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Research Article

Glia activation induced by peripheral administration of aluminum oxide nanoparticles in rat brains

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Abstract

With the wide application of nanoscaled particles, the risk of human exposure to these particles has been markedly increased. However, knowledge about their safety falls far behind the utility of these nanoparticles. Here we have analyzed the activation of brain microglia and astrocytes, which are sensitive to changes of brain environment after peripheral exposure to nanoscaled aluminum oxide suspension. Sprague-Dawley rats (six rats per treatment) were intraperitoneally injected once every second day for 30 or 60 days with nanoscaled aluminum oxide (NSAO; 1 mg/kg or 50 mg/kg), non-nanoscaled aluminum oxide (nNSAO, 1 mg/kg), or vehicle (saline). After 60 days' exposure the numbers of ED1$^+$, GFAP$^+$, and nestin$^+$ cells in cortex and hippocampus were significantly higher in NSAO-treated rats than nNSAO- or vehicle-treated rats; thus, compared with nNSAO, NSAO has potential effects on the innate immune system of rat brain. This should be considered when evaluating the toxicological effects of nanosized particles.

From the Clinical Editor: Sprague-Dawley rats were intraperitoneally injected with nanosized aluminum oxide, (NSAO); non-nanoscaled aluminum oxide, or vehicle (saline). The numbers of ED1+, GFAP+, and nestin+ cells in cortex and hippocampus were significantly higher in NSAO-treated rats than nNSAO- or vehicle-treated rats; thus, NSAO has potential effects on the innate immune system of rat brain.

Key words: Nanoparticles; Aluminum oxide; Microglia; Astrocytes; Nanotoxicology

Introduction

The term nanoparticle defines any particle less than 100 nm in at least one critical dimension. Because of their greater surface area, nanoparticles display properties not shown by their macroscopic counterparts; this has led to the rapid development of a new field, nanomedicine, which provides potent applications for diagnostic (e.g., imaging) and therapeutic (e.g., drug delivery) purposes.[1] However, due to their unique physicochemical properties, nanoparticles could also be highly reactive and pose potential risks to humans, compared with their normal-sized counterparts. Accumulated data show they have potential toxicity, particularly in vivo, and information about in vivo safety[2,3] and potential hazards of nanoscaled particles is urgently needed.

After entering the body by the respiratory route, skin, or gastrointestinal tract, nanoparticles may translocate and reach other tissues by various transfer routes and mechanisms. Their major routes involve transcytosis across epithelia and access to the blood circulation or via lymphatics,[4] all of which result in redistribution throughout the body. The reticuloendothelial system in liver and spleen are the major

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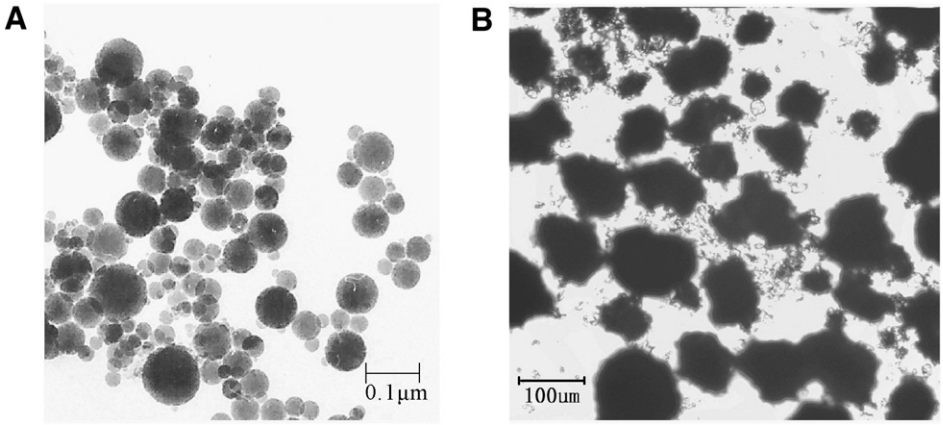

Figure 1. Size and morphology of nanoscaled aluminum oxide (NSAO) particles were analyzed. **(A)** The average particle size in the NSAO-water suspension was less than 100 nm (**B**) The morphology of nNSAO.

cleanup sites of nanoparticles via uptake by mononuclear phagocytes.[5] However, nanoparticles unrecognized by the reticuloendothelial system may have a long circulation half-life and relocate to other target organs, like brain, and induce inflammation via oxidative stress.[1,4,6,7]

Aluminum is implicated in disorders of various organs, including the hematopoietic system, liver, bone, and kidney, and is considered a neurotoxin. It has a role in dialysis encephalopathy and may contribute to the development and progression of Alzheimer's disease and other neurodegenerative processes.[8-15] Accumulation of aluminum in brain was reported in the 1970s,[15] and the neurotoxic effects of aluminum following occupational exposure have also been reported recently.[16] However, all of the reported toxic effects of aluminum are induced by soluble Al^{3+} ions or aluminum atoms[17]; until now, only in vitro safety information about aluminum oxide nanoparticles is available.[18] Nanoscale aluminum oxide particles, like other nanoparticles, might be small enough to access the central nervous system (CNS).[2] Therefore, the effects of nanoscale aluminum oxide (NSAO) on rat brains were investigated in this experiment.

Microglia and astrocytes are dominant glia and major immune cells in the CNS. They are sensitive to the changes to the microenvironment of the CNS and rapidly activated in virtually all conditions that affect normal neuronal functions.[19,20] Following activation, these cells upregulate or express certain molecules, which may induce an inflammatory response in the brain,[21] and can be sensitive markers to evaluate the toxic effects of xenobiotics. Here we analyzed activation of microglia and astrocytes in the cortex and hippocampus following peripheral administration of NSAO.

Methods

Characteristics of NSAO

The particle size and morphology of the NSAO (Plasmachem GmbH, Rudower, Germany) in water were analyzed by transmission electron microscope (TEM) (Tecnai G2 F20; Heidelberg, The Netherlands). The morphology of nNSAO was viewed by an optical microscope.

Animal experiments

Male Sprague-Dawley rats (70–90 g) (Wuhan, China) were housed with equal daily periods of light and dark and free access to food and water. All procedures were performed in accordance with the published International Health Guidelines under a protocol approved by the local Administration District Official Committee of Tongji Medical School of Huazhong University of Science and Technology.

Rats were randomly grouped (six per group) and received intraperitoneal injections of NSAO (1 mg/kg), non-NSAO (nNSAO; 1 mg/kg or 50 mg/kg; Fluka, St. Louis, Missouri), or 1 mL saline (vehicle control group) once every second day for 30 or 60 days, respectively. The weights of rats were measured every 2 days.

One day following the last injection, rats were deeply anesthetized with ether and perfused intracardially with 4°C, 4% paraformaldehyde in phosphate-buffered saline. Brains of three rats per group were quickly removed, post-fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, serially sectioned (3 μm), and mounted on silan-covered slides.

Immunohistochemistry

After dewaxing, brain sections were boiled (in an 850-W microwave oven) for 15 minutes in citrate buffer (2.1 g citric acid monohydrate/L, pH 6) (Carl Roth, Karlsruhe, Germany). Endogenous peroxidase was inhibited by 1% hydrogen peroxide in pure methanol (Merck, Darmstadt, Germany) for 15 minutes. Sections were incubated with 10% normal pig serum in Tris-buffered saline (Biochrom, Berlin, Germany) to block nonspecific binding of immunoglobulins and then with the mouse monoclonal antibodies: ED1 (1:100; Serotec, Oxford, Great Britain), glial fibrillary acidic protein (GFAP) (1:100; Chemicon International, Temecula, California), nestin

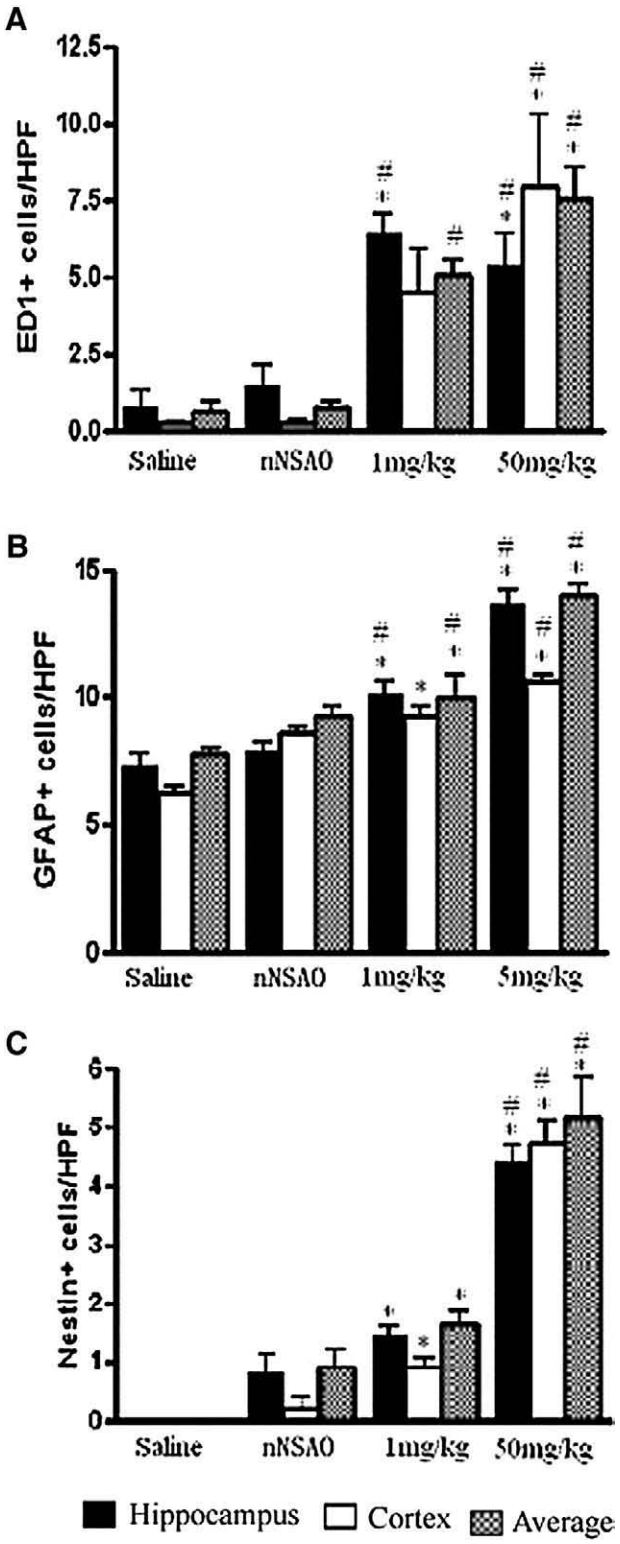

Figure 2. At the time point of 60 days brain sections of rats (three rats per group; $n = 9$) were immunostained with ED1, GFAP, and nestin to detect glia activation. The numbers of immunostained cells in hippocampus (filled bars) and cortex (empty bars) were calculated. The average number (hatched bars) is the average calculated of these two brain regions. (**A**) ED1 staining; (**B**) GFAP staining; (**C**) nestin staining. $*P < .05$ compared with saline-treated group; $^{\#}P < .05$ compared with nNSAO-treated group.

(1:50; BD Bioscience, Eindhoven, Germany) overnight at 4°C. Antibody binding to tissue sections was visualized with a biotinylated rabbit anti-mouse IgG $F(ab)_2$ antibody fragment (1:400; DAKO, Hamburg, Germany). Subsequently, sections were incubated with a horseradish peroxidise–conjugated streptavidin complex for 30 minutes (1:100; DAKO, Hamburg, Germany), followed by development with diaminobenzidine substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with Maier's hemalum. As negative controls, the primary antibodies were omitted.

Data evaluation and statistics

After immunostaining, brain sections were examined under light microscopy by three histologists who were blind to the treatments. Immunoreactivity of ED1, GFAP, and nestin was evaluated in the cortex and hippocampus. The numbers of $ED1^+$, $GFAP^+$, and $nestin^+$ cells of each rat brain section were counted in three nonoverlapping high-power fields (HPFs, 400× magnification) for each section, and sections of three rats per group were analyzed. The HPFs were selected from cortex and hippocampus areas that had a maximum of positive cells. In each field studied, only positive cells with the nucleus at the focal plane were counted. Results were given as arithmetic means of positive cell numbers per HPF and standard errors of means (SEM). Statistical analysis was performed by one-way analysis of variance followed by Dunnett's multiple comparison tests or nonparametric *t*-test (Graph Pad Prism 4.0 software, Tuebingen, Germany). For all statistical analyses, significance levels were set at $P < .05$.

Results

Size of NSAO particles

Particle size and morphology were analyzed by TEM. The particle size of NSAO in water suspension was less than 100 nm (Figure 1). The morphology of nNSAO is shown in Figure 1.

Microglia activation in the brain after peripheral NSAO administration

After 30 or 60 days' exposure, the survival, food consumption, and mean body weights of NSAO-treated rats were similar to rats of nNSAO and control groups.

Brain sections were immunostained with ED1 to detect microglial activation. Because number and activation of glial cell in the brain varies, two standard regions, cortex and hippocampus, were chosen for analysis. Morphologically, microglial cells were classified according to Ayoub and Salm[21] as follows:

Ramified microglia: normal-appearing soma with thin, delicate, and radially projecting processes

Hypertrophic microglia: enlarged, darkened soma and shorter, thicker, and less branched processes

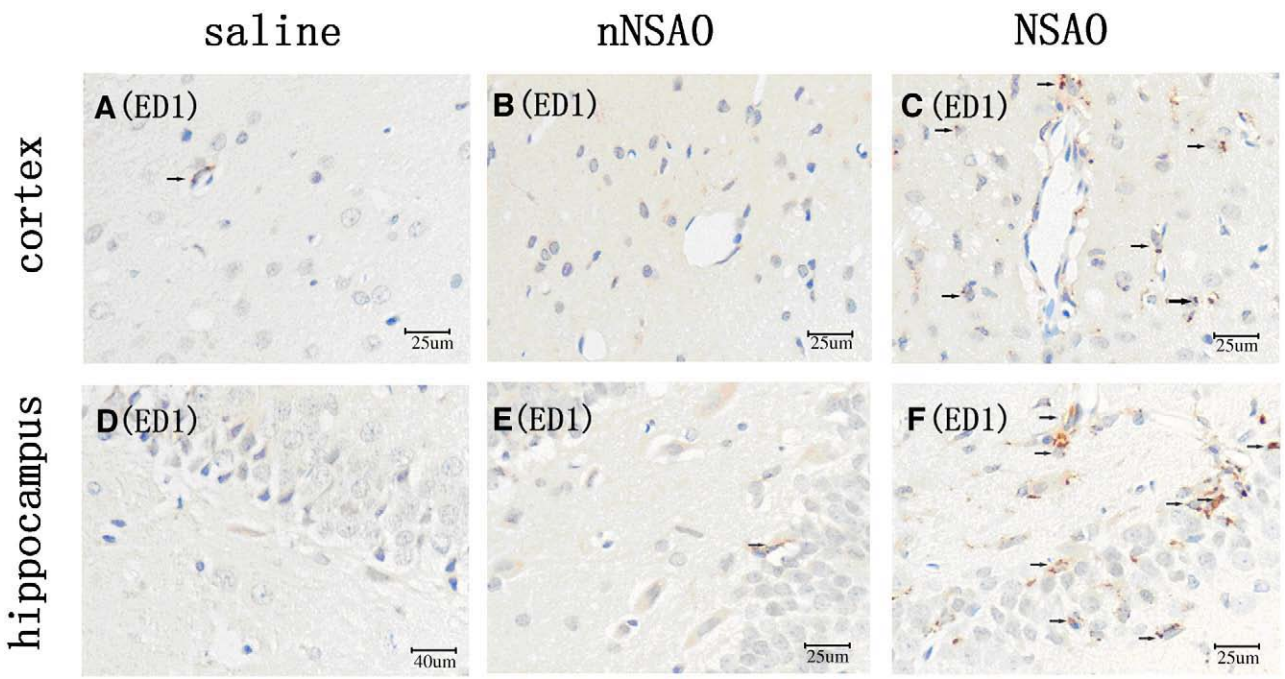

Figure 3. At the time point of 60 days the microglial activation of rat brains was analyzed by immunohistochemistry. For the NSAO-treated group, ED1 staining of macrophages/microglia was seen in cortex ($\mathbf{C_{(ED1)}}$) and hippocampus ($\mathbf{F_{(ED1)}}$), especially accumulating in the perivascular space of blood vessels. These cells had thick processes, which could easily be distinguished from the elongated perivascular cells situated adjacent to the basement membrane of medium to small vessels in the nNSAO group ($\mathbf{B_{(ED1)}}$, $\mathbf{E_{(ED1)}}$) and saline-treated group ($\mathbf{A_{(ED1)}}$, $\mathbf{D_{(ED1)}}$).

Ameboid microglia: enlarged cell bodies, few short processes if any, and often several filopodia.

Upon activation, microglial cells transform from ramified morphology to a rounded macrophage-like morphology.[21]

ED1 stains CD68, a lysosomal membrane protein, which is mainly found in phagocytosing macrophages and reactive microglia.[22] Upregulation of CD68 indicates that cells are phagocytically active, because they accumulate lysosomal vacuoles. ED1 staining has been widely applied to show microglia activation.[23,24]

Following 30 days' exposure no significant changes of glia activation were observed, but abundant reactive microglia and astrocytes were observed in the brains of NSAO-treated rats after 60 days of exposure.

In NSAO-treated rats (1 mg/kg and 50 mg/kg), numbers of $ED1^+$ cells in hippocampus were significantly increased as compared with saline and nNSAO groups ($P < .05$) (Figure 2). For the NSAO (1 mg/kg and 50 mg/kg) group, the density of $ED1^+$ microglial cells was 5.9 ± 0.8 and 5.4 ± 1.6 per HPF in hippocampus, 4.5 ± 1.4 and 7.9 ± 2.8 per HPF in cortex, and 5.2 ± 0.4 and 7.4 ± 0.9 per HPF for the average of these two brain regions. In brains of saline- and nNSAO-treated rats, ED1 immunoreactivity was rarely observed. However, in the brains of NSAO-treated rats, strongly stained $ED1^+$ monocytes were found in the meninges, the perivascular space, and the parenchyma. Most of the $ED1^+$ cells, which accumulated mostly near blood vessels, had thick processes (Figure 3, $A_{(ED1)}$, $F_{(ED1)}$), which could be easily distinguished from the elongated perivascular cells situated adjacent to the basement membrane of medium to small vessels (Figure 3, $A_{(ED1)}$).

Astrocyte activation in the brain after NSAO injection

Brain sections were immunostained for GFAP and nestin to detect astrocyte activation. In the brains of NSAO-treated rats the body of $GFAP^+$ astrocytes enlarged with thin, shortened processes (Figure 4, $C_{(GFAP)}$, $F_{(GFAP)}$) and the numbers of $GFAP^+$ cells in hippocampus were greater than in saline- and nNSAO-treated groups ($P < .05$) (Figure 2). For the NSAO (1 mg/kg and 50 mg/kg) group, the density of $GFAP^+$ cells was 10.0 ± 0.5 and 13.6 ± 0.8 per HPF in hippocampus, 9.2 ± 0.3 and 11.2 ± 0.3 per HPF in cortex, and average 9.6 ± 0.3 13.9 ± per HPF. Expression of nestin in brains was also observed in the NSAO-treated rats (Figure 4, $C_{(Nestin)}$, $F_{(Nestin)}$) but hardly seen in the saline-treated groups. For the NSAO (1 mg/kg and 50 mg/kg) group, the numbers of $nestin^+$ cells were 1.5 ± 0.2 and 4.4 ± 0.4 per HPF in hippocampus, 0.9 ± 0.1 and 4.8 ± 0.6 per HPF in cortex, and average 1.2 ± 0.2 and 5.2 ± 0.8 per HPF (Figure 2). The distribution of reactive astrocytes was in agreement with that of microglia, which was mainly in the perivascular areas.

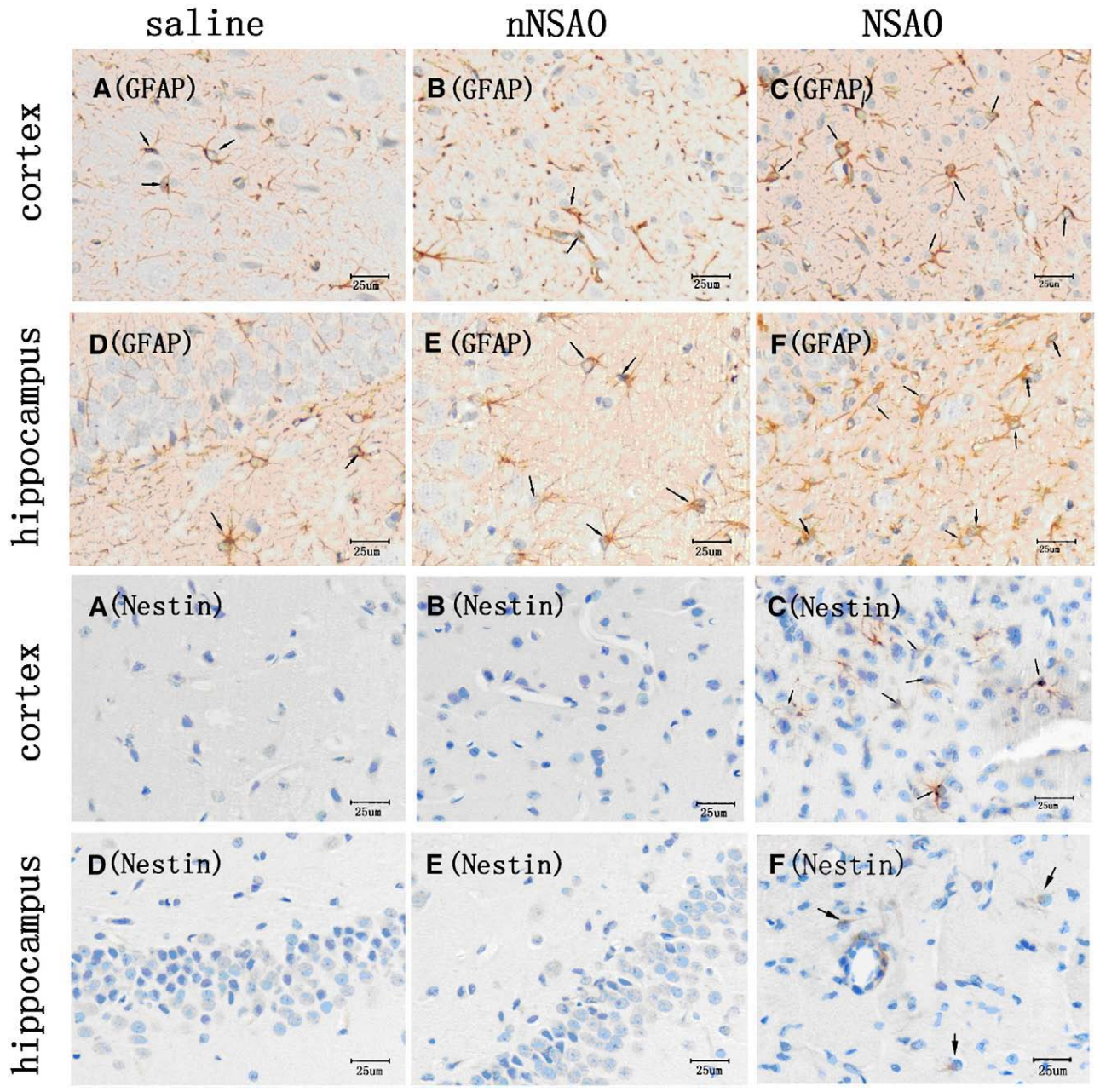

Figure 4. At the time point of 60 days the activation of astrocytes was analyzed by immunohistochemistry. For the NSAO-treated group, GFAP staining of enlarged astrocytes with thin, shortened processes and expression of nestin were seen in cortex **($C_{(GFAP)}$, $C_{(Nestin)}$)** and hippocampus **($F_{(GFAP)}$, $F_{(Nestin)}$)**. In the saline- **($A_{(GFAP)}$, $D_{(GFAP)}$)** and nNSAO-treated **($B_{(GFAP)}$, $E_{(GFAP)}$)** groups, only typical $GFAP^+$ cells with long and thin processes were seen. In saline- **($A_{(Nestin)}$, $D_{(Nestin)}$)** and nNSAO- **($B_{(Nestin)}$, $E_{(Nestin)}$)** treated groups, nestin was rarely observed.

Discussion

Numerous studies in rat have indicated that exposures to nanoscale or ultrafine particles (<100 nm) produce greater inflammatory and cytotoxic effects when compared to exposures to larger-sized particles at equivalent mass concentrations.[4,25] The biological activity of nanoparticles largely depends on the characterizations: size, shape, surface properties, and so on. The small size provides more opportunities to enter and/or interact with cells, even with subcellular structures.[4] Furthermore, mechanisms of toxicology for these nanoparticles is suggested to be in part inflammatory.[26-29]

Being the resident immune cells in the brain, microglia are highly abundant throughout the CNS and are considered as a key factor of many pathological alterations, such as infection, inflammation, trauma, ischemia, brain tumors, and neurodegeneration.[19] Reactive microglia is characterized by marked morphological changes, increased expression of surface molecules, and secretion of a wide

variety of soluble factors that include cytokines, oxygen and nitrogen free radicals, and chemokines. Their activation is a graded response process, characterized by morphological changes, proliferation, increased expression of certain cell markers, production of cytokines, and/or changes in function.[30,31]

Moreover, reactive astrocytes are key pathological features and may have a critical role in CNS inflammatory diseases.[32] The lysosomal marker ED1 is widely used to detect the activation of microglia/macrophages. ED1 expression is related to the upregulated phagocytic ability of microglia.

Furthermore, hyperplasia of reactive astrocyte and the positive expression of GFAP and nestin is a good indicator for CNS injury in adult rats. GFAP is a specific marker of astrocytes. Nestin is expressed only in stem cells of the subventricular zone and to a lesser extent in the choroid plexus in the normal adult CNS.[33] But re-expression of nestin was demonstrated in reactive astrocytes following certain types of brain injuries. This reversion to the immature phenotype may serve to protect the cells, perhaps by making them less susceptible to the attendant hypoxia that can occur after injury.[32] Similarly, re-induction of nestin has been reported in reactive astrocytes and endothelial cells in cerebral abscesses. This process is probably caused by pathogenic micro-organisms inducing inflammatory stress to the tissue.[32]

Therefore, we analyzed the expression of ED1, GFAP, and nestin in brain sections of saline-, NSAO-, and nNSAO-treated rats. In hippocampus and cortex of low-dose (1 mg/kg) NSAO-treated rats, significant accumulations of $ED1^+$ cells were observed in the perivascular space of blood vessels. Moreover, expression of GFAP and nestin suggested activation of astrocytes. In comparison with nNSAO and saline control groups, both low-dose (1 mg/kg) and high-dose (50 mg/kg) NSAO peripheral injection can induce activation of astrocytes and microglia/macrophages in various regions of rat brains, especially the perivascular area. In this experiment, glia located in hippocampus are more sensitive than those in cortex; moreover, ED1 and GFAP are both sensitive markers of glia activation.

Results demonstrate that exposure to NSAO produced significant inflammatory effects in the rat brain. However, it should be noted that both the nanosized and non-nanosized particles had almost the same inflammatory effect on liver and kidney tissues in our experiment (data not shown). It has been shown that nanoparticles are small enough to cross the blood-brain barrier (BBB) and reside in the brain parenchyma,[34] or interact with the BBB, inducing dysfunction.[3,34] It is thus possible that enhanced glia activation in the brains of the NSAO-treated group is directly and locally induced by the nanoparticles. This suggests that peripherally injected NSAO, even the low dose (1 mg/kg), might cross the BBB and induce a local inflammatory response that is not induced by systemic inflammatory effects.

In addition to inflammation, oxidative stress was considered as one of the major mechanisms of nanotoxicity. Titanium dioxide nanoparticles induce increased activities of antioxidases, such as GSH-Px, GST, GSH, and SOD, after 10 days exposure, and MDA, a product of lipid peroxidation, increased significantly in brain tissues of mice.[35] In vitro studies also showed that nanoparticles stimulated production of reactive oxygen species in the brain microglia cell line BV2.[36]

Briefly, significantly activated glia were induced in rat brain after NSAO administration. This should be considered in the usage of these nanoparticles, and some other nanoscaled particles may have the same adverse effect. The molecules expressed by glia could provide a potential means of assessing the neurotoxic effects of nanoparticles.

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