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RESEARCH ARTICLE

EVALUATION OF *IN VITRO* AND *IN-VIVO*ANTI-INFLAMMATORY PROPERTY EXHIBITED BY GOLD NANOPARTICLES USING INDOLE-3-CARBINOL

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ABSTRACT

Nanotechnology has emerged as an exciting approach in the drug development process and among the various nanoparticles, gold nanoparticles have been explored for its variety of medical applications. The present study demonstrates the *in-vitro* and *in-vivo* anti-inflammatory activity of biogenic gold nanoparticles using indole-3-carbinol (AuNPI3Cs). The anti-inflammatory activity of AuNPI3Cs was evaluated by *in vitro* methods and *in vivo* study were performed by carrageenan induced mice paw edema model to evaluate acute inflammation. AuNPI3Cs exhibited dose-dependent human red blood cell membrane stabilizing activity and prompted the inhibition of protein denaturation which was comparable to the standard anti-inflammatory drug, diclofenac sodium. *In vivo* study revealed that AuNPI3Cs significantly reduced mice paw edema after the induction of acute inflammation. The findings suggest that the synthesized gold nanoparticles from indole-3-carbinol could be a potent anti-inflammatory agent.

Key words: Gold Nanoparticles, Carrageenan.

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INTRODUCTION

Inflammation, which is a complex reaction of the immune system against infection and injury that leads to removal of unwanted factors and restoration of tissue structure and physiological function (Ricciotti and FitzGerald, 2011). The symptoms of inflammation are characterized by pain, heat, redness, swelling and loss of function. Inflammation can be classified into acute or chronic, based on the duration of the inflammatory reaction. It is initiated as a protective phenomenon, but loss of regulation of this complex process can lead to the development of various inflammatory disorders. The current pharmacological management of inflammation is occured mainly by two groups of drugs- the steroidal anti-inflammatory drugs and the non-steroidal anti-inflammatory agents. However these conventional drugs are associated with numerous side effects that has obliged the need for identification of alternative substances that can resolve inflammation in such a way that will be homeostatic, modulatory, efficient, and well-tolerated by the body (Recio *et al.,* 2012).

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One such alternative rationale for treatment of inflammatory disorders is phytomedicine-based drugs. Purified natural compounds from plants have aided in the synthesis of new generation anti-inflammatory drugs with higher therapeutic value and lower toxicity (Anilkumar, 2010). The major disadvantage of plant based drugs as pointed out by several research studies, is the lack of quality in the production and trade of phytomedicinal products (Rates, 2001). Another alternative method to develop newer anti-inflammatory agents with sustained release and better efficacy is the use of nanotechnology for drug development and delivery. Nanotechnology deals with the synthesis and fabrication of materials at the nanoscale level (1-100 nm) (Pitkethly, 2004). Metal nanoparticles can be prepared by physical, chemical and biological routes (Iravani *et al.,* 2011). Various metals like copper, titanium, gold, silver and iron were used for the synthesis of nanoparticle. Among the noble metals, gold nanoparticles (AuNPs) have become the focus of intensive research due to its wide ranges of application in public life and industry (Zhang *et al.,* 2016). Recently, biosynthetic methods employing naturally occurring reducing agents such as polysaccharides, biological microorganism or plants extract, have emerged as a simple and viable alternative to more complex physical and chemical synthetic procedures to obtain

AuNPs. In the recent decades, increased development of green synthesis of nanoparticles has become expectable because of its incredible applications in all fields of science Indole-3-carbinol, a phytochemical derived from the breakdown of glucosinolate, present at relatively high levels in most cruciferous vegetables such as brussels sprouts, broccoli, cabbage, cauliflower, turnips, collard greens and kale (Fujioka *et al.,* 2016; Licznerska and Baer-Dubowska, 2016). In recent timesI3C has become available as a nutritional supplement as well as an outstanding natural product for drug development in the pharmaceutical industry. It has been stated to displayvarious promising biological properties, with antioxidant, anti-atherogenic, anti-inflammatory, and anti-carcinogenic activities (Fuentes *et al.,* 2015; Maruthanila *et al.,* 2014). I3C suppressed the penetration of immune cells into the lung and formationof pro-inflammatory cytokines such as IL-6, TNF-α in broncho-alveolar lavage fluid in the lipopolysaccharide-induced acute lung injured mouse. IL-1β secretion was also suppressed by I3C in nigericin treated *in vivo* model (Jiang *et al.,* 2013). It has been revealed that I3C can suppress lipopolysaccharide-induced nitric oxide generation, iNOS and proinflammatory cytokines in macrophages (Chen *et al.,* 2003). The present study was undertaken to evaluate *in-vitro* and *in vivo*anti-inflammatory activity of synthesized gold nanoparticles using indole-3-carbinol.

MATERIALS AND METHODS

Chemicals and reagents: All the chemicals and reagents used in the present study were of analytical grade and procured from Sigma Chemical Co. (St. Louis, MO), USA and Merck.

Synthesis of bioengineered gold nanoparticles: Firstly, 0.05 mg ml^{-1} I3C was suspended in triple distilled water and then an aqueous solution of 0.5 mM hydro-chloroauric acid ($HAuCl_4$) was treated for 10-15 min at room temperature with the help of magnetic stirrer. After filtration, obtained gold nanoparticles using indole-3-carbinol (AuNPI3Cs) were centrifuged at 10,000 rpm for 10 min followed by redispersion of the pellet and was stored at 4°C for use. Thereafter, the purified suspension or powder of AuNPI3Cs was used to assess *in-vitro* and *in vivo* anti-inflammatory activity of synthesized gold nanoparticles using indole-3-carbinol (Pradhan *et al.,* 2016).

In-vitro anti-inflammatory activity

Human red blood cell (HRBC) membrane stabilization method: From healthy human individual, blood was collected and equal volume of Alsever's solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl) was added to it and it was centrifuged for 10 min at 3,000 rpm. 10% HRBC suspension was prepared by washing the obtained packed RBC with normal saline. Using distilled water, different concentrations (50,100,150, 200 and 500 µg ml^{-1}) of AuNPI3Cs were made and to each concentration, 1 ml of phosphate buffer, 2 ml of hypo saline (pH-4.5) and 0.5 ml of HRBC suspension were added and incubated for 30 min at 37°C. After centrifugation at 3,000 rpm for 20 min, the optical density was measured spectrophotometrically at 560 nm (Kamalutheen *et al.,* 2009). Diclofenac sodium was used in the experiment as a standard drug.

The percentage (%) of HRBC membrane stabilization was measured by the following formula:

$$\text{Percent Protection}(\%) = \frac{100 - \text{OD of drug treated sample} \times 100}{\text{OD of Control}}$$

Inhibition of protein denaturation

Briefly, to assess the inhibition of protein denaturation, 1% aqueous solution of BSA was added to AuNPI3Cs and all the reaction mixture was adjusted to pH 6.3 using small amount of hydrochloric acid (HCl) and the mixture was incubated at 37°C for 20 min. Then it was heated at 51°C for 20 min and cooled under tap water. The absorbance of the samples was noted spectrophotometrically at 660 nm (Deshpande *et al.,* 2009). Diclofenac sodium was used as standard drug and the experiment was repeated thrice.

The percent inhibition of protein denaturation was measured as follows:

$$\text{Percent Inhibition} = 100 - \frac{(\text{OD of test} - \text{OD of product control}) \times 100}{\text{OD of control}}$$

In-vivo anti-inflammatory activity

Assay of carrageenan-induced edema in rats

To study *in vivo* anti-inflammatory activity of AuNPI3Cs, mice paw edema model was considered.

Group I: Distilled water control (10 ml/kg body wt.)
Group-II: Carrageenan control
Group III: AuNPI3Cs (0.5, 1.5, 3, 5 mg/kg body wt.) treated in carrageenan induced mice
Group IV: Indomethacin (10mg/kg body wt.) treated in carrageenan induced mice.

0.1 ml of 1% sterile carrageenan in normal saline was administered sub-plantarly into the right hind paw of mice to induce paw swelling. At dose of 0.5, 1.5, 3 and 5 mg/kg body wt., AuNPI3Cs was administered orally 60 min before carrageenan injection (Winter *et al.,* 1962). Control group received the distilled water (10 ml/kg bwt) and standardanti-inflammatory drug indomethacin (10 mg/kg) was used as positive control. The inflammation was measured at 0, 1, 2, 3, and 4 h after carrageenan injection by calculating the volume displaced by the paw, using verniercalipers. The *in vivo* anti-inflammatory activity was calculated by using the following formula. Here, T, Thickness of paw in control group; T_0, Thickness of paw edema in the AuNPI3Cs treated group.

$$\%\text{ of inhibition of edema} = \frac{T - T0}{T} \times 100$$

Statistical analysis

Results were expressed as Mean ± SEM. Results were analysed using one way ANOVA. Differences were considered significant at $p<0.05$ level.

RESULTS AND DISCUSSION

Anti-inflammatory activity of AuNPI3Cs was found to be concentration dependent and HRBC cell membrane stabilizing activity of AuNPI3Cs was 84.2% at 500µg/ml dose that is comparable to that standard drug diclofenac sodium (89.2%) (Table 1).

Table 1. Human red blood cell (HRBC) membrane stabilization activity of AuNPI3Cs

Concentration (µg/ml)	HRBC membrane stabilizing activity (%)		
	Diclofenac sodium	I3C	AuNPI3Cs
50	75.45±.55	50±1.02	61±1.1
100	82.2 ±0.95	58.2±1.15	67.4±1.12
150	83.2 ±1.04	63.75±1.25	72.4±0.85
200	85.8 ±0.95	72.3±1.35	78.45±0.82
500	89.2 ±1.07	80.2±0.83	84.2±0.53

Table 2. Effect of AuNPI3Cs on protein denaturation

Concentration (µg/ml)	Percentage of Protein denaturation (%)		
	Diclofenac sodium	I3C	AuNPI3Cs
50	21.3±0.9	15.8±0.58	21.7±0.7
100	37.9±1.1	28.3±0.65	34.4±0.95
150	42.8±0.95	35.2±1.05	45.2±1.13
200	57.3±1.19	48.4±0.78	53.4±0.87
500	85.5±1.08	55.5±0.54	83.2±0.55

Table 3: Effect of AuNPI3Cs in carragenan induced paw edema model

Groups	Paw edema (mm)					
	0 hr	30 min	1hr	2hr	3hr	4hr
Carrageenan	1.61±0.022	3.4±0.083	4.1±0.12	4.02±0.15	3.9±0.09	3.98±0.12
Carrageenan + Indomethacin (10mg/kg bwt)	1.63±0.01	3.206±0.017	3.173±0.017	3.023±0.014	2.8±0.014**	2.526±0.014**
Carrageenan + I3C (50 mg/kg bwt)	1.65±0.07	3.3±0.05	3.21±0.01	3.156±0.023	3.03±0.08	2.92±0.02*
Carrageenan + I3C (75 mg/kg bwt)	1.65±0.03	3.3±0.03	3.22±0.02	3.1±0.04	3.05±0.01	2.95±0.05*
Carrageenan + I3C (100 mg/kg bwt)	1.68±0.03	3.35±0.03	3.28±0.03	3.1±0.02	3.01±0.01	2.92±0.05*
Carrageenan + I3C (150 mg/kg bwt)	1.6±0.017	3.38±0.014	3.32±0.04	3±0.01	3.07±0.04	2.85±0.01**
Carrageenan + AuNPI3Cs (0.5 mg/kg bwt)	1.65±0.017	3.283±0.020	3.16±0.04	3.056±0.023	2.9±0.028*	2.8±0.028**
Carrageenan + (AuNPI3Cs 1mg/kg bwt)	1.75±0.03	3.31±0.03	3.18±0.023	3.1±0.04	2.9±0.01*	2.8±0.05**
Carrageenan + AuNPI3Cs(2 mg/kg bwt)	1.65±0.017	3.1±0.014	3.3±0.014	3±0.011	2.7±0.014*	2.5±0.011**
Carrageenan+ AuNPI3Cs (4 mg/kg bwt)	1.623±0.014	3.05±0.017	3.1±0.011	3±0.011	2.6±0.02*	2.45±0.014**

In vitro Anti-inflammatory activity of synthesized gold nanoparticles (AuNPI3Cs) was studied by the inhibition of protein denaturation and stabilization of HRBC membrane activity. Few studies (Chandra *et al*., 2012) proved that destabilization of cell membrane especially lysosomal membrane, denaturation of proteins particularly blood proteins are the major causes of arthritis and inflammation. Human red blood cell membrane stabilizing activity was shown by AuNPI3Cs and this membrane stabilization effect of AuNPI3Cs is due to inhibition of hypotonicity induced lysis of erythrocyte membrane. Erythrocyte membrane is analogous to the lysosomal membrane and AuNPI3Cs stabilize erythrocyte membrane. So, it may be concluded that AuNPI3Cs can also stabilize lysosomal membranes (Kumar *et al*., 2011).

Stabilization of lysosomal membrane is significant to prevent the inflammatory process by the prevention of the release of lysosomal constituents of activated neutrophil that contains bactericidal enzymes and proteases which cause further inflammation and tissue damage after extracellular release (Yurugasan *et al*., 1981). Hypotonicity dependent haemolysis may be happened due to cell shrinkage as a result of osmotic loss of intracellular electrolytes and fluid components. AuNPI3Cs may arrest the process by decreasing the efflux of these intracellular components (Yang *et al*., 2010). In this study it was observed that protein denaturation inhibition by AuNPI3Cs was dose dependent and it showed 83.2% inhibition at 500µg ml-1 whereas standard drug diclofenac sodium showed 85.5% protein denaturation (Table 2).By application of stress or external compound, protein may be denatured and they lose their tertiary and secondary structure and fail to do their biological function. Inflammation causes Neutrophils are recognized to be a rich source of serine proteinase and are present at lysosomes. During inflammatory reactions leukocytes proteinase play a vital role in the development of tissue damage and noticeable level of protection was provided by proteinase inhibitors (Das and Chatterjee, 1995). In this study, *in vivo* anti-inflammatory effect of AuNPI3Cs was compared to carrageenan-control after 30 min which was comparable to standard drug indomethacin. After 4 h, significant decrease was observed in paw edema in AuNPI3Cs treated groups at 0.5, 1.5, 3 and 5 mg/kg bwt (Table 3) dose levels of AuNPI3Cs. Carrageenan-induced paw edema in rodent is an ideal model to study the acute anti-inflammatory effect of any test compound (Amdekar *et al*., 2015). AuNPI3Cs significantly inhibit paw volume in the carrageenan induced mice paw edema. The decrease in paw volume of the treated groups with respect to time is presented in Table 3. It was observed that anti-inflammatory effect of synthesized AuNPs started at 1 h and reached at peak at 4 h and the maximum inhibitory effect was found between 2 and 4 h. Carrageenan induced paw edema is a biphasic event involving different inflammatory mediators. In the first phase of inflammation that is after 2 h of carrageenan injection, histamine and serotonin play an important role, and in the second phase that is after 3-4 h of carrageenan injection, kinin and prostaglandins play vital role. Histamine, serotonin, kinin and prostaglandins (Yang *et al*., 2010; Shenoy *et al*., 2010) act as a chemical mediators. In our study it was observed that after administration of AuNPI3Cs, edema was inhibited from the first hour and the inhibition was continued in all phases of inflammation. It may be due to inhibition in release of various chemical mediators of inflammation. Carrageenan-induced paw edema is also sensitive to cycloxygenase inhibitors and is used to evaluate the effect of non-steroidal anti-inflammatory

agents like silver nanoparticles those primarily inhibit the cyclooxygenase, a catalysing enzyme for prostaglandin synthesis (Sarkhel, 2016). Both in in-vitro and in-vivo studies, it is evident that AuNPI3Cs possess potent anti-inflammatory effect.

Conclusion

This study has revealed that the green synthesis of gold nanoparticles using indole-3-carbinol has resulted in the formation of biologically active gold nanoparticles that possess prominent anti-inflammatory property. This finding suggests a novel nano-pharmacological rationale for the treatment of various inflammatory disorders.

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