Histol Histopathol (2014) 29: 447-456
DOI: 10.14670/HH-29.10.447

**http://www.hh.um.es**

**Histology and Histopathology**
*Cellular and Molecular Biology*

# Toxicological aspects of injectable gold-hyaluronan combination as a treatment for neuroinflammation

**Dan Sonne Pedersen[1], Linda J. Locht[2], Thao P. Tran[1], Sara Markholt[2], Jørgen Rungby[1] and Agnete Larsen[1]**
[1]Department of Biomedicine - Pharmacology and [2]Department of Biomedicine - Anatomy, Aarhus University, Denmark

**Summary.** Secondary inflammatory reactions to stroke or trauma contribute to irreplaceable loss of brain tissue of the affected patients. Likewise, neuroinflammatory processes are the main pathophysiological feature in Multiple Sclerosis (MS), a common neurodegenerative disease among young adults. In the search for safe and efficient ways to reduce inflammation within nervous tissue older immunosuppressive remedies have been re-investigated. The anti-inflammatory properties of gold salts are well known but result in uncontrollable systemic spread of gold ions, generating side effects such as nephrotoxicity, limiting their use. Recent studies have circumvented this obstacle by introducing metallic gold implants as a localized source of immune-modulating gold ions and suspension in hyaluronic acid (HA) enables injection of small amounts of gold in the natural spaces of the brain. By injecting >25 $\mu$m gold beads in HA intracerebrally we recently showed a slowing of disease progression in a rodent model of MS. The toxicological aspects were, however, not assessed. The present study investigates the viability of neuronal and macrophage cell cultures exposed to the gold/HA combination and the possible risk associated with unilateral gold/HA injection in young Balb/CA mice in the first 7 to 21 days of gold-exposure. Tracing by autometallography of gold accumulations throughout the brain exhibited sparse gold uptake in glia and neurons of hippocampus and cortex, and striatum and cerebellum were void of staining. No systemic spread of gold was seen in liver or kidney, nor were there signs of obstruction of the ventricular system. Both cell cultures of J774 macrophages and CCL neurons accumulated gold from gold/HA-exposure with no signs of reduced viability. In conclusion, our findings indicate that gold/HA is not overtly neuro- or cytotoxic, nor does intraventricular exposure result in widespread gold accumulation or tissue damage, warranting further studies into the pharmacological properties of this novel form of gold treatment.

**Key words:** Autometallography, Dissolucytosis, Hyaluronic acid, Metallic Gold, Neuroinflammation

*Offprint requests to:* Dan Sonne Pedersen, Department of Biomedicine - Pharmacology, Aarhus University, DK-8000, Denmark. e-mail: dspe@farm.au.dk

## Introduction

The paradigm that the brain is an immune-incompetent or inactive organ has long been overthrown and finding new ways to pharmacologically control the inflammatory response to insults of the brain are now of considerable interest to all branches of neurology. Increased inflammatory activity is seen not only in response to stroke and trauma but also in the pathophysiology of neurodegenerative diseases such as Alzheimer's and Parkinson's Diseases (Lucas et al., 2006; Amor et al., 2010). Moreover, considerable interest has been given to the role of autoimmune inflammation in Multiple Sclerosis (MS), a disease developing in response to a combination of genetic traits (McFarland and Martin, 2007) and environmental factors (Giovannoni and Ebers, 2007; McFarland and Martin, 2007). Although inflammation in the brain is a double-edged sword, i.e. a certain amount is useful in eliciting the regenerative responses (Das and Basu,

2008; Aharoni and Arnon, 2009; Ekdahl, 2012), uncontrolled inflammatory processes have devastating consequences in the long run (Lucas et al., 2006; Amor et al., 2010). The mechanisms are complicated but activation of microglia, breakdown of the blood-brain barrier (BBB) and invasion of additional immune-reactive cells inevitably leads to the formation of reactive oxygen species (ROS) detrimental to the surrounding tissue, causing irreversible loss of neurons (Mirshafiey and Mohsenzadegan, 2008; Lassmann, 2011). In autoimmune diseases in which the immune system reacts against itself, today's treatments try to modify this by immunosuppression. Especially in the treatment of autoimmune diseases such as rheumatoid arthritis (RA) the use of gold salts is well known and long validated (Forestier, 1934; Burmester, 2001). Among the known effects seen when using gold salts, e.g. sodium aurothiomalate (Myocrisin) as a source of pharmacologically active gold ions, is a reduction in the number of infiltrating inflammatory cells and inhibition of the release of lysosomal enzymes from macrophages (Persellin and Ziff, 1966). Gold ions are also believed to inhibit antigen processing and to suppress NF-kappaB binding activity and I-kappaB-kinase activation, causing a reduced production of pro-inflammatory cytokines (Yanni et al., 1994; Burmester, 2001). The current uses of conventional gold salts are, however, limited by side effects, as systemic spread of gold ions from gold salts may result in discoloration of the skin and hepato- and nephrotoxicity (Merchant, 1998).

In 2002, Danscher introduced metallic gold implants as a possible source of gold ions (Danscher, 2002) and it was soon established that macrophages adhere to such implants, resulting in a extracellular bio-liberation of gold ions, a process coined dissolucytosis (Larsen et al., 2007). To obtain maximal effect of the gold, i.e. a large gold surface with a limited amount of gold, small non-phagocytizable (>25$\mu$m) metallic gold beads were suspended in non-pyrogenic, sterile HA. As this combination has previously been shown to reduce the histological signs of trauma-induced inflammation (Larsen et al., 2008; Pedersen, 2009a,b), we recently tested if injection of gold/HA into the ventricular system of young female rats could reduce the progression of experimental autoimmune encephalitis (EAE) (Pedersen, et al., 2012), a well-established model of MS (Constantinescu et al., 2011). We saw a slowing of disease progression and an increased regenerative stem cell response in the gold-treated animals, indicating that this type of gold implantation could have clinical potential. However, introducing metallic gold to the nervous system as a permanent anti-inflammatory treatment could have undesirable consequences if vast amounts of gold are accumulated in nerve cells; a toxicological investigation of the gold/HA combination was needed to fully evaluate the pharmaceutical potential of this type of treatment. Using the autometallographic *in situ* gold visualization technique we thus investigated the bio-liberation of gold ions from metallic gold beads *in vitro* in J774 macrophages and CCL neurons *in vivo* in young Balb/CA mice receiving intracranial gold injections. *In vitro* cell viability assessments were made using the NucleoCounter 3000 platform.

## Materials and methods

### *In vitro cell cultures*

J774 Macrophages (ATTC, US) were grown as described in Stoltenberg et al. (2002). Cell media contained 89% RPMI 1640+10% FCS+1% penicillin/streptomycin. The cells were loosened by scraping before the experiments and prior to sub-cultivation 2-3 times per week. CCL neurons were grown in DMEM media +10% FBS+1% penicillin/streptomycin and trypsinized prior to experiments and for sub-cultivation once or twice a week.

### *Exposure of cell cultures to gold*

Cultures of J774 macrophages and CCL neurons were exposed to metallic gold beads for 5 days to investigate if their viability would be affected, using a NucleoCounter NC-3000 (ChemoMetec, DK) for counting of living and dead cells. The exposing environments were gold/HA, HA alone or control. The gold/HA suspension contained 463 mg gold beads in 0.85 mL HA (10 mg/mL, ProVisc, Alcon, Sweden). The amount was split into a six-well plate and carefully spread out covering the whole surface. The HA controls were treated in a similar manner. Control cells were grown directly on the bottom of the wells. Each viability test was performed three times, each with six replicates, and the results are presented as pooled data of those tests.

### *Collection of cells for viability tests and autometallography (AMG)*

After 5 days incubation cells were loosened in their old media, spun down and re-suspended in a concentration of $5x10^4$-$5x10^6$ cells/mL. To measure cell vitality, 95 $\mu$L of this cell suspension was mixed with 5 $\mu$L *solution 5* (ChemoMetec, DK) and 16 $\mu$L added per chamber into a 8 chamber NucleoCounter-slide (ChemoMetec, DK) and the overall cell viability (viable cells opposed to dead cells) measured using the NucleoCounter 3000 platform (ChemoMetec, Denmark). After viability tests, the remains of the same samples were spun down at 2000G for 5 min. The pellet was then smeared on glass slides, dipped into 4% paraformaldehyde and air-dried for subsequent AMG development.

### *Autometallography on cell cultures*

The glass slides were subjected to autometallo-

graphic development under a dark hood for 60 min. In short, glass slides were placed in a jar containing 120 mL of autometallographic developer placed in a slowly shaking water bath set at 26°C. Next, the process was stopped by replacing the developer with a stop solution (5% sodium thiosulfate) for 10 minutes. Finally, the glass slides were washed under running tap water for 20 minutes, air-dried, counterstained with toluidine blue and mounted for microscopy. The AMG developer consisted of 1) 120 mL gum Arabic solution 20 mL sodium citrate buffer (25.5 g citric acid*$2H_2O$ + 23.5 g + 100 mL water) thoroughly shaken before adding a silver donor (0.24 g silver lactate in 30 mL 40°C water) and a reducing agent (1.7 g hydroquinone in 30 mL 40°C water) immediately before use.

### *Animals*

A total of 11 female Balb/CA mice, 10 weeks old and weighing app. 22 g (Taconic, Denmark) were used. The animals were kept under standardized conditions of light and temperature, with a 12 hours day/night cycle and had free access to food and water. The animals were divided into three groups: A control group (n=2) and two gold/HA groups destined for 7d (n=6) and 21d (n=3) of survival. All animal experiments were conducted in accordance with the ethical guidelines of the Aarhus University and approved by the Animal Experiments Inspectorate, controlled by The Danish Ministry of Justice.

### Injection of gold/HA in the lateral ventricles

The procedures for gold treatment have previously been described in detail (Larsen et al., 2008; Pedersen et al., 2012). In short, 463 mg of 99.999% pure metallic gold beads 25-45 $\mu$m in size (Alfa-Aesar, Karlsruhe, Germany) were mixed with 0.85 mL of HA (10 mg/mL, ProVisc, Alcon, Sweden). Prior to gold injection the mice were deeply anesthetized i.p. with a mixture of Narcoxyl-Vet (Xylazin) and Ketaminol-Vet. Next, the mice were placed in a stereotaxic instrument (AngleTwo Dual, MyNeuroLab, USA) and 10 $\mu$L of gold/HA mixture were injected unilaterally in the right lateral ventricle using Hamilton syringes (Microliter, #702, Switzerland). Injections were placed -0.5 mm posterior to bregma, laterally 1 mm lateral from the midline and 2.0 mm ventrally from the surface, using Franklin & Paxinos' mouse brain atlas (Franklin and Paxinos, 2007). Post-operative the animals received analgesic treatment (Temgesic, Reckitt & Colman, Hull, UK, 0.5 ml/L water) for three days.

### *Tissue processing and autometallography of murine brain tissue*

After 7 and 21 days the mice were anesthetized (Mebumal, pentobarbital, 50 mg/mL, SAD, Denmark) and killed by transcardial perfusion with Zamboni's fixative (2% paraformaldehyde, 15% picric acid in 0.1 M sodium phosphate buffer, pH 7.4). After perfusion, the brains were removed, divided coronally near the gold injection points and allowed to post-fixate for 4 hours before being embedded in paraffin, using standard procedures. The two halves of the brain were then cut into 8 $\mu$m thick frontal serial sections. Every third section was subjected to 60 min of AMG staining along side control sections from sham-operated mice.

### *Evaluation of AMG-stained tissue*

The AMG-stained brain sections were investigated with focus on hippocampus, cortex, striatum and cerebellum. These areas were identified using the mouse brain atlas (Franklin and Paxinos, 2007) and assessed individually by going through serial sections from before, in and after the injection point, spanning from app. 1 mm anterior to bregma and all the way back and through cerebellum, -8 mm posterior to bregma (figure 22-97 in Franklin and Paxinos (2007)). Tissue sections were evaluated in a light microscope at 20x, 40x and 100x magnification. Likewise, the kidneys were systematically examined to evaluate if any systemic spread of gold had occurred. The amount of silver-enhanced grains was not quantified but evaluated as a whole.

### *Statistics*

The viability tests of the J774 macrophages were evaluated by Kruskal-Wallis due to a lack of normal distribution of data in the control and gold-exposed group and CCL neurons by one-way ANOVA. All cell data are presented as box plots. The level of statistical significance was set at $p<0.05$.

## Results

### *Viability of J774 macrophages unchanged by gold/HA but increased by HA alone*

The gold/HA-treatment did not change the viability of the macrophages compared to the control group (Fig. 1a). Interestingly, the viability tests of the J774 macrophages showed an increase in percentage of survival in the HA-treated control group, albeit not statistically different from the other two groups ($p=0.099$). An additional Mann-Whitney test revealed a difference between the control- and HA-exposed cells close to significant ($p=0.0606$), indicating that HA alone might improve the viability of the J774 macrophages.

### *Viability of CCL neurons unaffected by gold/HA and HA alone*

CCL neurons did not reveal any significant differences ($p=0.24$) between the controls, HA- and gold/HA-exposed groups (Fig. 1b).

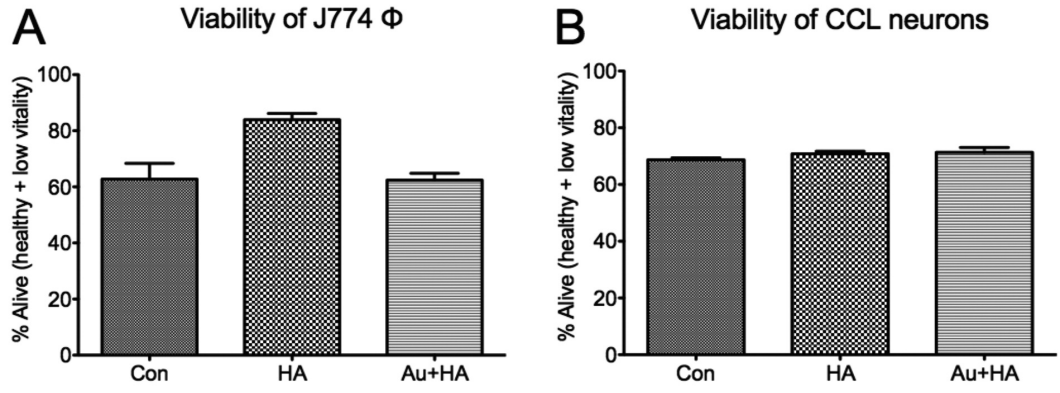


**Fig. 1.** Viability tests on J774 macrophages and CCL neurons exposed to gold beads. **A.** The viability of the macrophages was increased in the HA group but not significantly compared to the control and gold/HA-treated cells (p=0.099). **B.** None of the neuron groups were statistically significant different from each other (p=0.24).

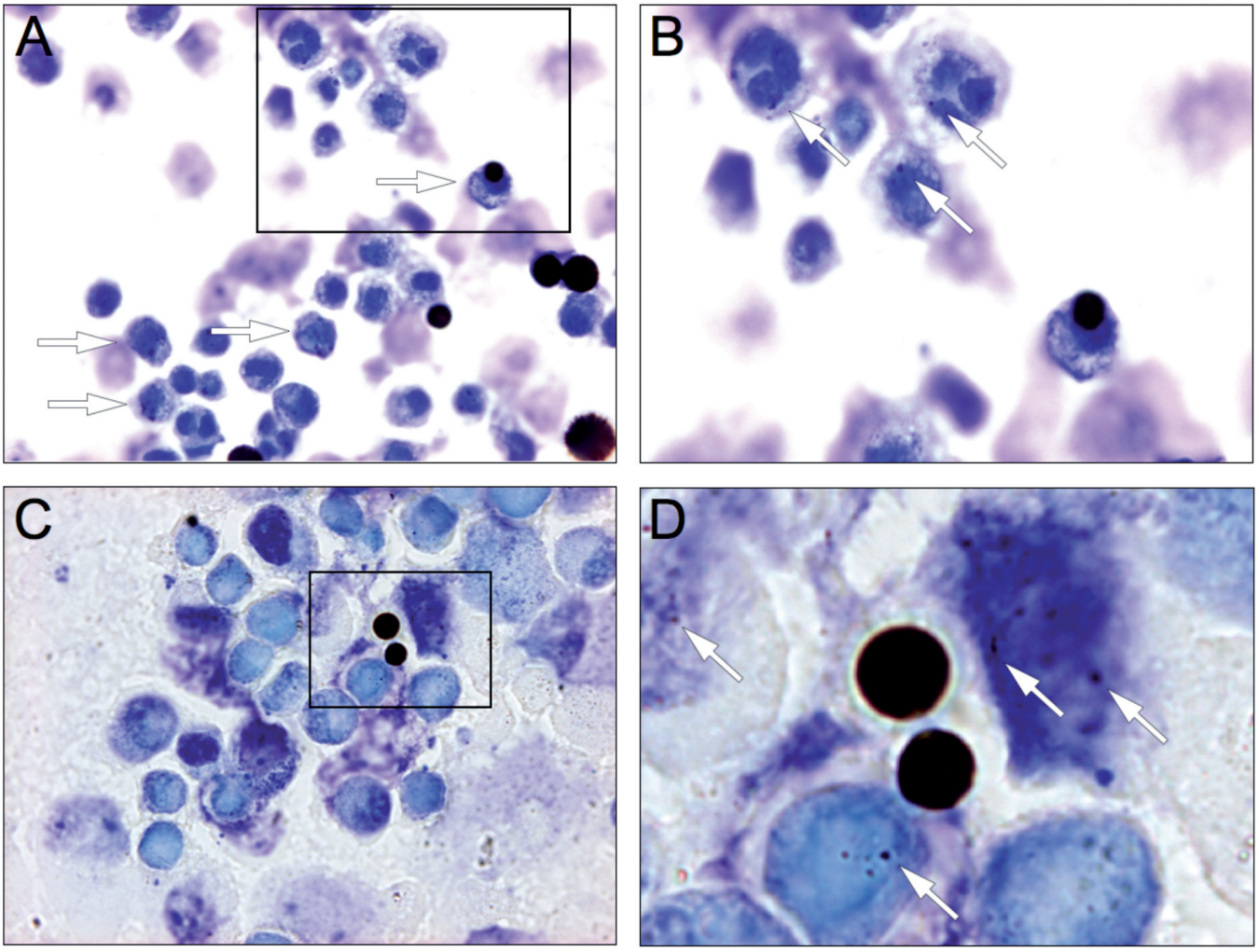


**Fig. 2.** Micrographs of AMG staining, counterstained with toluidine blue, of the gold bead-exposed J774 macrophages and CCL neurons from the viability tests. **A.** J774 macrophages containing silver-enhanced gold. Upper arrow denotes a macrophage in close contact with a gold bead, facilitating dissolucytosis. Lower arrows all denotes examples of macrophages in which gold grains can be easily spotted. **B.** Digital magnification of the square in previous panel, with arrows denoting gold-loaded macrophages. **C.** CCL Neurons around two gold beads. **D.** Digital magnification of the square in previous panel, showing uptake of gold ions, denoted by the arrows, by the surrounding neurons. A, C, x 40; B, D, digitally enlarged.

*AMG staining show gold-uptake by both J774 macrophages and CCL neurons*

AMG staining of fixated cells showed uptake of gold ions in both the macrophages and the neurons, demonstrating a dissolucytotic release of gold ions from the metallic gold beads (Fig. 2).

*Clinical observations: Quick recovery from gold implant procedure*

The animals recovered well from the surgical incisions the following 24h, returning to normal behavior; the animals were monitored during the next 7- or 21d and had a normal intake of food, well-groomed fur and did not display any unusual behavior.

*Ventricular morphology appears normal after gold/HA injections, as did cellular morphology*

Utilized as a deposit site for the gold beads, the lateral ventricles were investigated with respect to their appearance, i.e. signs of hydrocephalic-like morphology or any other deviations from normal morphology. Though only 10 $\mu$L of gold/HA were injected, the possibility of clogging the cerebrospinal fluid (CSF) drainage of the ventricular compartments was assessed looking at coronal serial sections from the area around the injection point. None of the animals were found to have enlarged or morphological deviating ventricles compared to the control animals, neither at 7- or 21d post gold injection. Comparing left and right ventricles, i.e. in the injection side versus the contra-lateral side, those of the gold-treated animals did not differ visually from the contralateral side (Fig. 3), no tissue loss was seen and the cells appeared normal.

*Limited bio-liberation of gold from gold beads*

The gold beads were distributed similarly in both the 7- and 21d animals, with most gold beads found in the ventricular system, although a few gold beads were also seen in other places, most notably in cortex and CC, some of them possibly escaping via the injection canal. Screening of silver-enhanced gold grains in the cortices, CC, hippocampus, striatum and cerebellum in the 7d animals showed no uptake of gold ions outside the immediate proximity of the gold beads and only rarely could AMG-enhanced gold grains be detected contra-laterally to the injection side. Few bio-liberated silver-enhanced gold ions were seen contra-laterally but it cannot be ruled out that the spread of gold beads via the ventricular system may have brought them into close vicinity to where the grains were found. The 21d animals revealed that escaped gold beads found in the CC trajectory above hippocampus had released a cloud of gold ions extending down towards the CA2 and CA3 regions of the hippocampus (Fig. 4), which seemed more pronounced after 21d compared to the 7d animals. In general more silver-enhanced grains were found in the dentate gyrus and CA-regions in the 21d animals as compared to the 7d animals, although no quantitative analyses were made. Only sparse intracellular traces of silver-enhanced gold grains were seen intracellularly in cells in CA2 (Fig. 5).

In the cortex, as with the hippocampus, most grains were found on the injection side, also intracellularly (Fig. 6), but some grains were also seen contra-laterally in both the 7- and 21d animals. Both the 7- and 21d animals seemed to display scarcely spread silver-enhanced grains, especially in the injection side, although the 21d animals had more grains.

The striatum had few located grains in areas adjacent

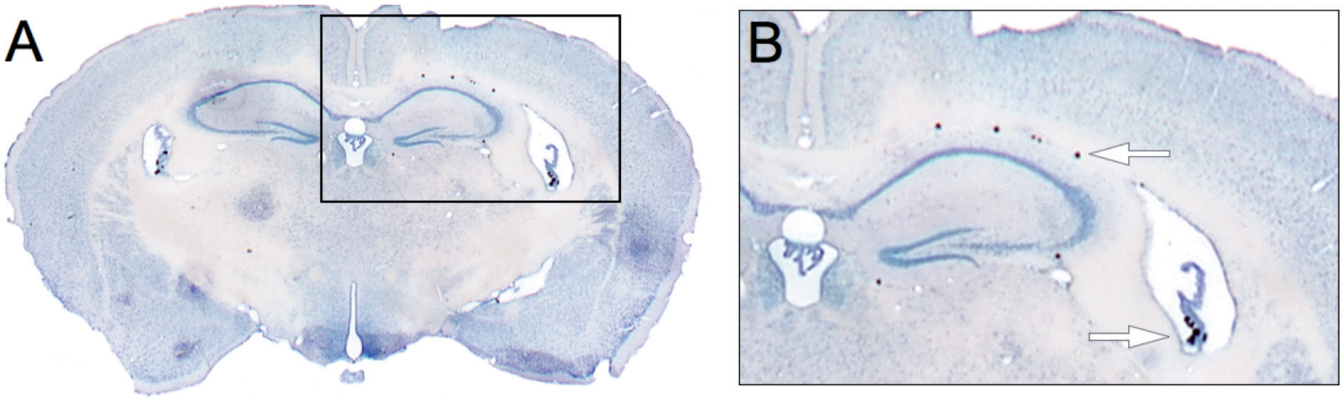


**Fig. 3.** Ventricular morphology and gold dispersion in gold-treated mice. **A.** High-resolution flat-bed scan of an AMG stained coronal section of a mouse brain 21 days after injection of gold/HA suspension. **B.** Digitally enlarged region of CA2 from the square in previous micrograph. Upper arrow denotes one of the stray gold beads found in corpus callosum. Lower arrow denotes a cluster of gold beads surrounded by plexus choroideus in the lateral

to the lateral ventricles and third ventricle in the 7d animals, with most in those slides in which gold beads could be seen in the ventricular system. In cerebellum in both the 7- and 21d animals metallic gold beads could be found near or in the fourth ventricle and, in some cases, a few were seen in arbor vitae, alongside silver-enhanced grains. Control animals were all devoid of signs of AMG staining.

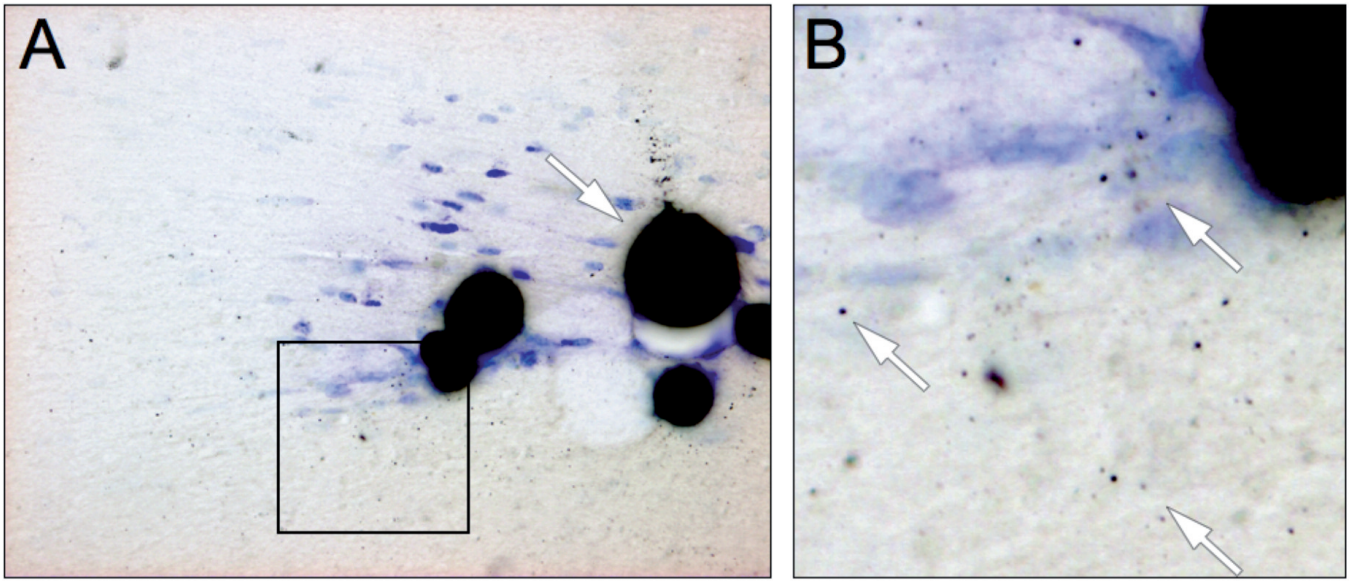


**Fig. 4.** Silver-enhanced intracellular gold beads and ions visualized in corpus callosum. **A.** Micrograph (from corpus callosum in the injection site of a gold/HA treated mouse at day 21. The arrow denotes a gold bead and left to the gold beads a cloud of silver-enhanced gold ions can be seen. **B.** Digitally enlarged region of the square in previous micrograph. Arrows denotes a few of the silver-enhanced gold ions, seemingly located in the white matter of corpus callosum. A, x 100; B, digitally enlarged.

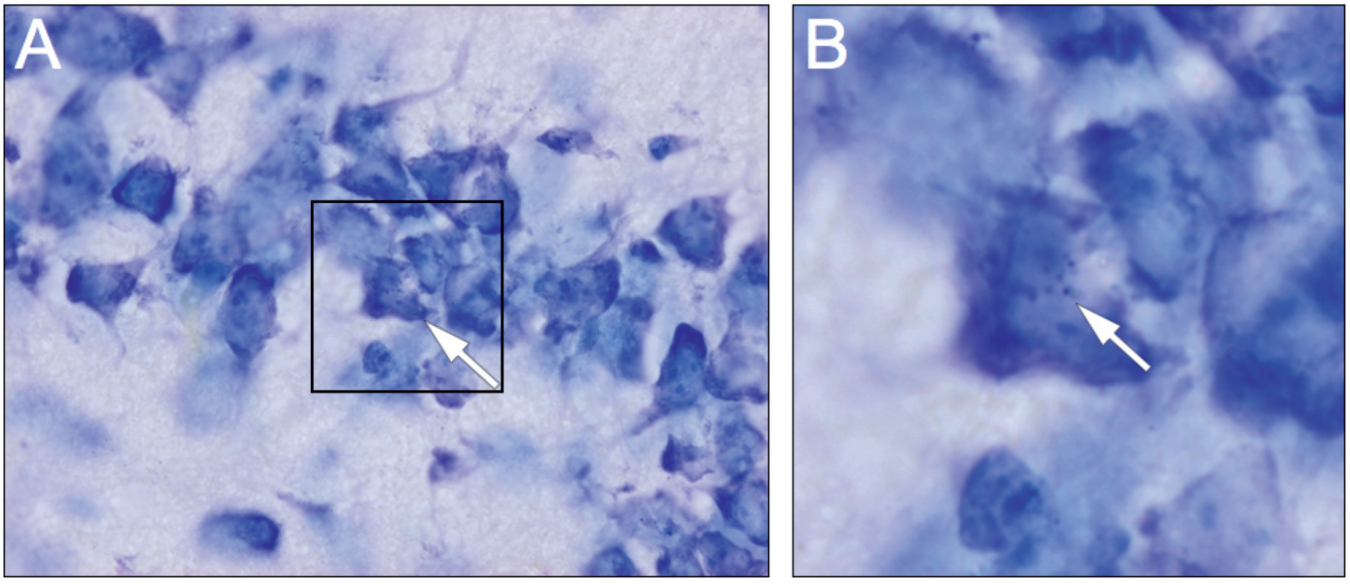


**Fig. 5.** Silver-enhanced intracellular gold grains visualized in hippocampus. **A.** Micrograph from the CA2 region in the injection site of a gold/HA treated mouse at day 7. The arrow denotes a neuron, in which small grains of silver-enhanced gold ions are seen. **B.** Digitally enlarged region of CA2 from the square in previous micrograph. The arrow points at a small cluster of silver-enhanced gold grains, seemingly located in the cytosol of the neuron. A, x 100; B, digitally enlarged.

*No systemic spread of gold to kidney and liver*

Neither the kidney nor the liver showed signs of silver-enhanced gold ions.

## Discussion

In the present study we have evaluated by means of AMG the extent of gold spread taking place in the CNS as a result of the injection of HA-suspended gold beads and the *in vitro* accumulation of bio-liberated gold in J774 macrophage and CCL neuron cell cultures exposed to HA-suspended gold. The AMG technique is an important tool in assessing whether bio-liberation of gold ions has taken place, as it is extremely sensitive in enhancing clusters of just a few nanoparticles of gold (Danscher et al., 1994; Danscher, 2002; Danscher and Stoltenberg, 2006). Unlike quantitative measurements such as atom absorption spectrophotometry, the AMG technique provides *in situ* information regarding the site of gold accumulation and it is useful in evaluating possible areas prone to a high degree of metal accumulation, even when the total amount of gold present is low.

*In vivo* we saw very limited gold liberation in this study, despite using the highly sensitive AMG technique. This marks a difference between gold and other heavy metals, such as silver and mercury, which are known to easily accumulate in brain tissue (Rungby, 1990; Danscher et al., 1994; Locht et al., 2010). However, although only a minute amount of metallic gold beads were injected, the mice in this study had 4 times more gold/HA injected per unit body weight i.e. 5.45 $\mu$g/25 g compared to previous EAE studies (Pedersen et al., 2012, 2013), in which rats treated with 1.36 $\mu$g/25 g gold/HA demonstrated a delayed disease development. Intracranial application of 1.36 $\mu$g/25g gold in a mouse model of brain trauma demonstrated beneficial effects in terms of anti-inflammation and neurogenesis (Larsen et al., 2008; Pedersen et al., 2009a).

It has thus been shown that this form of administration of metallic gold to the CNS under neuropathological conditions is capable of reducing inflammation, inducing astrogliosis and increasing the presence of growth factors such as VEGF, IGF, TGF-beta and Neurotrophin-4 (Larsen et al., 2008; Pedersen et al., 2009a,b). Adding to these histopathological signs of effect, a recent study showed that bio-liberated gold ions from gold implants are also capable of reducing clinical signs of neurodegenerative diseases such as disease-induced weight loss in experimental autoimmune encephalitis (EAE), a rodent model of MS (Pedersen et al., 2012). The advantage of metallic gold implants is that they serve as a permanent gold supply, which is also seen in veterinary studies employing gold beads as a modulator of pain in the long-term treatment of hip-dysplasia of dogs (Jaeger et al., 2006, 2007).

Despite previous results showing a beneficial immunosuppressive effect of metallic gold implants the potential toxicity of the use of metallic gold has not previously been investigated. Placing a permanent load of heavy metal deep within the nervous system might

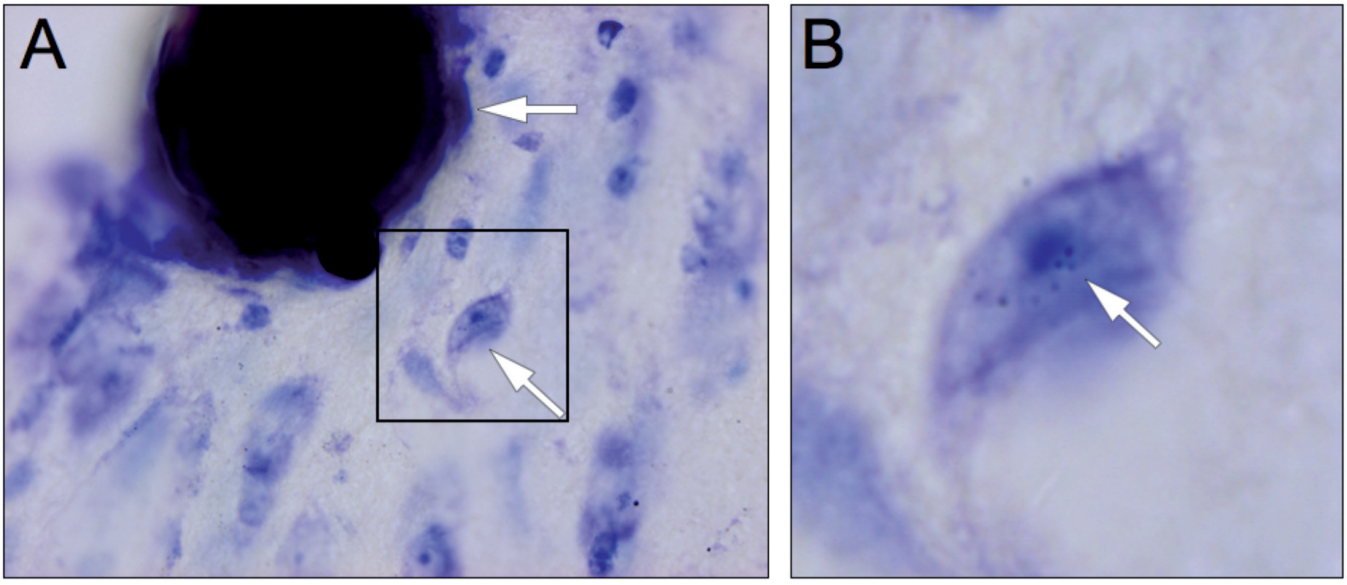


**Fig. 6.** Silver-enhanced intracellular gold bead and grains in cortex. **A.** Micrograph from cortex in the injection site of a gold/HA treated mouse at day 7. The upper horizontal arrow denotes a gold bead, probably a stray from injection canal. Next to the gold bead the lower arrow denotes a neuron, in which grains of silver-enhanced gold ions are seen. **B.** Digitally enlarged region of square in previous micrograph. The arrow points at a small cluster of silver-enhanced gold grains, seemingly located in the cytosol and in close vicinity of the nucleus of the neuron. A, x 100; B, digitally enlarged.

not be without risk. Zecca et al. (2008) previously reported that injection of 60 nm sized colloidal nano gold particles induced a moderate activation of microglia and a small loss of dopaminergic neurons. As such nano gold particles are subjected to immediate phagocytosis; thus, a higher and acute accumulation of gold in the injection-site cannot be excluded in contrast to the slow, gradual gold-liberation following dissolucytosis from larger (>25 $\mu$m) gold particles such as our gold beads. Furthermore, a certain amount of inflammatory response might occur simply as reaction to the presence of this size of nano particles (Sadauskas et al., 2009), thus contributing to the microglia activity.

However, it seems that in that sense the use of metallic gold is of an extraordinary character compared to other heavy metals such as highly reactive silver, which injected into CNS causes a massive loss of tissue (Locht et al., 2010). Numerous other studies report neuronal accumulation of heavy metals such as mercury and bismuth, which have been shown to accumulate in the lysosomes of long-living nerve cells (Moller-Madsen and Thorlacius-Ussing, 1984; Schionning et al., 1993; Schionning and Danscher, 1999; Stoltenberg et al., 2003; Larsen et al., 2005). This accumulation of heavy metals might possibly affect lysosomal stability, thereby leading to nerve cell death due to lysosomal leakage (Kurz et al., 2008; Terman et al., 2010), a phenomenon reported for many heavy metals, including bismuth (Stoltenberg et al., 2003), mercury (Sorensen et al., 2000) and silver (Rungby, 1990). Although gold accumulates in lysosomes (Moller-Madsen et al., 1984), the small amount of gold liberated will possibly be less of a threat to lysosomal survival. Furthermore, several studies indicate that gold ions do in fact increase lysosomal stability (Persellin and Ziff, 1966; Rodrigues and Galle, 1985; Graabaek and Pedersen, 1988).

A few reports in the literature have indicated that gold ions from gold salts can spread through retrograde transport Schionning et al. (1992) from muscle to dorsal ganglia cells and end up in both secretory and non-secretory cells in the anterior pituitary gland (APG) (Moller-Madsen and Thorlacius-Ussing, 1984). These localizations were not investigated here but the limited spread of gold even in the injection-near sites makes such a spread unlikely. Some studies have, however, connected gold salts to toxicological effects in the CNS. Gold-thioglucose has been used experimentally to induce chemical damage in ventromedial hypothalamus (VMH) to interfere with the long-term regulation of body weight (Debons et al., 1977; Bergen et al., 1996; Challet et al., 1999), due to capillary damage induced to VMH by gold-thioglucose and intense glucose-dependent gold uptake (Debons et al., 1979). These studies applied gold in the form of gold-thioglucose, thus enabling a highly localized and thus harmful accumulation of gold in glucose-responding neurons. Some neurological complications of treatment of rheumatoid arthritis (RA) with conventional chrysotherapy have manifested in terms of peripheral and cranial neuropathy in RA patients (Weiss et al., 1982; Fam et al., 1984), although this is a relatively rare contraindication and is related to a type of gold treatment, which causes massive gold spreading, including a discoloring gold accumulation in the skin (chrysiasis) (Merchant, 1998).

Thus, it does seem likely that adverse effects can occur in response to massive gold accumulation in nervous tissue; however, the investigated gold loads here lead to accumulations so small that it is feasible to believe that this type of permanent gold source will remain safe over time. Furthermore, no specific predication for high amounts of gold accumulation was found in this study exploiting the natural cavities of the brain as a gold reservoir. As shown in this study, metallic gold in contact with tissue releases more gold than might be expected from that staying in the ventricular system, altogether deeming this a safe route of administration.

Supporting the safety of the low dose gold/HA-treatment we found that neither the viability of the J774 macrophages nor CCL neurons were markedly affected by the presence of gold/HA, showing that the gold beads do not intoxicate any of the two cell types (Fig. 1a). The HA did, however, tend to increase the viability of J774 macrophages compared to controls, although insignificantly. Interestingly, this suggests that the anti-inflammatory effects seen in other studies are not due to the death of the macrophages but more likely are a result of an altered excretion pattern of inflammatory cytokines. This belief is based on our previous study, in which we found signs of reductions in the gene expression of the pro-inflammatory mediators Tnf-α, Il-1ß and Il-6 in macrophage cell cultures treated with gold beads (Pedersen et al., 2013).

To limit the amount of gold injected, we have developed a method using spherical unphagocytizable gold beads with a large surface suspended in viscous hyaluronic acid (or HA), a gel-like substance, allowing us to inject the combination into the lateral ventricles, the CSF-filled natural cavities of the brain. The HA used is a sterile compound known to be non-pyrogenic and used in the ophthalmologic clinic, e.g. for cataract surgery (Wilkie and Gemensky-Metzler, 2004), and is thus tested safe for use *in vivo*. However, the high viscosity of a compound injected directly into the ventricular system could constitute a risk of ventricular closure. Light microscopy revealed that the injections did not affect the ventricular morphology (fig. 3a), using the same concentration and amount of metallic gold beads suspended in HA as in our previous studies (Larsen et al., 2008; Pedersen et al., 2009a, 2012). Thus, it seems that the ventricular system does not clog up but sustains the CSF flow.

In conclusion, we saw that metallic gold implants - gold beads dispersed in HA-administered via the lateral ventricles - result in a limited, slow, long-lasting and highly localized release of gold ions in CNS. As the neuronal uptake of gold is minimal and gold appears to be non-toxic to neurons *in vivo* as well as *in vitro*,

further studies into a future use of metallic gold in the treatment of neurological disorders seems safe. Metallic gold in HA is non-toxic to cell cultures of macrophages, indicating that an altered macrophage response rather than a reduction of the macrophage load is the cause of the anti-inflammatory effects of gold, which we have previously reported.

*Acknowledgements.* Thanks to Albert Meier, Majken Sand and Heidi Schou Knudsen for their excellent technical assistance. We are grateful for financial support from the Aarhus University Research Foundation, Den Lægevidenskabelige Forskningsfond, Fonden af 17.12.1981 and Lykfeldts Legat.

## References

Aharoni R. and Arnon R. (2009). Linkage between immunomodulation, neuroprotection and neurogenesis, Drug News Perspect, 22, 301-312.

Amor S., Puentes F., Baker D. and van der Valk P. (2010). Inflammation in neurodegenerative diseases. Immunology 129, 154-169.

Bergen H.T., Monkman N. and Mobbs C.V. (1996). Injection with gold thioglucose impairs sensitivity to glucose: evidence that glucose-responsive neurons are important for long-term regulation of body weight. Brain Res. 734, 332-336.

Burmester G.R. (2001). Molecular mechanisms of action of gold in treatment of rheumatoid arthritis--an update. Z Rheumatol, 60, 167-173 (in german).

Challet E., Bernard D.J. and Turek F.W. (1999). Gold-thioglucose-induced hypothalamic lesions inhibit metabolic modulation of light-induced circadian phase shifts in mice. Brain Res, 824, 18-27.

Constantinescu C.S., Farooqi N., O'Brien K. and Gran B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br. J Pharmacol. 164, 1079-1106.

Danscher G. (2002). *In vivo* liberation of gold ions from gold implants. Autometallographic tracing of gold in cells adjacent to metallic gold. Histochem. Cell Biol. 117, 447-452.

Danscher G. and Stoltenberg M. (2006). Silver enhancement of quantum dots resulting from (1) metabolism of toxic metals in animals and humans, (2) *in vivo*, *in vitro* and immersion created zinc-sulphur/zinc-selenium nanocrystals, (3) metal ions liberated from metal implants and particles, Prog Histochem Cytochem, 41, 57-139.

Danscher G., Stoltenberg M. and Juhl S. (1994). How to detect gold, silver and mercury in human brain and other tissues by autometallographic silver amplification, Neuropathol. Appl. Neurobiol. 20, 454-467.

Das S. and Basu A. (2008). Inflammation: a new candidate in modulating adult neurogenesis. J. Neurosci. Res. 86, 1199-1208.

Debons A.F., Krimsky I., Maayan M.L., Fani K. and Jemenez F.A. (1977). Gold thioglucose obesity syndrome, Fed. Proc. 36, 143-147.

Debons A.F., Krimsky, I., From, A, Siclari E., Maayan M.L., Fni K. and Jiménez F.A. (1979). Action of gold thioglucose on pericapillary structures in the ventromedial hypothalamus. J. Pathol. 129, 73-81.

Ekdahl C.T. (2012). Microglial activation - tuning and pruning adult neurogenesis. Front. Pharmacol. 3, 41.

Fam A.G., Gordon D.A., Sarkozi J., Blair G.R., Cooper P.W., Harth M. and Lewis A.J. (1984). Neurologic complications associated with gold therapy for rheumatoid arthritis, J. Rheumatol. 11, 700-706.

Forestier J. (1934). Rheumatoid arthritis and its treatment by gold salts. The Lancet, 224, 646-648.

Franklin K.B.J. and Paxinos G. (2007). The mouse brain in stereotaxic coordinates. Academic Press.

Giovannoni G. and Ebers G. (2007). Multiple sclerosis: the environment and causation. Curr. Opin. Neurol. 20, 261-68.

Graabaek P.M. and Pedersen S.M. (1988). Effect of two gold compounds on lysosomes. Ann. Rheum. Dis, 47, 509-514.

Jaeger G.T., Larsen S., Soli N. and Moe L. (2006). Double-blind, placebo-controlled trial of the pain-relieving effects of the implantation of gold beads into dogs with hip dysplasia. Vet. Rec. 158, 722-726.

Jaeger G.T., Larsen S., Soli N. and Moe L. (2007). Two years follow-up study of the pain-relieving effect of gold bead implantation in dogs with hip-joint arthritis, Acta Vet. Scand. 49, 9.

Kurz T., Terman A., Gustafsson B. and Brunk U.T. (2008). Lysosomes and oxidative stress in aging and apoptosis. Biochim. Biophys. Acta 1780, 1291-1303.

Larsen A., Stoltenberg M., Sondergaard C., Bruhn M. and Danscher G. (2005). *In vivo* distribution of bismuth in the mouse brain: influence of long-term survival and intracranial placement on the uptake and transport of bismuth in neuronal tissue, Basic Clin. Pharmacol. Toxicol. 97, 188-196.

Larsen A., Stoltenberg M. and Danscher G. (2007). *In vitro* liberation of charged gold atoms: autometallographic tracing of gold ions released by macrophages grown on metallic gold surfaces. Histochem. Cell Biol. 128, 1-6.

Larsen A., Kolind K., Pedersen D. S., Doering P., Pedersen M.O., Danscher G., Penkowa M. and Stoltenberg M. (2008). Gold ions bio-released from metallic gold particles reduce inflammation and apoptosis and increase the regenerative responses in focal brain injury. Histochem. Cell Biol. 130, 681-692.

Lassmann H. (2011). Mechanisms of neurodegeneration shared between multiple sclerosis and Alzheimer's disease. J. Neural Transm. 118, 747-752.

Locht L.J., Pedersen M.O., Markholt S., Bibby B.M., Larsen A., Penkowa M., Stoltenberg M. and Rungby J. (2010). Metallic silver fragments cause massive tissue loss in the mouse brain. Basic Clin. Pharmacol. Toxicol. 109, 1-10.

Lucas S.M., Rothwell N.J. and Gibson R.M. (2006). The role of inflammation in CNS injury and disease, Br J. Pharmacol. 147 (Suppl 1), S232-S240.

McFarland H.F. and Martin R. (2007). Multiple sclerosis: a complicated picture of autoimmunity. Nat. Immunol. 8, 913-919.

Merchant B. (1998). Gold, the noble metal and the paradoxes of its toxicology. Biologicals 26, 49-59.

Mirshafiey A. and Mohsenzadegan M. (2008). Antioxidant therapy in multiple sclerosis. Immunopharmacol. Immunotoxicol. 31, 13-29.

Moller-Madsen B. and Thorlacius-Ussing O. (1984). Localization of gold in the anterior pituitary gland of rats exposed to sodium aurothiomalate. Exp. Mol. Pathol. 41, 74-80.

Moller-Madsen B., Mogensen S.C. and Danscher G. (1984). Ultrastructural localization of gold in macrophages and mast cells exposed to aurothioglucose. Exp. Mol. Pathol. 40, 148-154.

Pedersen M.O., Larsen A., Pedersen D.S., Stoltenberg, M. and Penkowa M. (2009a). Metallic gold treatment reduces proliferation of inflammatory cells, increases expression of VEGF and FGF, and stimulates cell proliferation in the subventricular zone following

experimental traumatic brain injury. Histol Histopathol. 24, 573-586.

Pedersen M.O., Larsen A., Pedersen D.S., Stoltenberg M. and Penkowa M. (2009b). Metallic gold reduces TNFalpha expression, oxidative DNA damage and pro-apoptotic signals after experimental brain injury. Brain Res. 1271, 103-113.

Pedersen D.S., Fredericia P.M., Pedersen M.O., Stoltenberg M., Penkowa M., Danscher G., Rungby J. and Larsen A. (2012). Metallic gold slows disease progression, reduces cell death and induces astrogliosis while simultaneously increasing stem cell responses in an EAE rat model of multiple sclerosis. Histochem. Cell Biol. 138, 787-802.

Pedersen D.S., Tran T.P., Smidt K., Bibby B.M., Rungby J. and Larsen A. (2013). Metallic gold beads in hyaluronic acid: a novel form of gold-based immunosuppression? Investigations of the immuno-suppressive effects of metallic gold on cultured J774 macrophages and on neuronal gene expression in experimental autoimmune encephalomyelitis. Biometals. 26, 369-385.

Persellin R.H. and Ziff M. (1966). The effect of gold salt on lysosomal enzymes of the peritoneal macrophage. Arthritis Rheum. 9, 57-65.

Rodrigues L.E. and Galle P. (1985). Lysosomotropism and anti-inflammatory action of gold salts. Rev. Rhum. Mal. Osteoartic. 52, 479-483 (in portugese).

Rungby J. (1990). An experimental study on silver in the nervous system and on aspects of its general cellular toxicity. Dan Med. Bull. 37, 442-449.

Sadauskas E., Jacobsen N.R., Danscher G., Stoltenberg M., Vogel U., Larsen A., Kreyling W. and Wallin H. (2009). Biodistribution of gold nanoparticles in mouse lung following intratracheal instillation. Chem. Cent. J. 3 16.

Schionning J.D. and Danscher G. (1999). Autometallographic mercury correlates with degenerative changes in dorsal root ganglia of rats intoxicated with organic mercury, APMIS, 107 , 303-10.

Schionning J.D., Poulsen E.H., Moller-Madsen B. and Danscher G. (1992). Autometallographic detection of gold in dorsal root ganglia of rats treated with sodium aurothiomalate. Exp. Mol. Pathol. 56, 239-247.

Schionning J.D., Danscher G., Christensen M.M., Ernst E. and Moller-Madsen B. (1993). Differentiation of silver-enhanced mercury and gold in tissue sections of rat dorsal root ganglia, Histochem J. 25, 107-111.

Sorensen F.W., Larsen J.O., Eide R. and Schionning J.D. (2000). Neuron loss in cerebellar cortex of rats exposed to mercury vapor: a stereological study, Acta Neuropathol. 100, 95-100.

Stoltenberg M., Larsen A., Zhao M., Danscher G. and Brunk U.T. (2002). Bismuth-induced lysosomal rupture in J774 cells. APMIS 110, 396-402.

Stoltenberg M., Schionning J.D., West M.J. and Danscher, G. (2003). Bismuth-induced neuronal cell death in rat dorsal root ganglion: a stereological study. Acta Neuropathol. 105, 351-357.

Terman A., Kurz T., Navratil M., Arriaga E.A. and Brunk U.T. (2010). Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging. Antioxid. Redox Signal. 12, 503-535.

Weiss J.J., Thompson G.R. and Lazaro R. (1982). Gold toxicity presenting as peripheral neuropathy. Clin. Rheumatol. 1, 285-289.

Wilkie D.A. and Gemensky-Metzler A.J. (2004). Agents for intraocular surgery. Vet. Clin. North. Am. Small Anim. Pract. 34, 801-823.

Yanni G., Nabil M., Farahat M.R., Poston R.N. and Panayi G.S. (1994). Intramuscular gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane, Ann. Rheum. Dis. 53, 315-322.

Zecca L., Wilms H., Geick S., Claasen J.H., Brandenburg L.O., Holzknecht C., Panizza M.L., Zucca F.A., Deuschl G., Sievers J. and Lucius R. (2008). Human neuromelanin induces neuroinflammation and neurodegeneration in the rat substantia nigra: implications for Parkinson's disease. Acta Neuropathol. 116, 47-55.

Accepted August 30, 2013