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Toxicity Assessment of Synthesized Nickel-Zinc-Ferrite ($Ni_{0.6}Zn_{0.4}Fe_2O_4$) Nanoparticles (NZFO NPs) on Swiss Albino Mouse *Mus musculus*, through Biochemical, Histological and Cytological Investigations

Anju Kaushal, Soorambali Keshava Shyama ** and Kavita Bakhale****

Abstract

The electromagnetic applications of synthesized NZFO NPs in electronics and medicine are because of their unique surface properties. Like other nanoparticles, NZFO NPs are also a material of investigations for hazards and risks associated with occupational and living populations. With the same objective, the present work deals with biochemical, histological and cytological approaches for explaining the activities of NZFO NPs in living systems. Both *in vitro* and *in vivo* studies were performed on swiss albino mouse *Mus musculus* at the exposure levels from 125 mg/l to 500 mg/ml of NZFO NPs. Oral route intake resulted in significant MDA (Malondialdehyde) equivalent level production after two weeks in liver and testes. By fourth week, level remained significant in liver only ($p<0.05$). After a month, tissues like blood, spleen and testes were highly affected. Examination of blood revealed that leukocytes reached high levels. The RBC count in million/cmm was reduced in lowest dose of 125mg/ml. Good cell viability was achieved (94-97%) upon studying for three consecutive days *in vitro* blood culture of mice (0.6 mg/ml-10 mg/ml). It is thus suggested that this nanoparticle may be carcinogenic, affects blood tissue and organs depending upon its size, shape, chemical composition and surface characteristics rather than applied concentrations.

1. INTRODUCTION

Toxicity assessments for both types of natural (NPs) and synthesized or engineered nanoparticles (ENPs) has been gaining ground since various international agencies like National Nanotechnology Initiative (National Research Council, 2002; Dunphy Guzman et al, 2006), United States Environment Protection Agency (US EPA, 2004), National Science Foundation (NSF), National Toxicological Program (NTP), National Research Council (NRC) (Service, 2004), National Institute of Occupational Safety and Hazards (NIOSH, 2005), National Institute of Environment and Health (NIEH, 2003) and few others raised concerns about their biosafety at occupational, environmental and consumer levels. Subsequently, they initiated and undertook various research projects involving the characterization and evaluation of the toxic and hazardous properties of both natural and synthesized NPs.

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$Ni_{0.6}Zn_{0.4}Fe_2O_4$ (NZFO) being magnetic in nature, are of high technological significance and have plenty of electromagnetic applications e.g., inductors, electromagnetic wave absorbers. This conserves considerable amount of energy (Tsutaoka, 2003). Ni-Zn ferrites find applications in electronics recording media heads, antenna rods, loadies coil, microwave devices and telecommunication applications (Bueno et al, 2007). As a semi conductive material, it responds to ethanol detection (Kapse et al, 2009). The nanocrystalline magnetic materials are used in biomedicine and biotechnology as contrast agent in magnetic resonance imaging (MRI) and also as drug carriers for magnetically guided drug delivery (Tartaj et al, 2003). With the introduction of new roles, the individuals in contact with NZFO NPs continues to increase in future. Al-Qubaisi et al, (2013) suggested their potential cytotoxicity against human colon cancer HT29, breast cancer MCF7, and liver cancer HepG2 cells. Paul et al, (2012) studied the interaction of BSA with NZFO for structural and functional changes. They found reduced functionality and binding of negatively charge domain I to NZFO carrying a positive surface charge. Believed to be toxic only in the loose form, Aitken et al, (2007) placed such metal oxides as priority one candidate materials for toxicology especially ecotoxicology. Blaise et al, (2008) reported that NZFO in ecosystem reflected eco sensitivity with a different mode of toxic action.

The present study is aimed at the toxicological investigations of NZFO by taking up several parameters such as lipid peroxidation, histopathological survey on various tissues, hematological, serum biochemical analysis (*in vivo*) and cell viability (*in vitro*) of mice at variable dose levels.

2. MATERIALS AND METHODS

$Ni_{0.6}Zn_{0.4}Fe_2O_4$ nanoparticles (NZFO NPs) were synthesized by self propagating auto-combustion of nickel zinc ferrous fumarato-hydrazinate complex. The precursor complex was characterized by chemical analysis, IR, AAS, thermal analysis and isothermal loss studies. The precursor on ignition underwent self-propagating auto combustion to give $Ni_{0.6}Zn_{0.4}Fe_2O_4$. The X-ray diffraction studies confirmed the single phase formation of nano size 'as- synthesized' NZFO. TEM observations showed the average particle size to be 20 nm. Infrared and magnetization studies and higher Curie temperature of 'as- synthesized' ferrite also hinted at the nanosize nature as reported by Gawas et al, (2007).

Swiss albino mice*, Mus musculus*, were taken as the representative animal model for the present investigations. Six week old mice were procured from M/S Sri Venketeshwara Laboratories & Animal suppliers, Mangalore. They were housed in polypropylene cages (290 X 220 X 140 mm) bedded with paddy husk and were maintained under 12 hour light /dark cycle at temperature of 28 (±2)^{o}C and 55 (±5)% humidity. They were fed with standard mouse pellets (Lipton India) and water *ad libitum*. Five animals were used for each treatment and control group. Freshly procured above mice were acclimatized in this laboratory conditions for two weeks.

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Experimentation

Eight weeks old mice, weighing 20-25 gms were divided into four group's viz. 1 to 4. Group 1 was used as control animals and was administered Millipore water through oral route in feeding bottles. Remaining groups viz. Group 2, 3 and 4 were used as exposed animals and were administered various concentrations, viz: 125, 250 and 500 mg /liter respectively of the NZFO added to Millipore water.

Lipid Peroxidation

Malonaldehyde content of the 10% homogenates (in 0.25M Sucrose containing 2mM $MgCl_2$) of liver, kidney and testis of both the control and exposed mice were estimated using Thiobarbituric acid (TBARS assay) (Uchiyama and Mihira, 1978; Stinson et al, 1992). The difference between absorbance at 534 nm and 520 nm was used as TBA value and extinction coefficient of 156 $mM^{-1}cm^{-1}$ was used to calculate the amount of malonaldehyde present. The statistical variations between exposed and control values were calculated using Student's 't' test.

Histopathological Survey

At the end of experiment i.e. on 28^{th} day, various tissues such as liver, kidney, lung, spleen and testes were collected from each mice and were processed for histopathological studies (Sood, 2006). They were studied for changes in cellular morphology, functioning and nuclear abnormalities.

Hematological Survey

Peripheral blood was collected from the heart and used to measure various hematological parameters such as HGB, Leukocytes, Platelet count, Erythrocytes, Reticulocyte count and Differential leucocyte count.

Serum biochemical analysis

SGOT was performed with Enzo kit (RFCL Limited New Delhi, India) by following the standard protocol mentioned in the kit. Total and direct bilirubin was done with kit from AUTOPAK (SIEMENS, Gujarat, India) following Jendrassik and Grof Method (Jendrassik and Grof, 1938).

Cell viability assay (*in vitro*)

Cultures were set with 0.6 ml of mouse blood collected aseptically from the widest part of posterior vena cava between the kidneys with 23-25 gauge needle and one ml heparinized syringe (Hoff, 2000).

Culture Procedure

Observing all the aseptic conditions, 2-3 drops of blood were gently dropped in vials containing 0.5 mg/ml-20.0 mg/ml concentrations of NZFO. Each culture vial already contained 4 ml of RPMI, 10% fetal bovine serum (1.0 ml) and 0.1 ml of PHA. NZFO was removed from each culture vials after 6-8 hours of incubation.

3. RESULTS AND DISCUSSION

Table 1: Dose intake NZFO (mg) / animal during a month studies.

Group (dose)	Lipid Peroxidation, Examination of Blood & Tissues			
	First week	Second week	Third week	Fourth week
1 (control)	-	-	-	-
2 (125 mg/l)	1.75	5.75	2.06	20.0
3 (250 mg/l)	5.0	13.75	6.2	25.0
4 (500 mg/l)	17.8	32.8	57.0	75.0

Intake of NZFO

The average amount of NZFO ingested by each mouse through oral route was calculated on daily basis and week wise (Table 1). By the end of 4th week, for lipid peroxidation, organs harvesting and blood examination, each mouse of Group 2, 3 and 4 had received 20 mg, 25 mg and 75 mg of NZFO respectively.

Active Behavior

Mice were not averse to drinking water laced with NZFO. They were active as usual with no sign of mortality, physical deformity and appearance of abnormal growth. There was no reduction in weight in any group during the study period.

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Table 2: Level of MDA equivalents in TBARS assay following NZFO exposure (first week).

S. No	Group (dose)	Tissue	First week	
			M mol MDA equiv/mg Protein ±SD	t' Value
1	1 (control)	Liver	-1.7154± 0.544	-
		Kidney	8.0952± 4.902	-
		Testes	13.214± 8.1816	-
2	2 (125 mg/l)	Liver	-8.5678± 8.12	-1.887 n.s
		Kidney	-60.653± 78.60	-1.955 n.s
		Testes	-14.718± 9.57	-6.305 n.s
3	3 (250 mg/l)	Liver	-7.2184± 3.236	-3.753 a
		Kidney	27.829± 21.26	2.06 n.s
		Testes	-8.4412± 5.25	-5.368 a
4	4 (500 mg/l)	Liver	-17.815± 3.125	-11.36 a
		Kidney	-21.231± 7.656	-8.171 a
		Testes	-10.456± 5.104	-5.515 a

ns: non- significant

-ve value of nmol MDA equivalent/mg protein means compound formed peak at 520 nm

-ve 't' value indicates control values > NZFO

a: indicates control value significantly higher than NZFO value at $p \leq 0.05$

b: indicates NZFO values significantly higher than control values at $p \leq 0.05$

Table 3: Level of MDA equivalents in TBARS assay following NZFO exposure (second week).

S. No	Group (dose)	Tissue	Second week M mol MDA equiv/mg Protein ±SD		t' Value
1	1 (control)	Liver	-0.782± 0.404		-
		Kidney	9.488± 2.267		-
		Testes	8.6208± 2.006		-
2	2(125 mg/l)	Liver	6.84± 1.654		10.02 b
		Kidney	10.440± 1.703		0.751 n.s
		Testes	19.054± 3.488		5.802 b
3	3 (250 mg/l)	Liver	4.244± 0.733		13.88 b
		Kidney	14.242± 2.761		2.978 b
		Testes	13.040± 2.146		3.368 b
4	4 (500 mg/l)	Liver	5.2932± 1.451		9.000 b
		Kidney	7.385± 0.748		-1.972 n.s
		Testes	14.892± 1.56		5.525 b

Table 4: Level of MDA equivalents in TBARS assay following NZFO exposure (third week).

S. No	Group (dose)		Third week M mol MDA equiv/mg Protein ±SD	t' Value
1	1 (control)	Liver	-18.373± 10.724	-
		Kidney	4.501± 2.879	-
		Testes	9.969± 2.926	-
2	2 (125 mg/l)	Liver	-28.849± 16.71	-1.286 n.s
		Kidney	-47.99± 38.68	-2.337 n.s
		Testes	-11.98± 10.24	0.266 n.s
3	3 (250 mg/l)	Liver	-23.427± 22.570	-0.477 n.s
		Kidney	-18.411± 12.24	-4.087 n.s
		Testes	10.784± 1.055	15.038 b
4	4 (500 mg/l)	Liver	-0.792± 8.796	1.289 a
		Kidney	-44.199± 16.613	-6.464 a
		Testes	-48.534± 42.90	-2.006 n.s

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Table 5: Level of MDA equivalents in TBARS assay following NZFO exposure (fourth week).

S. No	Group (dose)	Tissue	Fourth week	
			M mol MDA equiv/mg Protein ±SD	t' Value
1	1 (control)	Liver	-0.0122± 0.927	-
		Kidney	4.729± 1.130	-
		Testes	20.279± 4.50	-
2	2 (125 mg/l)	Liver	2.9142± 0.468	8.572 b
		Kidney	5.760± 0.743	1.7069 n.s
		Testes	21.622± 5.78	2.737 b
3	3 (250 mg/l)	Liver	-17.425± 2.56	-14.39 a
		Kidney	-13.445± 5.08	20.72 a
		Testes	22.565± 15.100	0.867 n.s
4	4 (500 mg/l)	Liver	3.646± 0.306	8.389 b
		Kidney	4.4134± 0.863	-0.497 n.s
		Testes	10.228± 1.172	-0.415 n.s

ns: non- significant

-ve value of nmol MDA equivalent/mg protein means compound formed peak at 520 nm

-ve 't' value indicates control values > NZFO

a: indicates control value significantly higher than NZFO value at $p \leq 0.05$.

b: indicates NZFO values significantly higher than control values at $p \leq 0.05$.

NZFO Induced Lipid Peroxidation

After first week, mice exposed to NZFO had lower values of MDA as compared to control, thus indicating no lipid peroxidation (Table 2). Values were mostly negative with possible reasons stated in the discussion. Liver and testes showed significant production of MDA at 5% critical level at the end of second week in all the groups. In Group 3, the values were significant in kidneys also (Table 3).

After three weeks values were either non-significant or significant only in testes of Group 3 against control at 5% critical level of significance (Table 4).

After a month, the values of MDA equivalents remained significant at 5% critical level only in liver of Group 2 and 4. The values in testes were significant in Group 2 only (Table 5).

Table 6: Examination of blood of mice exposed to NZFO to detect variations among different exposed groups and control.

S. No	Test(s)	Group(dose)	Result	Normal values
1.	WBC/cmm	4(500 mg/l)	19,400	600-15,000
2.	Retic count%	1(control)	1.2	2.5-13.3
		2(125 mg/l)	0.6	
		3(250 mg/l)	0.4	
		4(500 mg/l)	1	
3.	HGB g/dl	3(250 mg/l)	10.0	10.2-16.6
		2(125 mg/l)	9.4	
4.	RBC milli m/c mm	2(125 mg/l)	5.99	7.5-9.5
5.	Platelet count	4(500 mg/l)	5,64,000	16,0000-41,0000

WBC: White Blood Corpuscle

Retic: Reticulocyte

HGB: Hemoglobin

RBC: Red Blood Corpuscle

Examination of Blood Leukocytes Shoots Up

At the end of study, it was seen that WBC count had above normal values in Group 4. RBC count was low in Group 2. Amount of HGB also came down in Group 3 and 2. Retic count was below base line all groups including control. Platelet count had above normal values in Group 4. The results shown in Table 6 are of Groups and tests with abnormal values only. Differential count values were normal in all cases, thus not shown.

Serum Biochemical Analysis: Normal values

SGOT (1U/L) S. Bilirubin (mg/dl) values in all the groups including control were within the range of 54-298 and 0.1-0.9 respectively. These are not shown.

Histology: Appearance of Altered Morphology

Blood, spleen and testes were much affected in dosed individuals. Blood smears showed atypical lymphocytes in all the groups but they were most abundant in Group 4 and their severity decreases with decrease in dose. They were also not properly differentiated (Figure 1: A).

In lungs, nucleus appeared to be highly affected in exposed individuals with one or two circular chromatin bodies (Group 2,3,4) in otherwise clear nucleoplasm where as in control, the nucleus stained uniformly deep with no chromatin bodies observed (Figure 1: B).

At first observation, spleen appeared inflamed in exposed groups. In histological preparations, it appeared under high stress. It is tightly packed with cells. The intense blue stain all over showed greater divisional activity of cells (Figure 1: C, Group 4) whereas control individual showed healthy pink tinted spleen sections (Figure 1: C, Group 1).

Liver appeared to have normal fat storage (shown with pink stain) in all the cases. Nucleus appeared normal. Throughout the sections, the prominent brown spots were very clearly seen in exposed individuals, which could be accumulated NZFO (Figure 1: D, Group 3, 4). Kidneys appeared to be least affected with no observed difference between exposed and control individuals (Figure 1: E).

Upon careful examination in testes of exposed individuals, the normal activity was almost absent. Seminiferous tubules had shrunk in size with abundant gap in between. Internal lining appeared eroded with little spermatogenic activity and intraluminal mass (Figure 1: F, Group 2, 3, 4). In control, all the stages of sperm formation in healthy tissue were clearly visible (Figure 1: F, Group1).

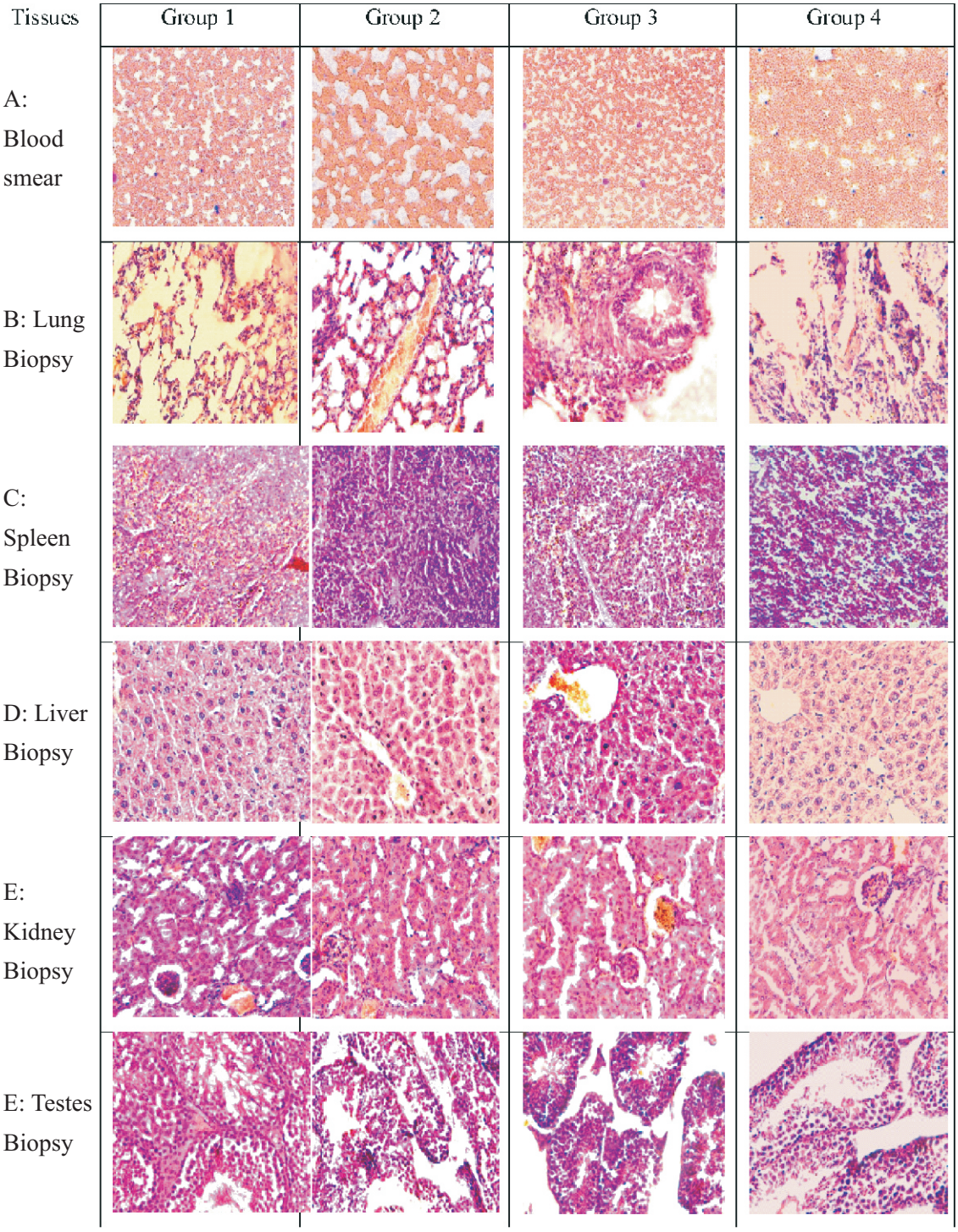

Figure 1: Histopathological survey of various tissues (A-F) of mice upon exposure to Group 1(control) Group 2(125 mg/litre) Group 3(250 mg/litre) and Group 4(500 mg/litre) to NZFO.

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Good Cell Viability Achieved *In Vitro*

Cultures remained healthy even at 72 hrs. NZFO has no deleterious effect on cell viability at 24 hrs. Cells appeared healthy, rounded, moving and at active pace. There was hardly any cell taking trypan blue dye. Although they had exposed to NZFO for 6 hrs at 42 hrs, cell viability came down marginally and remained at same level after 72 hrs. (Figure 2).

The most fascinating sight were the patterns at the bottom of cell culture vials. Upon examination under microscope, cell clusters were clearly observed with light brown nanoparticles inside them (Figure 3: B and C). It appeared that the cells have taken the NZFO. Since they are magnetic in nature they are attracted towards each other within the cells thus forming a rounded ball like masses. The phenomenon was observed at 48, 72 hrs long after NZFO has been removed from the vials. Cells could be counted after repeated pipetting.

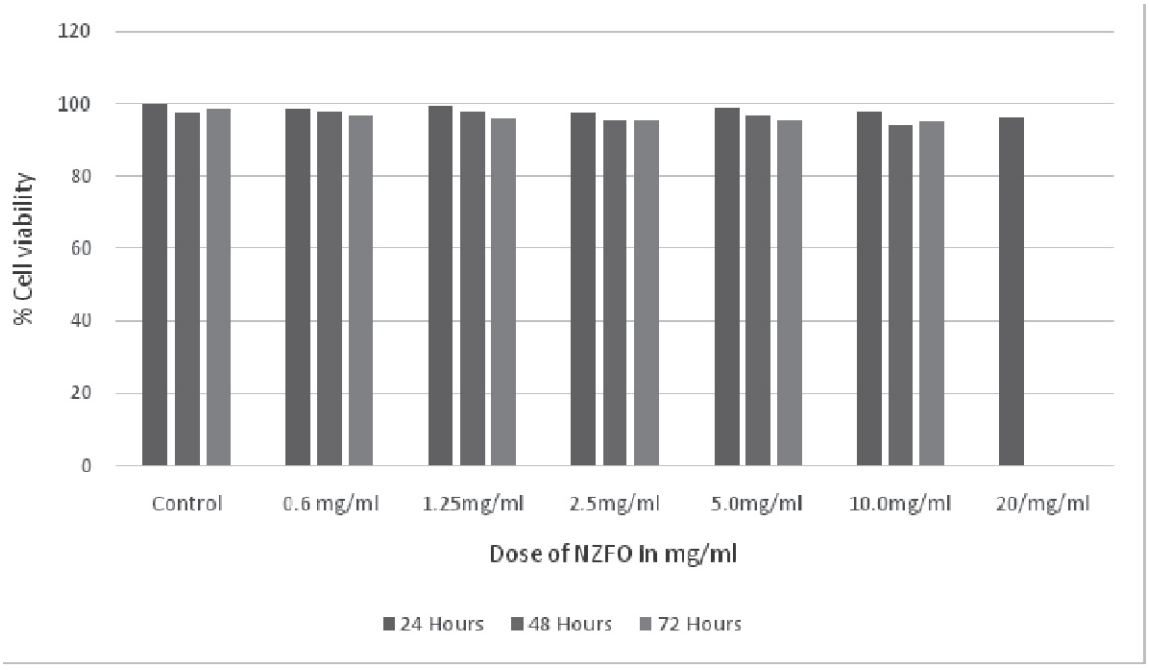
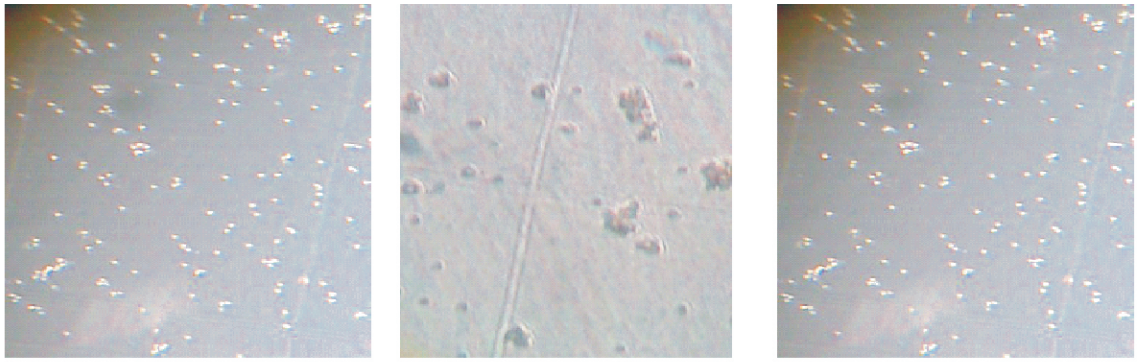


Figure 2: % viability of cells after exposure to various doses of NZFO at varying intervals of time.



(A) (B) (C)

Figure 3: View of blood cells of mice under microscope; Control blood cells (A) in culture. Clumped blood cells (B and C) storing NP in brown color at doses 10 mg/ml and 2.5 mg/ml respectively.

NZFO in lipid peroxidation behaved in unique way. The only positive evidence of significant ($p < 0.05$) malonaldehyde production was at the end of second week in liver and testes in all groups. In kidneys, the only positive evidence was found in Group 3 at the end of 2 weeks. Liver and testes values of malonaldehyde remained significant at one or two places at the end of four weeks. The values have been expressed as MDA equivalents because apart from malonaldehyde compounds like alk-2-enals and alk-2, 4-hydroxyalkenal, di-oxolanes, furan derivatives, acetaldehyde and sucrose, 4-dienals form red pigments absorbed at 532-535 nm (Kosugi et al, 1988). They are used as indices for evaluating stages of lipid peroxidation (Esterbauer ,and Zollern, 1989).

Normally the production of yellow orange and red pigmentation in stages (Kosugi et al 1987) was also observed in present investigations. The amount of MDA formed is dependent on the lipid content of the sample along with the amount of the metal ion contamination in the reagents and antioxidants present in sample. Several other compounds (e.g. other aldehydes, carbohydrates, amino acids and bile pigments react with TBA to form complexes that have absorption spectra overlapping with that of MDA- TBA complex (Gutteridge, 1986; Gutteridge and Quinlan, 1983; Gutteridge and Tickner, 1978; Gutteridge, 1981).

All the negative of difference between O.D's indicated that compound formed peak at 520 nm rather than at 535 nm. In the Kornblun-DeLa Mare rearrangement, ketones are prepared from peroxides and base derivatives of aldehydes and ketones; 2-4-dinitrophenylhydrazane has absorption max at 500-520 nm. Other compounds include uric acid and sulphuric radical adduct.

All can be said that the values of MDA equivalents hardly followed the dose dependent rules. The effect was not equal on all the tissues. Although the administration of NPs continued, the values decreased with time. Lipid peroxidation followed cyclic pattern during a month. Also, a variety of aldehydes, ketones and other compounds were generated at different time with different absorption maxima. Our nanoparticle contained Fe and it is reported to peroxidise lipids (Buzea et al, 2007).

Histopathological analysis showed that blood has abundant atypical lymphocytes. Our findings agreed with Xiao-Feng and Ting (2010), who observed the influence of Ferrite nano NiZn powders on blood and cells of rats. They found that the amount of leukocytes in the blood of the exposed group was more than the control but reverse in case with erythrocytes.

Spleen was enlarged thus more capacity to store erythrocytes. This resulted in decreased value of circulating erythrocytes as observed in Group 2. The amount of iron pigment hemosiderin, ferritin was increased in spleen. NZFO triggered immune response to which spleen responded well. It is seen packed with newly formed lymphocytes ready to burst into circulation. Thus spleen is holding excess of iron pigment, lymphocytes and lipid oxidation products (e.g. ceroid, lipofuscin).

Testes showed that constant exposure can cause male sterility. Changes in lung nucleus suggested that cytogenetic studies like micronucleus assay and comet assay might shed some light on genetic damage. NZFO was carried along with blood and is found accumulated in liver. Spleen appeared under stress to produce more lymphocytes; the effect could be seen as higher values of leukocytes in blood of group 4. The low value of hemoglobin and erythrocytes in blood of Group 2 is due to spleen storing more of them. Although NZFO contains iron nanoparticles which could have been even more absorbed by the body. Normal serum biochemical analysis values showed that nanoparticles did not affect enzyme action significantly.

94-97% cell viability indicated that nanoparticles do not affect cells sustenance *in vitro*.

4. CONCLUSION

In nutshell, it can be concluded that NZFO affected blood and germinal tissues and findings of present research work with lipid peroxidation, histopathology, and blood analysis supported each other. It has well been proved that NZFO causes harmful effect on blood like clumping seen only *in* vitro, excessive release of leukocytes in blood stream and since lipid peroxidation is positive, it may well be connected to carcinogenicity (Figure 4).

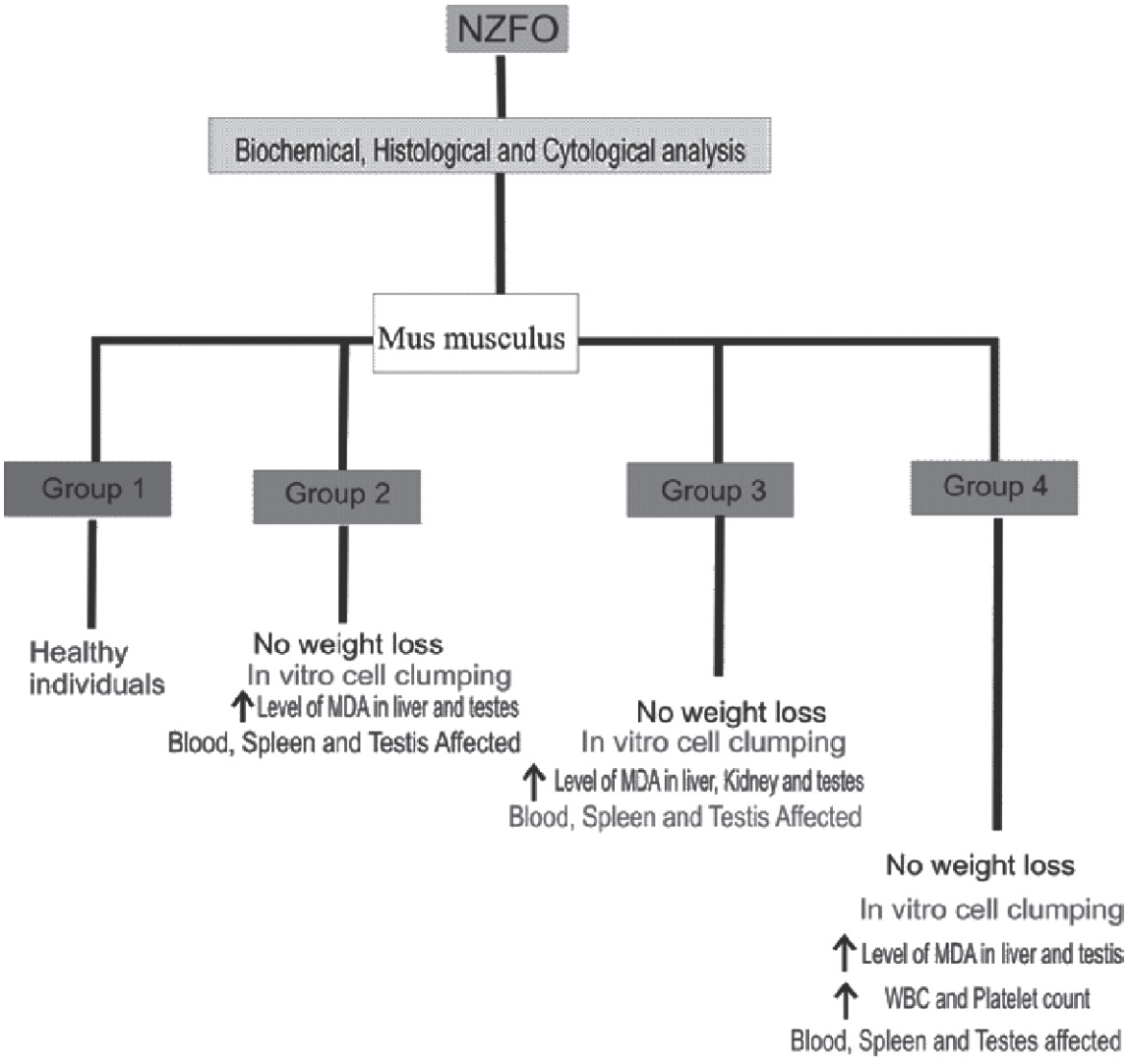


Figure 4: Summary of effects of NZFO on Swiss albino mice in a month old studies. Group 1 indicates control, Group 2 (125 mg/l), Group 3 (250 mg/l) and Group 4 (500 mg/l) dose levels administered orally.

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