Gold nanoparticles caused learning impairment in mice

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ABSTRACT

Aims : To investigate the toxic effect of gold nanoparticles (GNPs) to the learning and memory of mice.
Main methods: Naked GNPs of 17 nm and 37 nm were synthesized, purified, and injected intraperitoneally into BALB/C mice at a dose of 8 mg/kg/week. Passive avoidance test was performed to detect the possible leaning impairment. Biochemical analysis to examine levels of monoamine and acetylcholine in mouse brain was also performed. ICP-MS was performed to examine the presence of GNP in brain. Localization of GNP at hippocampus was surveyed by ex vivo Coherent anti-Stoke Raman scattering (CARS) microscopy. Transmission electron microscopy (TEM) was also performed to verify the cellular location of GNPs at hippocampus.
Key findings: Both 17 nm and 37 nm GNPs induced severe sickness in mice. However, only 17 nm GNP impaired the learning and memory of mice. GNP treatment elevated levels of dopamine from 114.5 ng/mouse brain to 143.6 ng/mouse brain for 17 nm ($p<0.01$) and to 138.2 for 37 nm GNP ($p<0.05$). Serotinin was significantly reduced by 17 nm GNP treatment from 57.2 ng/mouse brain to 44.3 ng/mouse brain ($p<0.05$). ICP-MS indicated the presence of GNPs in every part of the brain. CARS microscopy showed that 17 nm GNP was located at the Cornu Amonis regions of hippocampus where neuronal cells clustered, while 37 nm GNP was excluded from the cell clustered region. TEM and EDAX verified the presence of 17 nm GNP in the cytoplasm and in the dendrite of pyramidal cell, while 37 nm GNP was found in the extracellular region of neuronal cells. TEM image also indicated that both endocytosis and free diffusion coexisted for the invasion of 17 nm GNP.

Significance: The current study provided evidence that nanoparticles were capable of entering into brain and affecting normal brain function. The size-dependent invading ability of GNPs provides an extra dimension for drug delivery.

Keywords: gold nanoparticles, nanotoxicity, hippocampus, mice, learning impairment

1 INTRODUCTION

Nanocarriers have provided a novel platform for target-specific delivery of therapeutic agents. Over the past decade, several delivery vehicles have been designed based on different nanomaterials, such as polymers, dendrimers, liposomes, anotubes , and nanorods. Gold nanoparticles (GNPs) have recently emerged as an attractive candidate for delivery of various payloads into their targets. Several unique chemical and physical properties enable GNPs for transporting and unloading the pharmaceuticals. The gold core is essentially inert and non-toxic. Monodisperse nanoparticles can be readily synthesized with diameters ranging from 1 nm to 200 nm. Functionalization of GNPs is conveniently achieved through thiol linkages. In addition, the photophysical properties serve as an easy remote control to trigger drug release and clinical imaging.

Most pharmaceuticals cannot freely diffuse through the blood brain barrier (BBB) and require receptor-mediated transport through brain capillary endothelium to deliver their content into the brain parenchyma. Nanoparticles provide an unprecedented opportunity to carry drugs through BBB. The small dose requested for therapeutic efficiency could easily fit the loading capacity of nanoparticles and would not require the administration of large amount of potentially toxic materials.

The current study is based on the hypotheses that GNPs might be capable of passing through BBB and once get inside brain GNPs might be capable of altering the regular function of brain in a size-dependent manor.

2 MSTERIALS AND METHODS

2.1 Materials

HAuCl4, sodium citrate, NaBH4, HCl, HNO3, H2SO4, H2O2, and other chemicals of analytical grade were purchased from Sigma-Aldrich and Fisher. H2O was >18 MΩ from a Milli-Q water purification system.

2.2 Preparation of Gold Nanoparticles

Gold nanoparticles (GNPs) of diameter with 17 nm and 37 nm were synthesized as reported previously [1, 2]. The seed colloids were prepared by adding 1 mL of 0.25 mM HAuCl4 to 90 mL of H2O and stirred for 1 min at 25 °C. Two milliliters of 38.8 mM sodium citrate were added to the solution and stirred for 1 min, followed by the addition of 0.6 mL of freshly prepared 0.1 M NaBH4 in 38.8 mM sodium citrate. Different diameters of GNPs ranging from 3 nm to 100 nm were generated by changing the volume of seed colloid added. The solution was stirred for an additional 5-10 min at 0-4 °C. Reaction temperatures and times were adjusted to obtain GNPs of larger size. All synthesized GNPS were characterized by UV absorbance. The size of synthesized GNPs was verified by electron microscopy and atomic force microscopy. GNPs were

dialyzed against phosphate-buffered saline (pH 7.4) before injection into the animals.

2.3 Animal Treatment

Four-week-old male BALB/C mice were housed at room temperature of 22±2 C with 12 h light/dark cycle and fed standard rodent chow and water ad libitum. Mice were randomly assigned to 4 groups consisted of 8 mice including control group (not receiving any treatment), positive control (received scopolamine alone), 17 nm GNP-treated group, and 37 nm GNP-treated group. GNPs were administered in a weekly dose of 8 mg/kg body weight intraperitoneally. Passive avoidance test was performed on day 14 after the administration of GNPs. Animals were sacrificed at the end of experiment by cervical dislocation. Brain was isolated and weighted. Excised samples were washed with normal saline and stored at -70 º C for further assays.

2.4 Passive Avoidance Test [3]

The apparatus consisted of two compartments having a steel-rod grid floor (36 parallel steel rods, 0.3 cm in diameter set 1.5 cm apart). One of the compartments (48x20x30 cm) was equipped with a 20 W lamp located centrally at a height of 30 cm, and the other was a dark compartment of the same size, connected through a guillotine door (5x5 cm). The dark room was used during the experimental sessions that were conducted between 09:00 and 17:00 h. On the training trial, the guillotine door between the light and dark compartment was closed. When each mouse was placed in the light compartment with its back to the guillotine door, the door was opened, and simultaneously the time (step-through latency, STL) was measured with a stopwatch until the mouse entered the dark compartment. After the mouse entered the dark compartment, the door was closed. An inescapable scrambled footshock (1 mA for 2 s) was delivered through the grid floor. The mouse was removed from the dark compartment 5 s after the shock. Then the mouse was put back into the home cage until the retention trial. Twenty-four hours later, the retention trial was carried out. The mouse was again placed in the light compartment and, as in the training trial, the guillotine door was opened and the step-through latency was recorded and used as a measure of retention. An upper cut-off time of 300 s was set.

2.5 Analysis for Monoamine and Acetylcholine Concentration in Mouse Brain

Each tissue sample was homogenized in 1 ml of 0.4 M perchloric acid containing 0.1% L-cysteine. After being centrifuged, 10 ml of the supernatant was applied to a high-performance liquid chromatography (HPLC) system with electrochemical detection for determining the levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT, and 5-HIAA, according to the method of Magnusson et al. with a slight modification [4]. The HPLC system was composed of a pump equipped with a damper EP-300, Eicom, Kyoto, Japan., an electrochemical detector ECD-300, Eicom. with a graphite working electrode operated at 750 mV vs. an Ag–AgCl reference electrode RE-100, Eicom., and a reverse-phase column MA-5ODS, 2.1=150 mm inside diameter, Eicom.. The mobile phase was 0.1 M citrate–0.1 M sodium acetate buffer pH 3.9.containing 13% methanol, 1.0 mM sodium 1-octanesulfonate and 10 mM disodium ethylenediaminetetraacetic acid.

To determine the level of NE, a 10-ml portion of the remaining supernatant was applied to the HPLC system with the electrochemical detector operated at 450 mV and a reverse-phase column CA-5ODS, 2.1=150 mm inside diameter, Eicom.. The mobile phase was 0.1 M sodium phosphate buffer pH 6.0.containing 5% methanol, 2.5 mM sodium 1-octanesulfonate, and 50 mM disodium ethylenediaminetetraacetic acid.

The mice were decapitated and their brains were quickly removed. The brain samples were weighted and homogenized in 10 vol of 0.2 M perchloric acid containing 100 mM Na2-EDTA and 100 ng/ml isoproterenol in a Polytron homogenizer (Kinematica, Lucern, Switzerland) at a maximum setting for 20 s on ice. The homogenate was centrifuged at 15,000 g for 30 min. The solution of 1 M sodium acetate was added to adjust the pH to approximately 3.0. After filtration (0.45 mm), the samples were separated using an high performance liquid chromatography (HPLC) system. Monoamines and their metabolites were separated using an HPLC system at 30 oC on a reverse phase analytical column (ODS-80, 4.6 mm i.d. x 15 cm), and detected by an electrochemical detector (Model ECD-100, Eikom Co., Kypto, Japan). The column was eluted with 0.1 M sodium acetate-citric acid buffer (pH 3.5) containing 15% methanol, 200 mg/l sodium 1-octanesulfonate, and 5 mg/l Na2-EDTA, the following monomers and their metabolites were measured: norepinephrine, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT), 5-hydroxytyramine (5-HT, serotonin), and 5-hydroxyindoleacetic acid (5-HIAA).

2.6 TEM

Small pieces of unfixed tissue were fixed in 2.5% glutaraldehyde with 0.05 M sodium cacodylate-buffered saline (pH 7.4), at room temperature for 2 h. The primary fixation was followed by three 0.05 M sodium cacodylate-buffered saline washes (pH 7.4) for 20 min each. The samples were then placed into a 1% OsO4 in the same buffer at room temperature for 1 h. OsO4 post fixation was followed by three 20 min distilled-water washes and dehydration in acetone. The samples were transferred to 33% followed by 66% Spurr resin in acetone solutions for 30 min in each concentration. The samples were then transferred to 100% Spurr resin for 5 h and then overnight, with resin changes at the end of each period. Ultrathin sections were made by using an ultra microtome and sectioning the samples into 100-nm sections. The grids with the ultrathin sections were post stained with uranyl acetate

for 30 min followed by lead for 3 min. After the post staining procedure, a thin layer of carbon was evaporated onto the surfaces of the grids. Examination of ultra-thin-sectioned material was with a Jeol 1400 and 3200 FS.

3 RESULTS

3.1 Both 17 Nm And 37 Nm Gnps Induced Severe Sickness in Mice But Only 17 Nm GNP Caused Learning Impairment

17 nm and 37 nm GNPs were synthesized according to the published procedures (Brown et al., 2000, Liu et al., 2003). Synthesis of GNPs was monitored by UV absorbance, and the size was examined by electron microscopy (17±1 nm and 37±3 nm). The purified GNPs were injected intraperitoneally into BALB/C mice at a dose of 8 mg/kg/week. Injection of naked GNPs caused symptoms of toxicity in mice. The treated animals showed fatigue, loss of appetite, change in fur color, and weight loss. Starting from day 14, mice showed a significantly camel-like back and crooked spine. The majority of mice died before the end of the fourth week.

To explore if the injected GNPs cause learning impairment in mice, we examined passive avoidance performance of GNP-treated mice using scopolamine as positive control and untreated mice as negative control (Fig. 1). The device consisted of two compartments having a steel-rod grid floor. One of the compartments was equipped with a light source and the other was dark. On the training trial, mouse was placed in the light compartment. An electric shock was delivered as penalty for mouse entering into the dark compartment. Twenty-four hours after training trial the mouse was placed at the same location and the latency time was recorded. Scopolamine induced amnesia with an avoidance latency of 92 sec, a 50% reduction comparing to the untreated control ($p < 0.01$). Although both GNPs caused weakness in mice, insignificant reduction in latency was observed for 37 nm GNP-treated mice, while 17 nm GNP showed a latency of 81 sec in the passive avoidance performance ($p < 0.01$).

3.2 Monoamine and Acetylcholine Concentration Was Significantly Affected By GNPs

The formation and consolidation of learning and memory is associated with activity of neurotransmitter systems, such as acetylcholinergic, norepinephrinergic, dopaminergic, and serotonergic neurons. In some way, most neurotransmitter systems can influence learning and memory in mouse. GNP-treatment induced learning impairment which indicated that GNP might cause imbalance of neurotransmitters in the mouse brain (Fig. 2). Norepinephrine negatively regulates learning and memory process (ref2). Administration of scopolamine, 17 nm GNP, and 37 nm GNP did not affect levels of norepinephrine and its metabolite 4-hydroxy-3-methoxyphenylglycol (MHPG). Activation of dopaminergic system causes learning impairment (ref4). GNP treatment elevated levels of dopamine from 114.5 ng/mouse brain to 143.6 ng/mouse brain for 17 nm ($p<0.01$) and to 138.2 for 37 nm GNP ($p<0.05$). Serotinin was significantly reduced by 17 nm GNP treatment from 57.2 ng/mouse brain to 44.3 ng/mouse brain ($p<0.05$). GNP-induced learning impairment is correlated with the increase of dopamine and decrease of serotonin in mouse brain.

3.3 TEM Image Revealed the Cellular Location of Gnps in Hippocampus

TEM was performed to verify the cellular location of 17 nm and 37 nm GNPs in hippocampus (Fig. 3). For 17 nm-GNP treated mouse, the presence of 17 nm GNP in the cytoplasm of pyramidal cells was confirmed (Fig. 4). Compositions of Au and Cu were verified EDAX. The metallic nature of gold was also indicated by HR-TEM. The 17 nm GNP was found in the cytoplasm and also in the dendrite. In particular, 17 nm GNP was surrounded by coated pit-like structure in the cytoplasm. We highly suspected that 17 nm GNP entered cells through endocytosis. No endocytosis-related structure was found for the 17 nm GNP at dendrite. This evidence implied that GNP entered the dendrite through free diffusion. Here we show that alternative mechanisms coexist for the invasion of GNP as previously reported. Invasion of metallic particles like GNP in the neuronal cell and dendrite may seriously interfere with the electric signals transmitted through hippocampus and induce impairment in learning and memory.

The absence of 37 nm GNP in the neuronal cells was also examined by TEM. There are a few dark shadows located in the cytoplasm. Further examination with EDAX reveals composition of uranium which is possibly due to localized deposition of the staining material. There are quite a few 37 nm GNP located inside the dendrite structure of brain cells; however, no endocytosis structure were associated with 37 nm GNP in the dendrite. Apparently, 37 nm GNP may enter dendrite through free diffusion but not through endocytosis.

GNPs may have negligible toxicity for cultured cells. Here we show that given a sufficient dose, the invasion of seemingly nontoxic GNPs can impair learning and memory of mice. The reduction of cognitive ability is associated with the endocytosis of 17 nm GNP into the neuronal cells at the CA region of hippocampus. The observation that 37 nm GNP was found at the extracellular region of hippocampus is consistent with its inability to impair cognition of mice. Although both GNPs caused abnormal fluctuation of neurotransmitter levels in the brain, difference in the invasive ability seems to determine the destiny of the mice.

Learning and memory is not the only brain function. Since both GNPs affected monoamine profiles in brain, we

do not exclude the possibility that brain functions other than learning and memory be affected by the injection of GNPs. The invaded GNP might cause abnormal transmission of electric signal through neurons. It is also possible that engulfment of GNP might induce abnormal cellular response such as apoptosis or imbalance of intracellular electrolytes. Further experiment will be required to explore the damaging role of GNPs.

Nanoparticles of unknown origin are abundant in our environment. It was shown that nanoparticles may be found in the brain of bass. Our results indicated that nanopartilcles when invaded into brain affect normal brain function. The current study might provide molecular insights for abnormal behavior of wildlife.

Here we show that by changing the size of nanoparticle it is possible to deliver foreign particles to the selected area in the brain. While GNPs have been widely used for targeting and imaging in drug delivery, the current study might provide additional dimension to the design of drug carrier delivering into specific area of brain.

4 ILLUSTRATIONS

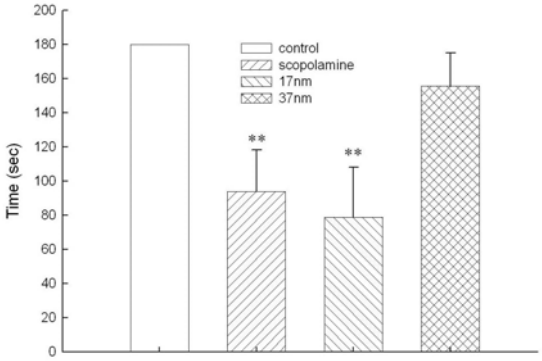

Figure 1: Learning impairment of passive avoidance performance in mice induced by scopolamine (1mg/kg, i.p.), 17 nm, and 37 nm GNPs. **: $p<0.01$ compared to control group.

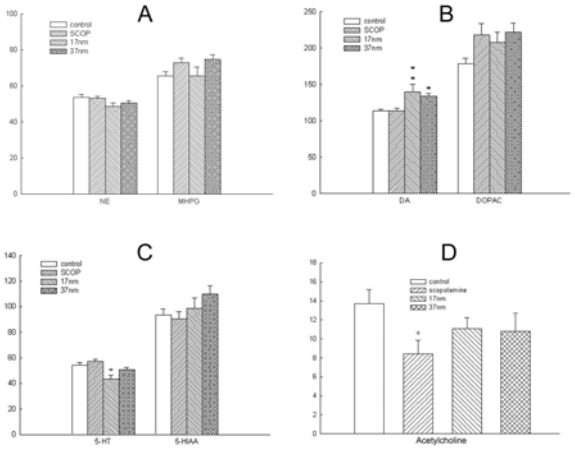

Figure 2: Levels of monoamines in mice treated with scopolamine, 17 nm GNP, and 37 nm GNP. (A) Norepinephrine (NE) and 4-hydroxy-3-methoxyphenylglycol (MHPG). (B) Dopamine (DA) and Dehydrophenylacetc acid (DOPAC) (C) Serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA). (D) acetylcholine. *: $p<0.05$ compared to control group. **: $p<0.01$ compared to control group.

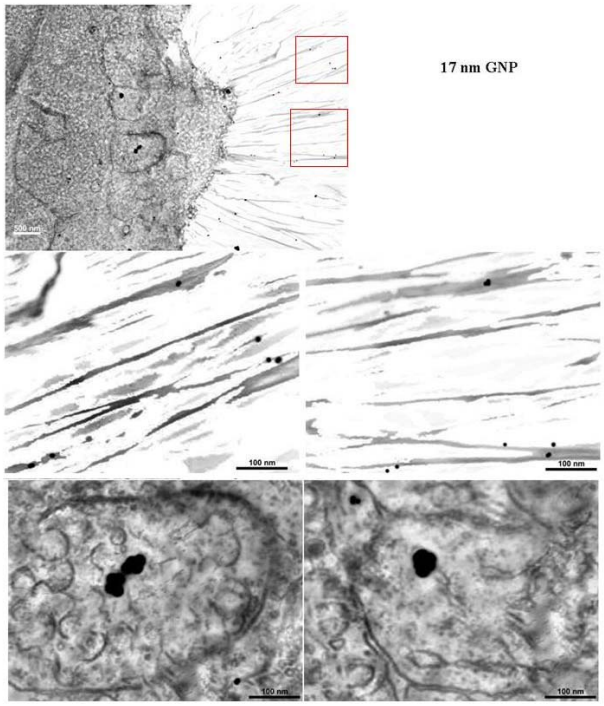

Figure 3: TEM image of 17 nm GNP-treated hippocampus.

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