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Survival, growth, behavior, hematology and serum biochemistry of mice under different concentrations of orally administered amorphous silica nanoparticle

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ABSTRACT

Silica nanoparticles (SiNPs) are used extensively in consumer products and biomedical research basically due to ease of production and low cost. However, insufficient literature is reported regarding the toxicity and biocompatibility of SiNPs. The present study aimed to investigate the potential role of amorphous SiNPs on survival, growth, behavioral alterations, hematology and serum biochemistry of mice at four concentrations (control, 50, 100 and 150 mg/kg/day) of an oral supplementation for a period of 3 months. Signs of toxicity (lethargy, nausea, coma, tremors, vomiting and diarrhea, etc.) were noted at 9:00 am and 9:00 pm (twice a day) and the body weight of each of these mice was measured every week. The data were subjected to mean, standard deviation (S.D). Moreover, One-Way Analysis of Variance (ANOVA) and Dunnett's test were applied for analysis of statistical significance between groups by using SPSS software, version 20. All the mice survived with minor alterations in behavior and no significant weight changes were observed during the stipulated time period. Complete blood count (CBC) analysis indicated non-significant ($P \geq 0.05$) systemic dysfunctions of organ systems. However, there was elevation in the level of AST and ALT in the analysis of serum biochemistry, while the values of all other examined parameters were not-significant ($P \geq 0.05$). The study concluded that orally administered large silica nanoparticles up to the dose level of 150 mg/kg/day are nontoxic for the in vivo use in mice.

1. Introduction

Nanoparticles are the objects ranging from 1 to 100 nm in at least one dimension but, according to FDA [21] if "a material or final product is designed to exhibit properties or phenomena, including physical or chemical properties or biological effects, which are attributable to its size, even if these dimensions are outside the nanoscale range, down to one micrometer (1000 nm)" is called nanoparticle. In spite of small size, have large surface area, resultantly they show unfamiliar physico-chemical properties when matched with micro scale particles [26,93].

Silica nanoparticles (SiNPs) are the components found naturally in the earth's crust [16] and these are included in top of the five commonly used nanoparticles in nanotechnology [35]. Many industries, such as construction, consumer products, electronics, food and medicines are making use of silicon-based materials due to their physio-chemical properties [57].

Numerous products, such as dietary supplements, bandages, lens, catheters, dental fillers and implants etc. that are basically manufactured for human use, are derived from silicon [2,66]. In current era of nanotechnologies, SiNPs are playing a key role owing to their unique characteristics such as small size, low density but high specific surface area, absorbing and encapsulating capacity [27,41].

In the industries of food production, NPs are playing a key role due to their nutritional and medical value [8,44,47,69,88]. The productivity of aquaculture can be enhanced by nanoparticles, by the activity of biomolecules with micronutrients [77,94], along with the application of treatment of diseases without any negative impact to health of humans [39]. In the list of nanoparticles, SiNPs show unique characteristics such as incredible biocompatibility [9], and drugs delivery [42,64].

SiNPs are also used in the industry of aquaculture for the production of white meat of fish, used as a tool for drug delivery to reduce the risk of outbreak of diseases in crowding [45], increasing the growth of diatoms,

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controlling the load of microbes [38].

In biomedicines, consequential research has been conducted by using SiNPs, such as in the diagnosis, controlling and curing of genetic syndromes and for enhancing life span [25]. In novel biomedical applications SiNPs have been used as supporters of enzymes [30], for the delivery and supply of drugs in a controlled manner [67], cellular uptake [71] and also as a biosensor [58]. There is a dire need to assess SiNPs toxicity as the exposure of humans to these nanoparticles is increasing day by day.

There are various types of SiNPs to be studied; mesoporous type (M-SNP) with an extremely large surface area are very useful for the delivery of drugs. However, in murine models they also intensify airway inflammation, having an adverse effect on human health [46]. PEGylated type (P-SNP) is a type of SiNPs with modified surfaces while, Spherical type (S-SNP) is a standard form of SiNPs [83]. Additionally, one of the most common SiNPs is of amorphous type, progressively used in cosmetics, foodstuffs, high molecule composite materials and industrial manufacturing [95]. The different ways of synthetic amorphous silica nanoparticles (thermal and wet, i.e., pyrogenic and precipitation respectively) produce SiO2 NPs (i.e., < 100 nm) [13] which can be aggregated and agglomerated variably depending on the conditions of production and its use (Young et al., 2016; [6]. SiNPs can be synthesized under controlled conditions with controlled size, crystallinity, shape, morphology and porosity. These parameters along with route of distribution modulate biodistribution patterns and toxicity [59]. The recent studies about the toxicity of amorphous SiNPs have pointed out that its toxicity is dependent on cell line, size, dose and time interval (Y. [50, 79]).

Essentially, less information is available about human beings who are exposed to some kinds of nanoparticles for regular and long term basis. The effect of orally exposed amorphous SiNPs on human health remains unknown, as very little information is available particularly about nanoparticles size and dosage of oral route of exposure [23,92].

Myriads of questions have been raised about the negative effect of SiNPs on human's health and its biocompatibility because these are practically applied on humans. The toxicity of SiNPs increases when its size decreases. Different body organs and tissues such as reproductive organs, lungs, liver, brain and blood vessels are affected by the toxicity of SiNPs [91]. When SiNPs of large size are given in low doses, the results are surprising and not favorable in terms of geno-toxicity, which reverses the concept that particles of small size cause more toxicity than particles with larger sizes when supplied in higher concentrations [52].

The microglial and neuromuscular functions could be affected by SiNPs [17,62]. It has been noted that SiNPs cause neurotoxicity when enter the central nervous system by crossing the barriers of brain blood [49]. Moreover, neuroglia and nerves distributing the neurological system can be interacted by these nanoparticles. There are evidences that SiNPs enhance degeneration of neurons and initiate neurotoxicity [31].

During in vivo studies variable results were noted in different experiments, when SiNPs were supplied in different ways. Myocardial ischemia and inflammation in pulmonary tract of old age rats were noted when SiNPs were supplied by inhalation way [33]. When SiNPs of 30 or 3000 nm were injected through intra-tracheal route, inflammation was caused, which was detected by microcomputed tomography of mice lungs [81] . SiNPs causes some kind of disorders and complications in humans health [32]. Many investigations concluded that SiNPs were deposited in the spleen and liver when injected to blood, most of them were cleared in less than one month. When mice were intravenously injected with nonporous amorphous SiNPs, an elevation of aminotransferase, inflammatory cytokines, hepatic necrosis was observed along with delayed clearance of SiNPs from liver [78]. However, when SiNPs of 20–25 and 80 nm were given by 2 and 3 mg/kg to mice, there were no signs of toxicity to organs; instead SiNPs were accumulated in various organs [34].

In sub chronic toxicity studies, a decreased gastrointestinal absorption was noted in rats at high doses of orally exposed amorphous SiNPs. At high concentrations silica was dispersed and gelation occurred. It points out the necessity of testing SiNPs at lower doses [86]. However, in all available oral studies very high dose level have been used making its significance questionable for human risk assessment of engineered nanomaterial in general and specifically of amorphous silica nanoparticles. There is a large use of amorphous SiNPs in food industry with insufficient information about their oral exposure. A dire need of research is required about the impact of low doses of SiNPs applied for a lengthy period to fill the knowledge gaps related to dosage and time duration. Thus it has become important to clearly identify the impact of large amorphous SiNPs in sub chronic oral exposure.

The recent work was therefore, accompanied to estimate the impact of large SiNPs given in sub chronic oral exposure to mice in order to examine its possible impact on survival, growth, behavior, different hematological and serum elements. Mice and humans are in the same clade (Euarchontoglires) having close resemblance in homology. Therefore, the present study was conducted to investigate the toxicity of silica NPs in mice as model animals. Thus, this study would be helpful for further implementation in human oriented research regarding usage of SiNPs in optimized method and dose.

2. Material and methods

2.1. Preparation of silica nanoparticles (SiNPs)

For the preparation of SiNPs, 'Stober method' was adopted. In this process tetraethoxysilane (TEOS) was used as a starting material. TEOS and ammonia water (25 wt%) were separately mixed with ethanol. Then, both of the separated solutions were mixed. The obtained solution was stirred by magnetic stirrer (78–1/JY, Changzhou, China) for about 24 h. By centrifugation through the centrifuge machine (TGL-16, JY/OEM, China) all the particles in the solution were collected and dried in hot air oven (YCO-NO1, Taiwan) for about 12 h. The concentration of SiO2 in solution was determined from the weight of heat treated (1000 °C) and collected particles versus the total weight of synthesized sol. The theoretical weight of SiO2 obtained from TEOS and the weight of obtained and heat treated (1000 °C) particles was measured by following formula:

$$\text{Percent weight} = \text{Actual weight/Theoretical weight} \times 100$$

To prepare silica particles suspensions, the synthesized particles were dispersed in deionized water. To prevent aggregation, sonication was done for 2.5 min at 400 W (Ningbo Scientz Biotechnology Co. Ltd, China) in an ice-water bath (HH-S4, SN: 90108283) to keep the suspensions from overheating.

2.2. Laboratory animals

The 40 male albino mice (5–6 week old), along with mice diet weighing about 25–26 g were kept in (polypropylene) stainless wire cages (260 W × 480 L × 180 H), at a temperature of 20–25 °C and 12 h' light and dark cycle, relative humidity of 45.5–58.5%, with light hours from 9 am to 9 pm and ventilation frequency 12–32 times per hours. Deionized water (to avoid the presence of minerals) was supplied to mice. The animals used in the experiment were quarantined for 1 week in a healthy environment.

2.3. Experimental Design

The mice were placed in 4 groups as G1, G2, G3 and G4. Each group received different doses of SiNPs as control (Saline treated), 50, 100 and 150 mg/kg/day respectively [55]. All groups of animals were housed separately, by placing 10 mice in each group. As the nanoparticles were not soluble in water or saline solution, so before the administration of

prepared suspension, it was vigorously vortexed to ensure the even distribution of colloidal solution. 24 G gavage needle was used to introduce 90 doses of SiNPs orally into the digestive tract of every mouse of treated groups (G2, G3 and G4) with dose level of 50, 100 and 150 mg/kg/day respectively for 90 days. 0.9% NaCl solution was introduced orally to the controlled group by gavage tube in measured quantity to their body weight so as they pass through the same stress condition as the animals of treated groups.

The study was performed in acquiescence with the regulations of care and use of laboratory animals based on animal protection act of 1890. The house temperature, supply of water and food, patterns of behavior, which are the clinical symptoms of toxicity were checked at 9:00 am and 9:00 pm (twice a day). The mice showing clinical signs were separated from the others. The body weight of each mouse was measured regularly during the whole experimental period.

3. Weight Observations

The initial mean body weight was determined by measuring the body weight of each mouse after acclimatization. While, the final mean body weight of each mouse was measured at the end of experiment. Mean body weight gain was measured by subtracting the initial mean body weight from final mean body weight. The feed consumption was measured by subtracting the leftover feed from total offered feed. The total feed intake was obtained by multiplying the one-day feed intake to the total no. of experimental days. After measuring all these parameters, the feed conversion ratio (FCR) was measured by dividing the intake feed to body weight gain as shown (Table 10).

4. Behavioral testing

4.1. Tests for anxiety induction

4.1.1. The open field test

An opaque wooden box having height, length and width of 36 cm, 38 cm and 56 cm respectively with a bottom having 16 quadrants and a square platform in the center was used in the test. As the mouse passed the central area line (considered most frightening to mice) the time was counted carefully. Each mouse was placed on the center of field and video was recorded for 20 min [90]. The ratio of total locomotion and locomotion within the quadrant for each mouse was calculated. The locomotion activity was assessed by the frequency of crossing through the peripheral and central quadrant.

4.2. The light dark box test

A wooden box partitioned into a black box (22 ×35 × 33 cm) and a white box (33 ×35 ×33 cm) was used in this test. To assess the experimental parameters, each mouse was placed in the center of light box and facing the opening of dark box [5]. The total time spent in dark and light boxes, number of entries between the boxes and exploring activities were calculated.

5. Tests for depression aggravation

5.1. The tail suspension test

The apparatus used for this test consisted of a rectangular wooden box (50 cm height, 53 cm width and 10 cm depth). The mice were passed through inescapable stress by suspending each mouse by its tail with tape [14]. In this test the measured parameters were; time consumed in immobility, time consumed in mobility, no. of rests and no. of escapes in the given time.

5.2. The forced swimming test

The apparatus used in forced swimming test consisted of a transparent tank having the dimension of (35 × 35 × 55 cm) with 25 cm water (25 ± 2ºC). The measured parameters were; time utilized in immobility, time utilized in mobility (swimming), number of climbing attempts and number of rests [96] .

5.3. Effects on memory and spatial learning

5.3.1. Morris water maze test

A circular tank (height 35 cm, diameter 100 cm), filled with water (25 cm) was used in this test. The mice were initially trained to swim. Each mouse was positioned in the pool to find visible platform. The measured parameters were; time utilized in mobility, time utilized in immobility, number of tries for climbing and escape latency (time of finding the platform).

5.4. The Y-maze test

The apparatus used in this test consisted of three equal arms designed in the shape “Y”. Each arm was spaced at 120º, having height of 25 cm, width 15 cm and length 50 cm. Food reward was placed in any of the arms then allowing the mice to find it. The measured parameters were; time spent in reward finding, reward preference memory, arms alterations and side preference [63].

5.5. Sample collection and hematological analysis

To check the health status of experimental mice a blood collection was done after the acclimatization period and before the oral administration of treatments. After the experimental period the blood of experimental animals was examined for detecting any systemic dysfunctions like changes in biochemical and hematological parameters. After 24 h of last oral administration the blood samples were collected from the animal’s heart by means of plastic syringes with 0.1 mm EDTA for hematological parameters and 5I U/ml of heparin sodium for serum analyses. HEMAVET (Drew Scientific, Dusseldorf, Germany) was used for the analysis of complete blood count. The collected blood was centrifuged for 15 min at the rate of 3000 rpm for 15 min at the temperature of 4 °C to harvest plasma. The level of ALT, AST, total protein, creatinine and total bilirubin in serum were determined by FUJI DRI-CHEM 4000i (Fujifilm Dusseldorf, Germany). While, the level of cholesterol, triglycerides, glucose, albumin, urea, globulin, sodium, potassium, chlorine, calcium, and phosphorus were measured by Microlab 300-EliTechGroup-Germany.

6. Statistical analysis

Data were subjected to mean and standard deviation. SPSS software version 20 was used for the analysis of data. The statistical significance was measured by One-way ANOVA (Steel et al., 1996). If the variance of data becomes homogeneous, it confirms the significance of differences; if not, the data were analyzed using Dunnett’s post hoc test. *$p < 0.05$ was taken as indicative of statistical significance. The probability ratio for significance was set as 0.05 (*$p \leq 0.05$) or 0.01 (**$p \leq 0.01$).

7. Results

7.1. Physicochemical properties of SiNPs

7.1.1. Size and size distribution of SiNPs

A dynamic light scattering (DLS) particle size analyzer was used to analyze the size, size distribution and intensity of synthesized SiNPs. The nanoparticles size was 5112 d. nm in average (peak 555.0 d. nm and Zeta Potential −34.2 mV) as shown (Tables 1 and 2). Size distribution

Table 1
Size and intensity of silica nanoparticle.

			Size (d. nm):	% Intensity:	St. Dev. (d. nm)
Z-Average (d. nm):	5112	**Peak 1:**	555.0	100.0	37.87
PdI:	1.000	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.769	**Peak 3:**	0.000	0.0	0.000

Table 2
Zeta potential, zeta deviation and conductivity of silica nanoparticle.

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-34.2	**Peak 1:**	-34.2	100.0	6.95
Zeta Deviation (mV):	6.95	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.0184	**Peak 3:**	0.00	0.0	0.00
Result quality:	**Good**				

and zeta potential distribution of SiNPs shown in Figures (Figure1 and Fig. 2).

8. Clinical observations for passive behavior

All the mice survived without any poisonous symptom in all doses of sub chronic toxicity studies as shown (Table 3).

9. The light-dark box test

All the mice exposed to different doses of SiNPs spent same time in the light box as compared to untreated mice. The time spent in finding the door, number of entries to dark box, time spent in dark box and the number of searching varied non-significantly ($P > 0.05$; Dunnet's post hoc test) resulting that SiNPs treated mice does not reduce locomotor activity (Table 5).

10. The tail suspension test

Analysis of results revealed that SiNPs treated mice non-significantly ($P > 0.05$) reduced the time of mobility and increased the time of immobility, while no change was observed in average no. of escapes and rests between the mice treated with SiNPs and saline treated mice (Table 6).

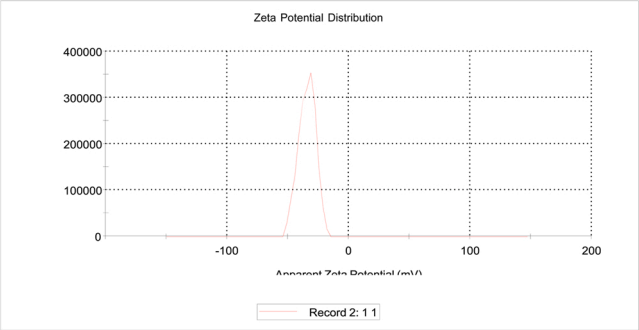

Fig. 2. Zeta potential distribution of silica nanoparticle.

11. The forced swimming test

SiNPs treated mice demonstrated non-significant ($P > 0.05$; Dunnet's post hoc test) increase in immobility without any neuropsychiatric and metabolic disorder as compared to controlled mice (Table 7).

12. Morris-water maze test

The findings of this behavioral test showed that SiNPs does not damage the hippocampus. So, no loss of learning and memory. A non-significant increase in latency was observed in treated mice. No significant differences were observed between the resulted values of treated and untreated mice. All the studied parameters varied non-significantly ($P \geq 0.05$) as shown (Table 8).

13. The Y-maze test

All the mice treated with SiNPs showed equal preferences to arms and equal numbers of side alterations with or without reward. It can be resulted that cognition and memory functions are not affected by SiNPs as shown (Table 9).

14. Weight Observations

During the whole experimental period there was an increase in body weight of all treated groups like the control group. A non-significant

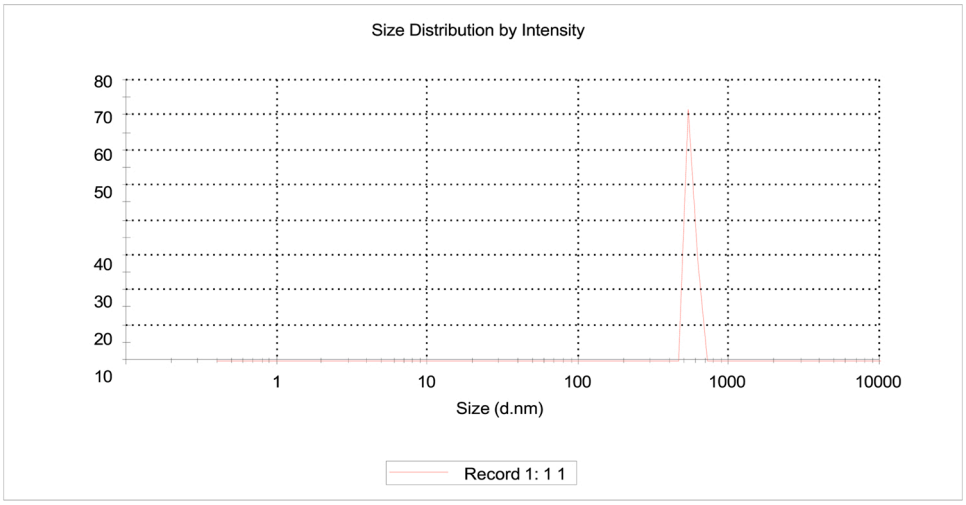

Fig. 1. Size distribution of silica nanoparticle (Result quality).

Table 3
Toxic symptoms in mice under different sub chronic treatments of SiNPs.

Symptoms of poisoning in mice under different sub chronic treatments of SiNPs									
Treatment	Dosage mg/kg/day	Status	Lethargy	Tremor	Coma	Hypopnea	Loss of appetite	Arching of back	Vomiting and diarrhea
Sub chronic	0	Good	No	No	No	No	No	No	No
	50	Good	No	No	No	No	No	No	No
	100	Good	No	No	No	No	No	No	No
	150	Good	No	No	No	No	No	No	No

The open field test
The values of results in this test varied non-significantly
($P > 0.05$; Dunnet's post hoc test) when compared the treated and untreated (controlled) mice as shown (Table 4).

Table 4
Observation of different factors of open field test under different sub chronic doses of SiNPs (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; AT, Average no. of thignotsaxis; AV, Average velocity; ALC, Average frequency of line crossing; ACQ, Average entry of central quadrant; ACR. Average no. of corner rest; ATC, Average time spent at the corner).

Groups/ Dosage	AT	AV (s)	ALC	ACQ	ACR	ATC (s)
G1	13.2 ± 5	7.55 ± 1.8	4.02 ± 0.9	1.38 ± 0.4	7.6 ± 1.9	38 ± 0.01
G2	13.0 ± 3	7.39 ± 1.9	4.05 ± 0.8	1.35 ± 0.5	7.8 ± 1.6	36 ± 0.04
G3	13.1 ± 4	7.45 ± 1.8	4.00 ± 0.2	1.34 ± 0.8	7.5 ± 1.7	37 ± 0.09
G4	12.9 ± 6	6.99 ± 1.6	3.98 ± 0.1	1.22 ± 0.2	7.0 ± 1.2	35 ± 0.06

Table 5
Observation of different factors of light-dark box test under different sub chronic doses of SiNPs. (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; TDF, Time spent in door finding; TLB, Time spent in light box; TDB, Time spent in dark box; NE, Number of entries; NS, Number of searching).

Group/Dosage	TDF	TLB	TDB	NE	NS
G1	8 ± 3	7/20 ± 0.2	13/20 ± 0.1	4 ± 0.8	7 ± 1.8
G2	9 ± 1	8/20 ± 0.1	12/20 ± 0.3	4 ± 0.7	6 ± 1.5
G3	8 ± 6	9/20 ± 0.3	11/20 ± 0.2	3 ± 0.9	7 ± 1.3
G4	11 ± 5	10/20 ± 0.2	10/20 ± 0.1	2 ± 1.2	5 ± 0.9

Table 6
Observation of different factors of tail suspension test under different sub chronic doses of SiNPs. (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; ATI, Average time of immobility; ATM, Average time of mobility; AE, Average no. of escape; AR, Average no. of rest).

Group/Dosage	ATI (s)	ATM (s)	AE	AR
G1	85 ± 26	275 ± 85	15 ± 6	16 ± 6.5
G2	88 ± 34	277 ± 66	13 ± 4.1	14 ± 4.5
G3	87 ± 29	274 ± 75	14 ± 3.9	14 ± 9.7
G4)	90 ± 27	270 ± 80	12 ± 0.8	11 ± 6.4

increase was observed in mean body weight gain and FCR values as shown (Table 10). So, in body weight changes there were no significant differences among treated and untreated mice as shown (Fig. 3).

15. Complete blood count analysis

Analysis of results revealed that all the studied parameters of complete blood count varied non-significantly ($P > 0.05$; Dunnet's post hoc test) when different experimental treatments were compared except white blood cells (WBC's) that were increased in all groups. The

Table 7
Observation of different factors of forced swimming test under different sub chronic doses of SiNPs. (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; TTS, Total time utilized in swimming; TS, Total no. of swimming; TSI, Total time spent in immobility; TR, Total no. of rest; CA, No. of climbing attempts).

Group/Dosage	TTS (s)	TS (s)	TSI	TR	CA
G1	330 ± 75	5 ± 1.5	23 ± 6	6 ± 1.3	5 ± 1.3
G2	331 ± 55	5 ± 1.6	23 ± 3	5 ± 22	4 ± 2.2
G3	328 ± 65	6 ± 0.2	24 ± 5	5 ± 1.9	5 ± 0.44
G4	326 ± 37	5 ± 1.0	22 ± 8	5 ± 0.21	4 ± 5.3

Table 8
Observation of different factors of Morris-water maze test under different sub chronic doses of SiNPs. (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; AEL, Average escape latency; ATS, Average time used in swimming; ATI, Average time used in immobility; ACA, Average no. of climbing attempts).

	AEL (s)				
Group/ Dosage	1st round	2nd round	ATS (s)	ATI (s)	ACA
G1	140 ± 60	110 ± 50	85/110 ± 0.30	25/110 ± 0.22	20 ± 3.3
G2	145 ± 35	109 ± 60	82/110 ± 0.36	28/110 ± 0.39	16 ± 5.4
G3	143 ± 22	116 ± 33	87/110 ± 0.26	23/110 ± 0.67	18 ± 7.4
G4	147 ± 34	122 ± 87	77/110 ± 0.39	33/110 ± 0.37	17 ± 4.6

Table 9
Observation of different factors of Y-maze test under different sub chronic doses of SiNPs. (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; NSA, No. of side alterations; SP, Side preference; PF, Preference of food).

Group/ Dosage	NSA	SP (no reward on either side)			PF	
		Right arm	Left arm	Re-visit	White cheese	yellow cheese
G1	20.40 ± 4.3	8.23 ± 2.1	8.19 ± 0.22	4.28 ± 2.2	3.80 ± 0.9	3.45 ± 0.6
G2	18.22 ± 3.4	7.36 ± 3.6	6.3 ± 6.2	5.33 ± 3.5	3.55 ± 0.5	4.34 ± 0.76
G3	19.49 ± 2.1	6.76 ± 4.6	9.34 ± 3.4	4.26 ± 4.2	3.34 ± 0.7	3.22 ± 0.87
G4	16.63 ± 1.1	7.56 ± 8.1	6.23 ± 2.3	3.11 ± 4.7	2.99 ± 0.8	3.05 ± 0.23

neutrophils, lymphocytes, monocytes, eosinophils and basophils showed various elevations. In red blood cells (RBC's) counts, there were non-significant differences in sub chronic treatments. Hemoglobin, and hematocrit were dropped in all the groups significantly (Table 11).

Table 10
Observation of different factors of body weight under different sub chronic doses of SiNPs. (G1, untreated; G2, G3 and G4 treated with 50, 100 and 150 mg/kg/day respectively; ±, Standard deviation; IBW, Initial body weight; FBW, Final body weight; MBW, Mean body weight gain; TFI, Total feed intake; FCR, Feed conversion ratio.

Parameters measured in relation of body weight gain and feed intake observations				
Measured parameters	G1	G2	G3	G4
IBW (g)	25.63 ± 0.27	25.72 ± 0.23	25.64 ± 0.25	25.51 ± 0.20
FBW(g)	33.99 ± 0.12	33.89 ± 0.10	34 ± 0.18	34.47 ± 0.10
MBW (g)	8.36 ± 0.15	8.17 ± 0.13	8.36 ± 0.07	8.96 ± 0.10
TFI	1386 ± 0.12	1422 ± 0.21	1377 ± 0.14	1395 ± 0.15
FCR	165.7 ± 0.1	174.05 ± 0.1	164.7 ± 0.09	155.6 ± 0.07

16. Serum analysis

The levels of ALT and AST in serum which are the signs of liver cell damage, were slightly elevated in treated mice. The level of protein total and globulin was decreased non-significantly (ns) from 6.16 ± 0.015 to 5.99 ± 0.019 and 3.20 ± 0.029 to 3.01 ± 0.037 respectively. There were minor differences in values of cholesterol level in treated and controlled mice (Table 12).

17. Discussion

Nanotechnology has become an important term of public interest due to its broad range of implementation [37]. Although nanotechnology is grooming due to its beneficial aspects but society cannot ignore its toxicological impacts [29,97]. Although SiNPs have been broadly established for uses in biomedicines but their toxicity has not been examined widely. The investigations about toxicity of SiNPs related to time period and quantity are still missing [49] .

It was reported that the age of animals, used in behavioral tests correlates with the results. Therefore, younger animals are used because of having developed brain with musculoskeletal maturity and social competence which is demanded for completing tasks [3,56,76]. A number of molecular and physiological sensations mostly take place in 1st three months of age [53].

Analysis of results of open field test showed that oral administration of different sub chronic doses of SiNPs cause no change in thigmotaxis, average no of velocity, line crossing frequency and no. of corner rests. Whereas, [89] reported against our results that anxiety was shown by rats during total time utilized in central area (%), reducing total locomotor activity and more time utilized in the corner of apparatus and peripheral zone.

The light-dark box test is a behavioral experiment that measures anxiety in animals[12]; [82]. The fear of an elevated, vast or open space is actually an anxiety-like behavior which is directly related to depression, both in humans and other animals [43,68,84]. Analysis of light-dark box test resulted in decreased rearing frequency in all treated as well as untreated mice which is an exploratory behavior in rodents. Similar results were reported by [40,74]. Rearing frequency is actually an exploratory behavior; a type of an innate behavior in which rodents stand on their hind legs [72]. [10] reported that treated animals learned helplessness and despaired behavior in tail suspension test which is used to assess the stress in experimental mice [14]. Our results showed that SiNPs treated mice non-significantly ($P > 0.05$) reduced the time of mobility and increased the time of immobility, while no change was observed in average no. of escapes and rests between treated and untreated mice.

In forced swimming test, SiNPs treated mice increase immobility (non-significantly) without any neuropsychiatric and metabolic disorder as compared to controlled mice. Our results are not in agreement with [11] reporting increase in immobility (significantly). [20] reported that in Morris water maze test due to damage of hippocampus; short term memory loss, disorientation symptoms and retention of new memories will appear in experimental animal but such results were not found in our study as the treated mice took same time to reach platform region as compared to untreated mice because hippocampus was not damaged during acquisition phase.

The Y- maze test becomes helpful in assessing the willingness of mice to find new environment [70]. Analysis of results of Y-maze test indicated equal no. of side alterations in treated and untreated mice and all the mice preferred the arm with reward compared to the arms without reward. Mice can decrease rearing frequency when initially exposed to new environment or to certain stimuli [48]. The animal's internal as well as external environmental factors strongly influence its behavioral performance such as synaptic plasticity and signal transmission which are neurophysiological properties of animal [24]. It helped us in resulting that the applied doses of SiNPs are not affecting the rearing frequency associated with the specific neuronal networks. [15] reported that growth of mice was not arrested under silica nanoparticles of up to 300 mg/kg with gradual increase in weigh. Similar results were observed in our study, showing a gradual increase in weight under different sub chronic treatments.

According to literature, SiNPs are becoming a major concern related to toxicity [16,59]. All the mice under observation in the current study survived despite of orally receiving large silica nanoparticles up to 150 mg/kg/day in sub-chronic treatments while, silica nanoparticles of 30 nm orally administered to albino rats at the dose level of 100 and 150 mg/kg/day exponentially raised toxicity [55]. When SiNPs are applied in large amounts these cause acute toxicity even death. Smaller

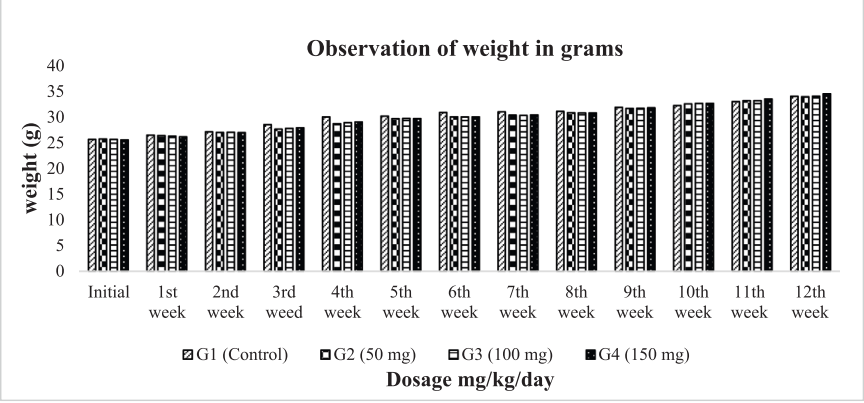

Fig. 3. Observation of weight gain under different sub chronic treatments of silica nanoparticles.

Table 11
Observation of different factors of complete blood count under different sub chronic doses of SiNPs. (WBC'S. White blood cells; RBC'S. Red blood cells; HGB. Hemoglobin; HCT. Hematocrit; MCV. Mean corpuscular volume; MCH. Mean corpuscular hemoglobin; MCHC. Mean corpuscular hemoglobin; RDW-SD. Red cell distribution; RET. Reticulocyte count; PLT. Platelets count; PDW. Platelets distribution width; MPV. Mean platelets volume.

Test	Unit	G1 (control)	G2	G3	G4
WBC'S	K/μL	8.49 ± 0.28	8.95 ± 0.32	9.14 ± 0.14	9.11 ± 0.32*
NEUT	K/μL	1.97 ± 0.03	2.05 ± 0.05	2.15 ± 0.04	2.42 ± 0.02
LYM	K/μL	3.96 ± 0.19	4.50 ± 0.06	4.84 ± 0.02	4.50 ± 0.29
MONO	K/μL	0.38 ± 0.01	0.40 ± 0.01	0.55 ± 0.01	0.38 ± 0.01
EO	K/μL	0.24 ± 0.01	0.27 ± 0.004	0.23 ± 0.01	0.25 ± 0.004
BASO	K/μL	0.07 ± 0.004	0.08 ± 0.001	0.07 ± 0.001	0.09 ± 0.002
RBC'S	M/μL	9.77 ± 0.25	8.96 ± 0.33	8.51 ± 0.20	8.09 ± 0.27
HGB	g/dL	15.2 ± 0.25	14.7 ± 0.18	13.9 ± 0.29	11.9 ± 0.36*
HCT	%	47.2 ± 0.38	47.2 ± 0.24	46.1 ± 0.18	42.2 ± 0.24*
MCV	fL	53.4 ± 0.36	54.1 ± 0.67	53.7 ± 0.62	55.7 ± 0.30
MCH	Pg	18.6 ± 0.23	19.9 ± 0.41	18.9 ± 0.49	20.7 ± 0.48
MCHC	g/dL	31.7 ± 0.27	32.5 ± 0.21	31.9 ± 0.38	32.2 ± 0.32
RDW-SD	fL	32.1 ± 0.39	35.9 ± 0.34	33.6 ± 0.47	35.8 ± 0.25
RET	K/μL	7.32 ± 0.28	8.67 ± 0.19	7.38 ± 0.34	7.37 ± 0.28
PLT	K/μL	599.5 ± 1.1	558.7 ± 3.9	506.6 ± 1.6	550.8 ± 0.8
PDW	fL	9.51 ± 0.17	10.6 ± 0.31	9.9 ± 0.31	11.8 ± 0.36
MPV	fL	7.66 ± 0.26	8.47 ± 0.24	8.85 ± 0.26	8.69 ± 0.24

Note: significant differences *$p \leq 0.05$ and non-significant differences (ns) as compared with controlled group.

Table 12
Observation of various studied parameters of serum biochemistry under different sub chronic doses of SiNPs.

Test	Unit	G1 (control)	G2	G3	G4
Cholesterol	mg/dL	67.06 ± 0.25	68.71 ± 0.46	69.57 ± 0.49	68.61 ± 0.32
Triglyceride	mg/dL	58.31 ± 0.31	57.03 ± 0.43	58.18 ± 0.42	56.92 ± 0.41
Glucose	mg/dL	138.72 ± 0.36	137.67 ± 0.38	136.32 ± 0.35	134.07 ± 0.35
Bilirubin Total	mg/dL	0.096 ± 0.0004	0.096 ± 0.0004	0.093 ± 0.0003	0.099 ± 0.0010
ALT	IU/L	37.52 ± 0.47	41.23 ± 0.77	39.69 ± 0.46	44.03 ± 0.74*
AST	IU/L	97.15 ± 0.43	101.95 ± 0.48	100.85 ± 0.59	102.05 ± 0.46*
ALP	IU/L	194.40 ± 1.26	202.68 ± 1.02	197.83 ± 1.42	204.32 ± 1.51*
Albumin	g/dL	4.52 ± 0.075	4.20 ± 0.059	4.03 ± 0.039	3.79 ± 0.034
Urea	mg/dL	27.06 ± 0.215	30.20 ± 0.462	32.95 ± 0.569	34.99 ± 0.604
Creatinine	mg/dL	0.45 ± 0.0037	0.51 ± 0.0062	0.50 ± 0.0061	0.53 ± 0.0047
Protein Total	g/dL	6.16 ± 0.015	6.09 ± 0.020	6.03 ± 0.004	5.99 ± 0.019
Globulin	g/dL	3.20 ± 0.029	3.16 ± 0.037	3.06 ± 0.042	3.01 ± 0.037
Sodium	mmol/L	151.66 ± 0.80	151.50 ± 0.70	150.30 ± 1.40	151.49 ± 0.64
Potassium	mol/L	5.05 ± 0.007	5.09 ± 0.010	5.03 ± 0.003	5.06 ± 0.006
Chlorine	mmol/L	95.30 ± 0.36	96.8 ± 0.46	97.30 ± 0.36	96.40 ± 0.42
Calcium	mg/dL	5.72 ± 0.008	5.67 ± 0.009	5.64 ± 0.005	5.67 ± 0.011
Phosphorus	mol/L	8.65 ± 0.006	8.64 ± 0.002	8.61 ± 0.004	8.63 ± 0.007

SiNPs caused death earlier than the larger SiNPs [75,91,98]. SiNPs mostly distribute in the resident macrophages of the liver (10.24% ID/g), spleen (34.78% ID/g) and lungs (1.96% ID/g). A very minute quantity of SiNPs in liver hepatocytes and kidneys' capillary endothelial cells was seen in imaging of TEM. The levels of ALT, AST, and LDH were elevated in SiNPs treated groups [65,73].

There was an elevation in the level of ALT, AST and ALP when silica nanoparticle was administered to Balb/c mice at a dose level of 140 g/kg for 10 weeks with fatty liver patterns but, the Si content in liver was same to control group and no significant difference was found on the health of treated mice [80]. In our study, we also observed minor changes in some biochemical indexes such as the significant elevated level of ALT and AST. This elevation was so minor that assuming no hepatocyte injuries. The level of ALT ($P > 0.05$), AST ($P > 0.05$) and ALP ($P > 0.05$) elevated up to.099 ± 0.0010, 44.03 ± 0.74 and 102.05 ± 0.46 respectively at 150 mg/kg/day of SiNPs. Unlike the results of this study [59] demonstrated that SiNPs of 10–15 nm can cause significant changes in albumin, cholesterol, triglyceride, urea, total protein, high density lipids (HDL), low density lipids (LDL), alkaline phosphatase (ALP) and aspartate aminotransferase (AST). [51] reported that damage and degeneration of kupffer cells occurred due to phagocytosis of SiNPs, which were taken up by other kupffer cells in liver, resulting the formation of granuloma due to release of chemokines and cytokines by kupffer cells with recruited neutrophils and lymphocytes. Unlike these results it was assumed that there was no granuloma formation in liver of mice treated with 150 mg/kg/day of SiNPs as the level of AST and ALT was not raised to a disastrous level. The discrepancy may be due to the difference in way of SiNPs administration, as SiNPs administered intravenously result in retention of SiNPs in liver tissues damaging the KCs.

Silica nanoparticles cause inflammation, infiltration, hepatic blooming and fibrosis. It also causes DNA damage due to liver damage in metabolic syndrome mice. However, SiNPs exposure led to improved insulin resistance in metabolic syndrome mice [54], opposite results were found in this study as the level of ALT and AST was not highly increased in different sub-chronic treatments. While, some investigators have reported an increase of AST and ALT activities in serum following silica nanoparticles administration [28]. The studies on the toxic effect of Nano and micron sized silica particles on mice indicated that Nano sized silica particles had a toxic effect on the liver with elevated levels of ALT [7].

The major function of a kidney is to remove waste products from the blood. So, in biological systems the size of these nanoparticles can affect their tissue distribution [36,47]. Clearance of injected SiNPs through kidneys is an important way for its elimination from body as kidneys are not in reticuloendothelial system (RES) [38,47]. It has been resulted that acute pathological and biochemical changes in renal tissues occur due to accumulation of SiNPs [18,36]. It was observed that 50-nm SiNPs after its distribution throughout the kidney can be eliminated through kidney by urine [19]. To evaluate renal damage, parameters such as levels of urea, creatinine and electrolyte (e.g., Na+ and K+) are used [4]. In the present study, significant increases in the serum urea and creatinine have been observed, while no significant differences were found in Na+ , and K+ content in SiNPs treated and control group. There was no obvious damage to kidney tissues when SiNPs were administered orally in sub chronic treatment, assuming that orally administered SiNPs up to the level of 150 mg/kg/day are not damaging the kidneys while, in heavy doses show toxicological effects on kidney tissues. Our results of orally administered SiNPs resemble to [61,87] with no or minimal damage to kidney cells.

According to the in vitro test, the cells incubated with silica nanoparticles resulted hemolysis and lysis of mice erythrocytes [85,91]. By these results the authors estimated SiNPs might cause anemia when given in vivo [15]. While in our study, there were minor changes in RBCs values of different sub chronic toxicity treatments assuming that there was no hemolysis and lysis of erythrocytes. An organism becomes

vulnerable to many infectious diseases due to decrease in immune system indicating the decrease in white blood cells count because the role of white blood cells is to protect the body from infectious diseases when it becomes susceptible to an infection [1]. Our results of sub chronic toxicity studies indicated no such disaster as the level of white blood cells was not lowered in all treated animals.

There were aggregation of platelets when SiNPs were applied on isolated human platelets [15]. The research concluded that nanoparticles size and platelet aggregation were inversely proportional to each other. It was noted that thromboxane-A2-mediated and matrix metalloproteinase- 2-mediated pathways cause platelets aggregation by applying SiNPs. In our results the level of platelets was also increased without any negative impact on mice receiving 150 mg/kg/day of SiNPs. [60] reported that more platelets were aggregated by the administration of larger doses of SiNPs to a cell model utilizing mice platelets, but no as such effect of dosing of SiNPs was observed in this study hence, no aggregation of platelets.

The in vitro toxicity assessment of SiNPs is dependent on type of cells, size and dose of nanoparticles. SiNPs synthesized by wet route show clearly different biological effects as compared to SiNPs synthesized by thermal route (particularly colloidal and stober). Because of unrealistic and improper dosing of in vitro and in vivo exposure, the results remain ambiguous about SiNPs' relation to toxicity, bioavailability and human health [22].

18. Conclusion

In the current study, no toxic effects were seen in mice under sub chronic treatments of SiNPs. Some minor physical and behavioral alterations were observed in the first week. However, growth was not arrested during the entire procedure which reveals that SiNPs are biocompatible in different sub-chronic treatments. Results indicated that subjection to SiNPs cannot alter the serum biochemistry of mice placed in the experimental group when compared with untreated group.

There are certain limitations in drawing the conclusions as the results of sub-chronic and acute treatments vary in relation to toxicity. Not only the time period, dosing and way of supplementations of SiNPs show variable results. There is insufficient data to conclude the limit of organs damage from hematology and serum biochemistry in relation to types of SiNPs. It is hazardous to translate its effects on human health due to unrealistic exposure and lack of data about epidemiology.

There is a dire need of unambiguous physio-chemical characterization of SiNPs so that, it can be directly implemented on humans. The long term consequences of bioavailability of SiNPs are required. There is necessity of comparison of different routes of administration in different doses with variable time periods.

Institutional Review Board Statement

The animal study protocol was reviewed and approved by Institutional Bioethics Committee (IBC), under the Office of Research Innovation & Commercialization (ORIC), The Islamia University of Bahawalpur, Pakistan with notification number (4227/ORIC-21-October-2021).

CRediT authorship contribution statement

Conceptualization, Amna Ismail and Nuzhat Sial; Methodology Amna Ismail, Nuzhat Sial; Formal analysis, Amna Ismail and Muhammad Shoaib Ismail; Investigation, Amna Ismail; Supervision, Nuzhat Sial, Resources, Rakhshanda Rehman and Muhammad Shoaib Ismail; Visualization, Sobia Abid and Amna Ismail; Writing-original draft, Amna Ismail; Writing-review & editing, Amna Ismail and Nuzhat Sial. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2023.05.006.

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