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Original Contribution

Enhanced antilipopolysaccharide (LPS) induced changes in macrophage functions by *Rubia cordifolia* (RC) embedded with Au nanoparticles ☆

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ABSTRACT

In this paper, we have shown that gold nanoparticles (Au (NPs)) embedded in *Rubia cordifolia* (RC) matrix (RC-Au (NPs)) exhibit a high therapeutic value relating to its anti-inflammatory characteristics. It was prepared by utilizing the reducing properties of RC to convert $HAuCl_4$ into Au (NPs). In order to compare its effectiveness, with respect to Au (NPs), the latter was synthesized separately by reducing $HAuCl_4$ with lemon extract. These Au (NPs) along with RC-Au (NPs) were characterized by X-ray diffractometry (XRD), transmission electron microscopy (TEM), and UV-visible spectroscopy. The enhancement in anti-inflammatory characteristics was assessed as its inhibitory potential for lipopolysaccharide (LPS)-induced nitric oxide (NO) release, by rat peritoneal macrophages. The RC-Au (NPs) significantly enhanced its potential to inhibit NO release, which was reported in terms of inhibitory concentration for 50% inhibition (IC_{50} = 11.98 ng/ml), as compared to either RC extract (IC_{50} = 47 × 10^3 ng/ml) or to Au (NPs) (IC_{50} = 587.50 ng/ml).

Introduction

Nanoparticles are used medically for enhancing the therapeutic potential of drugs by altered pharmacokinetics, biodistribution, or cellular uptake. RC has been in clinical use as an antioxidant and anti-inflammatory agent for a long time [1]. Gold has its own importance because of its relatively nonreactive and comparatively safe nature. In Ayurveda, the traditional system of Indian medicine, gold has been in clinical use for centuries and is termed as "Swarna Bhasma" for various ailments [2]. In the allopathic system also, gold salts are used to treat inflammation/arthritis [3,4]. Recently gold nanoparticles are being used for diagnostics and also as a drug carrier [5]. Nanostructures are defined as materials with overall dimension under 100 nm and can be synthesized in a variety of ways. One of them, which corresponds to "green synthesis," is through the reduction of the respective metal salts by plant extracts. This was first reported in 2004 [6], followed by other reports related to flavonoids as the reducing agent [7], and also as a stabilizer such as azacryptand [8]. Recently, Au (NPs) have been employed to boost the antioxidant characteristics of Trolox [9] and melatonin [10] and for enhancing the bioefficacy of antibodies. Studies also suggest their noncytotoxic, nonimmunogenic, and biocompatible properties on RAW 264.7 macrophages [9], along with a reduced rate of release in reactive oxygen and nitrite species. Antioxidants are of prime importance in preventing the progress of age-related chronic and degenerative diseases. Therefore, there is a demand for an efficient antioxidant. Many herbal products are being used. However, these are effective only when given in higher doses. Therefore, enhancement in their anti-inflammatory characteristic would be of great interest. Gold nanoparticles generally have enhanced bioactivity, and thus it is quite likely that their use, in conjunction with RC, may enhance anti-inflammatory characteristics. However, in this process, it is logical to study the toxicity of the nanoparticles, because while enhancing the efficacy, the associated toxicity also increases. Besides, some of the nanostructures, such as carbon nanotubes, by themselves, have also been associated with induction of inflammation [11]. In this paper, the HPLC-standardized-alcoholic extract of the roots of *Rubia cordifolia*

Abbreviations: Au (NPs), gold nanoparticles; EDAX, energy dispersive X-ray analysis; LPS, lipopolysaccharide; NO, nitric oxide; RC, *Rubia cordifolia*; RC-Au (NPs), *Rubia cordifolia*-embedded gold nanoparticles; SAED, selected area electron diffraction pattern

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Linn (RC) has been used [12]. Several preparations of RC are in clinical use as a blood purifier, analgesic, antiallergic, anticancer, and as an anti-inflammatory agent. In the past, we have also reported its antioxidant [13], anti-inflammatory [14], radioprotective, and anticancer properties [15–17]

In the present study, RC extract was ultrasonicated for 1 h with a standard solution of hydrogen tetrachloroaurate ($HAuCl_4$, 0.0005–0.001 M) to synthesize the gold nanoparticles, imbedded in RC matrix (RC-Au (NPs)). They were characterized by XRD, TEM, and UV-visible spectroscopy. They were then tested for bioefficacy, toxicity, and prophylactic response by using LPS-induced NO release in attached rat peritoneal macrophage culture.

Materials and methods

Synthesis of gold nanoparticles (Au (NPs))

In order to check the possible antioxidative properties of Au (NPs), we have synthesized Au (NPs) by reducing the solution of $HAuCl_4$ with lemon extract [12,18]. A solution of 50 ml, 0.001 M (0.39382 mg/ml) $HAuCl_4$ and 50 ml lemon extract, diluted in 50 ml of distilled water, was added together drop by drop and stirred on a magnetic stirrer for 3 h. The solution was ultrasonicated at a high frequency of 10 KHz for 1 h and then kept at a stationary position for 2 h at room temperature. The solution then turned red, indicating the precipitation of Au (NPs). The precipitate was filtered out and then analyzed by X-ray diffraction (X-Pert Pro Panalytical) and transmission electron microscopy (FEI-Tecnai 20 G^2).

Preparation of RC extract

An extract of dried roots of RC was prepared in a continuous Soxhlet extractor by using ethanol as solvent, for 20 h. The extract was distilled, and standardized by HPLC for further biological studies.

Synthesis of RC-Au (NPs)

We next attempted to grow Au (NPs) embedded within the RC matrix. For this, efforts were made to use RC extract itself as the reducing agent. The alcoholic extract of RC contains many polyphenolic compounds with an anthraquinone, known as rubiadin as a major compound of the extract. It is expected to act as reducing agent for $HAuCl_4$ in a manner somewhat similar to that of other reducing agents. The method of forming intracellular Au (NPs) was similar to that described earlier for the reduction of $HAuCl_4$ by lemon extract. For the preparation of Au (NPs) embedded in RC, 20 mg/ml RC extract and 0.001 M (0.39382 mg/ml) $HAuCl_4$ were mixed in an equal volume and sonicated for 3 h. Since the aim of this part of the investigation was to synthesize RC-Au (NPs), several variations such as concentration of RC and $HAuCl_4$, time, and rate of mixing of RC were carried out. The resulting products coming out of each run were investigated in TEM. Through extensive TEM investigations, it was found that Au (NPs) became embedded when the concentration of RC was optimized to be ~20 mg/ml and the time involved in the whole process was ~3 h. Repeated experiments revealed that the growth of embedded Au (NPs) was reproducible. We then proceeded to investigate the anti-inflammatory effects of gold nanoparticles embedded in RC, as against Au (NPs) alone.

Macrophage culture

Rat peritoneal macrophages were isolated by injecting 10 ml sterile ice-cold PBS, (devoid of calcium and magnesium ions) into the peritoneal cavity and squeezing the abdomen for 3–5 min. Then peritoneal fluid was aspirated out and its cells were isolated by centrifugation, followed by 2 washings with serum-free RPMI 1640 media. Finally these cells were suspended in a known volume of complete media with 5% fetal calf serum (FCS) for further studies. For experiments, appropriate numbers of cells were taken in each cavity of 96-well culture plates and incubated at 37 °C in 5% CO_2 atmosphere for 2 h to attach the living macrophages. Finally the culture supernatant was replaced with fresh complete media and these attached macrophages were used for various experiments. NO concentration was determined by the Griess method [19,20] and MTT assay was carried out by the Mosmann technique [21]. All protocols were approved by the Central Animal Ethics Committee (CAEC) at Institute of Medical Sciences, Banaras Hindu University (CAEC No. Dean/11–12/CAEC 563).

Evaluation of the effect of Au (NPs) for LPS-induced NO production

The antioxidant properties of all three samples, i.e., pure alcoholic extract of *Rubia cordifolia*, pure gold nanoparticles, and *Rubia cordifolia*-embedded gold nanoparticles (RC-Au (NPs)), were tested on an *in vitro* test model of lipopolysaccharide (LPS)-induced nitric oxide (NO) production by rat peritoneal macrophages. Here, attached macrophages (1×10^4 cells/well) in 96-well plates were preincubated for 30 min with all 3 agents [RC, Au (NPs), RC-Au (NPs)] separately in triplicate and then a fixed concentration of LPS (20 ng/ml) was added to each well. It was mixed gently and incubated for another 17 h at 37 °C in 5% CO_2 incubator, as per the protocol described earlier [22]. The next day, culture supernatant was saved in a separate plate and subjected to estimation of NO in terms of nitrite concentration. The experiment was independently repeated 3 times and each set of test concentrations was taken in duplicate wells.

The nanoparticles were diluted by mixing with 20% Tween 20 solution in double-distilled water. This Tween 20 is designated as drug vector. In the experimental control wells, only the drug vector was added which did not show any kind of protection. Since the whole process of protection starts within a few minutes of addition of LPS, the cells were preincubated with Au (NPs) for 30 min and then LPS was added. So the stability of these NPs for 30 min was well established because it showed the desired protective response.

Statistical analysis

All data are expressed as mean $\pm$ SD and Pearson's correlation analysis (SPSS 7.5 for Windows, SPSS Inc.) was used to test for the significance of correlation.

Results

Characterization of gold nanoparticles

The as-synthesized nanoparticles by reduction of $HAuCl_4$ with lemon extract were characterized by XRD and TEM. Fig. 1a is the recorded XRD pattern of synthesized material. This pattern has peaks at (2θ) 38.24, 44.37, 64.57, and 77.54° which correspond to diffraction from (1 1 1), (2 0 0), (2 2 0), to (3 1 1) planes of Au lattice structures, respectively. This analysis was further confirmed by extensive TEM investigation employing both the imaging and the diffraction modes. Fig. 1b shows a representative bright-field TEM image of the Au nanoparticles while the inset in Fig. 1b is the typical selected area electron diffraction pattern (SAED) of the gold nanoparticles. The size of Au (NPs) was found in the range between 15 and 30 nm.