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Dose dependent Effect of silica nanoparticles on rat central Nervous system: A behavioral and biochemical study: Research

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ABSTRACT

Occupational exposure to silica dust has been increasing the possible risk of varieties of pathologies. It has also been found extensive applications in biomedical and biotechnological fields such as medical diagnostics, drug delivery, gene therapy, biomolecules detection, photodynamic therapy and bio-imaging. This adds to the increasing industrial exposure to silica nanoparticles during production, transportation, storage, and consumer use by which human exposure and environmental burden were obviously increased. The purpose of this work was to investigate the neurotoxicity of two different dosages (40 and 80 mg /kg body weight) of silica nanoparticles (SiNPs) in Wistar rats for 14 days by intraperitoneal exposure. The neurobehavioral and neurochemical changes were analyzed. Spontaneous motor activity, catalepsy, gait and muscle incoordination were assessed. Thereafter brain was removed for investigation of lipid peroxide level and antioxidant enzymes (SOD and CAT). Our results showed SiNPs could pass through the blood brain barrier into the brain. Significantly increases the concentration of lipid peroxide levels and reduces the activity SOD and CAT and reduced behavioural activity were seen. The maximum changes were observed in rats who received 80 mg dose. We conclude that neuronal toxicity of silica nanoparticles could be related to the amount of the particle. Oxidative stress could be involved in cellular damage by lipid, protein and DNA and cognitive decline.

KEYWORDS : Silica nanoparticles, nervous system, free radicals, lipid peroxide

INTRODUCTION:

Silica nanoparticles (SiO(2)-NPs) are being used increasingly in diagnosis, imaging, and drug delivery for the central nervous system. However, to date, little is known concerning the potential adverse effects on the brain associated with exposure to SiO(2)-NPs.

There are multiple crystalline forms and one amorphous form of silica. Inhalation of the crystalline form of silica has been historically associated with the development of a severe respiratory disease, silicosis, which is a lung pneumoconiosis characterized by alveolar proteinosis and diffuses fibrosis resulting in progressively restrictive lung function.. Silicosis has been primarily associated with long occupational exposures to crystalline silica that typically occur in sandblasting, silica milling, rock drilling and tunnelling.

Excess silicon intake is generally regarded as non-toxic. However, in some patients high levels of Silicon has been associated with Alzheimer's disease. Toxicity of silicon can also lead to chronic fibrosis of the lungs. There is evidence that silica exposure can also be linked to the development of autoimmune diseases such as scleroderma (systemic sclerosis), rheumatoid arthritis, chronic renal disease and lupus (Cooper G S.et al., 2002). Additionally, silica inhalation is believed to be the cause of some rare lung cancers (Peretz A. et al., 2006), although significant relative risk (RR) of lung cancer is only associated with individuals that already have silicosis from silica exposure .The diversity of pathologies associated with silica exposure, it is unlikely that one common mechanism is responsible for all of the possible diseases. Although the exact sequence of events is not known. We hypothesized that the free radical damages initiated by silica may be one of the possible mechanics of the silica induced toxicity.

In the present study, we aim to evaluate the dose dependent effect of silica on different organ system in male albino rats. Moreover, the mechanism of free radicals production and defended by antioxidant will also be evaluate using biochemical investigation.

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In the present study, we aim to evaluate the dose dependent effect of silica on brain in male albino rats. Moreover, the mechanism of free radicals production and defended by antioxidant will also be evaluate using biochemical investigation.

METHODOLOGY

Animals:

In the present study, a total number of 18 male albino rats (weight-180 grams) were taken from Rajasthan University. All the rats were maintained on Hindustan Lever Food Pallets diet and water ad libitum in the animal house of the NIMS University, Jaipur on 12 hours light and dark condition. All the experimental procedure were approved by institutional animal ethical committee.

The rats were divided in to three following groups. Each group contains six rats.

Group - 1	Normal saline
Group - 2	SNPs 40

Group – 3 SNPs 80

Route of Dose:

The doses were given by intra peritoneal (IP) injection.

Experimental Procedure:

After 14 days of treatment; rats were allowed to move for behavioural study. Thereafter rats were sacrificed by cervical dislocation and brains were dissected out from cranium for further biochemical changes. A part of brain is preserved in 10% formalin solution for morphological study.

A. NEUROBEHAVIORAL STUDIES

Behavioural studies:

The Motor activity, behavioural changes, muscle coordination, sensory and motor reflex responses were assessed in Al administered rats and control rats as per the previously described protocol.

Spontaneous motor activity [SMA]: SMA was measured by scored on a scale of 0 – 9 in which SMA in control group was assigned score 4.

Righting reflex: Rats were placed on their back to see if the animal could quickly right itself and assume a normal posture. Neurological deficits were indicating by an inability to regain normal body posture within five seconds.

Catalepsy: A condition in which body or limbs remain passively in any position in which they can be placed. This test is used by placing forepaws on a metallic rod placed at height of 6 cm and forepaws were not observed withdrawn within 10 sec. catalepsy were consid-

ered positive.

Muscle coordination test (Rota rod): The period of stay on rotating rod (speed: 5 rotations / min; Total duration of test 2 min) for each control and treated rat were recorded by Rotamex (Techno Electronics, India). The rats were trained to stay for period of 2 min on rotating rod and only trained rats were included in the study. Motor was measured using Rota Rod at least 5s and it was rotated at speed of 10 rpm for 2 consecutive days on third day the time duration of each rotation speed was also recorded.

B. Biochemical Estimations

Tissue Homogenate Preparation

After 14 days of experiment rats were sacrificed. Their brains were removed and weighed individually. Ten percent (w/v) homogenate of the brain was prepared using York's homogenizer fitted with Teflon plunger in 0.1 M phosphate buffer (pH 7.1). The whole homogenate was first centrifuged at 2500 x g for 10 minutes in a refrigerated centrifuge. The pellet consisting of nuclear fraction and cell debris was discarded. The supernatant was further centrifuged at 11,000 x g for 15 minutes and mitochondrial fraction was separated. The clear supernatant was further centrifuged at 105,000 x g for 90 minutes and the resultant supernatant was used for determining enzyme activities.

PROTEIN ESTIMATION: (Lowry O. H., et al 1995).

Principle:

It is the most commonly used method for determination of protein in cell free extracts, because of its high sensitivity and quantities as low as 20µg of protein concentration be measured. The peptide bond in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue coloured complex. In addition, tyrosine and tryptophan residues phospho tungstate component of folin indicator regent to give blue product which contribute towards enhancing the sensitivity of this method.

Reagent:

- **Alkaline Na_2Co_3 reagent –A:** Prepare 2 % of Na_2Co_3 in 0.1 NaOH and makeup the volume up to 100 ml with 0.1N NaOH
- **Copper sulphate reagent –B:** Prepare 0.5% $CuSO_4$ in 1% Na-k tartrate solution.
- **Alkaline copper sulphate solution-C:** Add 50 ml reagent A and 2 ml reagent B to prepare reagent C. This mixture is unstable and make freshly.
- **Folin's reagent (1:2):** Folin + TDW (freshly prepared).

Procedure: Standard Protein Graph:

- Take six test tubes as Test ($T_1 – T_6$) and one as Reference (R).
- Place B. S. A. (50 µl, 100 µl, 150 µl, 200 µl, 250 µl, 300 µl) into Test tubes marked as ($T_1 – T_6$) except in Reference Tube (R).
- Add TDW (250 µl, 200 µl, 150 µl, 100 µl, 50 into test tubes ($T_1 – T_6$) & 300 µl into Reference Tube (R).
- Add 5 ml Reagent C in each test tube.
- Place the test tubes in incubator at 37 ^{0}C for 15 minutes.
- Add 500 µl Folin's in each test tubes.
- Place the test tubes in incubator at 37 ^{0}C for 40 minutes for development of colour.
- Read the colour at 660 nm vs. reagent blank.

Procedure: Protein estimation

- Take test tubes mark them as Test ($T_1 – T_6$) Reference (R).
- Place sample (100 µl) into Test tubes marked as ($T_1 – T_6$) except in Reference Tube (R).
- Add 100 ml TDW into Reference Tube (R).
- Add 5 ml Reagent C in each test tube.
- Place the test tubes in incubator at 37 ^{0}C for 15 minutes.
- Add 500 µl Folin's in each test tubes.
- Place the test tubes in incubator at 37 ^{0}C for 40 minutes for development of colour.
- Read the colour at 660 nm vs. reagent blank.
- **LIPID PEROXIDATION:** (Ohkawa H., Ohishi N. and Yagik. 1979).

Principle:

Acetic acid detached lipid and protein in the reaction mixture is dissolved by the addition of SDS, 2- thio barbituric acid (TBA) reacts with MDA, form colours additives with absorption mixture at 532 nm.

Reagent:

- Sodium deodyl sulphate (SDS) = 8.0 % (8 gm/100ml)
- Thio barbituric acid (TBA) = 0.8 % (0.8gm/100ml)
- Trichloro acetic acid (TCA) = 10 %
- Acetic acid glacial

Procedure:

- Take six test tubes and mark them as Test ($T_1 – T_6$) & one tube mark as Blank (B).
- Place 0.2 ml tissue homogenate in Tubes ($T_1 – T_6$) only.
- Add 1.0 ml TCA in each test tube.
- Add 0.5 ml SDS in each test tube.
- Add 0.5 ml Acetic acid in each test tube.
- Add 1.5 ml TBA in each test tube.
- Add 0.2 ml TDW in Blank (B) tube only to make final volume 3.7 ml.
- Place the test Tubes in water bath at 80 ^{0}C for 1 hour.
- Let the samples cool and centrifuge at 10,000 rpm for 15 minutes
- Take O D at 532 nm

Result = n moles/ml plasma

SUPER OXIDE DISMUTASE: (Mc. Cord & Fridorich., 1969).

Principle:

NADH reduce in the presence of phenozine metho sulphate (PMS) gives Super Oxide radicals (O_2). These Oxygen free radicals reduce Nitro blue tetrazolium (NBT) and form Farmazone having dark blue colour. When SOD source (biochemical sample) is added to above reaction mixture, these participate in another reaction to neutralize O_2 into H_2O_2 and therefore first reaction (reduction of NBT) showed down and indicatly give a measure of SOD in test samples.

Reagent:

Sodium pyrophosphate buffer – 909 mg/dl in TDW

Nitro blue tetrazolium (NBT) – 12.86 mg/10 ml in buffer

NADH (2.34 m mole) – 16.59 mg/10 ml

PMS (93µ mole) – 2.8 mg /100ml TDW

- Procedure:
- Take eight test tubes and mark them as Test ($T_1 – T_6$) & one tube mark as Blank (B) & other as Reference (R).
- Place 1.7 ml Buffer in Tubes ($T_1 – T_6$), Reference (R) Tube and 2.1ml buffer in Blank (B) tube.
- Add 0.2 ml NBT in each test tube.
- Add 0.2 ml PMS in each test tube.
- Add 0.02 ml tissue homogenate in tubes marked as Test ($T_1 – T_6$) only.
- Add 1.5 ml TBA in each test tube.
- Add 0.2 ml NADH in tubes, Test & Reference (R) but do not in Blank (B) tube.
- Place the test Tubes in incubator at room temperature.
- Add 1.0 ml Acetic acid in each test tube.
- Wait for 10 minutes
- Add 20 µl enzyme samples in reference tube
- Mix and read OD at 560 nm Vs reagent blank.

CATALASE: (Aebi H & Suter H., 1974).

Principle:

In UV range H_2O_2 shows a continual increase in absorption with decreasing wave length and maximum at 240 nm. The decomposition of H_2O_2 can be followed directly by the decrease in extinction 240 nm (E = 240 = 40 cm^2 µ mol). The decrease in extinction (E = 240) per unit time is the measured of the catalase activity.

Reagents:

H_2O_2 = 0.2 M

Phosphate Buffer = 0.01 M KH_2PO_4 = 681 mg/dl + K_2HPO_4 =

1.225gm/155ml

Procedure:
Take 2 ml of Phosphate Buffer and 1 ml of H_2O_2 into a cuvette; add 2 ml enzyme source (20 folds diluted in Buffer) & mix thoroughly. The decrease in absorbance at 240 nm will be recorded after every 30 seconds for 30 minutes. Results were expressed as Unit/mg protein

(D) STATISTICAL ANALYSIS
The raw data will be summarized and expressed as mean ± SEM and evaluated using the student T test.

RESULTS:
Behavioural study:

	Control (N = 6)	SNPs 40 (N = 6)	SNPs 80 (N = 6)
SMA	4.90 ± 1.03	4.01 ± 0.71	3.36 ± 0.31*
Catalepsy	11.5 ± 1.87	8.33 ± 1.21*	6.03 ± 0.83*
Gait	2.30 ± 0.74	1.90 ± 7.05*	1.02 ± 5.3*
Rota Rod	65.2 ± 11.5	49.2 ± 7.05*	42.9 ± 5.07*

The spontaneous motor activity were found to be significantly ($p<0.05$) reduced in SNPs80 when compared with the controls. Catalepsy was present in both the groups. The SNPs treated rats were unable to stay in metallic rod of more than 10 seconds. The significant reduction of time was observed in both SNPs 80 and SNPs 40 treated rats. Gait war also found to be reduced time to maintain posture in the edge of the cage. Both groups were showed significant ($p<0.05$) change when compared with the cont rol.The muscle coordination was performed using rota rod test. The time of staying at rod were found to be significantly reduced in both SNPs 80 and SNPs 40 groups when compared with their respective controls.

Body Weight:

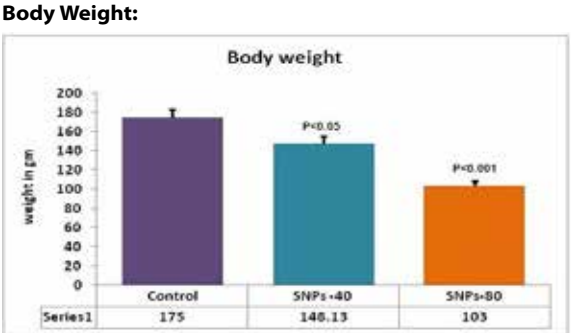

Fig.1 :The body weight were found to be significantly ($p< 0.05$) decreased by 15% in SNPs 40 treated rats while it was significantly ($p<0.001$) decreased by 41% in SNPs80 treated rats when compared with controls. The comparative difference between SNPs 40 and SNPs 80 was 30% in SNPs 80 when compared with SNPs 40.

Brain Weight:

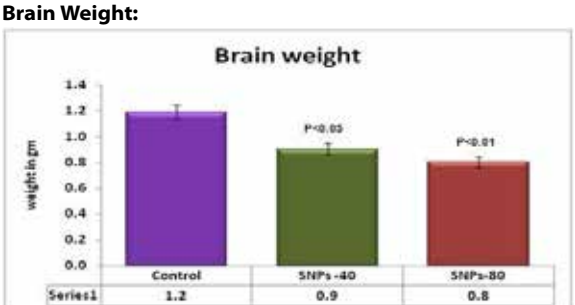

Fig 2 : The brain weight were found to be significantly ($p< 0.05$) decreased by 25% in SNPs 40 treated rats while it was significantly ($p<0.01$) decreased by 33% in SNPs treated rats when compared with controls. The comparative difference between SNPs 40 and SNPs 80 was 11% in SNPs 80 when compared with SNPs 40.

Protein Estimation:

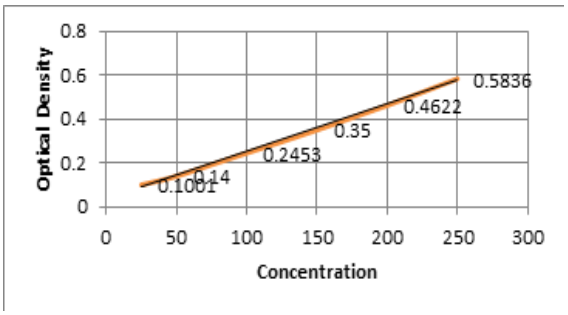

Fig. 3 Protein Standard Graph - The total protein content were found to be significantly ($p< 0.01$) decreased by 22% in SNPs 40 treated rats while it was significantly ($p<0.001$) decreased by 40% in SNPs treated rats when compared with controls. The comparative difference between SNPs 40 and SNPs 80 was 23% in SNPs 80 when compared with SNPs 40.

Lipid peroxidation:

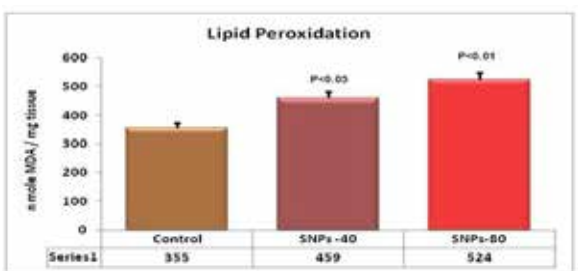

Fig.4 : The lipid peroxide was measured in term of n mole MDA/gm tissue following SNPs exposure for 14 days. The MDA level were found to be significantly ($p< 0.05$) increased by 29% in SNPs 40 treated rats while it was significantly ($p<0.001$) increased by 48% in SNPs 80 treated rats when compared with controls. The comparative difference between SNPs 40 and SNPs 80 was 14% in SNPs 80 when compared with SNPs 40.

SUPEROXIDASE DISMUTASE TEST:

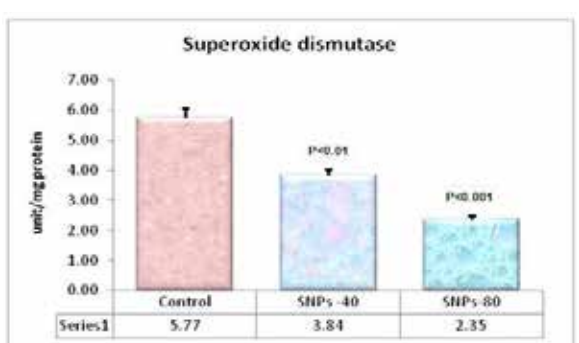

Fig.5

The superoxide dismutase activity was measured in term of unit / mg protein following SNPs exposure for 14 days. The superoxide dismutase activity were found to be significantly ($p< 0.01$) decreased by 33% in SNPs 40 treated rats while it was significantly ($p<0.001$) increased by 59% in SNPs treated rats when compared with controls. The comparative difference between SNPs 40 and SNPs 80 was 39% in SNPs 80 when compared with SNPs 40.

CATALASE TEST

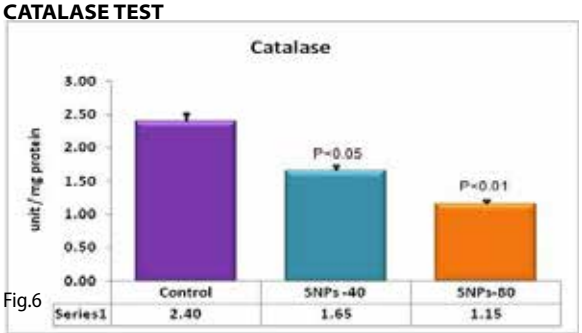

Fig.6

The catalase activity was measured in term of unit /mg protein following SNPs exposure for 14 days. The catalase activity were found to be significantly ($p< 0.05$) decreased by 31% in SNPs 40 treated rats while it was significantly ($p<0.001$) increased by 52% in SNPs treated rats when compared with controls. The comparative difference between SNPs 40 and SNPs 80 was 30% in SNPs 80 when compared with SNPs 40.

DISCUSSION & CONCLUSION

Cytotoxicity of two different doses (40 and 80 mg) was investigated in rat central nervous system. The effect of Si on body weight gain, food intake and feed efficiency was progressively increased during the experimental period of both the groups. The final body weight of intoxicated rats (Fig.1) with Si was significantly lower than that of the health normal group. These results clearly indicate that SiNPs cause a significant decrease in body weight. This harmful effect of SiNPs on the body weight gain was elevated paralleled with increase of SiNPs dose. The amount of food intake of all three groups was unchanged significantly. This means that the value of food intake was not paralleled to the rate of growth and feed efficiency. It is suggestive that loss of body mass indicated that SiNPs induces severe toxicity and it may be due to loss of lipid, protein and other biomolecules. In this study first time we evaluated neuromuscular dysfunctioning. The gross behavioral response i.e., spontaneous motor activity (SMA), Gait, catalepsy and rota rod are shown in the table 1.

Results demonstrated peroxidative damage of brain and blood lipids, as measured by the increased levels of lipid peroxidation products (MDA). This may be due to increased rate of ROS production. Moreover, as the membrane contains large amount of lipids that are rich in polyunsaturated fatty acids, they can readily react with free radicals and undergo peroxidation. Although SiNPs in biological tissues do not have any direct pro-oxidant properties but they may potentiate Fe to promote ROS formation and enhance peroxidative damage to lipids and proteins. Cellular endogenous defense system possess many antioxidant enzymes, among them SOD plays a major role as it prevents ROS mediated tissue damage and catalase are important antioxidants, in the cell which protect it from H_2O_2 mediated cellular damage and therefore decreased level of these biomolecules, as observed by us, may lead to increased severity of dose dependent SiNPs toxicosis.

Several mechanisms have been proposed to explain the adverse health effects of particulate pollutants. ROS production and the generation of oxidative stress have received the most attention. ROS, such as superoxides, hydrogen peroxide, hydroxyl and other oxygen radicals, are capable of directly oxidizing the DNA, proteins, and lipids (Yoshida et al., 2004). There are many evidences showing that nanoparticles increase ROS production and can cause cell death in different types of cultured cells (Becker et al., 2002; Peters et al., 2007; Pulskamp et al., 2007; Park et al., 2008). Furthermore, it has been well documented that antioxidant depletion and ROS production cause mitochondrial dysfunction and changes in expression of distinct genes and pathways related to inflammatory responses and apoptosis including MAPK/ERK kinase, NFjB, MIP-2, caspase-3, Bcl-2 (Kharasch et al., 2006). Thus apoptosis initiated by silica may be the result of increased ROS production and antioxidant depletion, leading to mitochondrial dysfunction, DNA damage, increased gene expression of death receptors and/or their corresponding ligands. In summary, our preliminary data has suggested that exposure of SiO_2 nanoparticles leads to cellular morphological modifications, mitochondrial dysfunction, and oxidative stress as indicated by elevation of intracellular ROS and TBARS, as well as depletion of antioxidant level (SOD and CAT), which may triggers neuronal damage further leads to cognitive decline. Further study is required to reveal the mechanism of SiNPs in other models.

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