Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

**Original Research Article** **DOI - 10.26479/2018.0403.02**

# IMPACT OF GOLD NANOPARTICLES ON ELECTROPHYSIOLOGY AND INTRACELLULAR CALCIUM IN CULTURED NEURON

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**ABSTRACT:** Nanomaterial stimulated alteration in the neuron is a great concern as the nanoparticles are widely used in the diagnosis of some neural disorders. Among the different nanoparticles, gold nanoparticles have a wide scope of applicability in the living system due to less toxicity. Gold nanoparticles can be used for cancer therapy especially for the killing of malignant cells in the brain. These particles can cross the blood-brain barrier and can change the physiological functions of the brain. Gold nanoparticles may cause the change of different membrane proteins that might be responsible for defective transport of chemical through the membrane and finally leads to cellular loss. The experiment has been designed to study the changes of membrane polarity and intracellular calcium level in gold nanoparticles exposed cultured neuron. Citrate-stabilized gold nanoparticles are prepared from chloro-auric acid using sodium citrate as a stabilizing agent. Gold nanoparticles are characterized by UV Vis-spectrophotometer. Intracellular calcium level is monitored using fluorescence dye fluo 3. Membrane depolarization is studied using voltage-sensitive dye $DiBAC_{(4)}3$. A higher level of intracellular calcium is observed in AuNP treated neurons. Elevation of intracellular calcium level might be due to the release of calcium from the intracellular storage or transport from extracellular media. Membrane depolarization and hyperpolarization study reveals that AuNP treated neurons membrane is present in depolarized state in comparison with the control neurons. A higher level of intracellular calcium and prolong depolarization in AuNP treated neuron may trigger some signaling mechanism leading to neuronal death

**KEYWORDS:** AuNP, intracellular calcium, neuron, depolarization

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## 1.INTRODUCTION

Nanoparticles have wide applicability in the biomedical field for diagnosis, drug delivery, and treatment. Among different nanoparticles used in the biological field, gold nanoparticles show less toxicity in living cells. These particles can alter physical and chemical properties of many exogenous and endogenous substances in the body that affect the brain and nervous system. In the field of neurosciences, gold nanoparticles are successfully tested for regeneration of damaged nerve [1] prevention of amyloid beta formation [2] etc. Gold nanoparticles with surface negativity may alter the excitability of the neurons. Earlier observation also suggested that intracellular Au NPs can increase neuronal excitability and aggravate seizure activation in hippocampal tissue [3]. These particles can be attached to neurons and used to stimulate the cells, without introducing any genes. Besides, gold nanoparticles are also used for mapping the brain electrical activity. Gold nanoparticles can change the intracellular composition of different chemicals including calcium, sodium, and magnesium [4]. Intracellular calcium concentration maintenance is very important for proper functioning of the cell. Calcium plays an important role in nerve physiology, impulse transmission and memory formation [5]. Changes in the cytosolic free calcium concentration are key regulators of almost any cellular function in eukaryotes. Nearly hundreds of proteins can bind calcium and consequently change their activity, thus modulating an intracellular signaling cascade. Alterations in synaptic function, neuronal excitability etc is also regulated by intracellular calcium level. Salinas and his group reported that there is an increase in firing rate approximately 17% in gold nanoparticle treated neurons [6]. Physicochemical properties of gold nanoparticles including size, shape, surface modification, and charges are closely related to their biological effect. The previous study states that membrane attachment of negatively charged particles is less than the positively charged AuNP. Gold nanoparticles with positive surface charge have much higher cell internalization ability than negative surface charge [7]. The biological half-life of gold nanoparticle is already determined and found to be $12.9 \pm 4.9$ [8]. Cellular membrane protein may react with the gold nanoparticles and can alter electrophysiological properties of the neuron [9]. Intracellular distribution of gold nanoparticles in different types of cells is studied and observed that 2 and 6 nm AuNP are found in the nucleus while 15 nm particles are found in the cytoplasm [10]. The hypothesis of this research work is that the surface negative gold nanoparticles may alter the movement of charged particles through the plasma membrane into the interior of the cell and disturb the interior chemical composition of cell. So, present research work has been designed to study the effect of citrate-stabilized gold nanoparticles on the calcium influx and intracellular calcium level. However, this research work has some limitations. First of all the exact numbers of gold nanoparticles applied in the experiment are not calculated. Secondly, the charge of the nanoparticles is not determined.

## 2. MATERIALS AND METHODS

**2.1 Culture of Neurons:** The experiment is conducted on Cultured Neurons: HT22 hippocampal neuron cell line is collected from NBRC lab. Cells are cultured in the Cell Culture Lab in culture plate kept 95% oxygen and 5% carbondioxide in $CO_2$ incubator at $37^0$ centigrade.

Test chemicals: Citrate stabilized gold nanoparticles are prepared and applied to hippocampal cells.

Calcium influx indicator dye: Fluo 3 is used to study the calcium influx and intracellular calcium level study. Loading dye concentration is 15 micromolar

**2.2 Preparation of citrate-stabilized gold nanoparticles**: 20 ml of 1.0 millimolar concentration gold chloride is boiled in a hot plate magnetic stirrer and immediately added 2ml of 1% sodium citrate. The change in color of gold chloride solution indicates the formation of gold nanoparticles. The solution is allowed to cool to room temperature. Gold nanoparticle solution is filtered with 0.22mm syringe filter and stored at $4^0$ centigrade. The absorbance of the solution is studied in Tecan Microplate reader. Highest absorbance is observed at 560 nm wavelength.

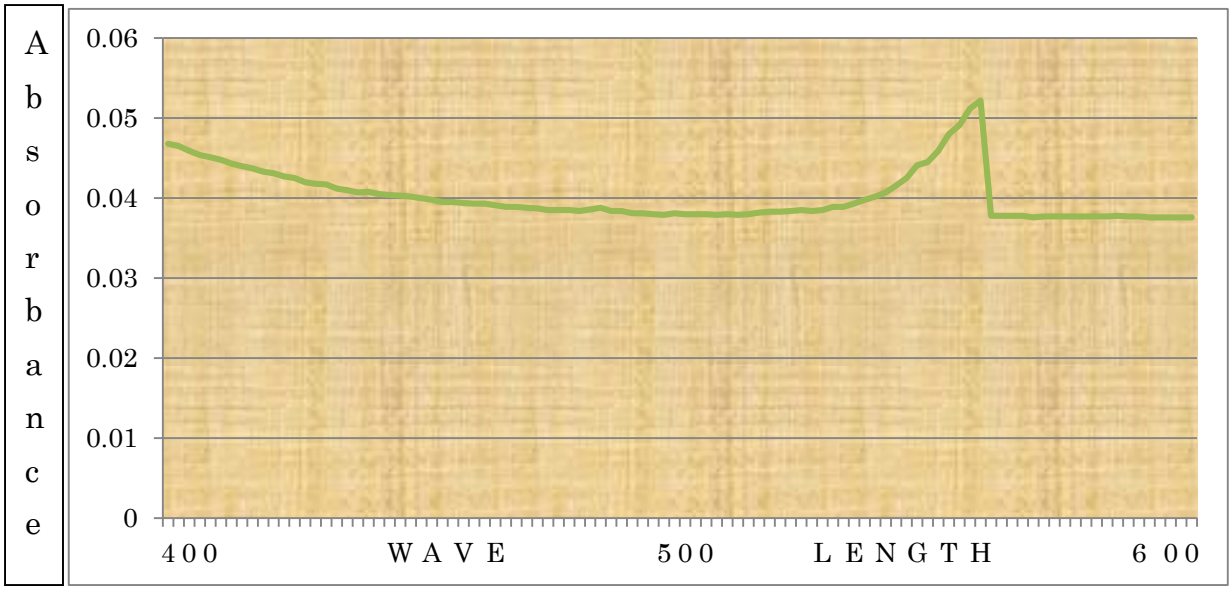


Fig 1: Absorbance of citrate-stabilized gold nanoparticles. (Peak absorbance at 560 nm)

**2.3 Application of Gold nanoparticle**: Gold nanoparticles are mixed with DMSO and applied at 100 microliter concentration in each well.

**2.4 For the study of calcium influx**; Nerve Cells (approximately 500) are seeded in chamber slide and are starved for 24 hours. Treated cells are given gold nanoparticles in PBS and control cells are given PBS without gold nanoparticles. Both the groups of cells are loaded with fluo 3 dye. Chamber slides are fixed in the live cell imaging devices and observed and recorded the signals. Data are collected every 1000 millisecond intervals.

**2.5 Study of membrane potential**: Membrane potential and depolarization in treated and control cells are studied by voltage-sensitive fluorescent dye $DiBAC_{(4)}3$. $DiBAC_{(4)}3$ is the bis-barbituric acid oxonol dye with excitation maxima at approximately 490 nm. Hyperpolarization results in extrusion

of the dye and then a decrease in fluorescence. Cells are seeded in 96 well plates and starved for 24 hours. AuNP is applied for 2 hours under controlled temperature. $DiBAC_{(4)}3$ is loaded both in treated and control cells at 150 nanomolar concentration and is kept in dark for 30 minutes at $CO_2$ incubator at 95% $O_2$ and 5% $CO_2$. Plates are fitted in a microplate reader and kianic acid is added. Fluorescence intensity is recorded immediately at excitation 490nm and emission 525nm. Data are recorded at 10 seconds interval for 3 minutes.

**2.6 Statistical Analysis**: Statistical analysis of calcium wave signal of single cell in both control and treated cells is done using Graphpad Prism 7 software. Nonlinear graph is transferred to sine wave form. Comparison between control and treated groups is done by Microsoft excel software.

Data are analysed using unpaired student T Test at 5 percent significant level.

## 3. RESULTS AND DISCUSSION

Calcium influx into the neuron is indicated by calcium signals recorded in the live cell imaging devices. A fluctuation of calcium influx is observed in both control and gold nano-particles treated neurons. AuNP treated neurons show statistically significant (at 0.005) higher level of fluorescence intensity than control neurons. Sine wave analysis clearly indicates higher amplitude in the treated neuron at the same wavelength which means very fluctuations in intracellular calcium. This clearly indicates a more depolarization and repolarization in treated neuron than that of the control. Phase shift difference in fluorescence intensity is also observed in control and treated neurons.

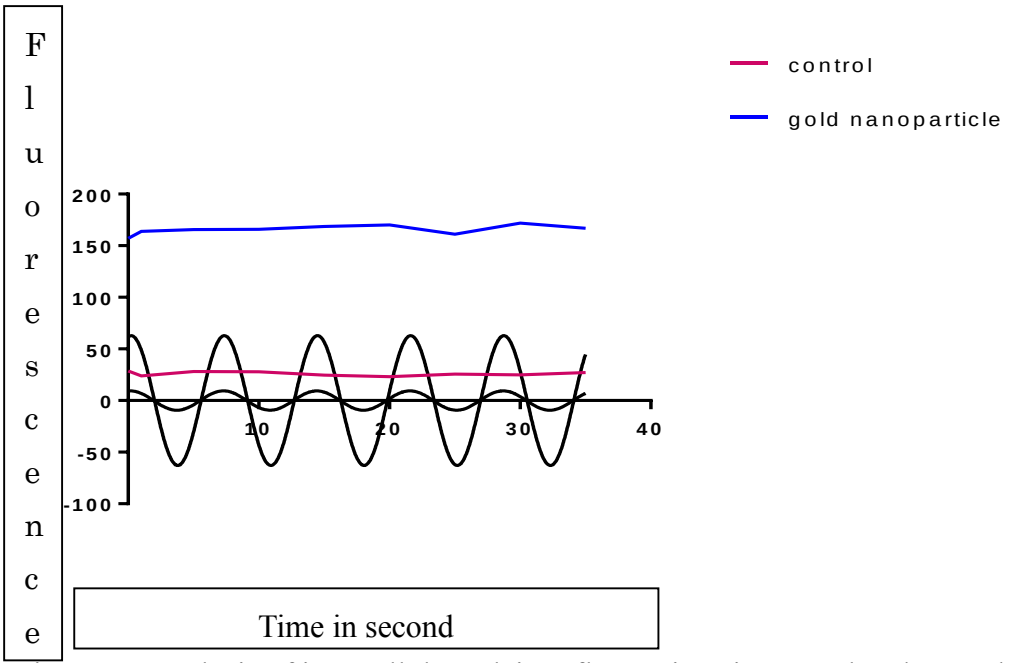


Fig 2: Sine wave analysis of intracellular calcium fluctuations in control and treated of neuron. Total 7 numbers of cells are studied.

| Standard sine wave | Control | AuNP |
|---|---|---|
| Best-fit values | | |
| Amplitude | 9.353 | 62.88 |
| Wavelength | 7.134 | 7.131 |
| Phase Shift | 1.425 | 1.364 |
| Frequency | 0.1402 | 0.1402 |

Sine wave data comparison between control and AuNP treated neuron

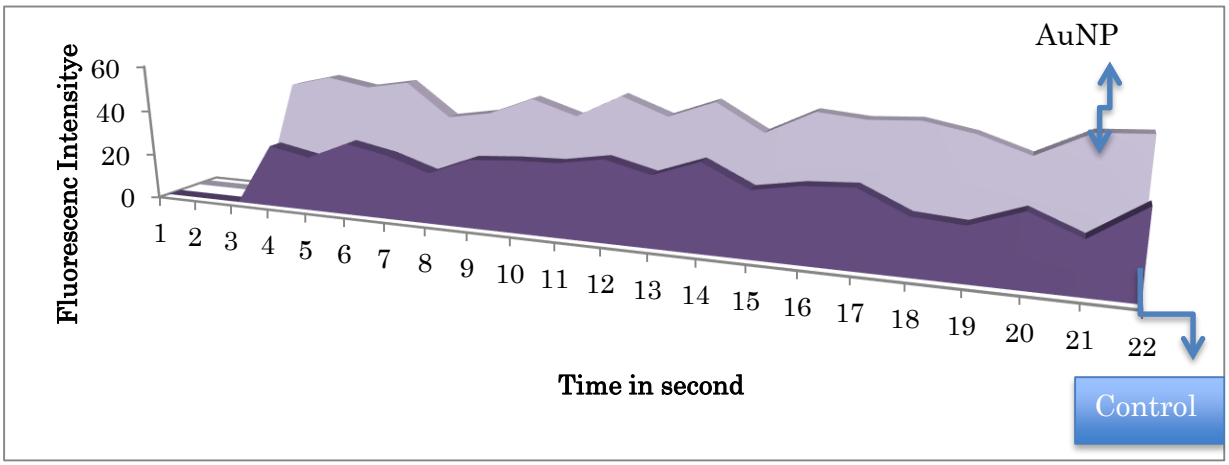


Fig3: Fluorescence intensity of control and AuNP treated cell

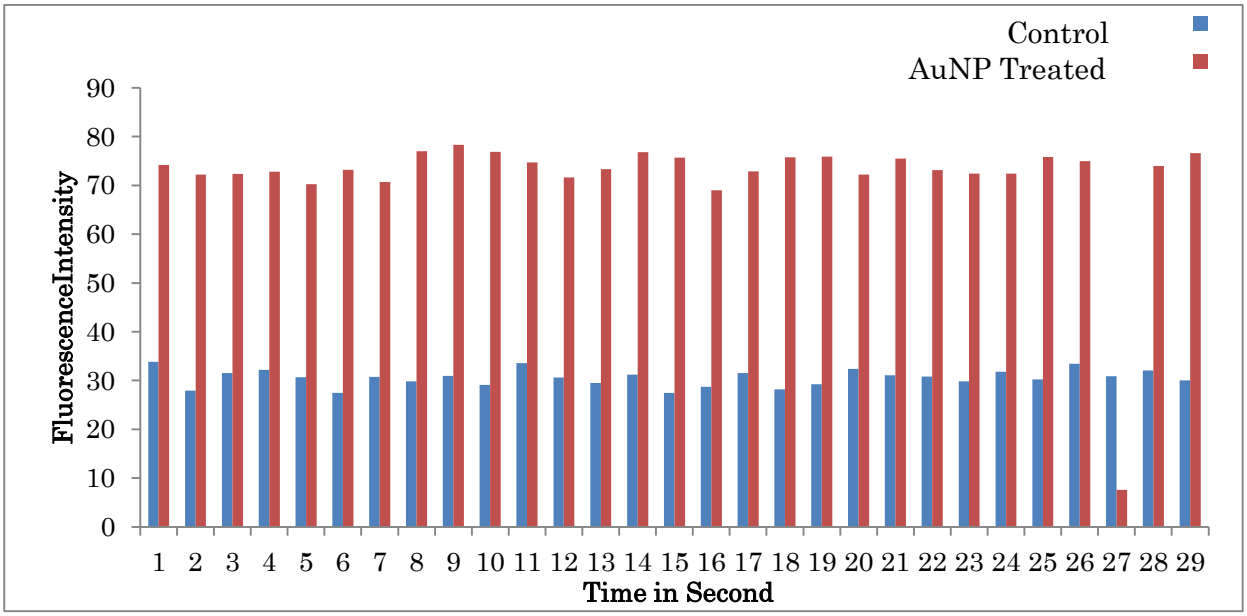


Fig 4: Intracellular calcium level expressed as fluorescence intensity in control and AuNP treated Neuron. Comparison the fluorescence between control and treated neuron is done using student t test. Statistically significant value P<0.005 is observed. (n=7)

**3.1 Depolarization study of control and treated neurons:** Depolarization study shows an increase in membrane potential after addition of Kcl as indicated by an increase in fluorescence activity of fluorescence dye Dibac $_{(4)}$3. In the AuNP treated neuron, the membrane is present in the depolarized state as shown by higher fluorescence intensity while in control neuron fluorescence intensity gradually increases. It is notable that the fluorescence intensity is found higher in AuNP treated cells indicated the entry of more dye into the cell. Entry of more dye indicated that the membrane was present in the depolarized state but the depolarization had no effect on mitochondrial release. In control neurons, the cells become gradually polarized and the dye enters in the control neurons slowly. After 120 seconds both control and treated cells become the same condition as both the curve touch at that point it means maximum potential state. DiBAC$_{(4)}$3 is slowly permeable dye in depolarized cells which slowly increases the fluorescence intensity after stimulation by kcl. In control neurons gradual increase in fluorescence intensity clearly indicates a sow change in membrane polarity while in gold nanoparticle treated cells membrane is found in polarized state due to the presence of nanoparticles. After 2 minutes of stimulation, the fluorescence intensity graph runs parallel and touches some points. This clearly reveals a maximum depolarization of both control and treated cells.

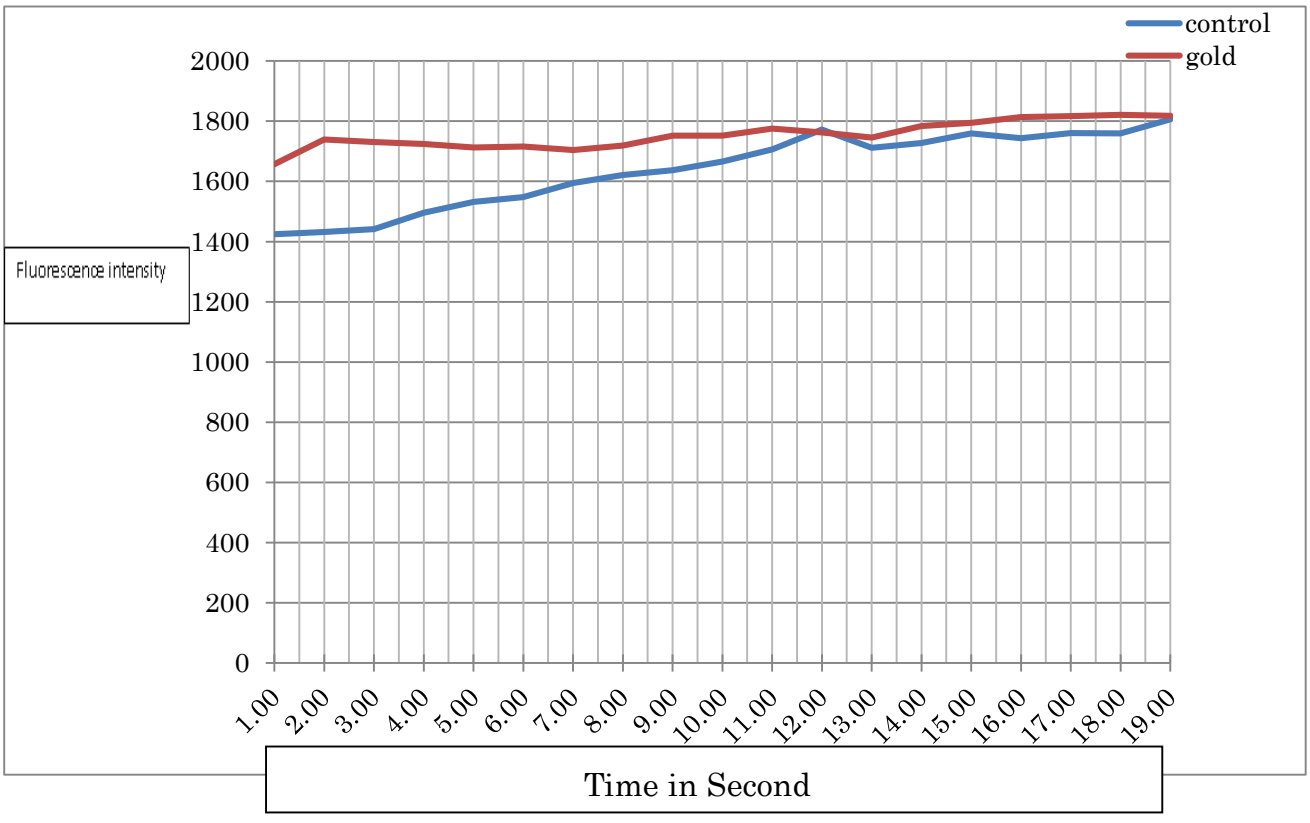


Fig 5: Post depolarized effect of control and treated neuron. Cells are loaded with voltage-sensitive dye Dibac $_{(4)}$3. Depolarization is initiated by Kainic acid.

Experimental findings of this research work reveal the effect of nanopartcles on biochemical constituents of the cell. Gold nanoparticle treated neurons show high intracellular calcium level in comparison with non-treated cells. Elevation of intracellular calcium levels after administration of different nanoparticles are reported by Meindl [11]. Another scientist studied and stated that cellular change in presence of nanoparticles depends upon the surface charge of the nanoparticles. Cationic nanoparticles cause more pronounced disruption of plasma membrane integrity stronger mitochondrial and lysosomal damage than anionic nanoparticles. Cellular uptake of nanoparticles also depends upon the surface charge of nanoparticles [12]. Besides, internal physiology of the cell also regulates the internalization of nanoparticles. Cellular bindings of different nanoparticles also depends upon the surface charge of nanoparticls [13]. A group of scientist investigated the correlation between zeta potential changes and cell membrane stability in bacterial cells. Changes of zeta potential may be correlated to the enhancement of membrane permeability and PDI and beyond a critical point, it may lead cell death [14]. Nanoparticles can cause the conformational change of different proteins [15]. This might be due to the influx of calcium from extracellular environment or release from intracellular storage. The previous report suggested the influence of gold nanoparticles on the intracellular environment that leads to cellular swelling. Cellular swelling might be due to a high level of intracellular calcium ion concentration. Previous works showed the effect of gold nanoparticles on the hippocampal neuron and also increased the seizure activity. Calcium ions play a role in neurotransmitter release. As the entry of calcium is also associated with depolarization so, calcium influx might be the cause of increase seizure. Gold nanoparticles may cause the increase electrical activity and final entry of calcium in to the cell [16]. Divalent calcium ion with positive charge controls different physiological processes in the cell and intracellular calcium level must be maintained for the proper functioning of the cell. Calcium concentration is very low in the intracellular fluid in comparison with the extracellular environment. The low level of calcium concentration is maintained by calcium buffers which include cytosolic-calcium binding proteins. Rapid spike in calcium concentration is due to the influx of calcium via calcium channels being modulated by calcium buffer. Positively charged calcium ion can interacts with the negatively charged inner side of the plasma membrane and can neutralize the negative charge. Gold nanoparticles are charged particles and can alter the calcium level and calcium spike. Calcium oscillations' are two types traveling waves and standing waves [17]. Sign wave analysis of control and treated neurons show higher amplitude and lower phase shift value. This finding clearly reveals the fluctuations of calcium level in gold nanoparticle treated neuron. It is also reported that the calcium release from the intracellular storage has not associated with any change of membrane potential [18]. As the average diameters of Ca2+, Na+ and K+ ion channels are in the range 0.9– 1.5 nm so, large size gold nanoparticles unable to cross through these channels easily. Previous work also suggests that the

penetration through ion channels is controlled by the affinity of the transported species to specific binding sites in the ion channel and not solely by size. The specificity of the ion channels is expected to prevent transport of AuNPs through them. Besides variation in the extracellular calcium ion concentration is a critical component in tight junction regulation in models of Ca2+ addition/depletion. After entering into the biological system, AuNP interacts with protein molecules and forms protein corona on their surfaces. Protein corona may significantly affect the cellular uptake of nanoparticles in living systems and might be causes toxicity in a living system [19]. The previous studies show that in the hippocampus, voltage-gated calcium channels (VGCCs) and N-methyl-D-aspartic acid (NMDA) receptors are two major players in the Calcium influx. Activation of postsynaptic NMDA receptors contributes to postsynaptic calcium influx that is essential for triggering LTP[20]. A group of scientists conjugating the AuNPs to some functional groups that specifically bind to external motifs of neuronal membrane proteins and observed that nonconjugated gold nanoparticles washed out from the neuronal membrane than conjugated AuNP [21]. AuNP and protein interaction serve as starting point to study the biological effects of Au NPs. Usually, the high abundance proteins first arrive at and adsorb on the surface of NPs, but they are eventually replaced by high-affinity proteins to form NP–protein complexes [22]. AuNPs in biological fluids may form NP–protein complexes that can be recognized by cell membrane receptors and then taken-up by cells. Au NPs can also be wrapped by the retracted cell membrane, and thus be directly transported into cells, which can directly affect the cellular responses. AuNPs can be internalized by cells in different ways: receptor-mediated endocytosis and phagocytosis pathways. The processes in both pathways include the formation of Au NP–protein complexes, recognition by cell membrane receptors, engulfment into a vesicle by the cells, being transported or penetrated into cells, the activation of signal pathway, sequential trafficking inside cells, and storage or elimination of Au NPs by cells. Studies on membrane potential in other cells indicate a change in membrane potential by BK channel opener [23]. Movement of sodium through potassium ions channels depend upon the energy level of the cell, and negatively charged particles may alter the energy level and change the movement of different ions leading to depolarization [24]. Moreover, toxicity of charged nanoparticles is more than the neutral nanoparticles might be due to electrostatic interaction [25]. In the present experiment, negatively charged nanoparticle is applied and these particles may interacte with plasma membrane and results in depolarization of neuronal membrane in resting condition. Experiment on physiological and biochemical effect of AuNP on cultured neuron reveals that the gold nanoparticle causes elevation of intracellular calcium level when compared with control neurons. This effect might be due to entry of calcium from culture media or from intracellular storage either from mitochondria or endoplasmic reticulum. Besides, gold nanoparticles also cause significant effect on neuronal membrane polarity.

## 4. CONCLUSION

In the experiment it is observed that citrate stabilized gold nanoparticle treated neuron shows higher level of intracellular free calcium in comparison with control neuron. Gold nanoparticle also causes the membrane of the neuron in depolarized state, however addition of depolarizing agent will not further increases depolarization.

## 5. ACKNOWLEDGEMENT

This research work was conducted in Dr. Shiv K Sharma's Lab at National Brain Research Centre, Manesar, Haryana, India, under DBT, National Associateship Award Programme. Author thankfully acknowledges the DBT for supporting this programme. Thankfulness is also expressed with equal sincerity to the Director, National Brain Research Centre for giving me the full facility to conduct this research work.

## 6. CONFLICT OF INTEREST

There is no conflict of interest

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