Nanomed. J. 6(1): 19-26, Winter 2019

RESEARCH PAPER

Antioxidant effects of gold nanoparticles on Schistosoma mansoni induced granuloma, in vitro

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ABSTRACT

Objective(s): Schistosomiasis and hepatitis C virus [HCV] co-infection is common among the Egyptian population. Co-infected patients have higher rate of chronic hepatitis, cirrhosis and hepatocellular carcinoma. The aim of the present study was to investigate the potential role of gold nanoparticles on granuloma in vitro.

Materials and Methods: In the current study, granulocytes were isolated from the blood of 50 Schistosoma/HCV co-infected patients and 25 healthy subjects. Granulocytes were used to induce granuloma in vitro in the presence of polyacrylamide beads coated with Schistosoma mansoni soluble egg antigen and interleukin-17. In addition, granuloma was treated on the 3rd day with gold nanoparticles alone or in the presence of human hepatocellular carcinoma cell line (Hep G2) as carcinoma model. Praziquantel (PZQ) was used as a positive control. Granuloma index was determined on the 7th and 14th day. Furthermore, the supernatants were collected to measure the granulocyte mediators including tumor necrosis factor alpha [TNF-α], hydrogen peroxide [H_2O_2] and nitric oxide [NO] by ELISA on the 7th and 14th day.

Results: Treatment with AuNPs in the presence of Hep G2 showed a significant reduction in granuloma index and granulocyte mediators including H2O2 and NO, while a significant elevation was observed in TNF-α level as compared to their corresponding values in the presence of IL-17 in both healthy individual and co-infected patients on the 7th and 14th day.

Conclusion: In conclusion, the presence of IL-17 accelerated the formation of granuloma and the treatment with AuNPs in the presence Hep G2 cells indicated that AuNPs were more effective antioxidant agents than PZQ.

Keywords: *Gold nanoparticles, Hepatitis, Interleukin 17, Schistosoma*

How to cite this article
El-Shorbagy A, Gamil E, Mohey M, Nady S. Antioxidant effects of gold nanoparticles on Schistosoma mansoni induced granuloma, in vitro. Nanomed J. 2019; 6(1): 19-26. DOI: 10.22038/nmj.2019.06.003

INTRODUCTION

Co-infected patients were characterized by higher hepatitis C virus [HCV] ribonucleic acid titers, histological activity, and incidence of cirrhosis and hepatocellular carcinoma [HCC] as well as higher mortality rates than patients with single infection [1].

Clinical studies in Egypt have shown that 70–90% of patients with chronic hepatitis, cirrhosis or HCC have co-infection of schistosomiasis and HCV [2]. The combination of chronic schistosomiasis caused by S. mansoni and hepatitis B virus or HCV may cause a higher risk of HCC as a result of increased viral load in co-infected patients leading to higher inflammatory activity as well as more advanced disease state. Likewise, it was reported that one of the most prevalent causes in the development of HCC in fibrotic patients was hepatitis resulting from viral infection [3].

Since, HCC occurs in frequent association with liver fibrosis, the two major factors noted in causing the pathogenesis of HCC were chronic hepatitis and hepatic fibrosis. In patients with viral hepatitis, prognosis was worsened in conjunction with schistosomiasis [4]; where the viral infection induced hepatitis, meanwhile, the liver fibrosis induced by schistosomiasis, and the development of HCC have been demonstrated. The association of virally induced hepatitis, liver fibrosis induced by schistosomiasis, and the development of HCC has been demonstrated [5, 6].

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Note. This manuscript was submitted on September 25, 2018; approved on November 15, 2018

The first attempts to establish in vitro HCV replication systems were conducted by infecting primary hepatocyte cultures [7, 8]. Due to restricted availability of primary hepatocytes, the immortalized human hepatocellular carcinoma [Hep G2] was later successfully used to host HCV replication in vitro [9, 10].

The effectiveness of praziquantel [PZQ] against Schistosoma sp. is well recognized but evidences are accumulated that it cannot prevent re-infection and may sometimes enhance it [11]. Meanwhile, in endemic areas, repeated chemotherapy has resulted in the emergence of drug resistant strains of schistosomes [12-15]. In addition, the search for bioactive natural products against Schistosoma has a great importance for establishing future strategies to control schistosomiasis [16-18].

Silver nanoparticles, gold, chitosan, and oxidized metals have growth inhibitory or cytotoxic effects on various parasites, including Plasmodium, Giardia, Leishmania, Toxoplasma and insect larva [19-21]. Nanoparticles could be used against parasites individually or in combination. Therefore, nanoparticles were recommended for destroying parasites [cytotoxic and inhibitory effect], providing more effective and less harmful medications and also beneficial vaccines for the prevention and control of the parasites [22].

The effects of gold nanoparticles [AuNPs] on schistosomiasis are associated with their oxidative stress by scavenging free radicals, which could result in a clinical use in the treatment of hepatic dysfunction in schistosomiasis [23]. In addition, an in vitro study of AuNPs treatment also induced upregulation of antioxidants, stress response genes and protein expression, thus AuNPs treatment may be useful for their ability to reduce granuloma formation [24].

METHODS

Patients

Fifteen Schistosoma/HCV co-infected patients (28 males with mean age=43.8±9.8 and 22 females with mean age=45.5±8.2) and 25 healthy subjects (12 males with mean age=29±5.4 and 13 females with mean age=38.6±13.6) were enrolled in this study. Participants were enrolled from Al-Qaser El-Ainy University Hospital, Cairo University, from October 2012 to June 2015.

Schistosomiasis was diagnosed by detection of S. mansoni ova in stool and seropositivity for anti-schistosomal antibodies (indirect hemagglutination; Femouz Laboratories, Asniéres, France). No other hepatic or intestinal parasites were found.

Hepatitis C virus infection was diagnosed by seropositivity for HCV antibodies, HCV RNA as assessed by PCR and elevated aminotransferase levels for 6 months. Liver biopsy samples showed evidence of chronic hepatitis. No serological markers for the presence of hepatitis A and B viruses, cytomegalovirus infection, and Epstein–Barr virus infection were found.

Healthy subjects had no past or current history for Schistosoma infection or any viral infection.

The study was approved by the research ethics committee of Cairo University, Egypt. All participants were given and signed informed consents.

Preparation of praziquantel (PZQ)

One tablet of biltricide (600 mg) was dissolved in 6 ml of Dulbecco's modified eagle medium (DMEM) and centrifuged at 2000 rpm for 10 min. The supernatant contained 100 mg of PZQ/ml. PZQ was used immediately or stored at 4 ºC until used according to the published method [25].

Citrate capped-gold nanoparticles (AuNP-citrate)

AuNPs were purchased from NanoTech (Egypt); the properties of AuNPs were selected according to previous study [26] with the following characteristics: appearance (color): Pink; appearance (form): Liquid; concentration: 1 mM Au3+; solubility: water soluble; optical properties: λmax = 520 nm; average size: 15 ± 5 nm; shape: spherical.

The human hepatocellular carcinoma cell line (Hep G2)

Hep G2 was purchased from VACSERA [Giza, Egypt]. Hep G2 cells were subcultured in a 75 2 cm flasks in DMEM supplemented with 2 mM L-glutamine [Biochrom], penicillin [Biochrom], streptomycin [Biochrom] and 10% heat inactivated fetal bovine serum [HyClone, UK] at 37˚C under a humidified atmosphere containing 5% CO_2 and maintained in an exponential growth state. The adherent cells were collected by 0.25% trypsin according to a previously published method [26].

Antigens

Schistosoma mansoni soluble egg antigen (SEA) was purchased as a lyophilized, endotoxin-

free preparation from Theodor Bilharz Research institute, Imbaba, Giza, Egypt.

Isolation of granulocytes from whole blood and in vitro granuloma formation

Granulocytes were isolated from whole blood and granuloma was induced in vitro, measured and classified according to recent published study [27].

Cytotoxicity assay

Cytotoxicity was determined by trypan blue dye; the granulocytes were counted and plated (1×105 cells/well) in 96-well culture plates. AuNPs-citrate were dispersed in cell culture medium, diluted at concentrations 0.625, 1.25, 2.5, 5, 12.5 and 25 µM. A negative control and diluted concentration were incubated at 37 ○C in a 5% CO2 atmosphere for 24 hours and the viability of granulocytes was determined by microscopic examination.

Optimization of Hep G2 count

Different counts (5×102, 1×103, 5×103, 1×104, 5×104 and 1×105 cells/ml) of Hep G2 cells were added to the cultured granuloma. The optimum count was 1×103 cells/ml which was determined by growth rate in 24-well flat-bottomed tissue culture plates. In addition, different concentrations of PZQ (0.1, 1, 5 and 10 mg/ml) were added to granuloma and incubated at 37 ○C and 5% CO2 in 24-well flat-bottomed tissue culture plates. The optimum concentration of PZQ was 0.1 mg/ml.

Measurement of granulocytes mediators

Tumor necrosis factor alpha (TNF-α) was measured using human TNF-α ELISA kit (Boster Immunoleader, USA) according to the manufacturer's instructions. Hydrogen peroxidase was measured using H2O2 colorimetric methods (Bio-diagnostic, Egypt) according to the manufacturer's instructions. Nitric oxide was measured as described previously [28]. Briefly, NO in the supernatant was assayed by the Griess reaction which has the ability to produce a chromophore with the Griess reagent. Reading of the color changes was measured using a microtiter platereader (Bio Tek, USA) at dual wavelength (450 and 640 nm). Standard curve was plotted to measure the concentration of nitrite.

Statistical analysis

Statistical analysis was performed by t-test to compare granulocytes functions of co-infected individuals with those of non-infected individuals using Graph Pad Prism 3 Software (San Diego, California, USA) as well as one way analysis of variance (ANOVA) was used for the analysis of AuNPs cytotoxic effect. Data are presented as % change to that of untreated granulocytes. Results with a P value of < 0.05 were considered significant.

RESULTS

Effect of AuNPs on granulocytes viability of healthy subjects

Fig 1 shows the viability of granulocytes treated with different concentrations of AuNPs [0.625, 1.25, 2.5, 5, 10, 25 µM/ml] for 24 h, as determined by trypan blue dye. The viability decreased significantly (P<0.05) as compared to their corresponding value of non-stimulated granulocytes (negative control) except at 1.25 and 2.5 µM/ml which were non-significant. The lowest granulocytes viability (61.9%) was observed with the highest concentration (25 µM/ml) of AuNPs while, the highest granulocytes viability (92.5%) was observed with 2.5µM/ml of AuNPs.

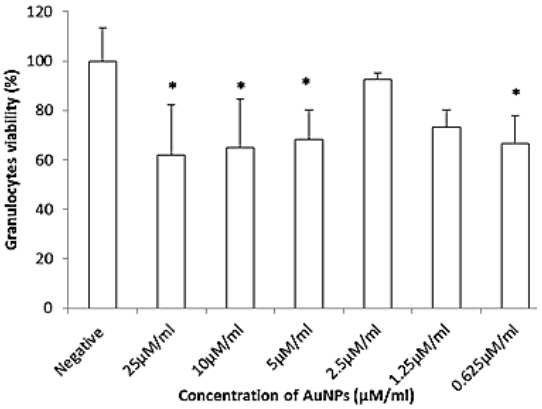

Fig 1. Effect of different concentrations of AuNPs on granulocytes viability of healthy subjects after 24 hours
Data is represented as % change to their corresponding value of non-stimulated granulocytes
* Significant at P<0.05 as compared to non-stimulated granulocytes

Effect of AuNPs on G.I. of granuloma produced by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17

The effect of PZQ (0.1 mg/ml) as positive control on granuloma produced by *S. mansoni* SEA-conjugated polyacrylamide beads in the presence

of IL-17 (125 pg/ml) caused reduction in G.I. formation after 7 and 14 days by healthy subjects' granulocytes (-11.3% and -9.4%, respectively) while this reduction for *Schistosoma*/HCV co-infected patients were -13.7% and -6.8%, respectively as compared to their corresponding values of G.I. formed by untreated granuloma (Fig 2).

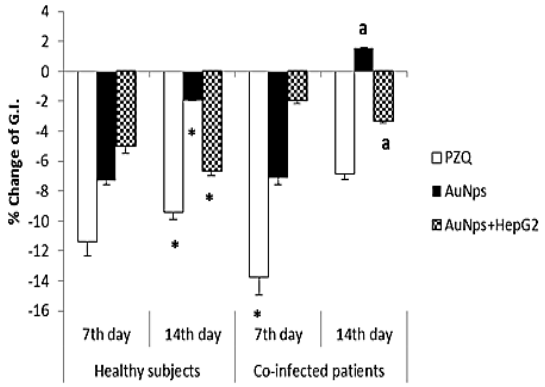

Fig 2. Effect of AuNPs on G.I. of granuloma formed by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17
Data is represented as % change to their corresponding value of G.I. of granuloma formed by granulocytes stimulated with SEA in the presence of IL-17
* Significant at P< 0.05 as compared to their corresponding value of G.I. of granuloma formed by granulocytes stimulated with SEA in the presence of IL-17
a: Significant at P< 0.05 as compared to their corresponding value of G.I. of granuloma treated by PZQ
[PZQ]: Praziquantel
[AuNPs]: Gold nanoparticles
[Hep G2]: Human hepatocellular carcinoma
[G.I.]: Granuloma index

Granuloma treated with AuNPs (2.5µM/ml]/ AuNPs in the presence of Hep G2 cells (1x10^3 cells) as carcinoma model showed decrease in G.I. of granuloma formation by granulocytes isolated from healthy subjects. However, G.I. of co-infected patients showed different responses on the 7th day as compared to their corresponding value of untreated granuloma (Fig 2). In addition, in comparison with AuNPs/AuNPs in the presence of Hep G2 cells treated with PZQ, a non-significant increase in G.I. of granuloma formed by granulocytes isolated from healthy subjects on the 7th and 14th day was observed, but a significant change took place on the 14th day in granuloma formed by granulocytes isolated from *Schistosoma*/HCV co-infected patients (Fig 2).

On the other hands, treatment with AuNPs/ AuNPs in the presence of Hep G2 cells as carcinoma model produced elevation in G.I. of granuloma induction by SEA in the presence of IL-17 with granulocytes isolated from co-infected patients as compared to their corresponding value of granulocytes isolated from healthy subjects (Fig 2).

Effect of AuNPs on TNF-α produced by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17

Granuloma induction by *S. mansoni* SEA-conjugated polyacrylamide beads in the presence of IL-17 treated with PZQ (0.1mg/ml) resulted in a significant elevation in TNF-α level by granulocytes isolated from healthy subjects (114.1%) on the 7th day. Moreover, a non-significant increase was recorded by co-infected granulocytes (45.6%) on the 14th day as compared to their corresponding value of untreated granuloma (Fig 3).

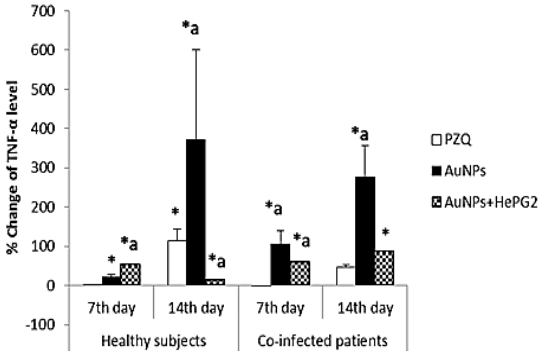

Fig 3. Effect of AuNPs on TNF-α produced by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17
Data is represented as % change to their corresponding value of TNF-α level production by granulocytes stimulated with SEA in the presence of IL-17
* Significant at P< 0.05 as compared to their corresponding value of TNF-α level production by granulocytes stimulated with SEA in the presence of IL-17
a: Significant at P< 0.05 as compared to their corresponding value of TNF-α level production by granulocytes of granuloma treated with PZQ
[PZQ]: Praziquantel
[AuNPs]: Gold nanoparticles
[Hep G2]: Human hepatocellular carcinoma
[TNF-α]: Tumor necrosis factor alpha

Granuloma treated with AuNPs (2.5µM/ml)/AuNPs in the presence of Hep G2 cells as carcinoma model produced a significant elevation (P<0.05) in TNF-α level by either granulocytes isolated from

healthy subjects (21.6% and 371.4% by AuNPs, 105.9% and 276.4% by AuNPs in the presence of Hep G2 cells) or granulocytes isolated from *Schistosoma*/HCV co-infected patients (52.3% and 13.8% by AuNPs, 59.0% and 87.3% by AuNPs in the presence of Hep G2 cells) as compared to their corresponding values of untreated granuloma (Fie 3). Moreover, granuloma treated with AuNPs/ AuNPs in the presence of Hep G2 cells showed elevation of TNF-α level on the 7th and 14th days by granulocytes isolated from either healthy subjects or *Schistosoma*/HCV co-infected patients as compared to their corresponding values of granuloma treated with PZQ (Fig 3). However, granuloma treated with AuNPs/AuNPs in the presence of Hep G2 cells resulted in inhibition of TNF-α production by granulocytes isolated from co-infected patients as compared to their corresponding value of granulocytes isolated from healthy subjects (Fig 3).

Effect of AuNPs on H_2O_2 level produced by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17

Granuloma induction by *S. mansoni* SEA-conjugated polyacrylamide beads in the presence of IL-17 and treated with PZQ (0.1 mg/ml) resulted in a significant inhibition in H_2O_2 production by granulocytes isolated from healthy subjects (-10.5%) on the 7th day and a non-significant decrease observed on the 14th day. Moreover, a significant decrease was recorded by co-infected granulocytes (-12.3% and -13.4%) as compared to their corresponding values of untreated granuloma (Fig 4).

Granuloma treated with AuNPs/AuNPs in the presence of Hep G2 cells produced a significant inhibition in H_2O_2 level by either healthy subjects' granulocytes (-17.0% and -10.6% by AuNPs, -14.2% and -11.9% by AuNPs in the presence of Hep G2 cells) or *Schistosoma*/HCV co-infected patients (-55.2% and -55.0% by AuNPs, -54.6% and -55.4% by AuNPs in the presence of Hep G2 cells) as compared to their corresponding values of untreated granuloma (Fig 4).

Treatment of granuloma with AuNPs/AuNPs in the presence of Hep G2 cells showed a significant reduction in H_2O_2 level on the 14th day by healthy subjects' granulocytes while a significant decrease was observed on the 7th and 14th day by co-infected granulocytes as compared to their corresponding value of granuloma treated with PZQ (Fig 4). On the other hands, granuloma treated with AuNPs/ AuNPs in the presence of Hep G2 cells resulted in a significant elevation of H_2O_2 level by co-infected granulocytes as compared to their corresponding value of healthy subjects' granulocytes (Fig 4).

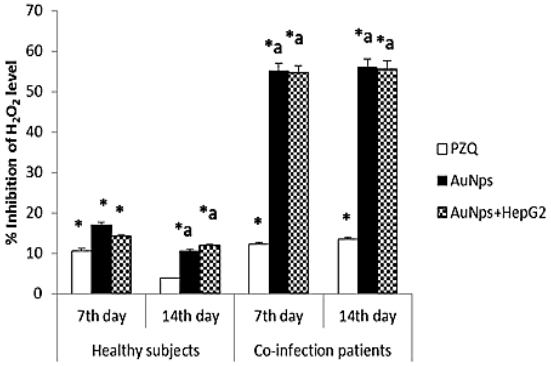

Fig 4. Effect of AuNPs on H2O2 produced by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17
Data is represented as % change to their corresponding value of H2O2level production by granulocytes stimulated with SEA in the presence of IL-17
* Significant at P< 0.05 as compared to their corresponding value of H2O2 level production by granulocytes stimulated with SEA in the presence of IL-17
a: Significant at P< 0.05 as compared to their corresponding value of H2O2 level production by granulocytes of granuloma treated with PZQ
[PZQ]: Praziquantel
[AuNPs]: Gold nanoparticles
[Hep G2]: Human hepatocellular carcinoma
[H2O2]: Hydrogen peroxide

Effect of AuNPs on NO production by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17

Data shown in Fig 5 indicated that PZQ treatment (0.1mg/ml) of granulocytes of healthy subjects stimulated by *S. mansoni* SEA-conjugated polyacrylamide beads in the presence of IL-17 producing granuloma induced a significant changes in NO level (-7.8%, 10.9%),. However, a non-significant change was observed in NO level by granulocytes of *Schistosoma*/HCV patients as compared to their corresponding values of untreated granuloma.

Granuloma treated with AuNPs [2.5µM/ml]/ AuNPs in the presence of Hep G2 cells resulted in a significant inhibition in NO level by granulocytes of healthy or co-infected subjects as compared to their corresponding value of untreated granuloma (Fig 5).

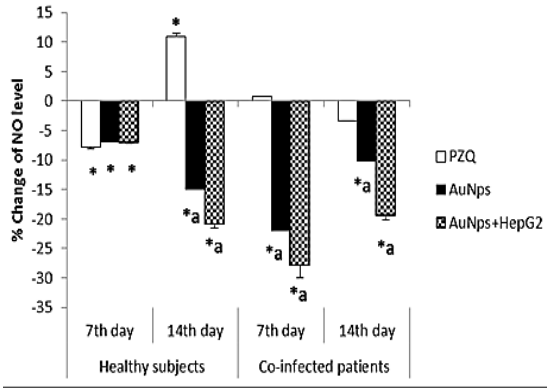

Fig 5. Effect of AuNPs on NO produced by granulocytes stimulated with S. mansoni SEA-conjugated polyacrylamide beads in the presence of IL-17
Data is represented as % change to their corresponding value of NO level production by granulocytes stimulated with SEA in the presence of IL-17
* Significant at P< 0.05 as compared to their corresponding value of NO level production by granulocytes stimulated with SEA in the presence of IL-17
a: Significant at P< 0.05 as compared to their corresponding value of NO level production by granulocytes of granuloma treated with PZQ
[PZQ]: Praziquantel
[AuNPs]: Gold nanoparticles
[Hep G2]: Human hepatocellular carcinoma
[NO]: Nitric oxide

Treatment of granuloma with AuNPs/AuNPs in the presence of Hep G2 cells showed a significant reduction in NO level on the 7th and 14th days by granulocytes of either healthy or co-infected subjects as compared to their corresponding value of granuloma treated with PZQ (Fig 5). However, granuloma subjects treated with AuNPs/AuNPs in the presence of Hep G2 cells showed non-significant changes in NO level in co-infected granulocytes as compared to their corresponding value of healthy subjects' granulocytes (Fig 5).

DISCUSSION

AuNPs in medicine has altered the methods of diagnosis and cancer therapy [29, 30]. AuNPs are accumulated in the tumor cells and show optical scattering. Thus they can act as the probe for microscopic study of cancer cells. They are also used in chemotherapy and diagnosis of cancer cell [31]. AuNPs have a great application not only in biosensing platforms but also in drug, gene and protein delivery [32]. The proliferation of hepatocellular carcinoma cancer cells was lower for cultures exposed to AuNPs/chemotherapy drugs conjugates, in comparison to cultures exposed to isolated cytostatic drugs. Aadditionally, small AuNPs (1 nm in diameter) can easily cross the cell membrane and nucleus, and attach to the deoxyribonucleic acid [33].

In the present study, granuloma induced by *S. mansoni* SEA-conjugated polyacrylamide beads in the presence of IL-17 was treated with AuNPs (2.5 μM/ml) in the presence of Hep G2 cells as carcinoma model indicated a decrease in G.I. of granuloma produced by granulocytes of healthy subjects. Different changes were found in G.I. of granuloma formed by co-infected granulocytes on the 7th day as compared to their corresponding value of untreated granuloma. The present results were similar to those reported previously [23, 34] in which treatment with AuNPs appeared to moderate inflammatory cellular infiltration and decrease the diameter of granulomas. Moreover, AuNPs treatment reduced the hepatic worm burden compared to the infected group. The treatment of infected *Schistosoma* mice with AuNPs reduced the extent of the histological disturbances evident in the brain of infected mice [34].

In the current study, the granuloma treated with AuNPs/AuNPs in the presence of Hep G2 cells produced a significant elevation in TNF-α level by granulocytes of either healthy or co-infected subjects as compared to their corresponding value of untreated granuloma or granuloma treated with PZQ. These results were in agreement with previous study [35], in which they demonstrated that both silver nanoparticles and AuNPs entered the cells, but only AuNPs up-regulated the expressions of pro-inflammatory genes (IL-1, IL-6, and TNF-α). They suggested that part of the negatively charged AuNPs might adsorb serum protein and enter cells *via* the more complicated endocytotic pathway, resulting in higher cytotoxicity and immunological response [35]/. Citrate stabilized and dihydrolipoic acid functionalized AuNPs neither induced apoptosis nor activated gene expression related to inflammatory response [TNF-α] while their decreased reactivity with biomolecules and cells provides a promising medical platform [36].

On the other hand, results of present study revealed that the granuloma treated with AuNPs/AuNPs in the presence of Hep G2 cells resulted in a significant inhibition of both H_2O_2 and NO levels by granulocytes of either healthy or co-infected subjects as compared to their corresponding

value of untreated granuloma or granuloma treated by PZQ. These results are in agreement with previous study [37]c in which they confirmed that antioxidant and anti-hyperglycemic effects of AuNPs were due to the inhibition of ROS production or balanced ROS generation in streptozocine-induced hyperglycemic mice. This inhibition showed antioxidant and free radical scavenging effects by elevating the level of antioxidant defense enzymes. AuNPs caused irreversible H_2O_2 reduction as shown below [37]c:

H_2O_2 Au $O_2 + H_2O$

In the same manner, comparable finding has been reported by others [23] who reported that the injection of AuNPs (0.25, 0.5, and 1 mg/kg) into *Schistosoma* infected mice resulted in a significant downregulation of inducible nitric oxide synthase mRNA expressions in hepatic tissue as compared to infected mice. Furthermore, AuNPs treatment of schistosomiasis promoted oxidative stress attributed to their ability to scavenge free radicals, and this action could find a clinical use in the treatment of hepatic dysfunction in schistosomiasis.

Furthermore, AuNPs have potential antioxidant activity effective in quenching reactive oxygen species, including H_2O_2 and superoxide anion radical in a dose dependent manner [38].

In the present study, the results of all measured parameters (G.I., H_2O_2, NO and TNF-α) in granuloma treated with AuNPs in the presence of Hep G2 cells (as carcinoma model) were higher than those treated with AuNPs alone.

This elevation may be resulted from the rapid growth of Hep G2 cells and hence their released mediators. In addition, the enhanced virus propagation by *S. mansoni* SEA in both Hep G2 cells and human blood cells were in well agreement with the results of a previous study [39].

The elevation level of TNF-α caused by immune response of granulocytes stimulated with *S. mansoni* SEA in the presence of IL-17 and also decrease in the H_2O_2, NO and G.I. levels of granuloma formation after treatment with AuNPs/AuNPs in the presence of Hep G2 cells may explain the mechanism of reduction in granuloma formation and liver fibrosis.

Treatment with AuNPs/AuNPs in the presence of Hep G2 cells compared to PZQ treatment caused an inhibition of H_2O_2 and NO production. These results indicated a decrease in oxidative stress.

CONCLUSION

AuNPs showed an antioxidant effects on granuloma formation *in vitro*. Further studies are also required to elucidate the exact mechanism of this modulatory response, and to study its potential therapeutic effects in more detail.

ACKNOWLEDGMENTS

The authors would like to thank the Egyptian Science and Technology Development Fund (STDF) for supporting this study through the grant No. 1814 awarded to Dr Soad Nady under the framework of Egypt/US cooperation program.

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