Research Article

Citrate Stabilized Gold Nanoparticles Induce Alterations in the Oxidative Parameters of Red Blood Cells

Chayanika Devi[1,2], Partha Pratim Kalita[3], Upama Baruah[4], Chadipiralla Kiranmai[5], Pankaj Kalita[6], Pichili Vijaya Bhaskar Reddy[1,*]

[1]Department of Life Sciences and Bioinformatics, Assam University, Diphu Campus, Diphu, Assam, INDIA.
[2]Department of Zoology, Pub Kamrup College, Baihata Chariali, Kamrup, Assam, INDIA.
[3]Program of Biotechnology, Assam Down Town University, Guwahati, Assam, INDIA.
[4]Department of Chemistry, Pandit Deendayal Upadhyaya Adarsha Mahavidyalaya, Amjonga, Goalpara, Assam, INDIA.
[5]Department of Biotechnology, Vikrama Simhapuri University, Nellore, Andhra Pradesh, INDIA.
[6]Department of Zoology, Eastern Karbi Anglong College, Sarihajan, Karbi Anglong, Assam, INDIA.

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ABSTRACT

Aim: Colloidal gold nanoparticles have been used in diverse biomedical applications due to their unique surface, electronic and optical properties. Because of the strong and size-tunable surface plasmon resonance, fluorescence and easy-surface functionalization, Gold Nanoparticles (AuNPs) have been widely used in biosensors, cancer cell imaging, photothermal therapy, and drug delivery approach. In general, the toxicity of gold nanoparticles depends on their physical dimensions (such as size and shape) and surface chemistry (such as coating). However, functionalized gold nanoparticles show obvious cytotoxicity. To clarify these problems, the cytotoxicity of gold nanoparticles in human blood cells (1st target during exposure) has been studied in the present experiment. **Materials and Methods:** Red Blood Cells were exposed with the Gold Nanoparticles (AuNPs) in a dose and time dependent manner *in vitro*. The level of oxidative stress biomarkers viz., Glutathione Reductase (GR), Glutathione Peroxidase (GPx), Glutathione S Transferase (GST), total thiols and malondialdehyde were determined. AuNPs were characterized with the help of spectrophotometer, FTIR, DLS technique, XRD, SEM EDx. **Results:** The current study was focused on investigating the effect of AuNPs on RBC. The results of our study demonstrated an altered activity of various oxidative stress markers in a time dependent and dose dependent manner. Malondialdehyde, a pro-oxidant and a by-product of lipid peroxidation was found to be significantly increased at differential degrees up on exposure to AUNPs for various time periods. While the MDA levels were increased, a concomitant decrease in the antioxidant markers was observed. Total thiols, Glutathione reductase, Glutathione S-transferase and glutathione peroxidase were significantly decreased at variable degrees. Effect of AUNPs was evident within 6 hr of exposure, 18 and 24 hr exposure resulted in a much greater increase that could be toxic to the cell. **Conclusion:** Surface negativity and small size of the AuNPs may be assumed to affect the biochemical and cellular architecture of the organs. Further molecular level analysis will reveal the usability potential of AuNPs in body system for different purposes.

Keywords: Gold nanoparticle, Citrate stabilized, Oxidative stress, RBCs, Cytotoxicity, *in vitro*.

Correspondence:
Dr. Pichili Vijaya Bhaskar Reddy
Department of Life Sciences and Bioinformatics, Assam University, Diphu Campus, Diphu, Assam, INDIA.

Email: vpichili@gmail.com

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INTRODUCTION

Nanoparticle research is an enthralling branch of science. Nanotechnology basically deals with materials at nanoscale level, which at least have one dimension less than 100 nm.[1] Nanoparticles have a wide continuum of prospective applications due to their distinctive properties

in the fields like medicine, environmental monitoring technologies, food quality testing, tissue engineering, biosensor development, cosmetics etc.,[2] Intrusion of nanotechnology in drug delivery, preventions of drug metabolism, diagnostic agent etc. dramatically replaced the past strategies with modern pharmacological advancements, thus creating nano-medicine as an important alternative to new drug therapy.[3] Studies reveal that nanoparticles have altered size, shape and surface chemistry in comparison to that of the parent compound. These unique properties of nanomaterials open a new era of research on their uses.[4] Nanoparticle size can influence the physiochemical properties and show variations in colours and characteristic properties. Due to its size and charge characteristics it has much more applicability like catalysis, drug delivery, disease diagnosis, imaging technique, energy based research etc. in health science research and in environmental applications, but still its toxicological assessment study in the body system is scanty.[5] Due to the small size and a large surface area per unit volume, in comparison to its bulk form, this novel characteristic shows different chemical and biological properties in nanomaterials.[6]

Red Blood Cells (RBC) are the 1st line of interacting cells in the body system for accessing the sensitivity of external exposure. In the blood circulatory system RBCs are abundantly present and due to its properties like biocompatibility, biodegradability and long circulating half-life, the RBCs are broadly used in drug delivery system;[7] thus playing the role of a novel target component to assess the toxicity of the foreign exposure. Rearrangement in the blood cell composition is also allied with diverse disorders like diabetics, Alzheimer disease, Wilson's disease etc.,[8] Some prototypes for nano mediated toxicity are inflammation, oxidative stress, cytotoxicity, genotoxicity, cell death etc.,[9] Because of the small size, the nanoparticles can interact with the membrane assemblies (mainly proteins) that lead to generation of Reactive Oxygen Species [ROS] in the cell.[10] When ROS level gets increased in the bodily defence mechanisms, they cause peroxidation of lipids and threaten cells, oxidations of proteins or damage to nucleic acids which finally results in various kinds of diseases along with necrosis and apoptosis.[11] These again cause an endogenous flux of ROS causing more stress in the cells.[12] Oxidative stress, thus production and accumulation of lipid peroxidation product, is a source of different ailments atherosclerosis, ischemia-reperfusion, heart failure, cancer, rheumatic arthritis and immunological disorders etc.,[13] Oxidative stress enzymes and its lipid product allow the analysis of possible toxicological markers in the body's vital organs in a non-targeted way and provides its usefulness of a new therapeutic stratagem akin to nano medicine.[14] Gold nanoparticles play a key role in modern biomedical science research and pharmacological applications.[15] Though its use in therapeutic is increasing, but imagining its toxic effect still lingers to be untangled.[16] Therefore, this study is designed to evaluate the lipid peroxidation level and the activity of RBC anti-oxidant enzymes when blood cells were exposed to gold nanoparticles *in vitro* at different doses and time duration. Lipid peroxidation level was determined by assessing the Malondialdehyde (MDA) and total thiol level; anti-oxidant enzymes were studied in terms of Glutathione Reductase (GR), Glutathione Peroxidase (GPx), Glutathione S Transferase (GST) activity in treated groups.

MATERIALS AND METHODS

Preparation of citrate reduced Gold nanoparticles

Preparation of Gold nanoparticle was done by taking 20 mL of 1.0 mM gold chloride and mixed with an excess amount (4-5 mL) of 1% tri-sodium citrate (reducing agent), to reduce the gold chloride which results in formation of nanoparticles. If pink color appears in the reaction mixture upon stirring, it means that nano gold is formed. The mixture was then syringe filtered using 0.22 mm syringe filter. A UV visible spectrophotometric absorption peak was observed (Eppendorf biospectrometer model) for the confirmation of gold nanoparticle.[17] Particle size and zeta potential of the gold nanoparticles was also characterized using Dynamic Light Scattering technique (DLS) (Malvern; Nano-zs90); for XRD analysis Philips X-ray Diffractrometer (X'Pert Pro) with Cu Kα1 radiation (λ=1.5406 Å)[18] and Scanning Electron Microscopy (SEM). The prepared samples were stored at 4°C for future use.[19]

Preparation of RBCs for the *in vitro* experiment

Blood samples were freshly collected by heart puncture from Swiss albino mice. The animal experiments were performed as per the approval of IAEC, Gauhati University. RBCs were separated using standard procedure and kept in a special buffer as described in previous literature.[20,21]

In vitro treatment of AuNPs with RBC

Different concentrations of AuNPs, viz.,1×10^{-3} mMol, 2×10^{-3} mMol, 3×10^{-3} mMol, 4×10^{-3} mMol and 5×10^{-3} mMol AuNPs were treated with 20 µL of RBCs, kept in special buffer and were incubated for 6 hr, 18 hr and 24 hr' duration at 37ºC. 1% SDS

(Sodium Dodecyl Sulphate) was used as positive control. All the experiments were performed in triplicate sets. Treated samples were then centrifuged at 2000 rpm for 10 min and subcellular supernatant portion was later used for estimating the level of lipid peroxidation and activity of antioxidant enzymes.

Biochemical assays

Estimation of lipid peroxidation level

The amount of lipid peroxidation brought on by cellular oxidative stress is indicated by the concentration of Malondialdehyde (MDA) in the sample. The conventional method was used to calculate the MDA concentration.[22] After being treated with 8.1% SDS, 20% acetic acid (pH 3.5), and 0.8% thiobarbutaric acid, the RBC subcellular fraction was incubated at 90°C for an hour. After cooling, the reaction mixture was centrifuged for ten minutes at 1000 g. The samples' absorbance was measured at 535 nM using malondialdehyde as the reference. The amount of MDA in the protein was reported in nmol mg^{-1}.

Estimation of Glutathione reductase activity

Glutathione Reducase (GR) activity was measured using the procedure that has been previously mentioned.[23] In summary, the reaction mixture contained 0.2 M sodium phosphate buffer pH 7.0, 0.1 M subcellular fraction of red blood cells, 0.2 M EDTA, 1 M oxidized Glutathione (GSSG), and 0.2 M NADPH. The values of the absorbances were represented as nmol min-1 mg protein and were measured at a wavelength of 340 nM.

Estimation of Glutathione peroxidase activity

Ganga *et al*., 2016 approach was utilized to assess the activity of Glutathione Peroxidase (GPx).[24] 1.59 mL of 100 mM phosphate buffer (pH 7.6), 0.1 mL of sodium azide (10 mM), 0.1 mL of EDTA, 0.1 mL of reduced glutathione, 0.01 mL of hydrogen peroxide (0.2 mM), and 0.1 mL of sample were used to prepare the reaction mixture. The protein content was reported as milli mol min^{-1} mg after the absorbance was measured at 340 nM.

Estimation of Glutathione S-transferase activity

By conjugating reduced Glutathione (GSH) with DCNB (1-Chloro-2,4-Dinitro Benzene), Glutathione S Transferase (GST) Activity was determined. The data were reported as nmol mg-1 protein and the absorbance was measured at 340 nM.[25]

Estimation of total thiol content

Total thiol content was measured according to the method of Sedlak and Raymond, 1968.[26] Using 0.1 mL of RBC sub-cellular fraction, 0.2 M Tris-buffer (pH 8.2) and a solution of 0.01 M DTNB on absolute methanol. After 30 min of incubation, the reaction mixture was centrifuged at 3000 rpm for 15 min. Sample absorbance (A412) was determined using a microplate reader with 492 nM filter. The total thiols values were expressed as nmol mg^{-1} protein using a molar extinction coefficient of 13,100.

Statistical Analysis

The results were obtained from five independent samples in triplicate experimental sets. Values are presented as mean±SD and analyzed by one-way analysis of variance followed by *post hoc* comparison using Tukey test. The values of significance were evaluated with p values. The statistical analysis showing $p<0.05$ was considered statistically significant.

RESULTS

In our previous systematic investigation, the citrate stabilized nanoparticles were characterized by different spectroscopic procedures where Dynamic Light Scattering (DLS) data regarding nanoparticle size was confirmed by TEM image with a particle size of 20-30 nM. Another study by us showed the effect of citrate stabilized AuNPs on a mice model where it was observed that swelling was increased in the RBCs[27,28] in a dose dependent manner. The following sections indicate the oxidative stress markers of RBCs induced by citrate stabilized AuNPs. Treatments were followed for different doses of AuNPs and time duration.

Effect of AuNPs concentration and exposure duration on RBC MDA level

The MDA level, a by-product of lipid peroxidation, is increased with the increasing concentration of AuNPs and time of exposure. Figure 1 shows that during 6 hr of AuNPs treatment, the level is increased by 49.90%, 49.90%, 54.07%, 74.24% and 114.37% with the treatment of 1×10^{-3} mMol, 2×10^{-3} mMol, 3×10^{-3} mMol, 4×10^{-3} mMol and 5×10^{-3}mMol AuNPs respectively and the increase was found to be statistically significant ($p<0.05$) (Figure 1). Similarly, when the same concentration of AuNPs was incubated with the RBC for 18 hr, the MDA level is increased significantly by 75.97%, 99.32%, 102.65%, 106.86% and 107.64% respectively while in case of 24 hr incubation period the level is more significantly increased by 114.4%, 123.72%, 137.06%, 140.67% and 154.2% (Figure 1). The positive control groups treated with SDS are increased by 58.29%, 139.36% and 197.52% in comparison to the negative control groups during 6 hr, 18 hr and 24 hr treated period respectively.

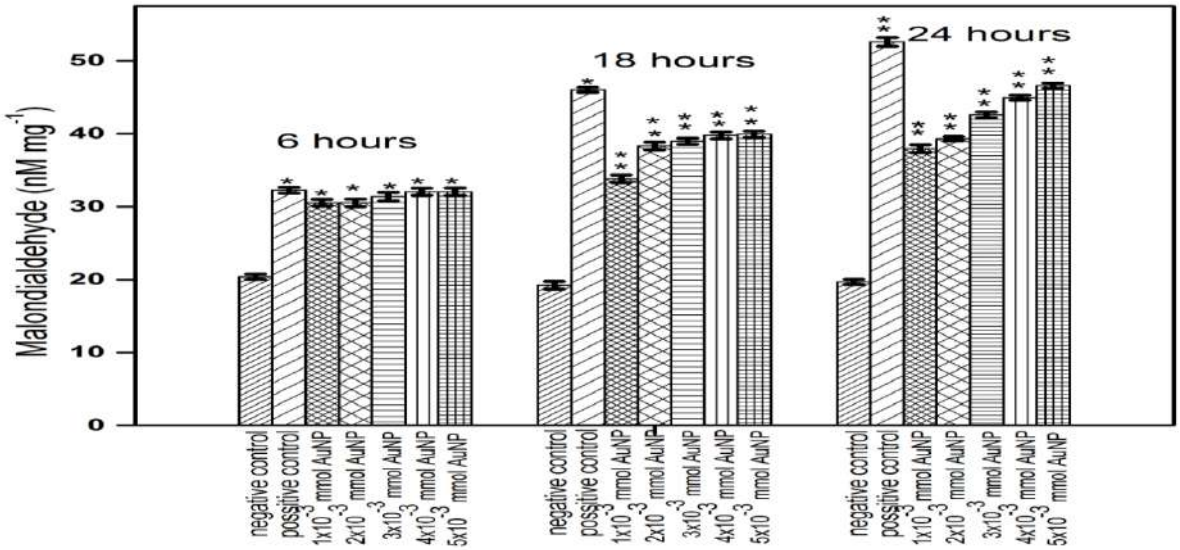

Figure 1: Effect of AuNPs on malondialdehyde in RBC depending on time and dose of exposure.

*Indicates significance level between the control group and treated group depending on exposure dose. **Indicates significance level is more (i. e. $p<<0.05$) in comparison to the * groups.

Effect of AuNPs concentration and exposure duration on RBC total thiol content

The total thiol level (Figure 2) decreases with the increasing concentration of AuNPs and time of exposure. During 6 hr of AuNPs treatment, a significant level is decreased by 34.70%, 47.45%, 50.19%, 74.90% and 80.78% in the 1×10^{-3} mMol, 2×10^{-3} mMol, 3×10^{-3} mMol, 4×10^{-3} mMol and 5×10^{-3} mMol AuNPs group respectively and the positive control group shows 78.62% decrement from the respective negative control group.

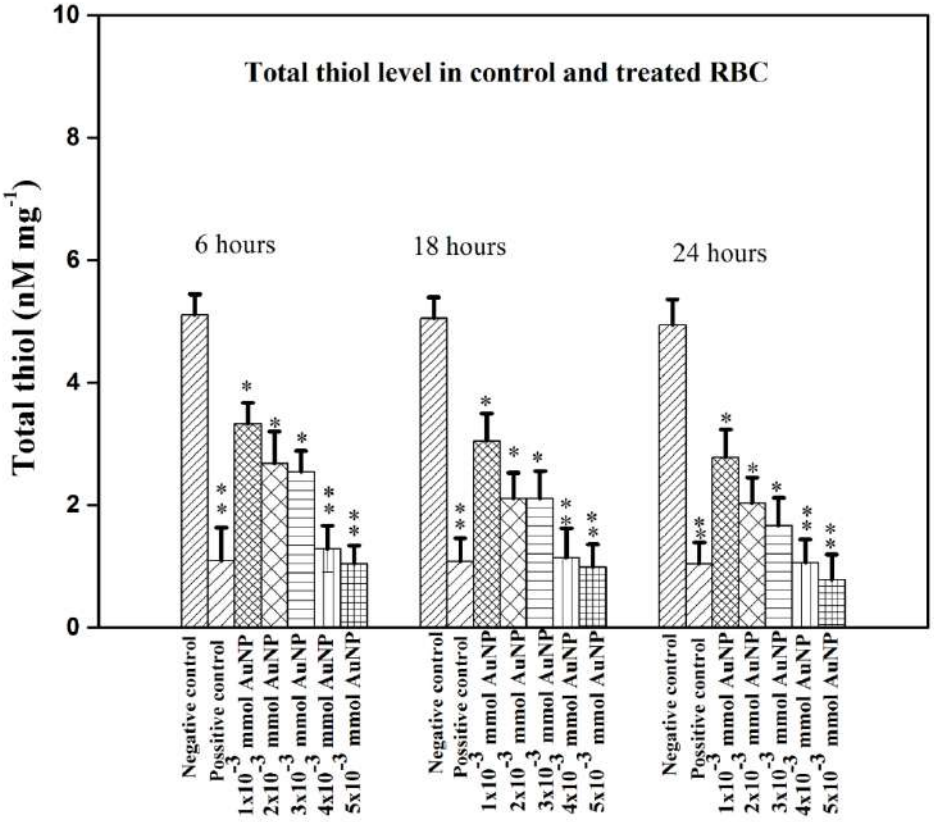

Figure 2: Effect of AuNPs on total thiol in RBC depending on time and dose of exposure.

*Indicates significance level between the control group and treated group depending on exposure dose.

**Indicates significance level is more (i. e. $p<0.05$) in comparison to the *groups.

Similarly, when the same concentration of AuNPs was incubated with the RBC for 18 hr, the enzyme level is decreased by 34.69%, 58.12%, 58.90%, 77.01% and 80.11% respectively. However, the positive control group shows 78.61% decrement. In case of 24 hr treatment, the level is again decreased by 43.74%, 58.90%, 66.39%, 78.54% and 84.21%. The positive control group decrement in this case is 80.70%. The change was found to be statistically significant ($p<0.05$) (Figure 2).

Effect of AuNPs concentration and exposure duration on RBC glutathione reductase activity

Figure 3 demonstrates that the glutathione reductase level is decreased significantly ($p<0.05$) with the increasing concentration of AuNPs. During 6 hr of AuNPs treatment, the level is decreased by 51.99%, 58.2%, 65.21%, 69.05% and 71.59% in the 1×10^{-3} mMol, 2×10^{-3} mMol, 3×10^{-3} mMol, 4×10^{-3} mMol and 5×10^{-3} mMol AuNPs group respectively and the decrease was found to be statistically significant ($p<0.05$) (Figure 3). However, the positive control group shows 84.71% decrement from the control. When AuNPs are incubated with RBC for 18 hr, the enzyme level is decreased by 53.48%, 60.92%, 68.43%, 70.36% and 80.19% respectively from the negative control group. The positive control group shows 95.7% decrement from the same negative control group. In case of 24 hr treatment the GR level is decreased by 62.75%, 66.38%, 75.71%, 77.19% and 84.62% and the positive control group shows 97.06% decrement from the negative control group (Figure 3).

Effect of AuNPs concentration and exposure duration on RBC glutathione S transferase activity

The GST level decreases significantly ($p<0.05$) with the increasing concentration of AuNPs; but seen to be insignificant with the exposure duration (Figure 4). During 6 hr of AuNPs treatment, the level is decreased by 30.15%, 42.85%, 58.85%, 69.32 % and 81.97% in the 1×10^{-3} mMol, 2×10^{-3} mMol, 3×10^{-3} mMol, 4×10^{-3} mMol and 5×10^{-3} mMol AuNPs group respectively in comparison to the negative control group. However, the positive control group shows 59.43% decrease from the same negative control group.

The decrement percentage was observed more or less in comparison to above when the same concentration of AuNPs was exposed with the RBC for 18 hr, the enzyme level is decreased by 36.08%, 52.74%, 64.78%, 77.6% and 87.46% respectively when compared with the negative control group and the decrease was found to be statistically significant ($p<0.05$) (Figure 4).

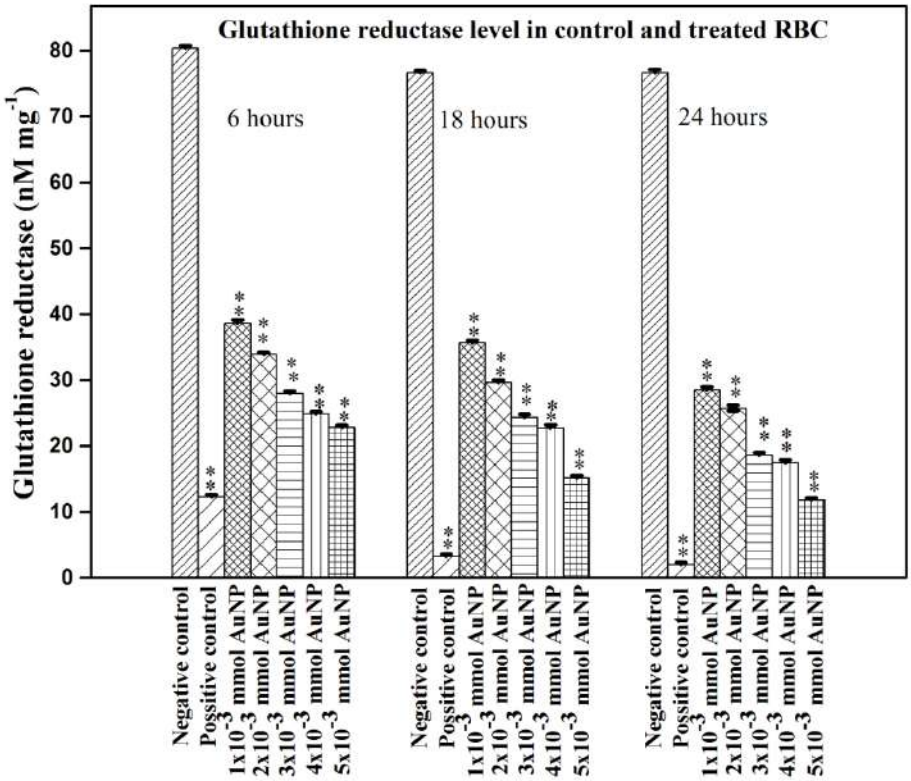

Figure 3: Effect of AuNPs on Glutathione reductase in RBC depending on time and dose of exposure.

*Indicates significance level between the control group and treated group depending on exposure dose.

**Indicates significance level is more (i. e. $p<0.05$) in comparison to the *groups.

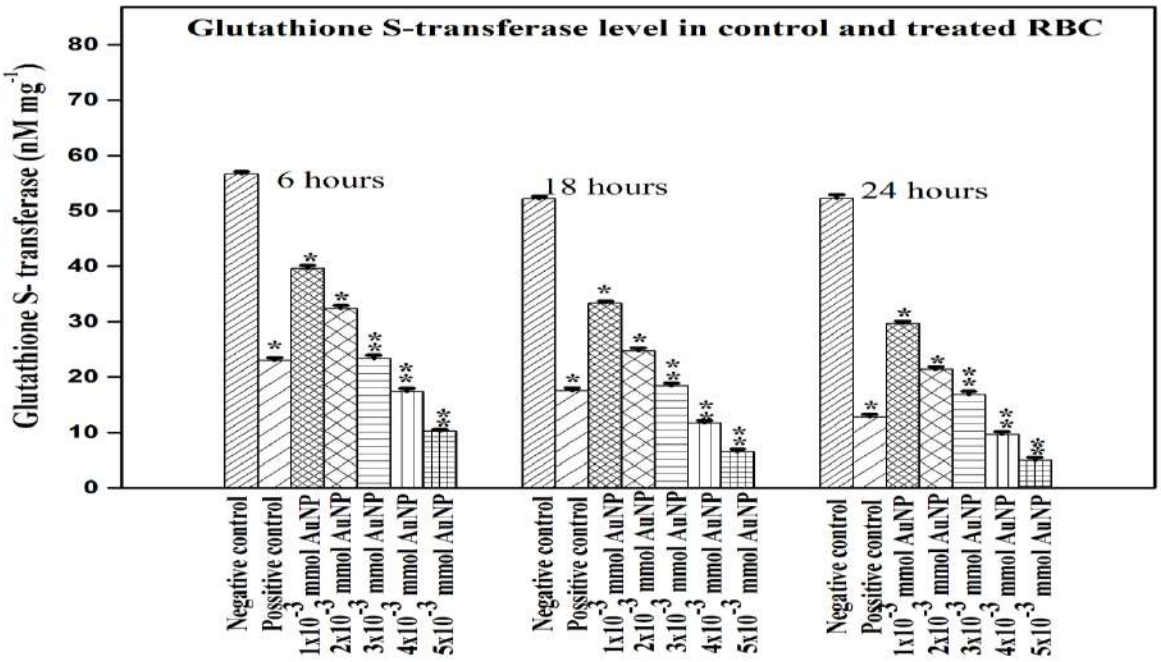

Figure 4: Effect of AuNPs on Glutathione S-transferase in RBC depending on time and dose of exposure.

*Indicates significance level between the control group and treated group depending on exposure dose.

**Indicates significance level is more (i. e. $p<0.05$) in comparison to the *groups.

In case of positive control group 66.37% decrement from the same negative control group is observed.

Next in 24 hr treatment the GST level is decreased by 43.15%, 59.02%, 67.69%, 81.49% and 90.50% in comparison to the 24 hr negative control and the positive control group shows 75.49% decrement from the same negative control group (Figure 4).

Effect of AuNPs concentration and exposure duration on RBC glutathione peroxidase activity

The GPx level is decreased significantly ($p<0.05$) with the increasing concentration of AuNPs; but seen to be not significant with the exposure duration (Figure 5). During 6 hr of AuNPs treatment, the level is decreased by 46.13%, 54.58%, 60.75%, 68.7% and 76.72% in the 1×10^{-3} mMol, 2×10^{-3} mMol, 3×10^{-3} mMol, 4×10^{-3} mMol and 5×10^{-3} mMol AuNPs group respectively in comparison to the negative control group. However, the positive control group shows 77.88% decrement from the same negative control group.

In the next case, more or less same decrement percentage was observed in comparison to the previous when the same concentration of AuNPs was incubated with the RBC for 18 hr. The enzyme level is decreased by 56.65%, 64.63%, 70.96%, 76.67% and 82.92% respectively when compared with the negative control group and the decrease was found to be statistically significant ($p<0.05$) (Figure 5). The positive control group shows 90.4% decrement from the same negative control group.

Again for 24 hr treatment the GPx activity is decreased by 62.20%, 68.27%, 74.58%, 79.52% and 85.42% in comparison to the 24 hr negative control and the positive control group shows 92.88% decrement from the same negative control group (Figure 5).

DISCUSSION

Red Blood Cell oxidative stress is induced by either increasing the reactive oxygen species or depleting the antioxidant defence mechanism of the cell. Erythrocytic oxidative alterations are regulated by a group of antioxidants and endomembrane enzyme system. RBCs are devoid of cell organelles like mitochondria (prime source of oxidative damage). During signal transduction process the cysteine residue of thiol protein in membrane system plays a crucial role. Thiols have the ability to react instantly with oxidizing agent like ROS thus, act as the antioxidant defence system.[29]

The mechanism of oxidative stress caused by AuNPs in RBCs are attributed to its several physico-chemical properties like its morphology, architecture, dose of administration and surface charge of the nanoparticles. Smaller sized gold nanoparticle has the higher haemolytic activity[30] which in turn cause increased lipid peroxidation product as there is less number of RBCs to protect the oxidative alterations.[31] RBCs are highly sensitive and prone to oxidative as it devoid of a proper oxidative damage repair mechanism.

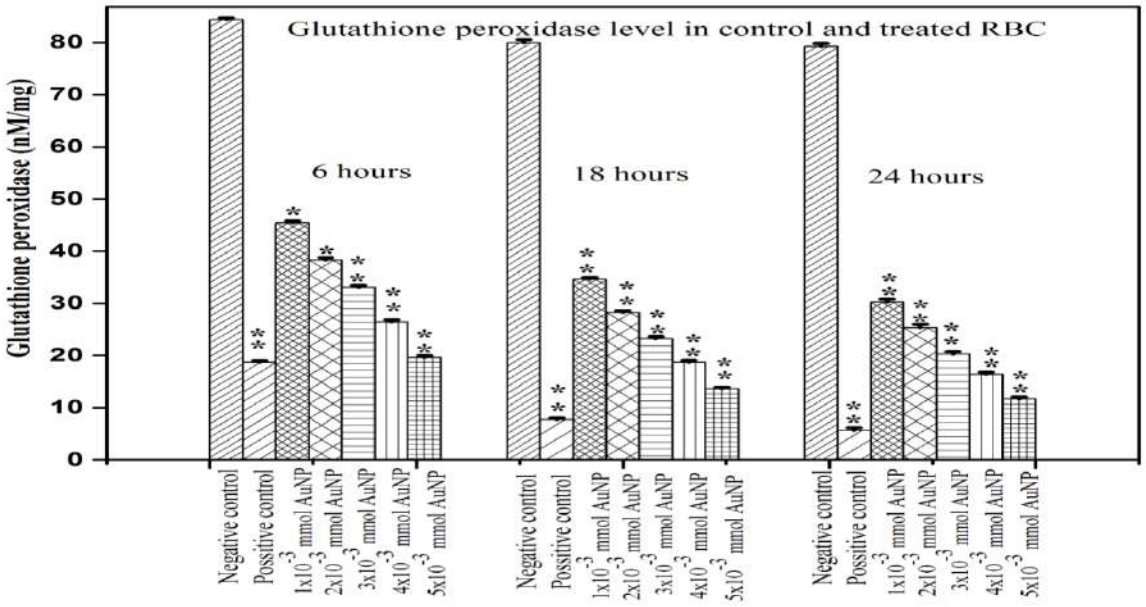

Figure 5: Effect of AuNPs on Glutathione peroxidase in RBC depending on time and dose of exposure.

*Indicates significance level between the control group and treated group depending on exposure dose.

**Indicates significance level is more (i. e. $p<0.05$) in comparison to the *groups.

Such oxidative alterations cause the thiol side chains of the polypeptide to form the disulphide bonds disturbing the delicate quaternary structure of haemoglobin.[32] Both reduced Nicotinamide Adenine Dinucleotide (NADH) and reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) methaemoglobin reductases are present in RBCs to convert methaemoglobin into oxyhaemoglobin. As there are no mitochondria in RBC, hence, the principal oxidative damages preventing mechanism is absent in RBC. Thus, the most important thiol oxidation leads to the disulphide bond formation enhancing more oxidative damages.[33] Our present study is in conformity with the previous studies[34-36] which describes more deterioration caused by smaller sized gold nanoparticles. From the present study, it is seen that smaller sized and higher concentrated dose gold nanoparticles have more cytotoxic effect on the RBCs because of their permeability in the cellular system. Because of their small size it can easily penetrate the membrane assemblies and can bind with end membrane biomolecules thus, deviating its own functioning leading to an altered effect. This effect is reflected as an oxidative damage in the RBCs, as the antioxidant mechanism is destructed by the smaller sized gold nanoparticles.[37] In conclusion, it is mentioned worthy that RBCs oxidative stress is the result of the balance between pathophysiology for oxidant production and enzymatic and non-enzymatic antioxidant defence mechanism prevailing in the body system.[38]

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The present study explores the effect of citrate stabilized gold nanoparticles on RBCs' oxidative stress in different doses. The effect of the citrate stabilized gold nanoparticles on five oxidative stress markers biochemically in blood samples was assessed. All the biomarkers of oxidative stress i. e. malondialdehyde, total thiol content, glutathione reductase, glutathione S-transferase and glutathione peroxidise are affected by the gold nanoparticles in a dose and time dependent conduct. An alternate approach for synthesis of gold nanoparticle with less toxicity will be helpful for its use in biomedical applications.

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