SECTION 3: NEUROTOXIC POTENTIAL OF SILVER NANOPARTICLES

Distinctive physical properties of NPs render them highly reactive and allow them to travel into intracellular compartments. They can interact with organelles leading to toxicity and enhanced generation of free radicals [199]. This condition leads to oxidative stress, where generation of harmful free radicals exceeds the threshold value [34]. Oxidative stress has thus been outlined as one of the major mechanisms responsible for NP induced manifestations. This may affect various organ systems, including nervous system, reproductive system, respiratory function, hepatic and renal system etc. Among various metal NPs, silver NP has been widely used in variety of applications. From coating of milk bottles to use of sulfadiazine creams on the skin of denuded patients are few examples [200]. Nano sized silver particles are major ingredients for electro conduit slurry, air purifiers, inks of inkjets etc [201].

In past, there have been reports indicating the toxic potential of these nanoparticles [204]. Kidneys, liver, spleen, lungs, brain and gastro intestinal tract (GI) tract, seems to be major target organs of Ag NPs [205]. Potential toxic effects are being reported as nanoparticles bind themselves to tissues, ultimately leading to cell death. Exposure to silver nanoparticles may lead to blood brain barrier disruption and ultimately causes impaired brain function. Besides numerous studies and scientific reports, impact of Ag NPs on human health, particularly on central nervous system has not been adequately evaluated. The present study was planned to investigate neurotoxicity following 10 days continuous exposure to silver nanoparticles.

Animals and treatments

Study was conducted in order to compare the toxicity of nanoparticles at three different doses of 20, 50 and 100 µM. The animals were divided into four groups of eight mice each and treated as below for ten days through oral gavage.

Group I - Control animals (received drinking water)
Group II - Silver nanoparticles; Ag NP, 20µM/kg (2 mg/kg/2 ml/day)
Group III - Silver nanoparticles; Ag NP, 50µM/kg (5 mg/kg/2 ml/day)
Group IV - Silver nanoparticles; Ag NP, 100µM/kg (10 mg/kg/2 ml/day)
Animals were dosed orally, once daily for 10 days and dosing was carried out at the same time between 1100 hours to 1200 hours. It is necessary to maintain regular dosing time to avoid any possible variations in results. After 10 days, blood was collected from orbital plexus in heparinized tubes and used for various biochemical assays before anaesthesia as induction of anaesthesia combined with orbital puncture can cause more distress. Animals were anaesthetised and euthanized by decapitation. Brain was removed, rinsed in cold saline, blotted, weighed, and used for various biochemical assays.

**Biochemical assays**

RBCs were isolated from blood using method of Steck and Kant (1974) [184]. RBCs were washed in phosphate buffer saline (0.1M) for three consecutive times. The packed cell volume (PCV) was divided into two parts. First part was diluted with chilled distilled water and kept for the analysis of ROS and reduced glutathione. Second part was used for the estimation of antioxidant enzymes i.e. glutathione peroxidase and glutathione-S-tranferase. Analysis of blood GSH concentration was done by the method described by Ellman, (1959) [160] and modified by Jollow et al., (1974) [161]. TBARS in red blood cells was measured according to a method by Stocks and Dormandy (1971) [224]. Tissue lipid peroxidation was measured by method of Ohkawa et al., (1979) [163]. Malondialdehyde (MDA) is the end product of lipid peroxidation which was measured in tissue homogenate on the basis of the reaction with thiobarbituric acid (TBA) to form a pink color complex. Determining the absorbance coefficient of the MDA-TBA complex spectrophotometrically at 535nm gives the amount of MDA produced. Reactive oxygen species (ROS) in blood and tissues was measured using 2’, 7’-dichlorofluorescin diacetate (DCF-DA). This dye gets converted into highly fluorescent DCF by the action of cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci et al.,(1999) [169]. Tissue GSH and GSSG levels were measured as described by Hissin and Hilf (1974) [164]. Detection of nitric oxide concentration by fluorometric kit (Nitric Oxide Assay Kit, Fluorometric; Catalog No. 482655, Calbiochem, Germany) using manufacturer’s protocol. Dopamine (DA), norepinephrine (NE) and 5-hydroxytryptamine (5-HT) were estimated according to the procedure of Jacobwitz and Richardson (1978) [171]. Frozen brain region samples were weighed. Acidified butanol was used to homogenize them. A 10% homogenate (w/v) was prepared in 0.25M sucrose.
Activity of acetyl cholinesterase (AChE) in brain was determined according to the method of Ellman et al. (1961) [172] using acetylthiocholine as substrate.

**Results**

*Effect of silver nanoparticles on body weight*

Table 4.5: Effect of various concentrations of silver nanoparticles on body weight changes in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Body weight gain or loss (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animal</td>
<td>25.10 ± 0.22</td>
<td>25.50 ± 0.10</td>
<td>+0.40 ± 0.003</td>
</tr>
<tr>
<td>Ag-20</td>
<td>25.34 ± 0.09</td>
<td>25.10 ± 0.16</td>
<td>-0.24 ± 0.010</td>
</tr>
<tr>
<td>Ag-50</td>
<td>25.62 ± 0.13</td>
<td>25.10 ± 0.10</td>
<td>-0.52 ± 0.006</td>
</tr>
<tr>
<td>Ag-100</td>
<td>26.80 ± 0.10</td>
<td>25.50 ± 0.11</td>
<td>-1.30 ± 0.020*</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n=8.

Table 4.5 shows the effect of NP exposure on body weight in mice. Pronounced reduction in body weight was observed in mice exposed to highest dose of silver nanoparticle (100µM). However no changes were observed in groups exposed to 20 and 50µM of doses observed. Maximum alteration was seen in case of highest dose that is, 100µM, compared to lower doses of 20 and 50µM.

*Effect of Ag Nps on blood biochemical variables*

Figure 4.17A and B depicts alterations in blood oxidative stress variables upon exposure to various concentrations of silver nanoparticles. Significant elevation in ROS and TBARS was observed in results. Fig 4.17C exhibits depletion of GSH, most significant at highest concentration (100µM), compared to all other lower doses.

*Effect of Ag Nps on brain biogenic amines*

Figure 4.18 represents the effect of Ag NPs on brain biogenic amines in mice. Nanoparticles significantly reduced the level of neurotransmitters (NE and DA). Levels of NE as well as DA enhanced significantly upon exposure to various concentrations of Ag NPs. However, maximum alteration/elevation in levels of NE and DA was observed in
case of highest concentration (100µM). At the same time not much of significant alteration was observed in case of 5-HT, even in case of highest concentration of Ag NP.

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**Figure 4.17 A: Effect of silver nanoparticles on blood ROS**

*Abbreviation used and unit: ROS; Reactive Oxygen Species as Fluorescent Intensity Unit (FIU). Values are mean±SE; n=8. *P=0.05 compared to normal animals.*

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**Figure 4.17 B: Effect of silver nanoparticles on blood TBARS**

*Abbreviation used and unit: TBARS: thiobarbituric reactive substances as µg/gm tissue. Values are mean±SE; n=8. *P=0.05 compared to normal animals.*
Figure 4.17 C: Effect of silver nanoparticles on blood GSH

**Abbreviation used and unit:** GSH, reduced glutathione as mg/g tissue, Values are mean±SE; n=8. *P=0.05 compared to normal animals.

Figure 4.18: Effect of silver nanoparticles on brain biogenic amines

**Abbreviation used and unit:** NE: norepinephrine as µg/g of tissue, DA: dopamine as µg/g of tissue, 5-HT: 5-hydroxytryptamine as µg/g of tissue. Values are mean±SE; n=8. *P=0.05 compared to normal animals.
**Effect of Ag NPs on alterations in brain AChE activity**

Figure 4.19 represents the effect of various concentrations of Ag NPs on alteration in brain AChE activity in mice. Ag NPs significantly reduced the activity of AChE. However as per the trend, maximum decline in activity was observed in case of highest concentration of Ag NP (100µM).

![Bar Chart](image)

**Figure 4.19: Effect of silver nanoparticles on AChE activity**

*Abbreviation used and unit: AChE, acetyl cholinesterase as nmol/min/mg protein. Values are mean±SE; n=8. *P=0.05 compared to normal animals.*

**Effect of Ag NPs on Brain NOS activity**

Figure 4.20 represents the effect of various concentrations of Ag NPs on brain NOS activity in mice. Ag NPs significantly increased the activity of NOS. However, maximum elevation in activity was observed in case of highest concentration of Ag NP (100µM).
Discussion

Manmade NPs have been widely used these days in every sphere of life such as, in electronics, cosmetics, medicines, fabrics etc. However awareness about their bad effects on human health lag much behind the rapid development of nanotechnology. NPs after exposure might reach into the brain directly (the olfactory bulb) via olfactory nerves [191]. Inhalation, injection, dermal penetration, and ingestion are various routes of nanoparticle exposure, through which they enter the body and then distribute by means of systemic circulation to various tissues including the brain. Studies have shown that any nanosize particle can reach brain and may be associated with various neurodegenerative diseases. Few NPs could not be eliminated physiological clearance systems and hence they accumulate within brain and gradually trigger toxic effects [191]. Exact etiology of such diseases remains unknown, but environmental pollutants, including NPs, may be an important risk factor.

Various toxicity studies have shown that Ag NP administered by various different routes were subsequently detected in blood and resulted in organ toxicity including brain [225]. Silver has received much attention because of its toxicity at high ionic
concentrations. The increased ROS levels in blood suggest free radical generations by silver nanoparticles (Figure 4.17A). Within blood, silver binds to albumin, enabling its transportation throughout the body leading to its toxic manifestation [215]. In addition, depletion of endogenous antioxidant GSH, following exposure to silver nanoparticles suggests it’s binding to GSH, or related enzymes involved in its synthesis [54]. Significant depletion in GSH and elevation in TBARS supports the generation of oxidative stress (Figure 4.17B and 4.17C). Following oral exposure, silver translocate from the gut enter into the bloodstream, and gets distributed to various organs including brain [91]. Administration of toxic chemicals disturbs the spontaneous activity of the cells and influences neurotransmitter turnover. Hence we evaluated the level of neurotransmitters in cerebral cortex region of mice. Exposure to increasing concentrations of Ag NPs led to an increased level of norepinephrine (NE) and dopamine (DA) in cortex region of mouse brain. This further suggests the weakened ability of brain to maintain an appropriate state of activation in the central nervous system due to toxicity. The level of 5-HT however remained unaffected following exposure of these nanoparticles, even at the highest dose (Figure 4.18). Neurotransmitters are known to play a crucial role in memory, awareness, thought, and consciousness. They are directly involved in alertness and thus guards against intense reflex reactions and other abnormal behavior. Our results suggest that accumulation of nanoparticles may result in altered synthesis and release of specific neurotransmitters and receptors in nerve cells, which may further lead to neural damage and hence progression of various neurodegenerative diseases. The resulting oxidative stress and brain injury is due to a cascade of reactions triggered by metal oxide nanoparticles, such as lipid peroxidation, decreased activity of antioxidant enzymes, release of nitric oxide, reduction of glutamic acid, and downregulation of acetylcholinesterase activity. Our findings clearly indicate down regulation of AchE activity, though maximum effect observed in case of exposure to highest concentration of 100µM (Figure 4.19).

Nanoparticles induce oxidative stress in the body by producing nitric oxide [209]. Upon contact with tissue or fresh blood serum, NPs catalyzes the generation of NO, which reacts rapidly with free radicals, leading to oxidation of DNA, proteins and lipid molecules [209]. Silver nanoparticles induced generation of oxidative damage is in agreement with our findings. These free radicals, particularly superoxide anions, react with nitric oxide (NO) generated in response to oxidative stress, leading to the formation
of harmful peroxynitrite species, which in turn oxidizes lipids, DNA and proteins [209]. Our results demonstrate increased generation of NO on exposure to nanoparticles, however a marked elevation in case of higher concentration of nanoparticles (100µM) was observed (Figure 4.20). These results in agreement with previous reports that show radical mediated damages include cell-signalling modifications along with oxidative injury [210, 211]. The results from our study provided some interesting observations, which are in agreement to the literature studies about the neurotoxic potential of silver nanoparticles. Silver nanoparticles possess the ability of damaging brain following introduction into the systemic circulation and thus leading to neurobehavioral abnormalities [226]. Our results demonstrate that highest concentration of Ag NP (100µM) leads to more pronounced damage to brain as evident by enhanced level of neurotransmitters and NOS and reduced AchE levels. However lowest concentration of 20µM was also toxic, as even at this concentrations biochemical and neurological manifestations were observed.

The study concludes that silver nanoparticles contribute significantly to the progression of various neurodegenerating conditions, as evident by results obtained. Also, higher dosage is responsible for greater toxic manifestations. But even at 20µM, toxic effects are visible. This dose was also specific in triggering NO generation and hence stimulates further damage. Therefore, the dose dependency of particle toxicity is an important issue.