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RESEARCH PAPER

Myelin enhancement of Multiple sclerosis model with gold nanoparticles into the corpus callosum

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ABSTRACT

Objective(s): With no substantial cost, we injected L-arginine into the rat's corpus callosum (CC) to create animal model of multiple sclerosis (MS) and investigated the pre-injection effect of gold nanoparticles (GNPs).

Materials and Methods: Adult male Wistar rat (250-300 g) was surgically cannulated at the CC, and after recovery it was injected L-arginine (3-200 µg/rat, intra-CC) once daily for 3 to 5 consecutive days. GNPs (0.001-0.01 µg/rat, intra-CC) were injected alone or prior to the L-arginine using the same procedure. Control group solely received saline (1 µL/rat, intra-CC). Brain was studied with luxol fast blue. Weight change was also analyzed via the analysis of variance (ANOVA).

Results: L-arginine significantly induced ($p< 0.05$) a reduction in the fiber density while the neurons increased ($p< 0.05$). Single GNPs reduced ($p< 0.05$) the fiber and neuron densities; however, pre-injection of NPs caused myelinated fibers and uniform density of neurons.

Conclusion: The L-arginine may trigger demyelination by pro-inflammatory nitric oxide (NO), and the GNPs may improve this effect.

Keywords: *Corpus callosum, Demyelination, Gold nanoparticle, L-Arginine, Multiple sclerosis, Nitric oxide, Rat*

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INTRODUCTION

Since 1986, after its identification as an endothelium-derived relaxing factor [1], nitric oxide (NO) was universally considered a messenger molecule in the central nervous system (CNS), as it transfers messages among neurons and glial cells [2]. The NO is a radical molecule, synthesized by nitric oxide synthase (NOS) from L-arginine by nitrogen oxidation of guanidino nitrogen to form L-citrulline [3]. This molecule also contributes to the pathogenesis of many diseases, including multiple sclerosis (MS) [4, 5] that is a chronic inflammatory demyelinating disease with high morbidity rates, especially among young adults [6, 7]. Several studies have confirmed inflammation, demyelination, and axonal degeneration as the main pathological features of the MS [8]. Demyelination leads to the attenuated conduction velocity or in the worst scenario to a complete conduction block. Since the function of the myelin sheath is to facilitate the conduction of electrical impulses through the axons, demyelination clearly slows the process of impulse conduction which finally results in function laesa [9]. Neurological diseases account for 13% of the global burden of disease. As a result, treating these diseases costs some billion a year. Nanotechnology, which consists of small (~1–100 nm) but highly tailorable platforms, can provide significant opportunities for improving therapeutic delivery to the brain [10]. Nanotechnology offers unique approaches to probe and control a variety of biological and medical processes that occur at nanometer scales, and is expected to have a revolutionary impact on biology and medicine. At a glance to modern sciences and findings, nanoparticles offer some unique advantages as sensing, delivery agents, and image enhancement. Nanoparticles (NPs) can increase drug solubility, overcome the blood–

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brain and brain (BBB) penetration barriers, and provide timed release of a drug at a site of interest. Gold (Au) nanoparticles (GNPs) have actively been investigated in a wide variety of biomedical applications because of their biocompatibility and easy conjugation to biomolecule [11]. Commonly, GNPs are employed as a carrier in drug delivery systems, including delivery into the brain [12-13]. However, the effect of the use of GNP per se in the brain in neurodegenerative disease such as MS is unknown. GNPs have also received a great deal of attention as anti-inflammatory agents because of their ability to inhibit the expression of NF-kB and subsequent inflammatory reactions [14-15]. Bearing in mind that the MS is a disabling chronic disease of the nervous system in which the myelin system of the central nervous system (CNS) is deteriorated, this study aimed to evaluate the GNPs for the improvement of demyelination. The animal was converted into an MS model by intra-corpus callosum (CC) microinjection of L-arginine, a precursor for pro-inflammatory agent, the NO.

MATERIALS

Subject

Adult male Wistar rats (weighing 250-300 g, bought from Pasteur Institute of Iran, Tehran) were housed in standard PVC cages in groups of four in a controlled colony room (temperature 21 ± 3°C). They were maintained on a 12-h light/dark cycle (lights on at 07.00 a.m.) with food and water *ad libitum*. Each animal was used only once and six animals were used in each experiment. The protocol was approved by the Local Ethical Committee for care and animal use under the supervision of the committee of the local University Graduate program.

Drugs

Gold (Au) nanoparticles (GNPs) were prepared through sonoelectrochemistry process and donated by Dr Abazar Hajnorouzi at the local university main campus; department of Physics as a stable suspension of nanoparticles with a mean diameter of 60 nm. Ketamine (100 mg/kg) and xylazine (20 mg/kg) were obtained from Veterinary Organization, Tehran, Iran and used i.p. to anesthetize the experimental animals. L-arginine was purchased from Merck Co., Germany. Luxol fast blue was bought from Sigma-Aldrich Co. Hematoxylin was bought from Farzaneh Arman Co., Tehran, Iran, and Entellan from Merck Co., Germany.

Drug administrations

According to the previous used L-Arginine to induce MS model [16], Male Wistar rats (250-300 g) were divided into the L-arginine dose groups (3-200 µg/rat; n=6 in each) and groups receiving nanoparticles (0.001-0.01 µg/rat; n=6) alone or pre-administered to L-arginine groups (3-200 µg/rat). All materials were injected intra-corpus callosum (intra-CC) once per day through a 3 to 5- day period. The control group solely received saline 1 µL/rat, intra-CC.

Surgery

All animals were anesthetized using ketamine (100 mg/kg) and xylazine (20 mg/kg) and were placed in a stereotaxic apparatus at zero flat skull position. When the ears in the ear bars and teeth in the incisor bar were fixed, a cut was made to expose the rat skull. A hole was drilled in the skull at stereotaxic coordinates: AP 1.2 mm anterior to bregma, L ± 1.8 mm, and V 3.2 mm according to the atlas of Paxinos and Watson [17]. A cannula (21 Gauge) was guided into the hole and lowered 2.5 mm from the surface of the skull to be positioned 1 mm above the target. The guided cannula was then anchored with a jeweler's screw, and the incision was closed with the dental cement. After the operation, the animals were under one-week recovery and then they were tested.

Intra - corpus callosum injection

Intracerebral injections were performed after the animal was fully recovered; the rat was allowed to move freely in a clean cage. After that with the gentle handling, while the injection cannula was placed in the guide cannula, the injection was performed during 60 sec. The injection setup was provided by the injection dental needle (27 Gauge) equipped by polyethylene tubing (0.3 mm internal diameter) connected to a 5-µL glass Hamilton syringe.

Histology

Verification of the cannula's place

After completion of behavioral testing, the animals were killed under deep anaesthetization. In Pilot studies, only 1 µL of aquatic methylene blue (1%) was injected into the corpus callosum with the help of the same injection kit as used for intra-CC injection of materials to make sure

that the target was in accordance with the atlas. After the main procedure, the rats' brains were collected in 10% formalin for 48 to 72 hours to be cut with a rotary microtome to provide the slices with 4 to 5 microns thickness. Then all the slices were examined by luxol fast blue staining. The slices were then assessed histopathologically for verifying the injection site and the placement of the cannula with the aid of the atlas of Paxinos and Watson [17].

Luxol fast blue staining

To perform the staining, the brain slices were deparaffinized at first with two xylene replacements. Then they were hydrated with decreasing concentrations of ethanol 96%, 70% and 50% each for 5 min. Subsequently, the slides were placed in aquatic luxol fast blue 1% and stayed overnight in the 45 ºC oven. After washing with distillated water, they were placed in hematoxylin stain for 30 min. The slides were then dehydrated by ethanol with increasing concentrations 50%, 70% and 96% each for 5-10 min, cleared in xylene and mounted with entellen, and were finally coversliped.

Image analysis

The tissue slides were observed under the light videophotomicrocopy (Olympus) and the images were taken under 4X or more magnification. The photographs were then assessed at 100-μm^2 units using the Image Tool program, the free image processing, and analysis program for Microsoft Windows (UTHSCSA, version 2.03) to provide quantification for the area unit.

Statistical analysis

Data are expressed as means±S.E.M (standard error of mean). Differences among groups were compared using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc*. All analyses were performed using the SPSS statistical package. Differences with $p<0.05$ were considered statistically significant.

RESULTS

Verification of site injection

Site for injection was at first revealed by injection of 1 µL of a methylene blue solution intra-CC using the same injection set up as used for injecting the L-arginine and/or GNPs. Furthermore, in the samples that were stained with the fast blue, the trace of the guide cannula could be detected.

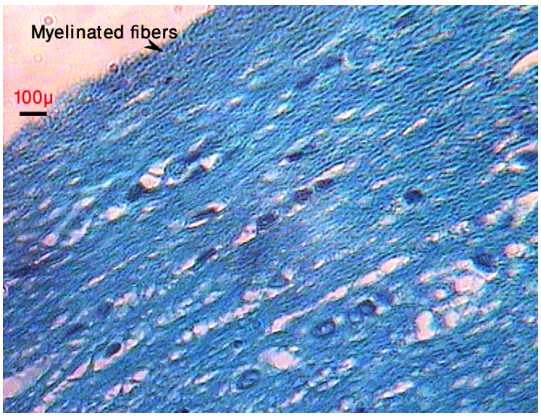

Fig 1. Injection of 1 µL of saline solution intra the corpus callosum (intra-CC). It indicates the normal density of myelin fibers and neurons as seen after the fast blue staining

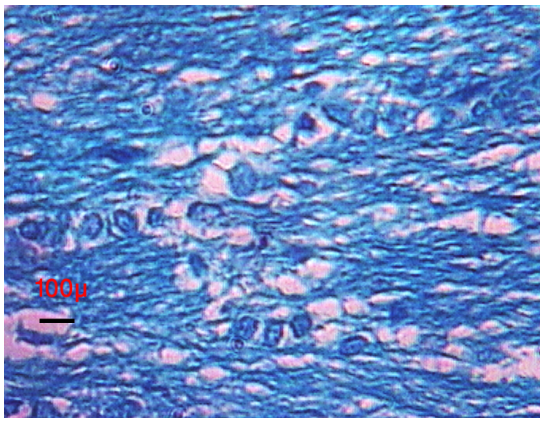

Fig 2. Reduction in fiber density, but, greater density of neurons in brain slices taken from the rat which received L-arginine (3-200 µg/rat, intra- CC) after staining by the luxol fast blue

Histopathological affects of L-arginine administration and/or GNPs intra the corpus callosum (intra-CC)

Light microscopic study displayed that higher doses of L-arginine, a precursor of nitric oxide (NO), caused significant reduction in fiber density, but it induced greater density of neurons in the corpus callosum (CC) of the experimental animals in contrast to that of the control (Fig 1 & 2). But, the injection of single gold nanoparticles (GNPs) showed less fiber and neuron densities. However, with pre-injection of GNPs into the site prior to injection of L-arginine both fibers and neurons had uniform density.

Effects of single L-arginine injection intra - corpus callosum on rat weight

Analysis of variance (ANOVA) of weight data after one to five- time injection of L-arginine (3-200 µg/rat, intra- corpus callosum) showed weight

loss of the treated groups in comparison with the control group (saline group: 1 µL/rat, intra-CC) ($p < 0.05$). Further analysis of data revealed that with increasing the dose of L-arginine (100-200 µg/rat, intra-CC) and increasing the number of daily injection (4 to 5 times), the weight loss was more significant (Fig 3).

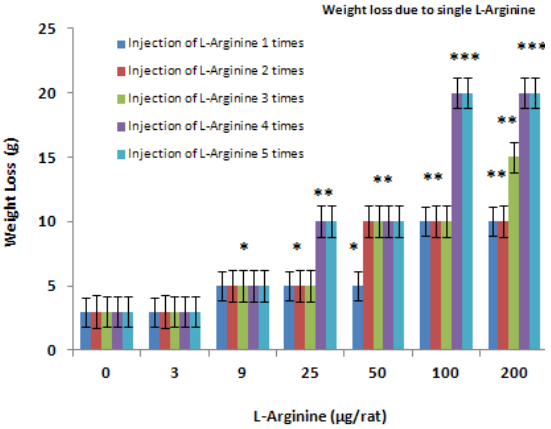

Fig 3. Weight loss in L-arginine (3-200 µg/rat, intra-CC) treated rats in comparison with the control (saline treated group: 1 µL/rat, intra-CC) legend 0. The more increase in the dose of L-arginine (100-200 µg/rat; intra-CC) along with the increasing of the number of injection (4 to 5 times, once per day) was observed, the more prominent was the weight loss. Between groups differences are shown by Tukey's test (*p<0.05, **p<0.01, ***p<0.001)

Effects of pre-injection of GNPs to L-arginine intra - corpus callosum on rat's weight

The analysis of results showed that due to pre-injection of GNPs (0.001 and 0.01 µg/rat, intra-corpus callosum) to L-arginine (3-200 µg/rat, intra-CC) in comparison with the control (saline treated group: 1 µL/rat, intra-CC, legend 0), the weight loss is significant ($p<0.05$). Regardless of the pre-injection of nanoparticles, the weight loss of rats occurred in the L-arginine injections (3-200 µg/rat, intra-CC). This was especially prominent from 3-time injections to more injections (Fig 4). According to the Tukey's test, the response was not significant at L-arginine 3 µg/rat.

Effects of single GNPs (0.001 and 0.01 µg/rat) injection intra - corpus callosum on weight of rat

Single GNPs were injected at different doses (0.001 and 0.01 µg/rat, intra - corpus callosum) for 1 to 5 times. Analysis of variance was significant ($p<0.05$). With further analysis, significant weight loss was observed at GNPs 5 times compared with the control (saline) group, and the response turned more significant with increasing dose (Fig 5).

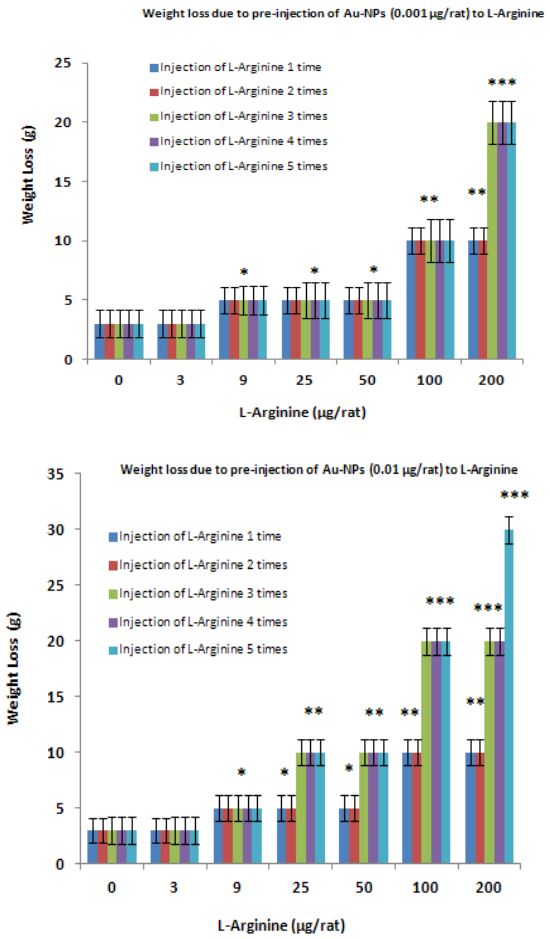

Fig 4. Weight loss in L-arginine (3-200 µg/rat; intra-CC) treated rats which received GNPs (0.001-0.01 µg/rat, intra-CC) prior to the L-arginine in comparison with the control group (saline treated group: 1 µL/rat, intra-CC) legend 0. Tukey's test revealed the significant differences between groups (*p<0.05, **p<0.01, ***p<0.001)

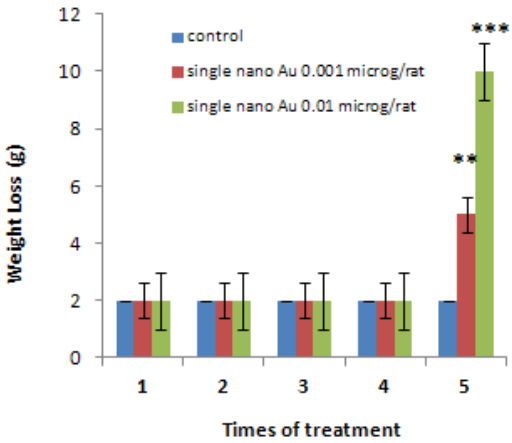

Fig 5. Weight loss in single GNPs (0.001 and 0.01 µg/rat, intra - corpus callosum) for 1 to 5 times in comparison with the control group (saline treated group: 1 µL/rat, intra-CC). Tukey's test revealed the differences between groups (**p<0.01, ***p<0.001)

MS signs due to L-arginine injection intra - corpus callosum

After administration of L-arginine at different doses (3-200 µg/rat, intra-CC), significant effects were determined in comparison with the control group (saline treated group: 1 µL/rat, intra-CC). According to analysis of variance (ANOVA), the signs were observed in about 50% of the animals which were given L-arginine (100-200 µg/rat) for 3-4 times.

This ratio for L-arginine (100 and 200 µg/rat, 5 times) was 100%. In the lower doses, it was insignificant (Fig 6).

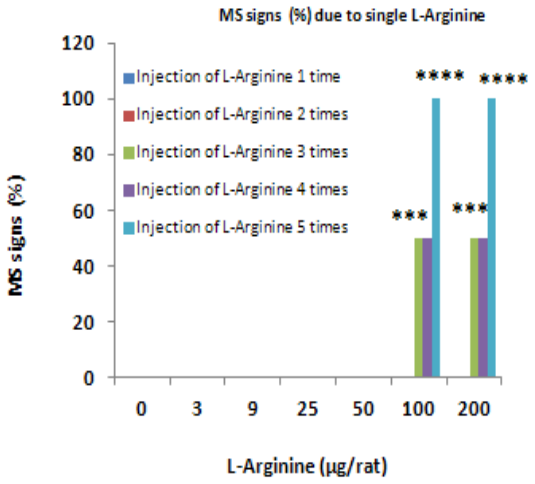

Fig 6. MS sings in L-arginine doses (3-200 µg/rat, intra-CC), in comparison with the control (saline treated group: 1 µL/rat, intra-CC). The signs (dragging of the immobilized limbs and tail and other fine motor features) were observed in about 50% of the animals given L-arginine (100-200 µg/rat) for 3-4 times. This ratio for L-arginine (100 and 200 µg/rat, 5 times) was 100%. In the lower doses it was insignificant. The differences between groups based on Tukey's test are as follows: ***p<0.001

MS signs due to pre-injection of GNPs (0.001 µg/rat) to L-arginine intra - corpus callosum

Analysis indicated that MS signs were observed in 50% animals due to GNPs (0.001 µg/rat) which were pre-injected to the L-arginine 5 times (100 µg/rat), and the L-arginine (200 µg/rat) 3 times, in comparison with control groups.

Also, with pre- injection of GNPs (0.001 µg/rat) prior to the injection of L-arginine (200 µg/rat) 5 times, the symptoms were seen in 100% of animals (Fig 7).

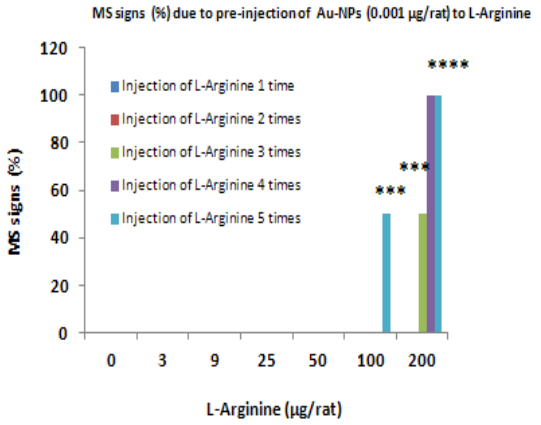

Fig 7. MS signs were observed in 50%of animals given GNPs (0.001 µg/rat) prior to injections both of L-arginine 5 times (100 µg/rat), and L-arginine (200 µg/rat, 3 times) in comparison with control groups. Also, from the pre- injection of GNPs (0.001 µg/rat) to the injection of L-arginine (200 µg/rat, 5 times), the symptoms were seen in 100% of animals (***p< 0.001, p***P<0.0001 based on the Tukey's test)

MS signs due to pre-injection of GNPs (0.01 µg/rat) to L-arginine intra - corpus callosum

Analysis showed that by administration of GNPs (0.01 µg/rat), only followed by injection of L-arginine (100 and 200 µg/rat, 3-4 times), but not the L-arginine (100 and 200 µg/rat, 5 times), the MS signs were returned (Fig 8).

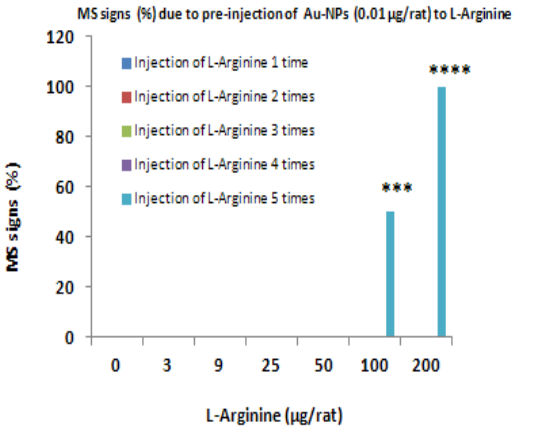

Fig 8. MS signs were returned by administration of GNPs (0.01 µg/rat), only followed by injection of L-arginine (100 and 200 µg/rat, 3-4 times), but not the L-arginine (100 and 200 µg/rat, 5 times). Tukey's test indicate significant differences; ***p<0.001, and ****p< 0.0001

DISCUSSION

As the goal of this study, we sought to demonstrate the improving effect of gold

nanoparticles (GNPs) on demyelinating effect of injection of L-arginine (intra-corpus callosum) as a progenitor of pro-inflammatory factor (nitric oxide) in the brain. Our results show that L-arginine (intra-CC) caused significant reduction in fiber density but increased the density of neurons in the target area of experimental animals in comparison with that of the control groups. But, the injection of single GNPs intra-CC caused reduction in fiber and neuron densities. . On the other hand, the pre-injection of GNPs to L-arginine caused myelinated fibers and neurons having uniform density comparing to the control groups.

Some studies have reported that NO may be involved in the pathogenesis of multiple sclerosis. Tang et al. [18] have argued that pathological process of NO in multiple sclerosis (MS) can be demonstrated in four aspects: 1: This substance acts as an essential stimulator of local inflammatory response to MS. It can disturb the structure of blood brain barrier (BBB) and directly increase barrier permeability, which can lead to inflammatory response, one of the main pathological characteristics of MS, 2: The substance causes neuronal apoptosis or necrosis and superoxide-triggered DNA damage leading to injury of axons, e.g. axonal swellings and transections, 3: It induces impairments of mitochondrial function and energy metabolism, especially in oligodendrocyte (OLs), and 4. The NO inhibits the expression of genes related to myelin formation and then indirectly accelerates death of OLs. In another study [16], it has been indicated that this substance (L-arginine, precursor of NO, intra - the corpus callosum) stimulates the inflammatory system to reduce in myelin concentration in the rat model.

Nowadays, some drugs are used to improve MS patients, but they are targeting different mechanisms in body and hence their use does not have specific effect [19]. So far, different types of nanoparticles have been used to treat the MS. They are capable to cross the BBB and increase the content of myelin, decrease oxidative stress, and improve remyelination [20].

To induce the MS model showing dragging of the immobilized limbs and tail and other fine motor features due to myelin loss using L-arginine, intra-CC, and to further investigate if direct application of GNPs into the brain areas of normal rats can improve demyelination, we have injected the GNPS (0.01-0.001 µg/rat) alone or prior to the L-arginine (3-200 µg/rat) into an extraordinarily myelinated area, the corpus callosum (CC). This approach showed the myelinated fibers and neurons having uniform density in the site of injection, but, the injection of single L-arginine had detrimental effect on myelin content. The injection of single GNPs also caused less fiber density and neurons. However, with pre-injection of the GNPs to the effective doses of L-arginine (100 and 200 µg/rat), the MS symptoms were returned, and the effect was diminished by increasing the injections times (5 times). Studies have accordingly shown improved effects of these materials. Liu, et al. [21] have argued that the GNPs can inhibit nitric oxide synthase (NOS) and thereby reduces NO following thet intracerebroventricular (i.c.v.) injection of lipopolysaccharide (LPS). In another study, it was reported that these materials (GNPs) blocked the activation of NF-kB by interacting with the cys-179 component of IKK-β and reduced the production of pro-inflammatory cytokines such as TNF-α and IL-1β [22]. Tsai, et al. [23], after working on the intraarticular use of GNPs in a rat model of rheumatoid arthritis, have observed the inhibition of proliferation and migration of inflammatory cells, along with the improvement in histological score, capillary density, macrophage infiltration, and expression of NF-kB, TNF-α, and IL-1β. Pedersen, et al. [24] evaluated anti-inflammatory effects of the GNPs with a diameter of 20 and 45 nm in the focal brain injury. Also, studies using GNPs have shown anti-inflammatory (decrease of proinflammatory cytokines) and antioxidant effects in dermal and muscle injury models [25-26]. The interactions of microglia and neurons with GNPs of three morphologies, spheres, rods, and urchins, coated with poly (ethyleneglycol) (PEG) or cetyl tri methyl ammonium bromide (CTAB) have also been demonstrated [27]. Muller, et al. have reported that GNPs prevent pathological events in the brain, cognitive deficits, oxidative stress and inflammation in a rat model of sporadic dementia of Alzheimer's type [28]. Similar explanations have also been indicated where GNPs carrying 2-(1'H- indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester and myelin oligodendrocyte glycoprotein showed significant disease remission [29]. Also, it has been found that GNPs encapsulated with potential antioxidant compounds epigallocatechin gallate, and α-lipoic acid have strong wound healing and migration along with an enhancement in the antioxidant

activity in vivo, having been featured as a treatment option for MS [30].

As a part of the protocol of working with animals to measure weight, the L-arginine at higher doses caused weight loss. This is probably due to the nitric oxide interactions in pain processes that remain unknown.

As a brief description of NP application in the MS, we should mention that the quantum dot complexed with metallopeptidase-9 (MMP-9) small interfering RNA (nanoplex) has significantly blocked the expression of MMP-9 in brain microvascular endothelial cells and leukocytes, demonstrating that the MMP-9 plays a pivotal role in disrupting the BBB, thereby attracting peripheral T-cells and thus initiating the neuroinflammation cascade [31]. Authors have encapsulated the tissue inhibitor of matrix metalloproteinase-1 in poly (lactic-co-gly- colic) acid NPs and they have shown strong neuroprotective activity in vitro by inhibiting the gelatinase-mediated MMP-9 activity [32]. The T-cell regulatory activity has also been improved in an autoimmune model with class I peptide–major histocompatibility complex-coated iron oxide NPs [33].

It is likely that these effects of nanoparticles are due to their ability to absorb and interfere with the electron, as in a review Cerium oxide (CeO) NPs are considered as potent antioxidants on account of their ability to either donate or receive electrons. It has also been shown that CeO NPs are extremely small size (2.9 nm) with enhanced brain uptake and reduced reactive oxygen species in vivo. The authors based on their data have explained that custom-made CeO NPs provide protection against autoimmune diseases associated with free radical toxicity [34]. There is also a report on neuroprotection in amyotrophic lateral sclerosis (ALS), where the researchers have stated that neuroprotection is possible with the implication of fullerenol (polyhydroxylated C60), in in the ALS model [35]. Other researchers have also made similar conclusions about the MS since they have reported that its diagnosis was even improved with the application of very small superparamagnetic iron oxide particles, which showed a characteristic accumulation in the inflammatory lesions [36]. And finally, in relation to animal weight loss, Gray, et al. [37] have accordingly reported daily weight loss of rats with the MS.

Generally, it should be noted that nanoparticles especially GNPs can be candidates for future studies to improve neurodegenerative diseases, e.g. the MS, and this research indicates that the L-arginine, producer of pro-inflammatory NO, may be responsive into the brain to induce demyelination. It can also be concluded that the application of GNPs may have an improvement effect on demyelination induced by central pro-inflammatory agent, the NO. Our study needs to be further developed at higher levels.

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