Effects of Gold Nanoparticles on Endotoxin-Induced Uveitis in Rats

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**Purpose.** This study evaluates the effects of the gold nanoparticle in endotoxin-induced uveitis in rats.

**Methods.** Adult male Wistar rats were divided into five groups: saline + saline, lipopolysaccharide (LPS) + saline, LPS + prednisolone, LPS + gold salt (GS) and LPS + gold nanoparticle (GNP). Two hours after LPS administration, prednisolone acetate 1%, GS, and GNP were topically applied to both eyes of rats and repeated every 6 hours for 24 hours. After 24 hours, rats were anesthetized and aqueous humor was sampled and the irides were removed. Aqueous humor TNF-α, myeloperoxidase activity were determined. Irides oxidative damage and content of toll-like receptor 4 (TLR4) and nuclear factor-xB (NF-xB) were determined.

**Results.** The administration of LPS-induced eye inflammatory response characterized by an increase in aqueous humor TNF-α, myeloperoxidase, and by irides oxidative damage. All these parameters were decreased by the administration of GNP. Since the inflammatory response secondary to LPS administration depends, in part, to the activation of the TLR4–NF-xB pathway we demonstrated here that a potential mechanism to explain the GNP effects was the decrease on TLR4 content and NF-xB activation.

**Conclusions.** These findings suggest that topical GNP decreases intraocular inflammation and oxidative damage by interfering in the TLR4–NF-xB pathway. (Invest Ophthalmol Vis Sci. 2012; 53:8036–8041) DOI:10.1167/iovs.12-10743

**U**veitis is an ocular condition characterized by intraocular inflammation, usually caused by several different etiologies and is a significant cause of visual loss,1–3 being highly prevalent worldwide.4 Various chemical mediators are shown to play key roles in ocular inflammation including pro-inflammatory cytokines,5 activation of intracellular signaling pathways5,6 and nuclear factor-xB (NF-xB),7 and leukocyte adhesion molecules.8,9 Oxidative stress is also suggested to be pathogenic by inducing inflammation in the eye.10–11 Endotoxin-induced uveitis (EIU) is an established animal model for ocular inflammation. These animals develop acute bilateral anterior inflammation, characterized by a breakdown of the blood–ocular barrier and accumulation of inflammatory cells.12 Oxidative biomarkers are shown to be elevated in EIU, suggesting that inflammation and oxidative stress cooperatively contribute to its pathogenesis.13

Over the past few decades, gold nanoparticles (GNP) have become the object of special interest due to their anti-inflammatory activity.14 Gold compounds have been used as anti-inflammatory treatment against rheumatoid arthritis for more than 50 years.15–17 The molecular mechanisms of gold actions include modulation of pro-inflammatory, as well as inactivation of phagolysosomal enzymes and inhibition of NF-xB.18,19

In view of these anti-inflammatory properties of gold compounds, the present study investigates the potential applications of GNP in the modulation of the inflammatory response in an animal model of uveitis.

**Materials and Methods**

**GNPs Preparation**

GNP were prepared as described by Turkевич et al.20 In brief, glass vials were washed in aqua regia and rinsed in ultrapure water, then aurochloric acid solution (0.2 mM) was gently warmed up to 90°C and sodium citrate (39 mM) was added. After 20 minutes of vigorous stirring and refluxing, the solution was cooled at room temperature (20 ± 2°C) and the GNP were purified by serial centrifugations at 9000g for 10 minutes, followed by supernatant removing and ultra pure water rinsing. The resulting powder was resuspended in saline solution (NaCl 0.9%) and stored at 7°C until use.

The UV-visible measurements were performed employing a Shimadzu UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan). A Varian AA240Z atomic absorption spectrometer (Varian, Loveland, CA) was used to evaluate the concentration of gold (Au). The GNP were

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The irides were excised and stored at −80°C prior to analysis. The morphology and average particle size were determined by electron transmission microscopy analysis (TEM) using a JEOL Titan 80-300 kV and X-ray diffraction (XRD) (Jeol, Tokyo, Japan) equipped with a Shimadzu XRD-6000 diffractometer with Cu X-ray source with a wavelength of 1.5406 Å as radiation wavelength. 30 kV voltage and 30 mA current (Shimadzu, Tokyo, Japan). The average particle size was also calculated from XRD measurements applying Scherrer’s equation, \( L = \frac{h}{\beta \cos(\theta)} \), where \( k = 0.94 \), a constant characteristic of near spherical nanoparticles, \( \lambda \) is the wavelength of the radiation used, \( \beta \) is the full width at half maximum of the peak in radians and \( \theta \) is the Bragg angle, as described previously. \(^{21}\)

**Animals**

Male Wistar rats weighing 300 to 350 g obtained from Universidade do Extremo Sul Catarinense breeding colony were housed individually under standard conditions (12-hour light/dark cycles with room temperature of 22–24°C). All studies were performed in accordance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with the approval of the Ethics Committee from Universidade do Extremo Sul Catarinense. Experimental animals were first randomly divided into five groups (12 animals per group): saline + saline, lipopolysaccharide (LPS) + saline, LPS + prednisolone, LPS + GS, and LPS + GNP.

**EIU and Treatments**

EIU was induced by the administration of LPS (Escherichia coli, serotype 055: B5, Sigma-Aldrich, St. Louis, MO) 100 µg/100 µL pyrogen-free 0.9% sodium chloride into subcutaneous tissue. \(^{22}\) Two hours after LPS administration, saline, prednisolone acetate 1% (Latinofarma Pharmaceutical Industries, Cotia, Brazil), GS (aurochloric acid 2 mM) and GNP (40 mg/mL) were topically applied to both eyes of rats and repeated every 6 hours for 24 hours. Both GS and GNP were prepared under sterile conditions as isotonic solutions. After 24 hours, rats had been anesthetized with ketamine chloride hydrochloride (50 mg/kg) and xylazine 2% (20 mg/kg), and aqueous humor was sampled bilaterally by aspiration with a 30-gauge needle under microscopic visualization.

**Aqueous Humor Inflammatory Response**

As an index of neutrophil infiltration it was measured myeloperoxidase activity as previously described. \(^{23}\) Briefly, aqueous humor was homogenized (50 mg/mL) in 0.5% hexadecyltrimethylammonium bromide and was centrifuged at 15,000 g for 40 minutes. One aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM hydrogen peroxide. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37°C.

TNF-α levels in aqueous humor were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

**Oxidative Damage in Irides**

The formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction is widely adopted as a sensitive method for measurement of oxidative damage. \(^{24}\) The irides homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid, and heated in a boiling water bath for 30 minutes. TBARS were determined by absorbance at 532 nm. Results were expressed as malondialdehyde (MDA) equivalents (nmol/mg protein). Protein oxidation was measured by the determination of carbonyl group content based on the reaction with dinitrophenylhydrazine (DNPH), as previously described. \(^{25}\) Proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in DNPH, and absorbance was monitored at 370 nm.

**Western Blot Analyses for Toll-Like Receptor 4 (TLR4) and Nuclear Factor–κB in Irides**

NF-κB determination was performed in nuclear extracts and TLR4 in whole tissue homogenates from irides. The samples were centrifuged at 11,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 40 minutes to remove insoluble material, and the supernatants were used for protein quantification, using the Bradford method. \(^{26}\) Extracted proteins were denatured by boiling in Laemmli’s sample buffer containing 100 mM dithiothreitol (DTT), run on SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked, probed with anti-TLR4 and anti-NF-κB antibodies (Santa Cruz Biotechnology, Inc.,

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**FIGURE 1.** Gold nanoparticles characterization. (A) Representative image of a transmission electron microscopy analysis of gold nanoparticles (300 kV). (B) X-ray diffraction spectrum of gold nanoparticles.
Santa Cruz, CA) and developed as described previously.28 The blots were exposed to preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens (Kodak Brazil, Sao Paulo, Brazil) at \(-80^\circ\text{C}\) for 12 to 48 hours. Band intensities were quantitated by optical densitometry (Scion Image software, ScionCorp, Frederick, MD) of the developed autoradiographs.

**Statistical Analysis**

Results are expressed as mean \(\pm\) SD. Differences between groups were determined by ANOVA followed by the Tukey's test. A \(P\) value less than 0.05 was considered statistically significant. All statistical analyses were performed using a statistical package (SPSS 20.0 for Windows; SPSS, Inc., Chicago, IL).

**RESULTS**

**Characterization of GNP**

The UV-Visibile spectrum for the GNP solution shows a band with maximum absorption at 520 nm due to Surface Plasmon Resonance (SPR) that can be attributed to the collective oscillation of free conduction electrons by an interacting electromagnetic field.29 This narrow band is typical of monodispersed and spherical GNP.21 Atomic absorption analysis reveals a 36 ppm Au concentration. TEM image (Fig. 1A) shows predominantly near spherical gold nanoparticles with narrow size distribution and a mean diameter of 30 nm. As seen in Figure 1B, the XRD analysis of the nanoparticles, performed between 30° to 70°, showed large peak close to 38°.
and two minor \(200\) and \(220\) corresponding to Bragg's reflection (JCPDS number 4–0784). The \(111\) peak was used to estimate the average size of the nanoparticles, employing Sherrer's equation. The average size obtained, 29 nm, is very close to the value from TEM images. In addition, the \(111\), \(200\), and \(220\) reflections suggest a face centered cubic structure of metallic gold.

**Effect of GNP on EIU-Induced Inflammatory Markers**

As shown in Figure 2, there were a significantly increase on the levels of TNF-\(\alpha\), and myeloperoxidase activity after LPS administration in the aqueous humor. All treatments were effective in decreasing TNF-\(\alpha\) levels (Fig. 2A), but the effect of GNP was more pronounced when compared with prednisolone and GS (Fig. 2A). The administration of GNP, but not GS and prednisolone, was able to slightly decrease myeloperoxidase activity (Fig. 2B) after LPS.

**Effect of GNP on EIU-Induced Oxidative Damage in Irides**

LPS administration resulted in an increase in the levels of TBARS (Fig. 3A) and protein carbonyls (Fig. 3B) in irides. Both GNP and prednisolone, but not GS, were able to decrease irides oxidative damage induced by LPS.

**Effects of GNP in TLR4 and NF-\(\kappa\)B Levels**

Once GNP exert potent anti-inflammatory effect in this model it was decided to verify if this effects involves, at least in part, the down-regulation of TLR4 and the activation of NF-\(\kappa\)B. LPS administration increases TLR4 levels and GNP was able to
attenuate this (Fig. 4A) and also decrease the nuclear translocation of NF-κB (Fig. 4B).

**DISCUSSION**

In the present study the administration of GNP 2 hours after LPS injection significantly reduced inflammatory parameters and oxidative damage in the eye. In addition, GNP seems to be superior to prednisolone in this model.

Corticosteroids have been the gold standard treatment to uveitis, however its frequent side effects can limit its use. Indeed, many alternative approaches have been shown to prevent ocular inflammation in experimental animals.\textsuperscript{30,31} Recent research, suggests that TLR4–NF-κB signaling pathways might be involved in the pathogenesis of uveitis.\textsuperscript{32,33} Here, we showed a decrease in protein levels of TLR4 after GNP treatment in an animal model of EIU and a consequent reduction of NF-κB nuclear content. Ma et al.\textsuperscript{34} showed that GNP suppressed LPS-induced activation of NF-κB in RAW264.7 cells through the inhibition of protein kinase B (Akt) activity.

The NF-κB activation consequently results in the induction of various pro-inflammatory cytokines, chemokines, and the recruitment of leukocytes to the site of infection. Among these inflammatory mediators, TNF-α reached a peak concentration in the early stages of inflammation; thus, plays an important role in the initiation of the inflammatory response. Studies reported that the concentration of TNF-α in the aqueous humor of uveitis patients was significantly higher than that of a normal control group.\textsuperscript{35,36} In our model, the administration of GNP attenuated the TNF-α levels in the aqueous humor and this effect was superior to that of prednisolone and GS. In fact, corticoids could paradoxically up-regulate some aspects of the inflammatory response.\textsuperscript{37} Koizumi et al.\textsuperscript{38} showed that administration of TNF-α-inhibitor reduces leukocyte rolling and adhesion significantly in EIU, and this is in accordance with our data on myeloperoxidase activity in the aqueous humor.

Reactive oxygen species (ROS) could be mediators of inflammation induced by cytokines and chemokines, which in turn induce intracellular ROS generation by mitochondrial respiratory chain reaction, membrane-bound superoxide-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and arachidonic metabolic reaction. GNP treatment reduced oxidative damage in the irides after LPS, this could be a result of the reduced activation of macrophages, which are a main source of ROS during inflammation. Furthermore, the macrophage-derived ROS are partially mediated by TNF-α, which stimulate the production of ROS by initiating the nuclear translocation of NF-κB, with subsequent induction of inducible nitric oxide synthase and cyclooxygenase 2.\textsuperscript{39,40} These mechanisms could explain both the reduction in TNF-α and antioxidant effects seen in this study, since NF-κB is involved in macrophage activation, ROS formation and transcriptional regulation of inflammatory cytokines.

Some limitations of our study must be pointed out. We do not present data concerning clinical scores, thus, we cannot ascertain whether the observed alterations in inflammatory response contributed to an improvement in parameters with more clinical significance. Since inflammatory response is a major responsible for the occurrence of clinical symptoms in uveitis, we do believe that the observed alterations probably induced improvements in clinical signs of uveitis. The GNP doses were based on pilot studies, thus, we did not present a large dose-response study analyzing all inflammatory and oxidative parameters, and this may limit a more direct comparison of GNP effects with prednisolone. In addition, we did not measure gold concentration in different eye compartments then we can not ascertain the distribution of gold in different eye structures. Despite all of this, it is well known that GNP is readily diffusible and can be distributed to several different components of the eye.\textsuperscript{41} The long term toxicity of GNP is not well known. It is suggested that GNP have, in general, low acute toxicity both in vitro and in vivo, and its toxicity depends on particle size, concentration, and surfaces properties.\textsuperscript{42} Specifically in the eye there is some data on GNP toxicity. In different animals models the intravenous, intravitreal, or corneal administration of GNP seem to have no evident retinal and/or corneal toxicity.\textsuperscript{41–44}

In summary, the present study provides evidence that topical GNP can have anti-inflammatory affects that are, at least in part, secondary to the down-regulation of the TLR4–NF-κB pathway in an LPS-induced uveitis model.

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**References**

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