Nanoparticles as Alternative Strategies for Drug Delivery to the Alzheimer Brain: Electron Microscopy Ultrastructural Analysis

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Abbreviations: BBB, blood brain barrier; SiNPs, silver nanoparticles, AD, Alzheimer Disease; EC, endothelial cells;

Abstract

One of the biggest problems and challenges in the development of new drugs and/or treatment strategies for AD is the difficulty of passing the drugs across the blood brain barrier (BBB). The use of nanoparticles in drug delivery therapy holds much promise in targeting remote tissues, and as a result many studies have attempted to study the ultrastructural localization of nanoparticles in various tissues. However, there are currently no *in vivo* studies demonstrating the ultrastructural distribution of nanoparticles in the brain. The aim of this study is to address how intravenous injection of silver nanoparticles in the brain leads to leaking on the interendothelial contact and luminal plasma membrane, elucidating the possibility of penetrating into the areas of the brain which are most affected by AD (vascular endothelium, perivascular, neuronal and glial cells). Therefore current study indicate that the silver nanoparticles can be conjugated and used to deliver the drugs into the cell cytoplasm of the damaged brain cells which being able to diminish the lesions that occur in AD or other neurodegenerative damaged effected brain.

Keywords: Silver nanoparticles, Electron Microscopy, Drug delivery, Dementia, Vascular Endothelium, Blood-brain-barrier, Alzheimer Disease

Introduction

According to statistical reports by well known authentic centres such as Centre for Disease Control and Prevention (CDC), as well as the National Centre for Health, Alzheimer disease (AD) has surpassed cardiovascular, diabetes, and cancer as a leading

cause of death. AD, the most common form of dementia, is an exasperating health disorder characterized by a progressive decline in cognitive function [1]. AD affects millions of peoples in this era of modern science and technology all around the world. It is a progressive neurodegenerative disorder of hitherto ***"unknown"*** etiology leading progressively to severe incapacity and ultimate death, has been described as the pandemic of the 21st century [2]. AD is epidemic with an estimated 33.9 million people worldwide having the disease [3]. With the nation facing an unprecedented population shift of aging baby boomers and AD poised to strike 10 million of them, it is clear this escalating epidemic must be addressed [4].

Several common occurring risk factors of AD such as obesity, diabetes mellitus, hypertension, renal disease, and smoking will increase the incidence even more [5] and interventions that prevent, stabilize, remediate, or cure AD are essential. As there is no effective and currently available cure for this disease to date, new strategies are necessary to provide apposite therapeutic measures. One of the biggest problems and emerging challenges in the development of new drugs and/or treatment strategies for AD is the difficulty of passing the drugs across the blood brain barrier (BBB). In addition, an increased BBB permeability in AD is also likely since structural damage of brain vascular endothelial cells (EC) is quite frequent in AD brain [6-8]. Thus, enhanced drug delivery in AD is strongly needed to induce neuroprotection and therapeutic success [9].

Creation and synthesis of inorganic materials designed to react with biological tissues can be utilized to plan novel treatment strategies that would extend the biomaterial functionality. Nanotechnology is an emerging multidisciplinary field, which will greatly help in developing novel therapeutic strategies to treat AD cases. The use of nanoparticles (NPs) in drug delivery therapy holds much promise in targeting remote

tissues, and as a result many studies have attempted to study the ultrastructural localization of NPs in various tissues [10-15]. NPs conjugation can therefore be used as an alternate route for the drug delivering system to the damaged neurons, as for example, by using conjugation of silver nanoparticles (SiNPs) with selective mitochondrial antioxidants. Indeed, SiNPs are progressively finding more applications in medical research [16] among other fabricated NPs such as gold, iron, titanium, copper, silica, and zinc [17]. There are currently no *in vivo* studies about the ultrastructural distribution of SiNPs in the brain tissues. This is important since drug penetrating into the brain is the key for therapy success, especially in those areas affected by AD that able to give us proper information not only the pattern but also effectiveness of the treatment. Therefore, investigating the cellular, subcellular, and biochemical pattern of biomaterials, such as the silver ion distribution in the neuronal and non-neuronal cells of brain cellular compartments can expose a new venue of biomedical therapeutic strategies to improve the outcome in AD.

Materials and Methods

Preparation of Silver nanoparticles (SiNP) solution:

The SiNPs used in this study has been obtained from the Sigma , St. Louis, USA. The SiNPs was prepared according with the manufacture protocols, where SiNP dissolved in sterile 0.9% NaCl physiological solution (0.001% final concentration) before intra peritoneal injection.

Animals and treatment protocols

Adult (6-8 months old) Wistar albino male and female rats were purchased from Charles River Laboratories USA (Colombian branch) and kept on a 12:12 h light/dark schedule and received food and water *ad libitum*. Animals were handled in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals, the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience about protection of experimental animals. Experimental procedures were approved by Javeriana University, Bogota, Colombia institutional animal use and care committee before any experimental procedure. Special care was taken to minimize suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

The animals where divided into the following groups: n=7 for each experimental group: control animals with vehicle injection; SiNP 1 and 4 days after the injection. For control experiments, animals were given the same amount of vehicle treatment (0.9% physiological solution) for 1 and 4 days. The SiNP ((silver ion, 5 nm, 0.001% final concentration in 0.9% physiological solution) has been i.p. administered in animals, and 1 and 4 days after the injection. By the end of experiments animals under the terminal anesthesia animals were perfusion fixed via the heart and brain immediately removed and replaced into the fresh solution of the same fixative for the future electron microscopic ultrastructural analysis.

Electron microscopy ultrastructure analysis

Rats were deeply anesthetized with pentobarbital (60 mg/kg) and perfusion fixed as we described earlier [18, 19] with balanced phosphate buffer (PB) containing 1000 IU commercial heparin and followed perfusion fixed with 4% of paraformaldehyde in PB at least 30 min. By the end of perfusion the brains were quickly removed, and immediately harvested with 4.0% paraformaldehyde and kept at 4°C at least for 72 hours and/or until used. After several subsequent washes with 0.1M phosphate buffer (0.1M PB), brains were sliced with vibratome to produce 50-100 mkm randomly selected hippocampal sections, post-fixed with 2.5% glutaraldehyde in 0.1 M PBS for 24 hr, washed again with 0.1 M PB, and exposed to 1% osmium tetraoxide (OSO_4)in 0.1M PB buffer (pH 7.3) for 1 hr. After several additional washes, the tissues were dehydrated in stepped absolute ethanol solutions and flat embedded in Spur's embedding media as we have described earlier [18, 19]. Randomly selected ultrathin sections of the CA1 and CA3 areas with and without lead citrate and uranylacetate count staining were examined using a transmission electron microscope (Jeol 1230) operating system at 100kV. Images were randomly selected and captured using a CCD camera [18, 19].

Results

Control animals which received vehicle injection revealed typical ultrastructural morphology of brain microvessels and neurons (Fig. 1). On the contrary, the animals that received i.p nanoparticles injection after 1 day showed different degrees of brain ultrastructural alterations (Fig. 2). Moreover, main characteristics of the hippocampal neurons 1 day after the nanoparticles injection appeared to be the presence of a SiNPs in the neuronal cell body (Fig. 2). Especially when samples were prepared without exposition to osmium tetraoxide, clear localization of the SiNPs (silver nanoparticles) was seen throughout the neuronal cell body (Fig. 2). The vascular wall cells and neuronal

ultrastructural alterations (especially cellular organelles such as mitochondria, granular and agranular endoplasmic reticulum, Golgi etc) correlate with the presence of nanoparticles in the cytoplasmic matrix of the different hippocampal cellular compartments. Furthermorer, increased density of the presence of silver nanoparticles observed throughout neuronal cell body 4 days after the nanoparticles injection. Neuronal cell body reveals association of silver nanoparticles with mitochondrial alteration. The presence of silver nanoparticles in the neuronal nucleus were also noted (Fig. 3). Very often the presence of varying sizes of silver aggregates was seen throughout the neuronal cell bodies after 4 days of silver ion injection. The main characteristics of the neuronal cell bodies appeared to be the absence of organelles, including mitochondria, which coexisted with the presence of the high density silver particles and/or their aggregates (Fig. 4). Moreover, the accumulation of the silver particles was also associated with the extracellular matrix, which was observed to coexist in the presence of a *"flake-like"* structure surrounding the neuronal tissue after four days of silver injection, and appeared to be a key and permanent feature of the different hippocampal vascular, neuronal and glial and macrophages (Figs. 4 and 5). The presence of silver ions accumulation in extracellular matrix was associated within the presence of flake–like precipitate in the cell body and/or surroundings tissue. A very interesting and key observation in our study is that brain microvessels at different levels show the interruption of the interendothelial contacts in an area where silver ions were present (Fig. 5). These results indicate that using nanoparticles in *in vivo* conditions has clearly undesirable opportunity in the treatment for the neurodegeneration and that expanding this study in the future to include multiple dosages and different drug conjugation for comparison is necessary.

Detailed ultrastructural analysis showed that main characteristics of hippocampal microvessels from the rat brain 4 days after silver nanoparticles injection appeared to be heterogeneity of the tissue response to the action of the silver ions. We have found that microvessels with minimal and severe damage was seen throughout hippocampus (Fig. 5 A and B respectively). Moreover, severely damaged microvessels characterized by absence of luminal endothelial cell plasma membrane (Fig. 5 B).

Discussion

We investigated the subcellular distribution of NPs in the rat brain hippocampal tissue after i.p. injection of SiNPs. Our main goals is to determine the ability of SiNPs to cross BBB on rat brain to account for the effects of SiNP on BBB homeostasis and potentially conjugation of the neuroprotective drugs that could be open new and effective treatment strategies against brain lesions that occur during the course of neurodegeneration, and perhaps other brain diseases. Therapy for central nervous system (CNS) diseases, such as AD, requires drugs that can cross the dynamic interface between the CNS and the peripheral tissues called BBB [20, 21]. BBB represents one of the greatest challenges in the delivery of drugs to the brain tissue as a formidable barrier in the treatment of AD [5]. To date, many strategies have been devised to meet this challenge such as lipid soluble drugs, BBB disruption, and use of transport systems [22-29]. Drugs that normally cross the BBB may be taken up differently by the AD brain than by the normal BBB, thus complicating the treatment of CNS conditions such as pain, depression, psychoses, and delirium in the AD population [5]. It is estimated that many drug trials for over 400 drugs

investigated to treat AD failed because of the lack of BBB penetration to reach deep brain targets [5]. The current study assessed for the first time that intraperitoneal injected SiNPs crossed the BBB and are able to penetrate into the brain cells cytoplasm (neurons, vascular endothelium, perivascular pericytes, astrocytes, macro and microphages) and induce underlying cellular changes, which can be used for drug delivery. Even though little is known about the mechanism of CNS penetration of therapeutic agents under the pathological conditions, this study clearly proposes the application of SiNPs in the treatment of AD and provides a "window of opportunity" through which drugs that do not normally cross BBB are able to do so.

Use of NPs in drug delivery therapy holds much promise in targeting remote tissues, including the brain. Nano-enabled drug delivery has already been successful in delivering drugs to specific tissues within the body, and promises capabilities that will enhance drug penetration into cells, as well as other means to improve drug activity. NPs based targeted drug delivery has already been implemented in cancer treatment [27, 30]. Nanotechnology-based systems can be used to deliver therapeutic entities such as small-molecule drugs, peptides, proteins, and nucleic acids, either alone or in combinations. Also, NPs based drug delivery system has been reported as a strategy to overcome the major barriers in drug action such as multi drug resistance, lack of selectivity and less solubility [31].

A promising prospect of the use of NPs is its use in targeted drug delivery and also *"multi-targeting"*, which is essential in the case of several diseases [32]. In this present study, we observed the significant advantage of SiNPs in crossing BBB and to reach brain hippocampal cellular compartments. SiNPs have been widely used as a novel therapeutic agent as anti-microbial, anti-fungal, anti-viral and anti-inflammatory [17, 32].

The targeting of cancer cells using SiNPs has proven to be effective [32]. Also, various formulations of SiNPs have been investigated for delivery of genes in gene therapy [31]. Therefore, our findings showed for the first time by using electron microscopy appears to reveal the benefit of already established treatment strategy for a disease that has currently no established treatment and possible cure. In this context, NPs distribution can therefore be used as an alternate route for the drug delivering system to the damaged neurons, as for example, by using conjugation of SiNPs with selective mitochondrial antioxidants. Indeed, SiNPs are progressively finding more applications in research and medicine [16].

The ultrastructural characteristics of the brain tissue damage are also quite similar to those which we have showed recently in different animal models of brain damage such as in aged rat brain [33], in a 2 vessel occlusion of the brain hypoperfusion [34], as well as transgenic mouse model of human AD [19] and human AD [18, 35, 36]. In the present study, vascular and neuronal damage correlates with the presence of NPs in hippocampal tissue. This might be due to chemically synthesized NPs used for these approaches. However, recent reports suggested that biologically synthesized NPs have numerous advantages over the chemically synthesized ones [37, 38]. For example, biological synthesis of NP allow good control over size distribution of nanoparticles, while chemical synthesis may exert toxic effects [39, 40].

The biomedical applications of SiNPs can be effective by the use of biologically synthesized NPs, which minimize the factors such as toxicity, cost and are found to be exceptionally stable, especially those synthesized from microbes [32]. It has been reported that the particle size and shape of the NP affect the toxicity and those are dependent on various conditions, such as the culture supernatant, NPs type, reaction temperature and reaction mixture composition [32]. In this context, a size-controlled

synthesis is advisable, which is very much necessary when it comes to cellular interactions, suggesting that different formulations of SiNPs could be tested in future. In this study, the disruption of the inter-endothelial contacts in an area where silver ions were present would expand this study in the near future to include multiple dosage comparison with different formulations of NPs. Future considerations for this approach will enable us to open new pathways, not only for the better understanding of BBB homeostasis, which most likely plays a key role in the development of AD, but also for the development of new and more specific treatment strategies that will be more powerful and effective in bringing a cure for this devastating disease.

Conclusions

Our study by using electron microscopy indicates for the first time that injected (i.p.) SiNPs are definitely able to cross the BBB and can penetrate into the cell cytoplasm and induce underlying cellular changes. This approach can be used for drug delivery system after controlling of toxicological aspect of NPs. Further research in this area should include more specific cellular and subcellular mechanisms such as targeting mitochondria, which appears to be a main target for cell viability, and therefore a prime target for selective treatment strategies particularly in case of AD.

CONFLICT OF INTEREST

The authors declare that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure Legends:

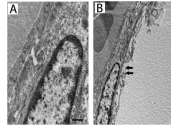
Figure 1. The ultrastructural characteristics of hippocampal neurons from the control rats with vehicle injection. Hippocampal neuron shows typical intact neuronal ultrastructural morphology. Original magnification (x5, 000 respectively A and B) Abbreviations: N-Neuronal nucleus.

Figure 2. The electron microscopic pattern of the hippocampal neurons 1 day after the silver nanoparticles injection. Samples were prepared without exposition to osmium tetraoxide. The presence of silver nanoparticles was seen throughout the neuronal cell body (dark dots, arrows). Original magnification X12, 000, X 40,000 and X50, 000 respectively A, B and C.

Figure 3. Electron microscopic features of the hippocampal tissue 4 days after the nanoparticles injection. The presences of silver nanoparticles throughout the neuronal cell body were noted . Neuronal cell body also reveals association of silver nanoparticles with mitochondrial alteration. The presence of a silver nanoparticles in the neuronal nucleus were also seen. No Osmium Teraoxide counstaining. Original magnification: 20,000, 25, 000 and 25,000 respectively A, B and C).

Figure 4. The ultrastructural characteristics of the hippocampal neuronal tissue 4 days after the nanoparticles injection. The presence of silver nanoparticles was seen throughout hippocampal tissue. Neuronal cell body reveals association of silver nanoparticles with neuronal ultrastructural alteration (arrow in figure A. Original magnification X12,000. B-D, High magnification shows the accumulation of silver nanoparticles in the extracellular matrix spaces that associate with the presence of flake–like precipitate (arrows). Original magnification: B: X60, 000. C: X15, 000. D X50, 000. No Osmium Teraoxide counstaining.

Figure 5. Ultrastructural characteristics of the hippocampal microvessels from the rat brain 4 days after nanoparticles injection. Microvessels with minimal and severe damages (A and B, respectively). Severely damaged microvessels, characterized by absence of luminal endothelial cell plasma membrane (double arrows). Magnification, 30,000 and 8,000 respectively A and B. No Osmium Teraoxide counstaining. Abbreviation used in figures. EC indicate- endothelial cells; N- Cell Nucleus; VL-Vessel lumen.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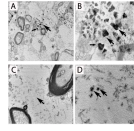
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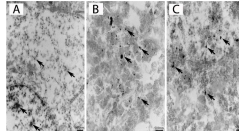
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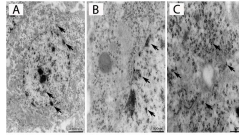
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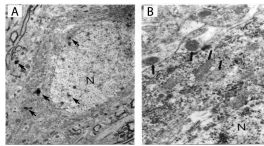

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