3_ (Forward), 5_-GTGGTCCATGGAATCCTGAA-3_ (Reverse);

2.5. Statistical analysis

Data are expressed as Mean ± Standard deviations (SD) of experiments done in triplicate. Data were analyzed using ANOVA for multiple comparisons to determine the significance between different groups and the results were considered statistically significant when $p<0.05$.

3. Results and discussion

Amount of interleukins in different aliquots of cells (untreated control cells, LPS-treated cells and LPS+GFS-SNR- treated cells) are displayed (Table 1). LPS treated cells showed a significant increase in the IL-1 cum IL-6 level when compared to control cells. However, the cells treated with LPS+GFS-SNR showed the reverse trend as revealed by a significant decrease in IL-1 cum IL-6 level when compared to LPS-treated cells.

Table 1. Concentrations of Interleukins (IL-1, IL-6) and MCP-1in pg/ml in different aliquots of A375 (Human malignant skin melanoma) cells.

Tested Groups	**IL-1**	**IL-6**	**MCP-1**
Control	315±5.2	336±4.6	22±1.6
LPS	860± 10.5	895±10.9	73± 1.1
LPS+GFS-SNR	542± 7.8	482±7.1	32± 1.5

The amount of MCP-1 in the cell supernatant is shown in the Table 1. The MCP-1 protein level was highest in LPS-treated cell, which were significantly turned down on pre-treatment with GFS-SNR. That is, the MCP-1 value was equivalent to near normal values (i.e. near untreated control values) in LPS+GFS-SNR treated cells.

Previous, report suggests that skin cells are not only a major target for wounds but also the target for many infections that leads to inflammation. Kurane *et al*., [18] and Gabay *et al*., [19] have reported the correlation between the upregulation of inflammation markers during infection and inflammation response. LPS is used to induce inflammation in cells *in vitro* and *in vivo* because it mimics the natural inflammation process by producing the inflammatory markers [20]. Therefore, it is logical to perform anti-inflammatory studies in LPS-treated skin cell line model. We intended to study the anti-inflammatory activity of GFS-SNR and explore its mechanism using LPS-treated skin cell. Our results reveal that the GFS-SNR prevents LPS-induced inflammation by decreasing the expression of IL-1, IL-6 and MCP-1 mRNA and simultaneously decreasing the levels of IL-1, IL-6 and MCP-1.

Cytokines play an important role in the pathogenesis of a wide variety of inflammations and tissue injury. IL-1 and IL-6 belongs to Cytokine family. Monocyte chemoattractant protein-1 (MCP-1) is a member of C-C chemokine family, which is a primary chemotactic for mononuclear leukocytes. In our study, LPS-treated skin cell line showed a significant increase in IL-1, IL-6 and MCP-1 levels. Cytokines and the Chemokines are over expressed in inflammatory cells. Recently, Zhong *et al*., [21] have reported altered levels of interleukins and the chemokine MCP-1 during LPS- inflammation *in vivo* and *in vitro* in renal cells. Anand *et al*., [22] also reported that MCP-1 secretion by human dermal microvascular endothelial cells and human umbilical vein endothelial cells increased upon treatment with LPS. We therefore suggest that LPS has upregulated the levels of IL-1, IL-6, and MCP-1 in skin cell lines.

We have observed a concomitant decrease in the levels of IL-1, IL-6 and MCP-1 in the media of GFS-SNR+ LPS treated cells (GFS-SNR pretreated cells). The concentration of these proteins can be used to assess the anti-inflammatory activity of test compounds of interest. For example, Comalada *et al*., [23] and Heras *et al*., [24] have proven the anti-inflammatory activity of polyphenolic compounds, quercetin and terpenoids using interleukins and macrophage chemoattractant proteins as markers. Those studies have reported the down regulation of interleukins and MCP-1 by anti-inflammatory compounds. Therefore, we have also used these markers to analyze the anti-inflammatory efficacy of our test compound, GFS-SNR and our results coincide with the previous findings. Hence, we report that GFS-SNR inhibits LPS-induced inflammation and protects skin cells by down regulating the expression of IL-1, IL-6 and MCP-1.

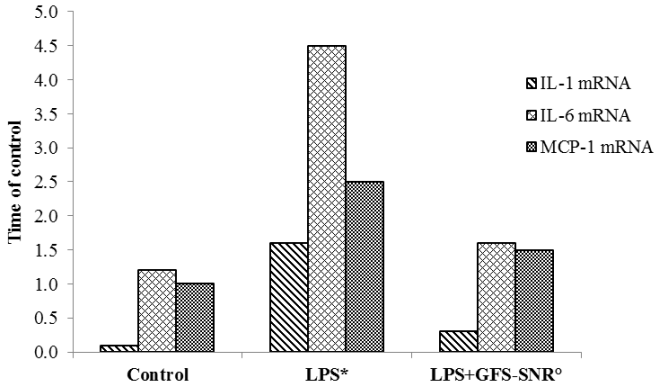

*Fig. 1 Levels of IL-1, IL-6 and MCP-1 mRNAs in different aliquots of A375(Human malignant skin melanoma) cells. (Values plotted are average of experiment done in triplicates). * Values differ significantly from Control group. °Values differ significantly from LPS treated groups.*

We have determined the expression of IL-1 mRNA, IL-6 mRNA and MCP-1 mRNA in different groups of cells (Figure 1). In LPS-treated cells, the expression of all the three types of mRNAs increased significantly as compared to control cells. These results suggest that LPS-mediated inflammation is due to long term activation mechanism. In GFS-SNR+LPS treated cells, the expression of IL-1, IL-6 and MCP-1 mRNAs were reduced when compared to LPS treated cells, indicating the attenuation of LPS- induced inflammation by GFS-SNR in A375 (Human malignant skin melanoma) cells. FTIR spectrum of GFS-SNR reveals the presence of phenolic coat on the surface of the silver nanorods [1]. Zhong *et al*., [21] have proved the anti-inflammatory effect of phenolic compound on LPS-induced inflammation *in vivo* and *in vitro*, and suggested that phenolic compound function as anti-inflammatory agent by inhibiting the expression of interleukin and MCP-1 mRNAs. Wong *et al*., [25] has reported the anti-inflammatory activity of silver. Correlating these reports with our present findings, it could be suggested that GFS-SNR functions as anti-inflammatory agent by decreasing the expression of mRNA of IL-1, IL-6 and MCP-1. Our hypothesis is further supported by the fact that, decrease in the expression of mRNAs of IL-1, is associated with a decrease in the MCP-1 protein in GFS-SNR+LPS treated cells.

4. Conclusions

This is the first study to prove the anti-inflammatory activity of the therapeutic dose of GFS-SNR. From this study we speculate that the anti-inflammatory activity of the GFS-SNR may be due to: i) its increased surface to volume ratio contributed by its nanoscale dimension ii) the surface-bound phytochemicals such as polyphenols, contributed by the germinated fenugreek seeds iii) rapid membrane transport contributed by its size iv) inhibition of mitochondrial dehydrogenase pathway, contributed by the surface bound phenolic moieties. We have already optimized the synthesis protocol and disclosed the anti-cancer activity of GFS-SNR. Taken together, we conclude that the GFS-SNR could emerge as a potential therapeutic tool for wound healing, cancer and other diseases which is related to inflammation.

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