WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 2, 845-857. Research Article ISSN 2277– 7105

EVALUATION OF SUBACUTE ORAL TOXICITY OF ZINC OXIDE NANOPARTICLES IN WISTAR RATS- A PILOT STUDY

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Article Received on
24 Nov. 2019,

Revised on 14 Dec. 2019,
Accepted on 04 Jan. 2020,

DOI: 10.20959/wjpr20202-16623

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ABSTRACT

Nanotoxicology is the study of interactions of nanostructures with biological systems. The rapidly developing field of nanotechnology is likely to become yet another source for human exposures to engineered nanoparticles. Present study is aimed at evaluation of toxicological and pharmacological effects of Zinc oxide nanoparticles. Wistar rats of either sex were treated with ZnO nanoparticle saline suspension at a dose of 100 mg/ 10 ml orally for 28 days. Random allocation of animals 8 rats (4 Males and 4 Females) in Test group and 4 rats (2 Males and 2 Females) in Control group was done. The blood samples of both test and control group animals were drawn on 7th, 14th, 21st and 28th days in the morning on laboratory platforms. There were no statistically significant changes in hematological and biochemical parameters. Histopathology of major organs was also without significant changes. In the present preclinical study, possible pharmacological effects of nanoparticle of fixed size have been studied at fixed dose. ZnONP (< 50 nm size) if administered orally at a dose of 100 mg/kg have no clinically observable adverse effects in animals. ZnONP (< 50 nm size) if administered orally have effects on lungs parenchyma with reduced relative weight of the organ with similar study showing perivasculitis and peribronchitis in Lungs.

KEYWORDS: Nanopharmacology, Zinc Oxide nanoparticles, Subacute toxicity.

INTRODUCTION

The field of nanotechnology is rapidly expanding. The development of newer nano-pharmaceuticals has changed medical treatment.[1] Current standards in biomedicine and environmental contamination define nanoparticles as being <100 nm in at least one dimension.[2] The behavior of the nanoparticle in biological systems requires much of research before they can be utilized in human beings. This is especially required while designing the pharmacokinetics that is absorption, distribution, metabolism and excretion which affect dosing parameters. Drug is organic molecule while nanoparticle is somewhat a discrete entity comprised of atomic scale parts, so there is difference in delivery of them. This difference is due to the quantum effects and electronic interactions that predominate at the nanoscale.[1]

Whereas such dimensional thresholds between nanoscale and bulk materials are useful for research and scientific inquiry, they are not completely applicable to toxicology and risk assessment. When looking at a particle as a whole, its dimensions may not meet the above criteria for a nanoparticle while its construction may have important nanoscale variability that requires it be studied as a nanoparticle.[2]

Nanopharmacology further becomes complex as it is difficult to establish the behaviour of nanoparticles within the conventional pharmacological parameters of pharmacokinetics and pharmacodynamics. Due to the quantum effects and electronic interactions that predominate at the nanoscale, we need to alter the way in which we think about pharmacological parameters, to adapt to nanoscience. Therefore in nanopharmacology, there is a need of investigations in conventional ADME parameters done carefully and modify them if necessary.[1] Most of the nanomaterials submitted for preclinical evaluation either have unacceptably high toxicities during in vitro or in vivo testing, or they fail to meet the minimum criteria for bioavailability according to their ADME profile.[3]

Nanotoxicology is the study of interactions of nanostructures with biological systems.[4] The rapidly developing field of nanotechnology is likely to become yet another source for human exposures to engineered nanoparticles (NPs)—by different routes: inhalation (respiratory tract), ingestion [gastrointestinal (GI) tract], dermal (skin), and injection (blood circulation). The diversity of engineered nanomaterials and of the potential effects represents major challenges and research needs for nanotoxicology.[5]

AIMS AND OBJECTIVES

- To study the toxicological effects of Zinc oxide nanoparticles (< 50 nm) at specific dose of 100 mg / kg.
- To study the pharmacological effects of Zinc oxide nanoparticles (< 50 nm), if any, at specific dose of 100 mg/kg.

MATERIALS AND METHODS

Institutional Animal Ethics Committee approval was taken before commencement of study. All study procedures were conducted following CPCSEA guidelines.

Animal Husbandry

Total 12 adult healthy Wistar rats of same developmental age, weight and bred at National Toxicology Centre, Pune were procured Animals of both the sexes (6 Males and 6 Females) weighing 180- 200 gm were included in the study. Random allocation of animals 8 rats (4 Males and 4 Females) in Test group and 4 rats (2 Males and 2 Females) in Control group was done. Animals were housed in separate metabolic stainless steel cages measuring 34 x 23 x 15 cm with two animals in each cage and males and females were separated. Cages were kept in a ventilated animal room maintained at relative humidity of 50 %, temperature of 25 - 30 C with 12 hour light/dark cycle. Distilled water and sterilized food for rats was made available ad libitum.

Treatment to Test and Control group

For test group, ZnO NP saline suspension was prepared as follows. Spherical ZnO NPs of < 50 nm size were procured from Sigma Aldrich. Stock formulation ZnO NP saline suspension was prepared in 0.9% normal saline with a concentration 100 mg/ 10 ml by sonication for 30 seconds. The suspensions was kept on ice for 15 seconds and sonicated again on ice for a total of 3 minutes at a power of 400 W. The ZnO NP saline suspension was diluted to desired concentrations before use with 0.9% normal saline so that approximately 2 ml test substance was administered to animal. All samples were prepared under sterile conditions. Proper mixing of sample was done with vortex method before administering the dose.

After mixing with a vortex, a single dose of ZnO NP saline suspension was administered orally using gavage technique. Similarly Control group animals were administered with 0.9% normal saline of the volume proportional to body weight. The test and control group animals

were administered with ZnO NP saline suspension and 0.9% normal saline respectively, daily till 28 days with same dose and same route of administration.

Blood samples of both test and control group animals were drawn from retro-orbital plexus with capillary method at 1st, 3rd, 6th and 24th hours of administration of ZnO NP saline suspension or normal saline on first day. The blood samples of both test and control group animals were drawn on 7th, 14th, 21st and 28th days in the morning on laboratory platforms. Similarly, fecal and urine samples were collected 2nd, 7th, 14th, 21st and 28th days in the morning.

Behavior, symptoms and mortality

Body weights, Neuro-behavior and mortality of both Test and Control animals were monitored and recorded carefully on 0th and 28th days after treatment. Neuro-behavioural features were observed such as Temperature, Convulsions/tremors, Lacrimation, Piloerection, Salivation, Urination/Defection, Gait, Mobility (Depression/ Anxiety), Righting reflex and other significant findings. Difference in eating and drinking patterns, their physical activity and other parameters were noted.

Anesthesia and necropsy

After 28 days of treatment with ZnO NP saline suspension, both Test and Control group animal were anaesthetized with Ketamine and Xylazine 1:1. One of the male animals (TM3) from test group died on due to overdose of anaesthetic medication. Rest of the animals from both test and control groups were alive and well clinically.

On 28th day, blood samples were collected from the Retro-orbital plexus. Serum was obtained by centrifugation at 3500 rpm for 10 minutes and stored at -20˚C until usage. Neurobehavioural and clinical symptoms of animals were studied and urine and fecal sample were collected. The animals were then euthanized by cervical dislocation. Photoghraphy of gross dissection of animals was done. Selected wet organs of both test and control animals were weighed. Major organs such as Liver, Intestines, Stomach, Kidney, Gonads and Brain were sampled for histopathology.

Organ weight/Body Weight coefficients

All major organs of both test and control group animals were weighed. Then the organ to body weight coefficients of were calculated as organ weight (wet weight, mg)/BW (gm) x 100%.

Heamatology and Biochemistry

Blood samples of both test and control group animals were withdrawn on 28th day and were subjected for Blood biochemistry and haematology using automated koulter machine (Triscan Operon KT-6360). Hematological examination included Complete Blood Count, Blood Sugar Level (BSL), Total proteins, SGOT, SGPT, Alkaline Phosphatase (ALP), Total Bilirubin, Blood Urea, Creatinine, Electrolytes Sodium (Na) and Potassium (K), Coagulation tests (PT, PTTK) was done as blood biochemistry.

Statistical analysis was done using Un-paired t - test between test and control groups with 95% confidence interval.

RESULTS AND OBSERVATIONS

Body weights

There is consistent gain in body weights in both test and control groups over 28 days. In control group weight is increased from 201 (SD = 16.63) to 247 (SD = 21.80) gm so mean weight gain in control group is 46 gm (SD = 5.17) corresponding to 22% increase. In test group weight is increased from 202.07 to 229.43 gm so the mean weight gain is 27.36 gm (SD = 8.32) corresponding to 13.53% increase. Although there is no statistically significant difference between test and control animals; there is lesser weight gain in test group as compared to control group and there is consistent reduction in *p* value from 0.918 on 0th day to 0.248 on 28th day.

There was no observable difference in clinical parameters. Eating and drinking patterns, their physical activities and other parameters observed were normal in both test and control groups. Gross stool examination of the animals show stools were semi solid to liquid in consistency in animals with test group while all animals with control group were having normal stools.

Absolute and relative organ weights

Major body organs were measured as absolute organ weight and their organ weight to total body weight coefficients were calculated as relative body weight. There was no significant difference between test and control groups in absolute and relative organ weights. Mean absolute organ weights of Lungs in control animals was 1.33 gm (SD = 0.09) and that in Test is 1.70 gm (SD = 0.48) with *p* value of 0.091.

Heamatological parameters

Statistically significant difference was observed in Red Blood Corpuscles (RBC) count, Heamoglobin (Hb) and Haematocrit (Hct) between test and control group (p value 0.043, 0.048 and 0.028 respectively). All the three parameters were higher in Test group 13.3 gm%, 43.76% and 8.34 lac/mm^3 respectively as compared to those of control group 11.48 gm%, 38.15% and 7.31 lac/mm^3 respectively. But there was no statistically significant difference in blood indices. The mean MCV in control and test group were 52.23 (SD = 1.93) and 52.53 (SD = 2.58) with p value of 0.827. The mean MCH in control and test group were 15.65 (SD = 0.66) and 15.87 (SD = 0.94) with p value of 0.659. The mean MCHC in control and test group were 30.03 (SD = 0.39) and 30.29 (SD =0.53) with p value of 0.376.

No significant difference was observed in platelet count and WBCs. Statistically significant difference was also observed in total granulocyte (p = 0.031) and percentage granulocyte (p = 0.025) along with percentage lymphocytes (p = 0.021). The total granulocytes were 1.48 (SD = 0.81) and 2.86 (SD = 0.77) and percentage granulocytes were 28.73% (SD = 3.17) and 38.39% (SD = 8.39) in control and test group respectively. While percentage lymphocytes were 68.08% (SD = 2.78) and 57.76% (SD = 8.72) in control and test group. All these parameters were increased in test group as compared to control group. No significant difference was seen in total lymphocyte count.

Biochemical parameters

Statistically significant difference (p = 0.021) was observed in total proteins between control (mean = 3.73 and SD = 0.73) and test groups (mean = 5.93 and SD = 1.83) with increase in total proteins in test group as compared to control group. There was no significant difference in Blood Sugar Levels (BSL) with mean BSL 83.25 (SD = 21.67) and 104.14 (SD = 25.89) in control and test group respectively.

No significant changes in biochemical parameters such as Liver Function Tests and Renal Function Tests were observed. There was also no significant difference in Coagulation parameters like Prothrombin time and Thromboplastine time. There was also no significant difference observed in serum electrolytes such as Sodium and Potassium.

Histopathology

Histopathological examinations of organs like Brain, Kidneys, Gonads, Liver, Intestines and Stomach of both test and control animals were done. There were minimal histopathological

changes in liver and kidneys in the form of congestion and hemorrhages and Focal cellular swelling, granular and vacuolar changes in cytoplasm. Mild to minimal changes in the stomach and intestine were observed. In intestine they were in the form of Goblet cell hyperplasia, vacuolar changes of mucosal enterocytes. In stomach necrotic changes were observed with loss of length of mucosa, congestion of submucosal blood vessels and inflammatory cell infiltration at mucosa and submucosa. No abnormalities were detected in the brain and gonadal tissues. The changes observed in stomach were mainly focal and some changes were also observed in control animals.

TABLES

Table I: Changes in haematological parameters in Control and Test animals.

	RBC in lacs/mm^3	Hb in gm%	Hct in %	MCV in fl	MCH in pg	MCHC in gm/dl
Control Mean	7.31	11.48	38.15	52.23	15.65	30.03
Control SD	0.50	0.90	3.21	1.93	0.66	0.39
Test Mean	8.34	13.30	43.76	52.53	15.87	30.29
Test SD	0.79	1.68	4.86	2.48	0.94	0.53
p value	0.028	0.043	0.048	0.827	0.659	0.376

Table II: Changes in haematological parameters in Control and Test animals.

	WBC	LYM	MON	GRAN	LYM %	MON %	GRA %	Platelets
Control Mean	5.38	3.73	0.23	1.48	68.08	3.20	28.73	536.00
Control SD	3.34	2.42	0.05	0.81	2.78	0.54	3.17	39.10
Test Mean	7.81	4.66	0.30	2.86	57.76	3.84	38.39	527.86
Control SD	2.75	2.12	0.14	0.77	8.72	0.69	8.39	123.59
p value	0.267	0.546	0.239	0.031	0.021	0.128	0.025	0.876

Table III: Changes in biochemical parameters in Control and Test animals.

	BSL mg%	TP in gm%	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	T. Bil. in (U/L)
Control Mean	83.25	3.73	73.75	224.50	484.50	0.39
Control SD	21.67	0.73	10.36	60.39	113.19	0.14
Test Mean	104.14	5.93	76.76	325.57	349.71	0.62
Control SD	25.89	1.83	15.43	126.68	163.07	0.26
p value	0.193	0.021	0.708	0.108	0.144	0.087

Table IV: Changes in haematological parameters in Control and Test animals.

	Na in mmol	K in mmol	Urea in mg/dl	Creat in mg/dl	APTT	PT
Control Mean	151.0	7.69	27.35	0.73	16.20	26.05
Control SD	7.62	1.28	5.12	0.22	2.99	1.56
Test Mean	151.1	7.31	26.45	0.84	17.13	23.27
Control SD	12.32	2.13	1.66	0.22	2.58	4.93
p value	0.982	0.718	0.753	0.428	0.623	0.207

FIGURES

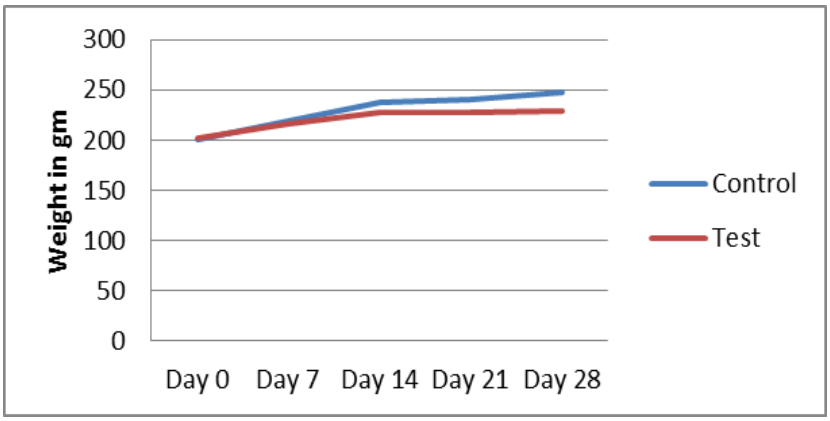

Fig. 1: Showing body weight pattern in test and controls animals during treatment.

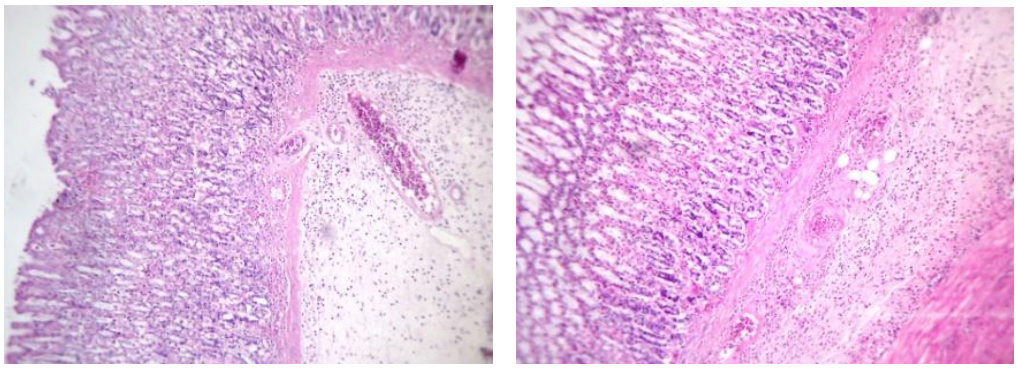

Fig. 2: Showing histopathology of stomach of Control and Test animals.

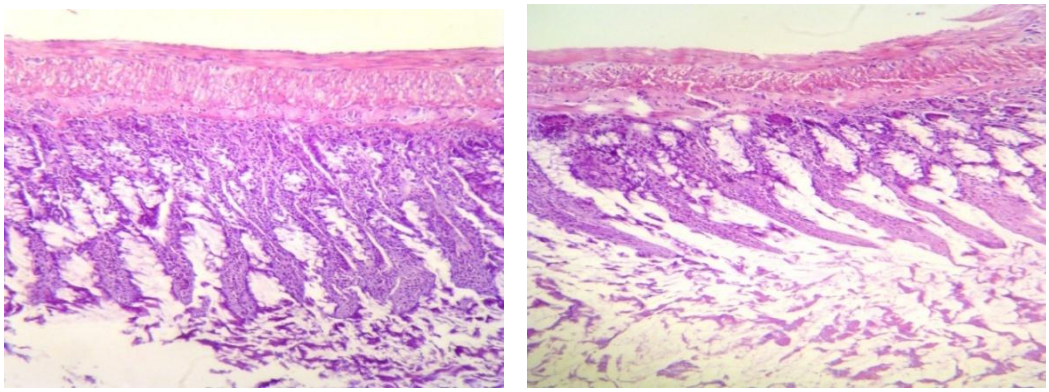

Fig. 3: Showing histopathology of intestine of Control and Test animals.

DISCUSSIONS

Nanoparticles research is an area of intense scientific interest due to wide variety of potential applications in biomedical fields. ZnONP is prototype of commercially available nano materials with medical applications in diagnostics, therapeutics drug delivery system etc.[6]

Nanoparticles have larger 'surface area to unit mass' ratios so they are likely to have toxic effects that are unusual and not seen with larger particles of the same material. In contrast to delivering organic molecule like drug, nanoparticle is comprised of atomic scale parts. Due to which, quantum effects and electronic interactions become significant at the nanoscale. Also smaller size of nanoparticle renders them the property of faster and easy entry into human body. So there is possibility that pharmacological parameters may be altered.[1]

ZnO NP are unstable and get dissolved in aqueous solutions, releasing zinc ions from the particles. As far as this property is considered, ZnO NP differ from other metal oxide nanoparticles, such as titanium dioxide, cerium oxide, and iron oxide, The solubility of ZnO NP depends on pH, concentration, particle size, and the presence of organic compounds.[7-10]

In the present preclinical study, possible pharmacological effects of nanoparticle of fixed size have been studied at fixed dose. In a similar study conducted using ZnO NP, three different doses 100 mg/kg, 200 mg/kg and 400 mg/kg were used for oral administration. The size of ZnO NP used was 20 nm and study was conducted for 14 days. It was observed that there was rise in serum inflammatory markers like TNF Alpha and IL – 6 levels. There was rise in IgG levels in all doses and LDH level was highest in high dose group (400mg/kg) as compared to other groups. All animals survived at the end of experiment and none of them shown any features of toxicity externally.[11] Considering the size dependent toxicity, larger size of nanoparticles that is 50 nm is used with lowest dose (100mg/kg) in above mentioned study and hence the same dose was used for present study.

On Histopathology, major features were perivasculitis and peribronchitis of Lungs in study with 20 nm sized ZnO NP at three dose levels. Animals on low dose have shown these changes (60%) of mild grade while animals on high dose levels have shown 40% of animal with same changes each of moderate and severe variety. Comparing these changes with present study there were minimal changes in liver and kidneys, minimal to mild changes in stomach and intestine. While there were no changes in Brain and gonads.[11]

Another study was comparing oral versus intravenous route with single dose and two concentrations (3mg/kg and 30mg/kg) of ZnO NP with size of 35 nm in Sprague Dawley rats. The study was aimed at their toxicokinetics and toxicity. Toxicity determined by serum biochemistry and histopathological analysis occurred mainly in the rats treated intravenously with a high dose of ZnO NP. Significant elevation in levels of AST, CPK, total proteins, BUN, and Creatinine were observed in the intravenous group, while glucose and albumin / globulin ratio were decreased. Significant increase in total proteins seen in present study with increase in total proteins in test group as compared to control group and it is consistent with results of present study. However, serum biochemical changes evident at day one have disappeared by day seven. No changes in serum biochemical parameters were observed in rats orally administered with ZnONP. WBC, Hb, and lymphocytes increased in rats orally administered with the high dose, but monocytes and MPV decreased compared to control.[8]

This finding is consistent with present study in which higher levels were observed in Heamoglobin, Haematocrit and RBC counts in test group compared control group with statistical significance. Although the rise was significant, it is not biologically relevant as values are within normal range. neutrophils and eosinophils were raised, while lymphocytes and monocytes fell in the rats treated with 30 mg/kg intravenously. Similarly, in present study total and percentage granulocytes and percentage lymphocytes were raised in present study.[12]

Other two studies were conducted in similar way using ZnONP of 20 nm[13] and 100 nm[14] sized particles with 90 day oral administration in Sprague Dawley rats. ZnO NP with 20 nm size used dose levels of 500, 250 and 125 mg/kg while 100 nm size ZnONP was used with 500, 125 and 37.5 mg/kg dose levels. Dose level 125 mg was observed to be a No Observed Adverse Effect Level (NOAEL) for 20 nm sized and 37.5 mg/kg was NOAEL for 100 nm sized nanoaparticles.

In a study conducted with 20 nm ZnO NP, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were all significantly decreased but RBC count is significantly increased in both 250 and/or 500 mg/kg groups compared with the controls. Total proteins and albumin were significantly reduced in both groups. Rest all biochemical parameters didn't differ significantly in both groups.

In a study conducted with 100 nm ZnONP, the MCV and Hb levels were significantly lower in rats receiving 125 mg/kg and Hb, MCV, MCH, and MCHC levels were significantly lower in rats receiving 500 mg/kg. Total RBC count is significantly reduced during treatment phase but it is significantly increased during recovery phase in both groups. Total proteins and Albumin were significantly reduced in both groups.

Eosinophil count was significantly raised in both 20 nm and 100 nm ZnO NP groups with 250 mg/kg dose and 125 mg/kg respectively.

Histopathological changes were seen in Stomach, Pancreas and Retina in dose dependent manner in animals administered with 20 nm sized ZnO NP. But reduction in incidence and grades of lesions were observed in recovery group. In 100 nm group, significant lesions in the stomach, pancreas, eye, and prostate gland were observed in the 500 mg/kg groups, they exhibited dose dependency except in pancreas where lesions not found in group other that 500 gm/kg dose.

CONCLUSIONS

- ZnONP (< 50 nm size) if administered orally at a dose of 100 mg/kg have no clinically observable adverse effects in animals.
- ZnONP (< 50 nm size) if administered orally at a dose of 100 mg/kg have heamatinic effect evidenced by statistically significant rise in Heamoglobin, Haematocrit and RBC counts between test and control group and supported by literature. But exact reason for hematinic activity is yet unclear.
- ZnONP (< 50 nm size) if administered orally have effects on lungs parenchyma with reduced relative weight of the organ with similar study showing perivasculitis and peribronchitis in Lungs.
- On Histopathological studies mild to minimal changes were observed with ZnONP (< 50 nm size) after oral administration.

ACKNOWLEDGEMENT: None.

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