Nanoparticles show potential to retard bradyzoites in vitro formation of Toxoplasma gondii

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Abstract: Toxoplasmosis is a common parasitic disease caused by Toxoplasma gondii (Nicolle et Manceaux, 1908), an obligate parasite capable of infecting a range of cell types in almost all warm-blooded animals. Upon infecting an intermediate host, the parasites differentiate into tachyzoites which rapidly infect host tissues. Usually, the invading parasites are cleared by the immune system and administered drugs, but some tachyzoites differentiate into bradyzoites forming tissue cysts. These tissue cysts could serve as a source for re-infection and exacerbations. Currently, treatment for toxoplasmosis is limited and, moreover, there are no drugs for treating the dormant cyst stages is lacking. The bradyzoite-containing tissue cysts of T. gondii are responsible for chronic toxoplasmosis. Current treatments for toxoplasmosis include the use of pyrimethamine, sulfadiazine and clindamycin (during pregnancy), which can suppress the active infection (Kamau et al. 2012); however, therapy that targets the dormant cyst stages is lacking. The bradyzoite-containing cysts of T. gondii are responsible for chronic toxoplasmosis. Despite the high prevalence of toxoplasmosis, the discovery of new therapeutic targets has been challenging. Accordingly, new approaches in the treatment of toxoplasmosis are needed. Recently, the application of nanoparticles not only possesses anti-tachyzoite potential but they also have anti-bradyzoite potential.

Keywords: cyst, infection, medicinal biochemistry, nanomedicine, toxoplasmosis

Toxoplasma gondii (Nicolle et Manceaux, 1908) is the causative agent of toxoplasmosis, which affects nearly one-third of the human population (Black and Boothroyd 2000). In healthy individuals, the primary infection can be asymptomatic, but may prove fatal in those who are pregnant or immunocompromised (Black and Boothroyd 2000, Skariah et al. 2010). The parasite transits between the tachyzoite and bradyzoite (cystic) forms, thus making the life cycle complex (Black and Boothroyd 2000). Current treatments for toxoplasmosis include the use of pyrimethamine, sulfadiazine and clindamycin (during pregnancy spiramycin), which can suppress the active infection (Kamau et al. 2012); however, therapy that targets the dormant cyst stages is lacking. The bradyzoite-containing cysts of T. gondii are responsible for chronic toxoplasmosis. The cysts are sequestered in tissues and upon reactivation, can be life-threatening particularly for the immunocompromised patients. Many potential new molecular targets have been identified (Maubon et al. 2010), but, no drug candidate with anti-cystic activity has been reported to date. Therefore, the discovery of new therapeutic agents that are effective on cysts is an important challenge in toxoplasmosis control.

Accordingly, new approaches in the treatment of toxoplasmosis are needed. Recently, the application of nanotechnological approaches for biomedical purposes has rapidly expanded (Curtis and Wilkonson 2001) and nanoparticles are becoming attractive as potential treatment options for various diseases (Debbage 2009, Allahverdiyev et al. 2011). The small size and large ratio of surface area to volume are unique characteristics of nanoparticles, which can be exploited for medicinal value. These properties enhance reactivity and aids easy cellular penetration which might result in interaction with cellular biomolecules in a number of ways (Adeyemi et al. 2017). We recently showed that inorganic nanoparticles have strong activity against the tachyzoites form of T. gondii in vitro and these nanoparticles significantly suppressed parasite growth by ≥ 80% possibly through generation of reactive oxygen species (Adeyemi et al. 2017). Herein, we sought to determine whether nanoparticles can prevent and/or eliminate the cystic forms of T. gondii in vitro.

MATERIALS AND METHODS

Inorganic nanoparticles (NPs) including gold (AuNP, 5 nm), silver (AgNP, 10 nm) and platinum (PtNP, 3 nm) were supplied...
by Sigma-Aldrich (St. Louis, MO, USA). The nanoparticles were used as supplied after they were evaluated to confirm the supplier’s specifications. All reagents were of analytical grade and used as supplied unless otherwise stated.

In vitro effect of NPs on cyst formation as assessed by use of an immunofluorescence assay

ME49 strain of *Toxoplasma gondii* strain was used for this study unless otherwise indicated. The parasite was maintained by repeated passages in monolayers of human foreskin fibroblast (HFF) cells cultured in Dulbecco’s Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) and supplemented with GlutaMAX™-I (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10% (v/v) fetal calf serum (FCS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and penicillin and streptomycin (100 U.ml⁻¹; Biowhittaker, Leicestershire, UK).

In order to obtain parasite suspension, host cells infected with *T. gondii* tachyzoites were lysed by passing them through a 27-gauge needle. The cell lysates were then filtered and washed three times in fresh culture medium to yield a tachyzoite suspension free of host cell debris. The parasite density was measured with a hemocytometer and adjusted for *in vitro* experimental infection analysis.

To assess the effect of the NPs on cyst formation, we examined *in vitro* differentiation by using an immunofluorescence assay (IFA) under two different conditions. For bradyzoite induction, ME49-infected HFF cells were treated with 1% FCS in DMEM containing 25 mM HEPES [pH 8.1] but no NaHCO₃ from 24 h after parasite inoculation, in humid air, for 72 h. From our preliminary experiments, cyst formation *in vitro* was achieved (>50% cyst wall positive of total parasites) after at least a 48-hr incubation under these bradyzoite-inducing cell culture conditions. NP concentrations were selected on the basis of a previously published report of the effective concentrations for tachyzoite death (Adeyemi et al. 2017).

First, we sought to determine the effect of NPs on cysts *in vitro*. Briefly, ME49 strain of *T. gondii* was inoculated onto confluent HFF monolayers on coverslips at a multiplicity of infection (MOI) of 0.5. The coverslips were incubated at 37°C and 5% CO₂ for 24 h to allow for invasion and infection. The infection medium was then removed and replaced with bradyzoite-inducing medium (pH 8.1, containing 1% FCS in DMEM). Further incubation at 37°C and 0% CO₂ was then carried out for 72 h. Thereafter, the NPs were added in fresh bradyzoite-inducing medium and the coverslips were incubated at 37°C and 0% CO₂ for 24, 48 and 72 h. At each of these time points, the coverslips were fixed with 4% paraformaldehyde (PFA) and stained with an anti-CST-1 Salmon E monoclonal antibody as described elsewhere (Tomita et al. 2013). The total parasites were detected by staining with rabbit anti-*T. gondii* RH strain polyclonal antibodies (1 : 1,000) (Bio-Rad Laboratories, Hercules, CA, USA) and nuclear staining was achieved by using DAPI (4’,6-diamidino-2-phenylindole, 1 : 1,000). Secondary staining was achieved by antibody solutions containing 1 : 1,000 ALEXA 488 goat anti-mouse (green signal for cyst wall) and ALEXA 594 goat anti-rabbit (red signal for total parasites) in 2% FCS-PBS. At least 25 random views were counted using a Nikon Eclipse E400 (Tokyo, Japan). The number of bradyzoites was estimated as a percentage of the total parasites.

In the second experimental condition, we assessed whether the NPs could prevent cyst wall formation *in vitro*. Briefly, ME49 strain of *T. gondii* was inoculated onto confluent HFF monolayers on coverslips at a MOI of 0.5. The coverslips were incubated at 37°C and 5% CO₂ for 24 h. The NPs were added in bradyzoite-inducing medium (pH 8.1) 24 h after parasite inoculation and the coverslips were incubated at 37°C and 0% CO₂ for 48, 48 and 72 h. At each of these time points, the coverslips were fixed, the coverslips were incubated at 37°C and 0% CO₂ for 72 h. After 72 h (75 – 88% of total parasite was cyst wall positive), NPs were added and the incubation continued for 24, 48, and 72 h under bradyzoite-inducing culture conditions. For explanation – see text, page 2.

The graph shows averages for independent experiments performed in triplicates and normalised to the number of cysts present in the untreated control sample. Data are presented as the mean ± SEM; statistical significance relative to the control is indicated by a *p* value of <0.05.
parasites (PLK/DLUC_1C9) per well. Treatment with NPs (concentration range, 0.01–200 µg/mL) was initiated at 24 h post-inoculation and continued for 48 h under bradyzoite-inducing culture conditions. To normalise for bradyzoite formation, the parasite expressed Renilla luciferase under the control of the α-tubulin promoter. Data are presented as the mean ± SEM. The experiment was performed three times independently. α is significant at \( p < 0.05 \) relative to the control.

**Data analysis**

Data were analysed using one-way ANOVA (GraphPad Software Inc., San Diego, CA, USA) and are presented as the mean ± SEM. Differences among the group means were determined by using the Tukey post-hoc test; \( p \) values < 0.05 were considered to be significant. The concentration of the nanoparticles causing a 50% reduction in BAG-1 promoter activity (i.e. EC\(_{50}\) value) was estimated by using Prism 5 (GraphPad; CA, USA); non-linear regression analysis was used to fit the curve.

**RESULTS**

**NPs retard tachyzoite to bradyzoite conversion in vitro**

Under the first experimental condition, AuNP and AgNP treatment led to a time-dependent reduction in cyst wall positive vacuoles (Fig. 1A). For PtNP, the reduction in cyst wall positive vacuoles do not seem to be time-dependent. Altogether, the finding indicate the potential of NPs to reduce bradyzoite burden.

Under the second experimental condition, we found that the NPs significantly reduced parasite cyst wall formation in vitro (Fig. 1B). Bradyzoite formation was retarded but not in a time-dependent manner. This further confirms the anti-bradyzoite potential of the NPs.

**NPs decrease BAG-1 promoter activity**

Using a parasite expressing luciferase activity, PLK/DLUC_1C9, we tested the effect of NPs on BAG-1 promoter activity under two different conditions. Under the first condition, whereby the parasite was cultured under bradyzoite-inducing culture conditions, treatment with NPs decreased BAG-1 promoter activity relative to the untreated control (Fig. 2A–C). However, this reduction in BAG-1 promoter activity waned with increasing concentrations of NPs. In a parallel experiment under normal cell culture conditions, NP treatment increased the BAG-1 promoter activity only mildly relative to the untreated control (Fig. 2D–F). Under the same conditions, 80 µM PDTC increased the BAG-1 promoter activity by >3-fold relative to the control (data not shown), thus validating the assay. Furthermore, the treatment with NPs significantly reduced the total parasite number (Fig. 3A–F), suggesting that the anti-bradyzoite effect of the NPs is linked to its anti-tachyzoite potential.
DISCUSSION

The large global burden of toxoplasmosis and the lack of effective therapeutic options underscore the urgent need for better anti-

*T. gondii* drugs. Current therapy for toxoplasmosis suppresses the active infection but does not cure the latent infection (Black and Boothroyd 2000, Boothroyd and Dubremetz 2008). An ideal anti-

*T. gondii* drug would be potent, nontoxic and eliminate the latent infection (Kamau et al. 2012). Recently, we demonstrated that inorganic nanoparticles showed promising activity against the tachyzoite stage of *Toxoplasma gondii* (Adeyemi et al. 2017). In the present study, we provide evidence that NPs have anti-bradyzoite capability.

First, we evaluated the effect of NPs on already formed cysts *in vitro*. In this experiment, tachyzoites-infected cells were cultured under bradyzoite-inducing culture conditions for 72 h before treatment with the NPs. The NPs caused a dramatic and significant reduction in the percentage of bradyzoites relative to the control. We also tested whether the NPs could prevent cyst formation and found that NPs have the capacity to limit bradyzoite formation, even under bradyzoite-inducing culture conditions. Taken together, these findings suggest that NPs have anti-bradyzoite potential.

![Fig. 3. A–C – 48 hpi, bradyzoite-inducing culture conditions. In vitro effect of NPs on total parasite number HFF cells were infected with 10,000 parasites (PLK/DLUC_1C9) per well. Treatment with NPs (concentration range, 0.01–200 µg/mL) was initiated at 24 h post-inoculation and continued for 48 h under bradyzoite-inducing culture conditions; D–F – 48 hpi normal cell culture conditions. Total parasite number is the same as the luminescence of Renilla luciferase. Data are presented as the mean ± SEM. PLK/DLUC_1C9 expressed firefly luciferase under the control of the bradyzoite-specific BAG-1 promoter. To normalise for bradyzoite formation, the parasite expressed the Renilla luciferase under the control of the α-tubulin promoter. A known bradyzoite-inducing agent, ammonium pyrrolidinedithiocarbamate (PDTC; Sigma-Aldrich) served as a positive control. The experiment was performed three times independently.](image-url)
bradyzoite-inducing culture conditions might have triggered the mild bradyzoite formation.

Furthermore, the role of stress induced by NP treatment is underscored if we consider that, under bradyzoite-inducing culture conditions, the reduction in BAG-1 promoter activity gradually waned with increasing NP concentrations. This may indicate that increasing the concentration of the NPs effectively reduced the total parasite number, but failed to concomitantly decrease the BAG-1 promoter activity due to the stress imposed by the NP treatment. Beyond this, data may also indicate that NPs have activity against both the tachyzoite and bradyzoite forms of T. gondii.

Taken together, our data provide evidence for the anti-bradyzoite potential of NPs but demonstrate that the anti-T. gondii potential of NPs may not necessarily preclude bradyzoite formation. Our findings support the anti-parasitic action of NPs and are consistent with other reports that have demonstrated the anti-protozoal (Ahmad et al. 2015, Rahul et al. 2015, Saad et al. 2015, Yah and Simate 2015, Saini et al. 2016), including anti-T. gondii (Leyke et al. 2012, Adeyemi et al. 2017, 2018a), potential of NPs.

However, to our knowledge, this is the first study to provide evidence in support of the anti-bradyzoite potential of NPs. Moreover, the effective doses (EC_{50}) values of nanoparticles reported in the present study are far below the dose (≥20 µg/mL) required to cause cytotoxicity in HFF monolayers (Adeyemi et al. 2017).

In the separate investigations, in vitro cellular toxicity by gold, silver or platinum nanoparticles have been reported at doses ≥100 µg/mL (Jiao et al. 2014, Adeyemi and Sulaiman 2015, Adeyemi et al. 2018a,b). This dose is higher than the effective doses of nanoparticles used in the present study. Additionally, in vivo toxicity studies have shown that gold, silver and platinum had oral LD_{50} of ≥2000 mg/kg, ≥1,000 mg/kg and ≥1,000 mg/kg body weight, respectively (Pokharkar et al. 2009, Maneewattanapinyo et al. 2011, Adeyemi and Faniyan 2014, Adeyemi et al. 2014, 2016, Sulaiman et al. 2015).

In conclusion, although the NPs did not completely prevent bradyzoite formation, our data show that NPs have the capacity to limit the bradyzoite burden in vitro. These novel findings not only support the anti-parasitic potential of NPs but further strengthen the prospects of exploring NPs as promising alternative anti-parasitic agents.

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