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Assessment of Defensive Role of Citrus juice Against Zinc Oxide Nanoparticles-Inducing Pulmonary Toxicity in Female Swiss Albino Mice

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Abstract

In pursuit of separating the mysteries of zinc oxide nanoparticles (ZnO-NPs) and their potential toxicity during pregnancy, a study was conducted using Swiss albino mice as experimental models at the Institute of Zoology, University of Punjab, Lahore. The research aimed to explore the effects of ZnO-NPs on pregnant female mice and their F1 generation offspring, as well as the protective role of fresh orange juice through co-administration. Forty pregnant female mice were divided into different groups: a control group, a group treated with ZnO-NPs, a group given both ZnO-NPs and fresh orange juice, and a group administered only with fresh orange juice. The administration of ZnO-NPs during organogenesis resulted in decreased body weight and increased lung weight in the pregnant mice. Biochemical analysis showed disruptions in LDH levels and fluctuations in CBC parameters. Histopathological examination revealed structural abnormalities in the lungs of ZnO-NPs treated mice and their fetuses, including distorted bronchi and undifferentiated alveoli. Morphometric analysis showed reduced body weight and increased lung weight in the ZnO-NPs group compared to controls. Elevated LDH levels, WBC count, and abnormalities in lymphocytes and platelets were observed in both mothers and F1 mice. The study concluded that ZnO-NPs could be toxic to pregnant mice and their offspring, potentially causing lung toxicity by crossing the placental barrier. However, the research also highlighted the protective potential of fresh orange juice, attributed to its antioxidant properties as determined by FRAP and TPC analysis. In essence, this study sheds light on the intricate interplay between ZnO-NPs, pregnancy, and

lung toxicity in mice, emphasizing the importance of understanding nanoparticle toxicity and exploring natural remedies for mitigation.
Keywords: ZnO, Nanoparticles (NPs), Ferric Reducing Antioxidant, Total Phenolic Content Assay, Antidote, Lactate Dehydrogenase Test, Lymphocytes

INTRODUCTION

Nanoparticles such as zinc oxide (ZnO) are semiconducting metal oxides nanoparticles which are frequently use for biomedical applications such as anticancer drugs, imaging tools, and cosmetics (Sharma *et al*., 2012). Nanotechnology improves the healthier packing of food and makes it healthier, safer, and better tasting, resulting in a fast-developing food category known as nano food (Chen *et al.,* 2015). Nanoparticles are small particles (less than 100 nm) exhibiting specific characteristics when comparison to their bulk counter parts (Kabir *et al*., 2018). In medicine, environmental remediation, pharmacology fields, electronics, veterinary, and renewable energy are just a few of the applications for nanoparticles (NPs) (Schirmer & Auffan, 2015). Because of their vast production and uncontrolled discharge, NPs have become important in obtaining a complete and mechanistic understanding of ecosystems (Ke & Lamm, 2011; Bilal[a,b] et al., 2024). The widespread use of ZnO nanoparticles in various fields necessitates research into its toxicity, particularly in the cells of the mammalian and bacterial (Wang *et al*., 2010). They have a broad application in other fields also like cosmetic materials, sunscreens (Schilling *et al*., 2014), food packing, agriculture and biomedical applications (Rasmussen *et al*., 2010). ZnO nanoparticles are widely used as nano sensors, catalysts (in automotive tail gas remediation and refineries), and absorbers of UV. Solar type cells, LCDs, pigments, and rubber manufacturing all use ZnO NPs (Chang *et al*., 2011). Zinc oxide nanoparticles are effective in cosmetic such as sunscreens (due to its UV inhibiting characteristics), materials about foot care, and topical ointments. Exposure of humans to ZnO NPs occurs through a variety of routes, including cutaneous, inhalational, and orally (Kermanizadeh *et al*., 2015). Such particles can enter the respiratory pathway and harm pulmonary cells, causing genotoxicity and inflammation (Hussein *et al*., 2020).

Humans are exposed to NPs by breath, ingesting, and cutaneous absorption by their small particle size and greater surface area (Nohynek & Dufour, 2012). Its toxicity is determined by the production of reactive oxygen species (Hsin *et al*., 2008). Prenatal humans are more vulnerable to hazardous chemicals than mature humans (Yamashita *et al*., 2011). The passage of particles from the mother through the placenta indicates the potential fetal toxicity, making NPs penetration a significant component in veterinary and medical research (Adair, 2009). Diffusion, facilitated diffusion, biotransformation via enzyme metabolism, and either phagocytosis or pinocytosis are the four processes that control the transfer of molecules (Syme *et al*., 2004). Fibrosis,

carcinogenesis, and cardiovascular problems are all possible outcomes (Aschberger *et al*., 2010).

Before accessing tissues, NPs must pass through live cells. Interception, impaction, sedimentation, and diffusion are all known ways for inhaled particulates to deposit in distinct parts of the respiratory system (Geiser & Kreyling, 2010). The process of endocytosis, during the process of the phagocytosis, and may be transcytosis across the cells which are epithelial and endothelial type of cells determine how materials are carried through the lungs (Chuang *et al*., 2014).

Furthermore, macrophages are thought to make a significant contribution in NM redistributing in lungs by material which are transporting towards the lymph nodes which is thoracic (Konduru *et al*., 2014). The phagocyte-mediated evacuation of NPs is connected to the pro-inflammatory cytokines release, reactive oxygen species, and plethora of some other mediators and the chemicals type such as chemokines. The oxygen reduction stages are produced by the phagocytes during the particulate engulfment material. Neighboring cells launch a cascade of responses that can end in apoptosis or tissue injury in the presence of abundant or continuous activation, recruitment of different other leukocytes (such as macrophages), and the generation of toxic substances. In reality, the any type of potential pulmonary pathogenicity consequences of inhaling Nanoparticles are contingent towards a significant over capacity of the lung, that is evaluated by site and the deposition extent to lung, as well as subsequent clearance (Pauluhn & toxicology, 2014).NPs are thought to be throughout the lung deposit, reaching towards the alveolar parts in lungs (Hoet *et al*., 2004)). Mucociliary transport clears NPs in the upper lung pathways, while pulmonary macrophages clear NPs in the alveoli (Madl & Pinkerton, 2009).

Orange juice is increasingly frequently utilized to cure a variety of health conditions, and the juice firm has responded by increasing its output in response to demand. There are numerous elements in orange juice, including carbs, fats, proteins, carotenoids, phenolic compounds, fiber, and vitamins (Sharma *et al*., 2012). Natural veggies and fresh juices are being consumed in large quantities, and this is helping to eliminate undesirable situations (Block, 1991). Several citrus liquids, including lemon juice, orange juice, red blood orange juice, grapefruit juice, and other varieties that are high in vitamin C have been studied, but nothing is known about their health benefits (Aviram *et al*., 2000).

Orange juice is utilized in a variety of fruit liquids around the world (Meléndez-Martínez *et al*., 2007). Taking fresh natural juices has been shown to minimize oxidative stress while also improving blood cholesterol levels, which helps to prevent platelets from clumping together in the bloodstream (Hallfrisch *et al*., 1994). Fresh juices are increasingly being used to manage oxidative stress in the body (Tonin *et al*., 2015). This protection is provided against oxidative stress. Vitamin C is the most abundant water-soluble antioxidant in the body and contributes to antioxidant defence

against oxidative stress. This fact is associated with its ability to operate as a reducing agent as well as an oxygen scavenger in a variety of situations (Kitts, 1997). Citric acid, garlic acid, ferulic acid, vitamin A and C, naringenin, hesperidins, and folic acid are all found in fresh orange juice, along with other nutrients (Arabi *et al*., 2017).

MATERIAL AND METHOD

Chemical to be Used in Study

A sub lethal dose of ZnO NPs of known size was prepared accordingly.

Animal Rearing

Swiss Albino mice was reared in steel cages at room temperature of 25± 1º C. Two females were caged with one male in different cages for breeding purposes under 12 hour's light/dark cycles. Animals was given clean mineral water and commercially prepared food (feed no 12; National Feed Lahore, Punjab, Pakistan) containing protein and vitamins daily. Sperm positive smear on the vagina was checked regularly in the early morning which was indicating first-day post-coitus. Animals were of the same age and free of any environmental treatment. Forty pregnant females were divided into four groups. The total gestation period of mice is 21 days. Dose administration started from the 2nd week of gestation.

Doses

25 mg/kg body weight sub-lethal dose was used in the experiment.

Grouping

Four groups were designed, and each group contains ten experimental animals.

Group (1) served as a control group and was treated with normal H2O and food (ad libitum)

Group (2) was treated with (1 ml of ZnO NPs of 25 mg/kg body weight) sub-lethal dose.

Group (3) was treated with doses of zinc oxide NPs (1ml of 25 mg/kg BW) and fresh orange juice (2 ml) as an antidote.

Group (4) was treated with (2 ml) Fresh Orange Juice as antidote.

Observations

All pregnant females were observed daily throughout the gestation period for clinical signs (mortality, morbidity, general appearance, and behavior). Maternal body weights, feed consumption and water consumption were measured daily from GDs0–20.

Execution of Experiment

After the ingestion of doses, on GD 20, 50% of mothers were subjected to the cesarean section and pups were recovered, labelled, and preserved for histological study and used for biochemical analysis. The other 50% of mothers were allowed to give natural birth to pups and these pups were reared under a controlled environment for 1-2 weeks to record their behavioral responses. After that, pups were sacrificed, and lungs tissues were proceeded for histological study and biochemical analysis.

Recovery of Maternal Tissue

After the recovery, Lungs tissues of mother were recovered and preserved in Bouin's fluid for histological study.

FRAP And TPC Assays

An assay called FRAP was utilized for measure the antioxidant capacity of lungs level and as of orange juice. Reactive oxygen species and antioxidant electrons in lungs and freshly prepared orange juice are primary mechanisms suffered in reducing the power of Fe+ (colorless) to ferric ion (dark blue). Antioxidant capacity was determined by comparing the standard and FRAPS values. The FRAP values were derived from spectrophotometer measurements of optical density (OD).

Total Phenolic content was used to determine the fresh orange juice's total phenolic content (antidote). According to Franco (2002) and Asami (2001), TPC was tested using the Folin-Ciocalteau assay (FCA) (2003). Oxidative stress was lowered by the total phenolic content of fresh orange juice in response to reactive oxygen species.

Analysis Techniques

Toxic effect of ZnO NPs will be analyzed by using different techniques which are as follows biochemical and histological study.

Biochemical Analysis

The biochemical assay is an analytical procedure that was used to assess the toxicity of ZnO NPs by measuring concentrations of different enzymes in the blood. Such as L.D.H.

Histological Study

The paraffin sectioning technique is a vital method for studying embryos and organs histologically. It involves steps such as fixation, washing, clearing, embedding in paraffin, trimming blocks, sectioning, drying slides, and mounting sections. Each step is crucial in preparing tissue samples for detailed analysis under the microscope.

Fixation

Recovered embryos and lungs were fixed in Bouin's fluid for 48 hours. After fixation, the sample was washed with 70% ethanol.

Washing and Dehydration

To prepare the tissues for paraffin embedding, they underwent a series of steps involving ethanol and xylene. The specimens were washed with varying concentrations of ethanol (30%, 50%, 90%, and finally 100%) for several hours to remove moisture. Following dehydration, the specimens were exposed to xylene to clear any remaining fluids. Subsequently, the tissues were treated with a mixture of xylene and paraffin wax before embedding to ensure proper infiltration. These meticulous steps are essential for preserving tissue integrity and preparing samples for microscopic analysis.

Embedding

Finally, embryos were treated with paraffin wax. For this purpose, paraffin wax was allowed to melt in an incubator at 60º C. Then tissues were allowed for paraffin infiltration at 60º C. This step is very important for proper sectioning.

Block Preparation

After infiltration, blocks were prepared with the help of molten wax. In this process, molten wax was poured carefully into the mold. The specimen was positioned properly and then bubbles were removed from wax with a red-hot needle. Wax was allowed to solidify completely and then it was separated carefully from the mold.

Trimming and Mounting of Paraffin Block

A thin layer of molten wax was applied on wood for complete adhesion before mounting block on the wood piece. Then the block was trimmed in a trapezoid shape with the help of a sharp blade and then mounted on a wooden block.

Sectioning of Blocks

Sections of the block were cut with microtomes. The blade was cleaned with xylene. The size of the sections was 4μm on the scale. Long stretched ribbons were obtained through microtome.

Mounting of Sections

After cutting, sections were mount on slides. These slides were arranged by adding a thin layer of Mayer's Albumin adhesive. Ribbons were allowed to stretch in warm water and shifted on slides.

Staining of Slides

After drying, sections were stained by using hematoxylin and eosin staining techniques by de-waxing, dehydration, and staining.

De-hydration

After complete staining, slides were dipped one by one in 70 and 90% alcohol for dehydration.

Clearing

Slides were treated again with xylene and then allowed to dry in a dust-free environment.

Study of Slides

After complete drying, microphotography was done.

Microphotography

Prepared slides were studied under a microscope at 40X for further studies.

Statistical Analysis

All the data was compiled and deciphered with the help of SPSS version 20. To compare the geometric mean and standard error of the control group and the other treatment groups, a one-way ANOVA was utilized.

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RESULTS AND DISCUSSION

Morphological Analysis

Measurement of Body Mass, Body Feed Consumption and Water Consumption

Figure 1 shows a significant decrease in body mass of Female mice F0-M dissected on gestation day 18 treated with ZnO NPs in a correlation with Orange juice administration. Figure 2 shows a significant decrease in body mass of Female mice F0-M(AP) dissected after parturition treated with ZnO NPs in a correlation with orange juice administration. Figure 3 shows a significant decrease in body feed consumption of Female mice F0-M dissected on gestation day 18 treated with ZnO NPs in a correlation with Orange juice administration .Figure 4 shows a significant decrease in body feed consumption of Female mice F0-M(AP) dissected after parturition treated with ZnO NPs in a correlation with Orange juice administration ..Figure 5 shows a significant decrease in body water consumption of Female mice F0-M dissected on gestation day 18 treated with ZnO NPs in a correlation with Orange juice administration. Figure 6 shows a significant decrease in body water consumption of Female mice F0-M(AP) dissected after parturition treated with ZnO NPs in a correlation with orange juice administration.

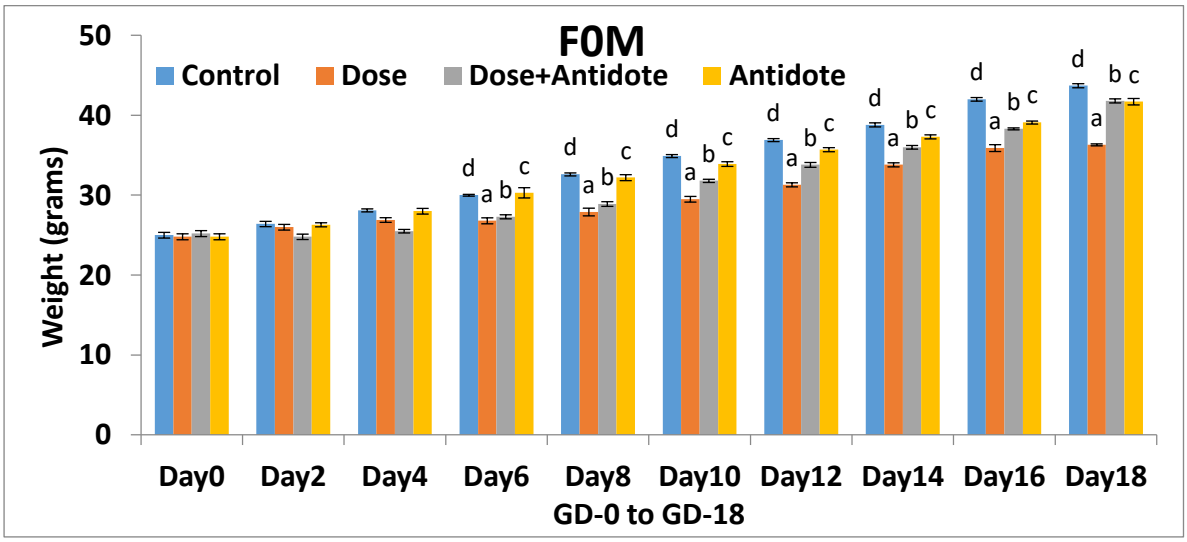

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Fig 1*:* Standard Mass of body of Pregnant Female Mice (F0-M) Sacrifice on GD-18 of all groups

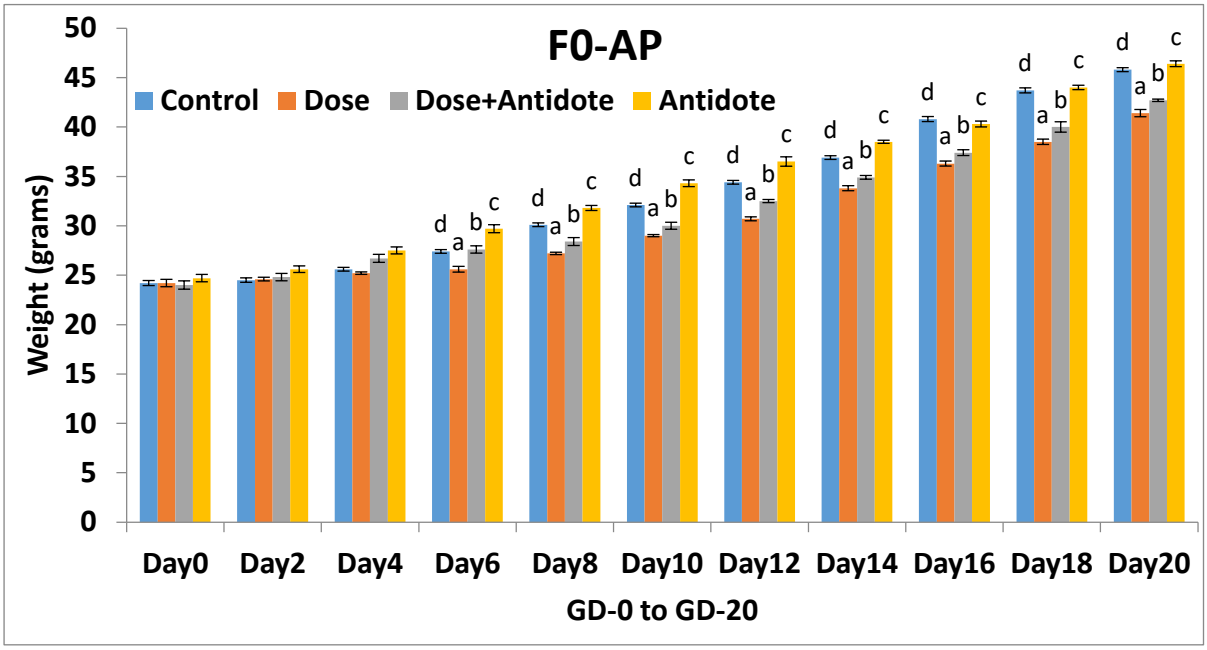

Fig.4.2*:* Standard Mass of body of Pregnant Female Mice (F0-M(AP) Sacrifice After parturition of all groups

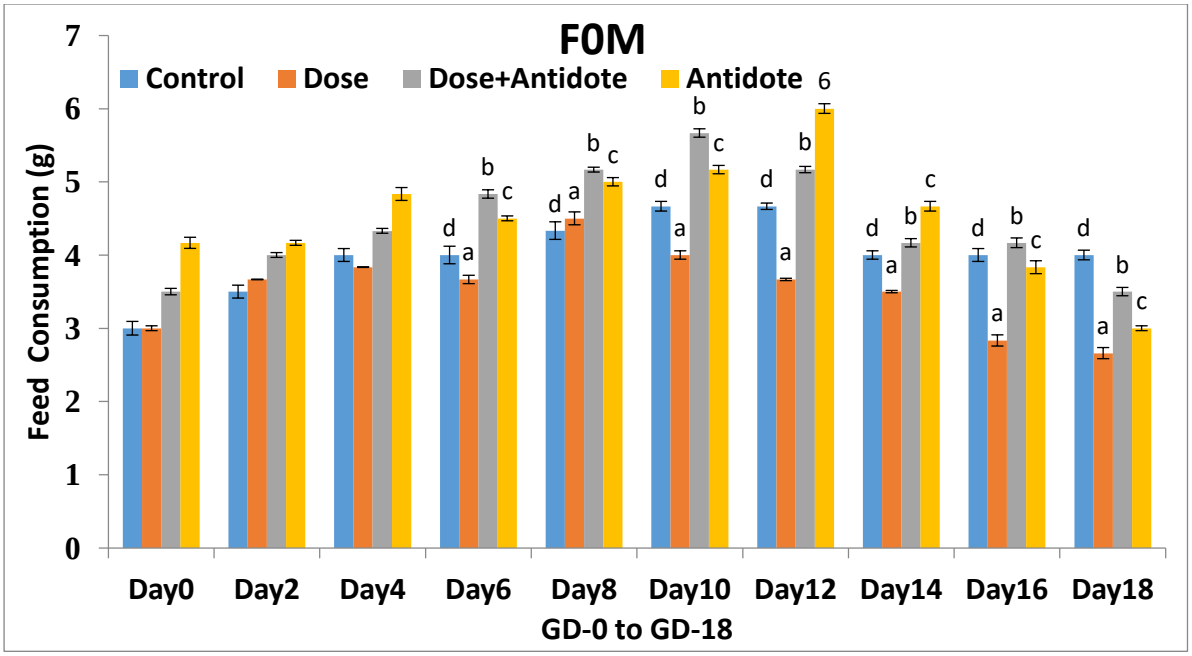

Fig.3*:* Standard Feed consumption of Pregnant Female Mice (F0-M) Sacrifice At GD 18 of all groups

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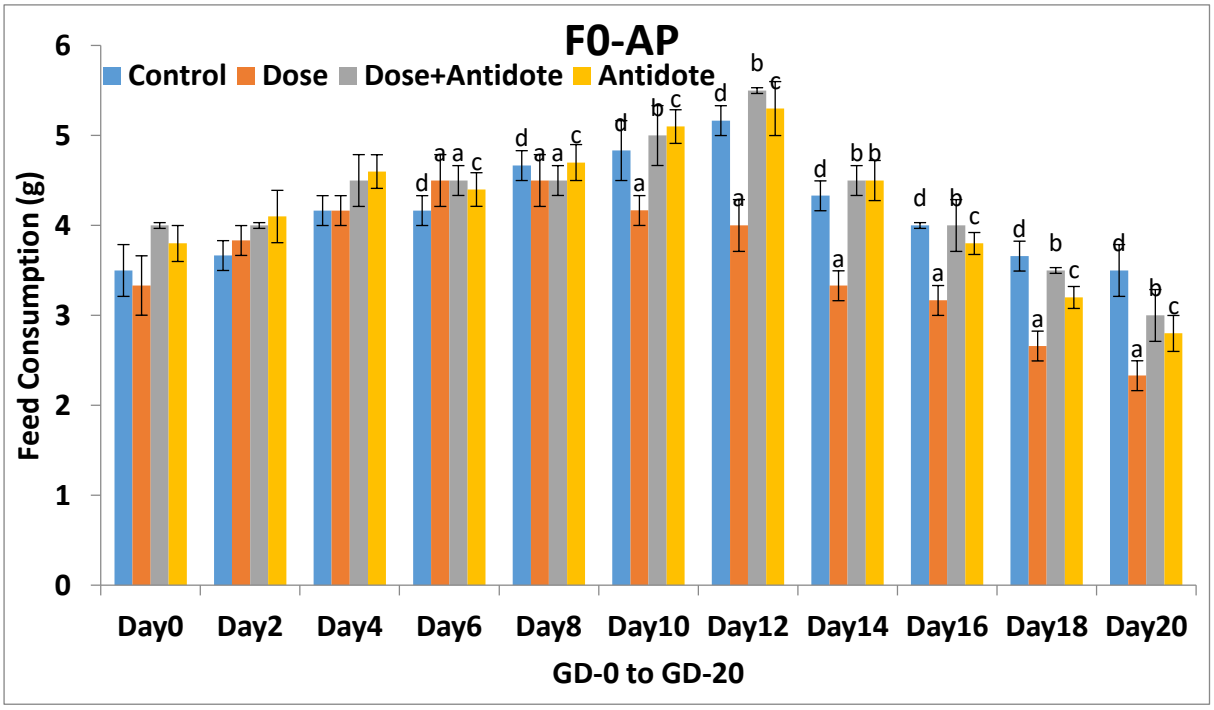

Fig.3*:* Standard Feed consumption of Pregnant Female Mice (F0-M(AP) Sacrifice after parturition

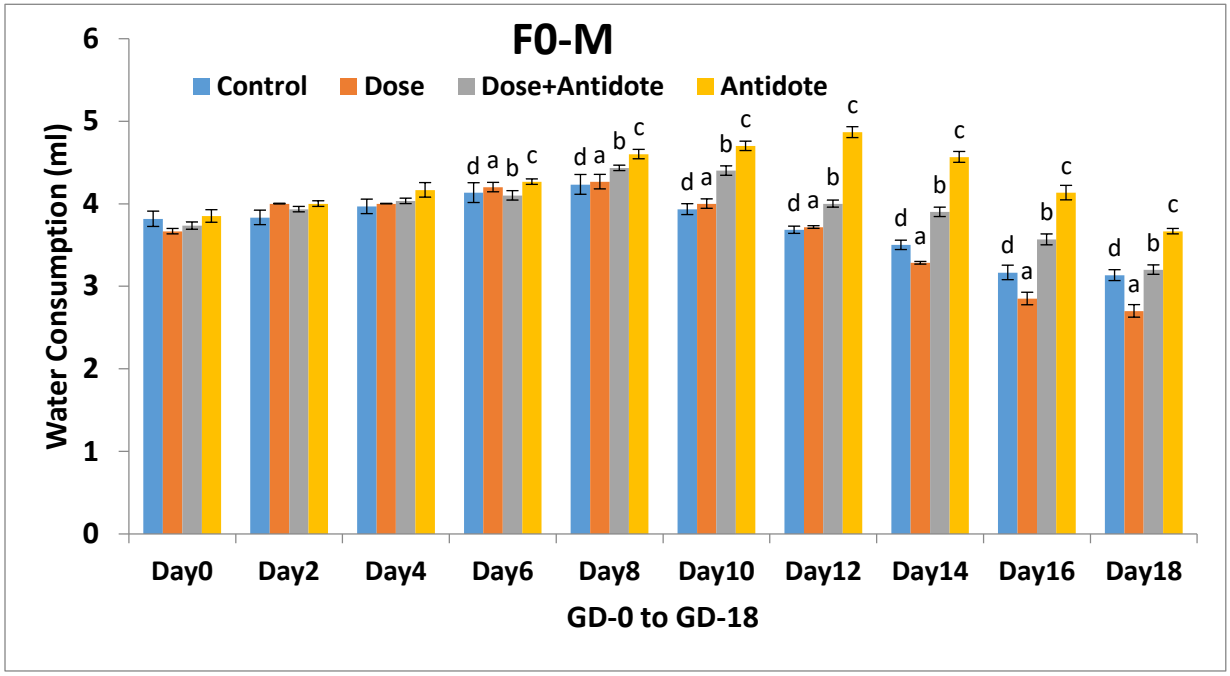

Fig.3*:* Standard Water consumption of Pregnant Female Mice (F0-M) Sacrifice At GD 18 of all groups

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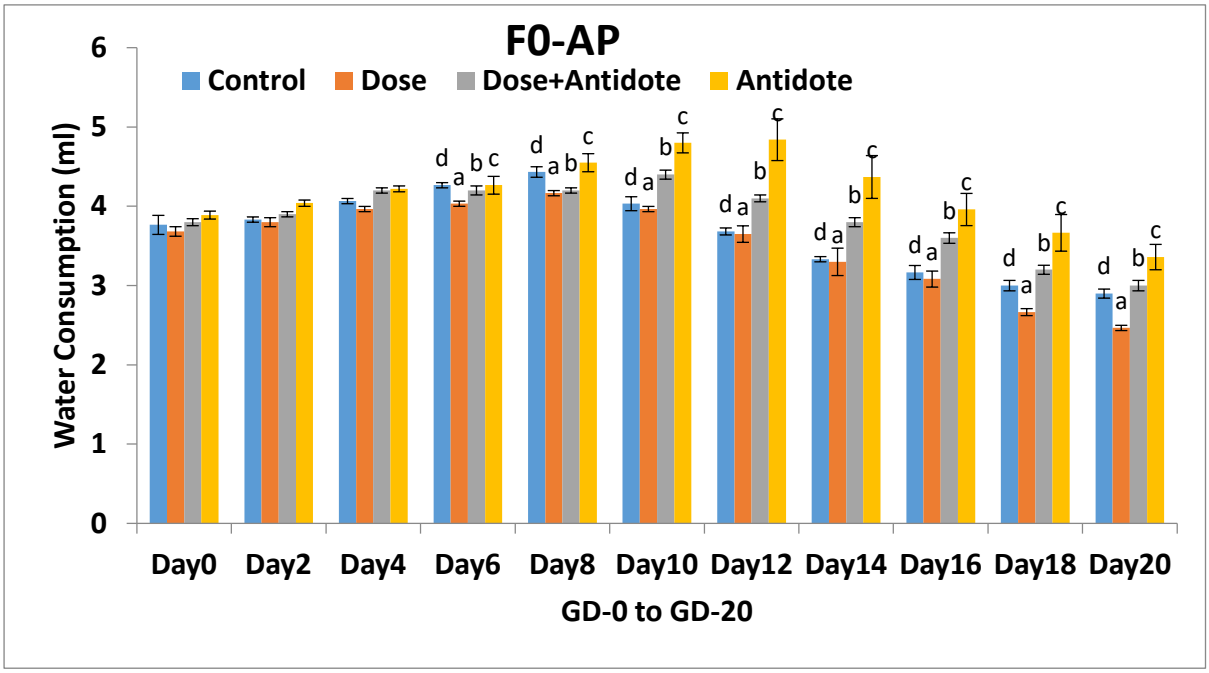

Fig.6*:* Standard Water consumption of Pregnant Female Mice (F0-M(AP) Sacrifice after parturition

Organ mass measurements and macrographs

Figure 7 shows the mean mass of female lungs given with ZnO-NPs move upright considerably as compared to Control and Antidote group. Figure 8 shows the Morphology of Lungs recovered from All groups.

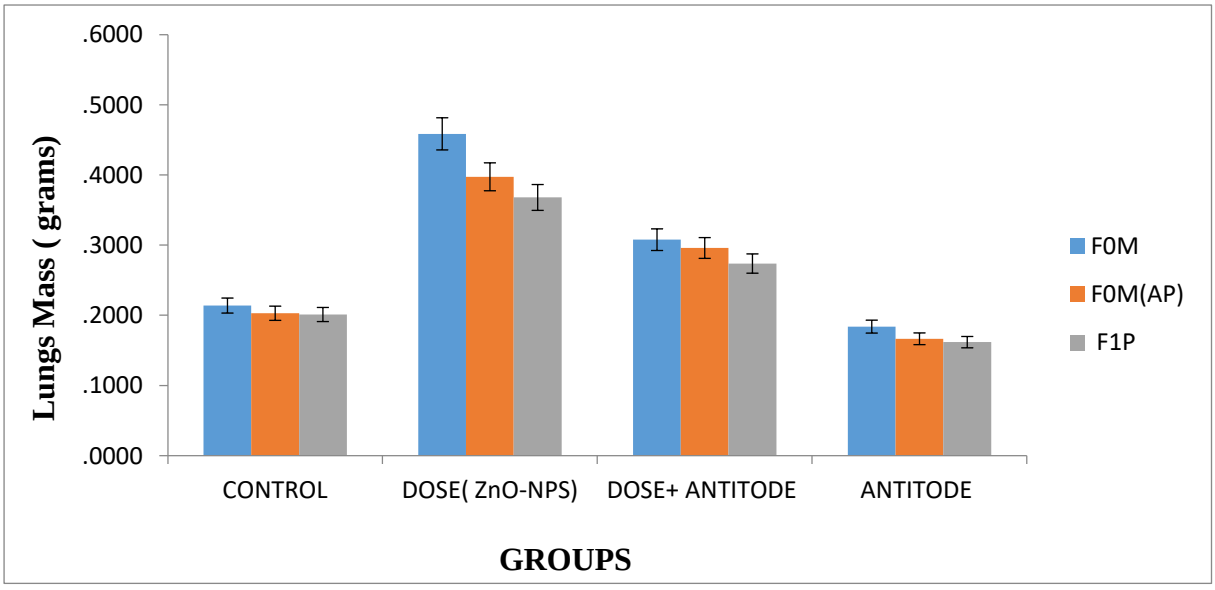

Fig 7: Standard mass of Lungs recovered from Mothers of all groups

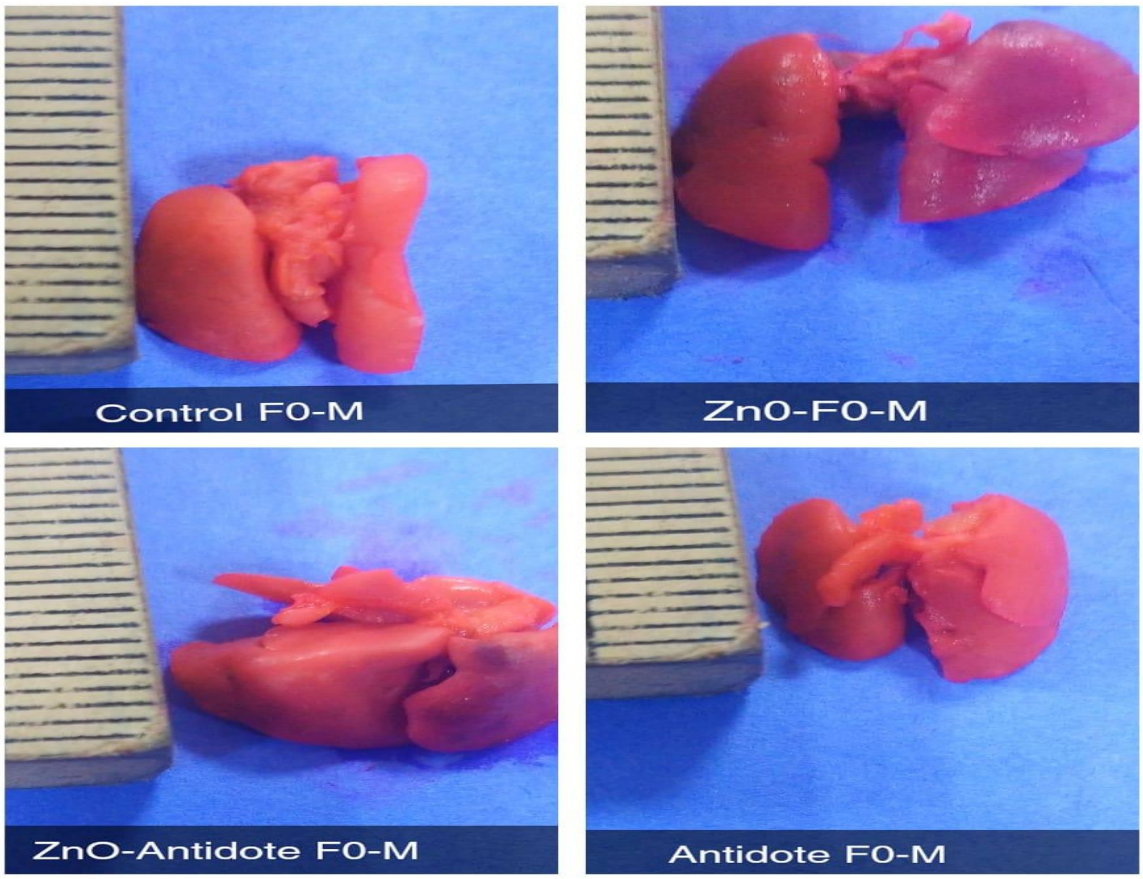

Fig 8: Macrographs of Lungs recovered from mothers of all groups

Foetus recovered from mothers of all groups

Figure 9 shows the fetus retrieved from the mother of the zinc oxide nanoparticles treated group had amelia, which is a birth abnormality in which one or more limbs are missing and the tail is short, when it was dissected on the 18th day of gestation Embryos from the ZnONPs and fresh juice treated displayed bleeding and hyperextension, indicating that the treatment was effective the fetus retrieved from mother of the orange juice treated group was found to have hyperflexion.

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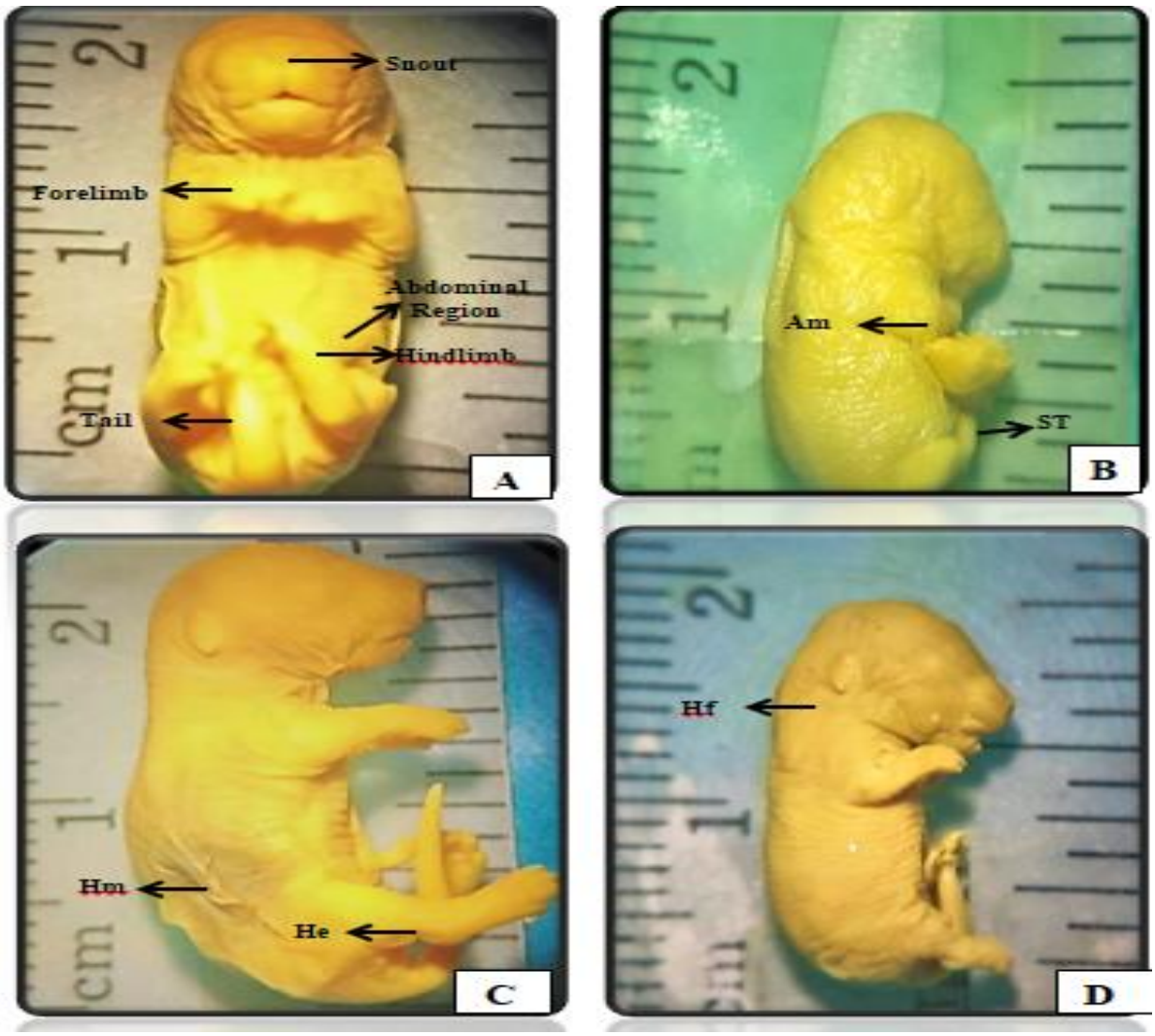
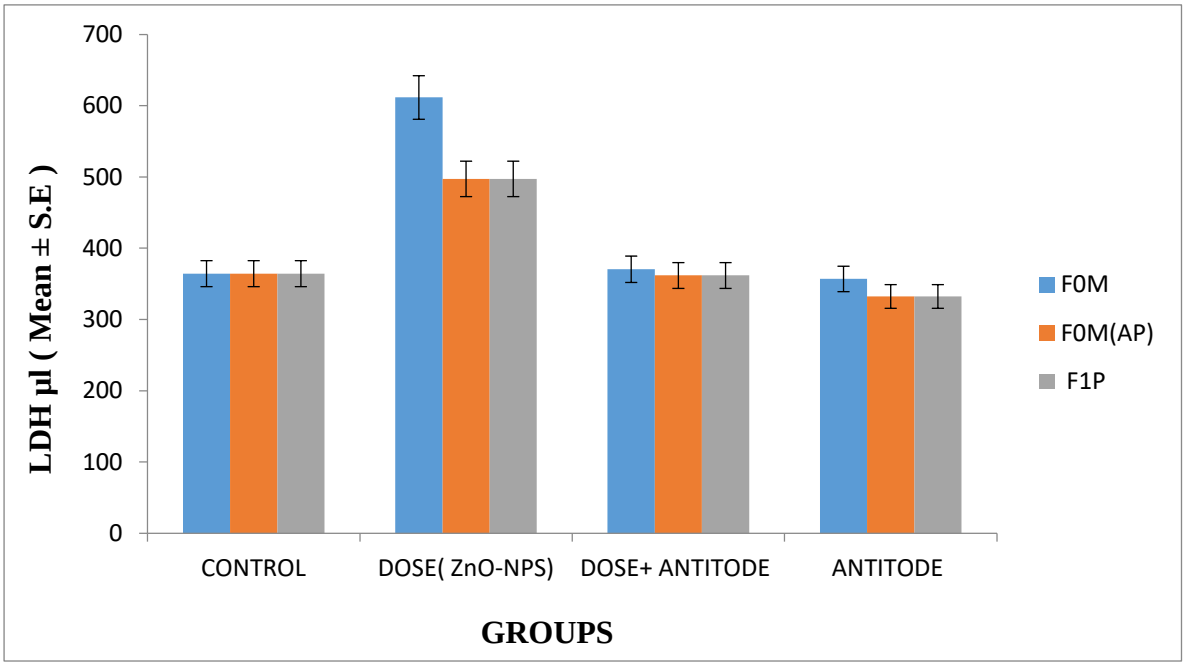


Fig 9: Micrographs of fetus **(A)** From control mother,**(B)** From ZnONPs mother, , **(C)** From ZnO & OJ treated mother **(D)**From Orange juice treated mother
Am: amelia, ST: short tail,Hm :hemorrhage, He:hyper-extension Hf : hyper flexion

Biochemical Analysis

Lactate Dehydrogenase Test (LDHT)

Figure 10 shows the elevated level of Lactate Dehydrogenase in ZnO NPs treated females as compared to control and Antidote group.



Complete Blood Count Analysis

Figure 11 shows Clustered Graph represents the complete Blood count of mice F0(dissected at day 18th of gestation) influenced by ZnO NPs administration along with Fresh orange juice and without fresh orange juice against control group. Superscripts were added by using different alphabets for showing significant differentiation among groups ($p \leq 0.05$).

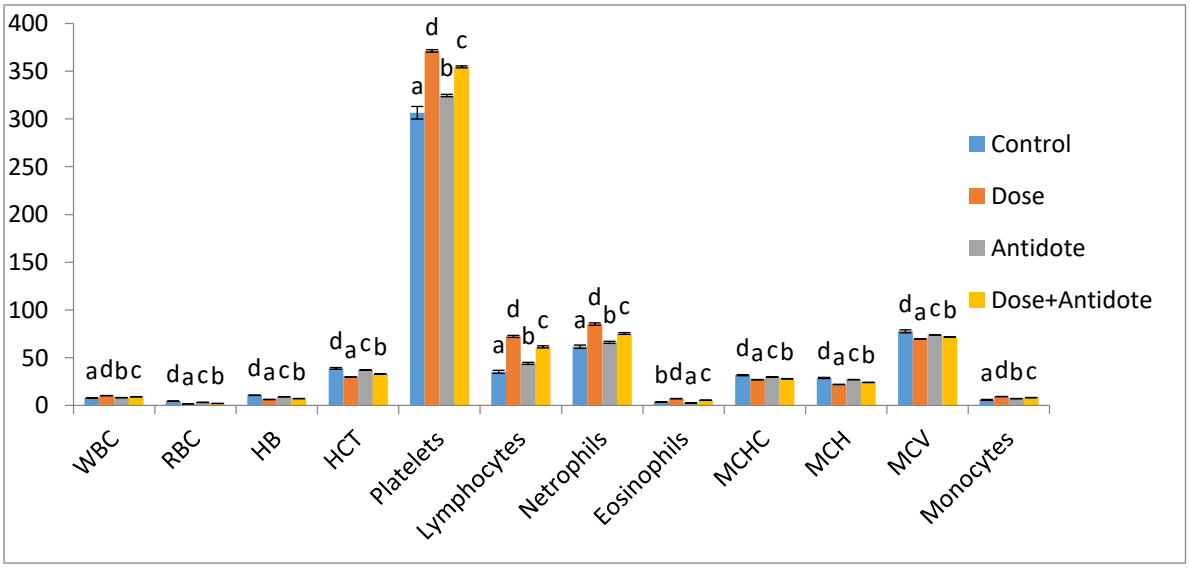


Figure 12 shows Clustered Grapgh of Complete Blood Count of F0 mice(dissected at post parturition week 8)influenced by ZnO NPs administration along with Fresh orange juice and without fresh orange juice against control group. Superscripts were added by using different alphabets for showing significance difference in all groups at ($p<0.05$) significance value.

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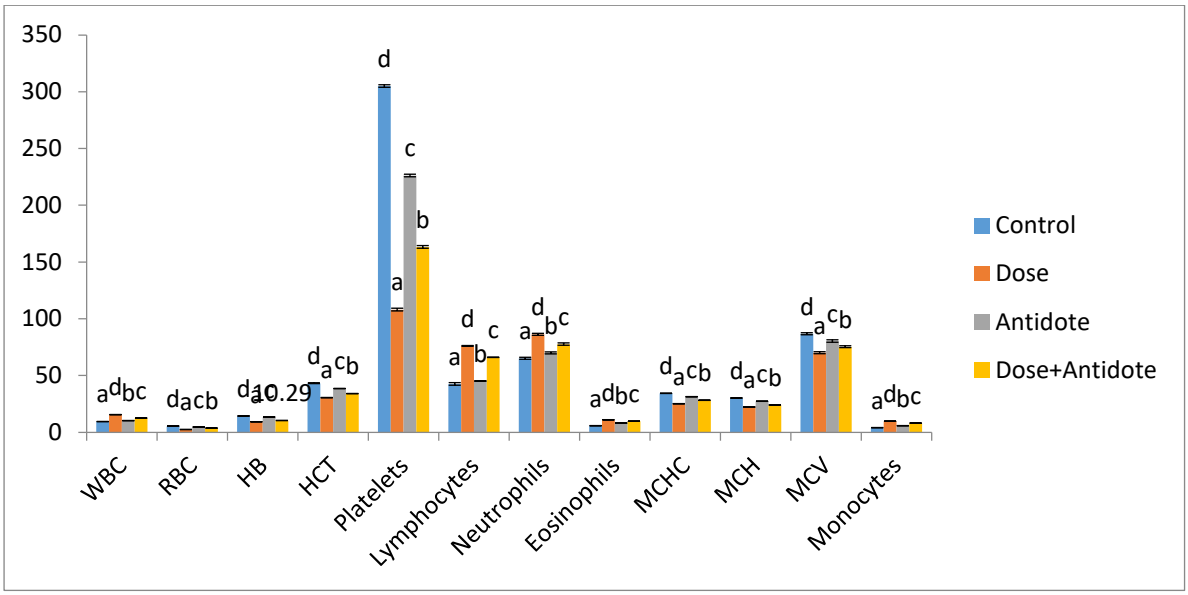

Figure 13 shows Clustered Graph of Complete Blood Count of F1 Pups influenced by ZnO NPs Prenatal administration along with Fresh orange juice and without fresh orange juice against control group. Superscripts were added by using different alphabets for showing significance difference in all groups at ($p<0.05$) significance value.

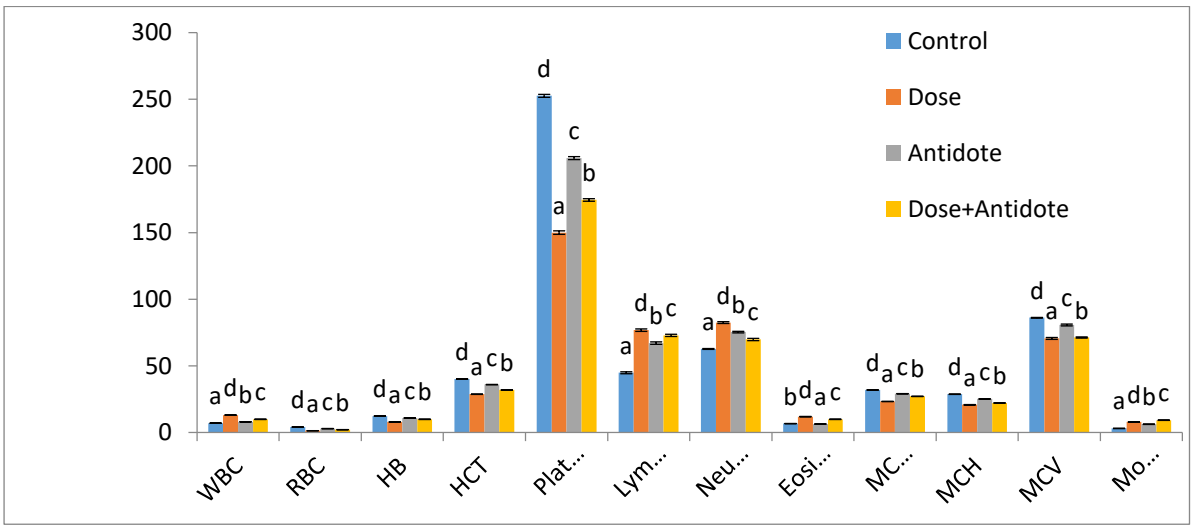

Table1:.L.D.H And Complete Blood Count Analysis of F0-M Mother sacrificed at GD-18 For all groups

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L.D.H\CBC	Control	Dose	Dose+Antidote	Antidote
L.D.H	364.4[a]±25.14	611.6 [b]±46.37	370.6 [ac]±17.1	357 [ad]±10.67
Haemoglobin(g/dl)	10.84±0.14[a]	9.50±0.18[b]	10.36±0.12[a]	12.16±0.14[a]
RBCs(µ/l)	5.63±0.06 [a]	4.29±0.05 [a]	5.10±0.11[b]	5.78±0.10 [a]
HCT (%)	41.84±0.74 **[b]**	28.26±0.60 [a]	34.70±0.23 [a]	44.42±0.72[b]
MCV (fl)	74.23±0.66 [a]	65.92±0.94 [b]	68.12±1.8[b]	76.36±0.45 [a]
MCH (pg)	19.24±0.26 [a]	22.14±0.28 [b]	20.52±0.54 [b]	20.88±0.16 [a]
MCHC(g%)	25.94±0.42[b]	33.56±0.24 [b]	30.20±0.08[a]	27.42±0.17 [a]
WBCs(µL)	4.90±0.15 [b]	9.14±0.30 [a]	5.92±0.20 [b]	5.02±0.10[c]
Platelets(µL)	671.20±13.76	785.40±17.63	688.4±8.21 [b]	1036.6±29.4 [c]
Neutrophils (%)	53.0±1.79 [c]	63.6±1.36 [a]	56.4±1.63 [c]	60.4±1.89 [b]
Lymphocytes (%)	34.60±1.08 [b]	30.80±1.11[a]	35.60±1.08[b]	30.60±2.08 [a]
Mononcytes (%)	7.80±0.37 [a]	3.20±0.37 [c]	5.0±0.45 [b]	5.40±0.25 [a]
Eosinophils (%)	4.20±0.37 [c]	1.80±0.49 [c]	2.60±0.25 [b]	3.0±0.32 [a]
Basophils (%)	0.80±0.20 [a]	0.60±0.25 [b]	0.40±0.25 [b]	0.50±0.25[c]

Table 2: L.D.H And Complete Blood Count Analysis for F0-M(AP) Mother sacrificed after parturition

L.D.H\CBC	Control	Dose	Dose+Antidote	Antidote
L.D.H	364.4[a]±25.93	497.4[b]±16.29	361.8[ac]±15.1	324 [ad]±16.63
Haemoglobin(g/dl)	10.70±0.29 [a]	27.20±17.70 [b]	10.36±0.12 [b]	11.40±0.12 [b]
RBCs(µ/l)	5.89±0.11 [a]	4.62±0.08 [a]	4.92±0.12 [b]	5.99±0.06 [a]
HCT(%)	43.42±0.55 [a]	32.04±0.77 [b]	32.12±0.75[a]	45.34±0.50
MCV(fl)	73.62±0.72 [a]	64.02±0.69 [b]	65.48±0.76 [b]	75.62±0.54 [a]
MCH(pg)	18.16±0.17[b]	20.66±0.24 [a]	20.52±0.54 [b]	20.88±0.16 [a]
MCHC(g%)	24.62±0.49 [b]	32.24±0.12 [a]	32.34±0.46 [b]	25.22±0.19 [a]
WBCs(µL)	5.70±0.15 [b]	8.10±0.14 [a]	5.28±0.28 [b]	5.10±0.26 [a]
Platelets(µL)	586.0±21.92 [b]	749.40±21.03 [a]	667.80±16.97 [b]	959.0±21.41 [a]
Neutrophils (%)	62.80±1.56 [a]	59.0±1.52 [b]	54.8±1.07 [a]	49.8±0.86 [b]
Lymphocytes (%)	26.0±0.84 [a]	32.40±0.98 [b]	35.80±1.20 [a]	37.20±0.58 [b]
Mononcytes (%)	7.20±0.58 [a]	4.80±0.86 [b]	5.60±0.68 [a]	8.0±0.32 [b]
Eosinophils (%)	3.40±0.51 [b]	3.0±032 [a]	3.20±0.49 [b]	4.40±0.40[a]
Basophils (%)	0.60±0.25 [b]	0.80±0.20[b]	0.80±0.20 [a]	0.40±0.24[b]

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Table.3: L.D.H And Complete Blood Count Analysis for F1 Pups from F0-M(AP) Mother sacrificed after parturition

L.D.H\CBC	Control	Dose	Dose+Antidote	Antidote
L.D.H	364.4[a]±25.93	497.4 [b]±16.29	361.8 [ac]±15.09	332.4 [ab]±16.63
Haemoglobin(g/dl)	11.64±0.25 [b]	9.70±0.20 [a]	10.22±0.15 [a]	11.80±0.16 [b]
RBCs(µ/l)	5.40±0.12 [b]	4.57±0.13 [a]	4.71±0.08 [a]	5.91±0.07 [b]
HCT (%)	39.50±0.97 [b]	29.94±0.78 [a]	32.56±0.25 [a]	45.92±0.43 [b]
MCV (fl)	73.11±0.48 [b]	65.46±0.50 [a]	68.44±0.84 [a]	77.72±0.67 [b]
MCH (pg)	21.56±0.04 [b]	20.52±0.94 [b]	21.79±0.50 [a]	19.90±0.23 [b]
MCHC(g%)	29.47±0.147 [a]	32.40±0.29 [b]	31.70±0.48 [a]	25.70±0.15 [b]
WBCs(µL)	5.86±0.36 [a]	7.46±0.22 [b]	6.50±0.23 [a]	4.36±0.13 [b]
Platelets(µL)	483.0±13.16 [a]	762.6±14.53 [b]	657.6±14.84 [a]	839.2±14.52 [b]
Neutrophils (%)	61.80±1.88 [a]	60.40±0.93 [b]	49.80±2.73 [a]	48.60±1.08 [b]
Lymphocytes (%)	27.60±1.29 [a]	30.60±1.29 [a]	37.40±1.47 [b]	37.20±0.86 [b]
Mononcytes (%)	6.60±0.51 [a]	5.80±0.66 [a]	7.20±0.58 [b]	8.20±0.37 [b]
Eosinophils (%)	3.20±0.37 [a]	2.60±0.25 [a]	4.20±0.58 [b]	5.0±0.63 [b]
Basophils (%)	0.80±0.20 [a]	0.60±0.25 [a]	1.40±0.40 [bs]	1.0±0.01 [b]

FRAP and TPC analysis

Figures 14,15 And 16 show the FRAP AND TPC Analysis to determined antioxidant capacity of Lungs of all groups and Fresh orange juice. There is co-relation between ZnO NPs treated group and Antidote group.

Table 4,5 and 6 show FRAP Analysis for Lungs of Mothers of all groups.

Parameters FRAP (N=10)	**Capacity of antioxidants in Lungs of Mother sacrificed on GD-18 Mean ± S.E.M**
Control	163.33[a]±4.02
Dose	89.2[b]±4.93
Dose+ Antidote	181.0 [c]±3.52
Antidote	175.6 [ad] ±2.66

Parameters FRAP(N=10)	**Antioxidant capacity of Fetus Lungs recovered from Mothers of all groups on GD-18 Mean ± S.E.M**
Control	167 [a]±4.16
Dose	92.4[b]±3.23
Dose + Antidote	186.6 [ac]±2.89
Antidote	167.4 [d]±1.29

Parameters FRAP(N=10)	**Antioxidant capacity of Fetus Lungs recovered from Mothers of all groups on GD-18 Mean ± S.E.M**
Control	167 [a]±4.16
Dose	92.4[b]±3.23
Dose + Antidote	186.6 [ac]±2.89
Antidote	167.4 [d]±1.29

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Parameters FRAP(N=10)	Capacity of Antioxidants in Lungs of Mother (who gave natural birth to pups) Mean ± S.E.M
Control	167.4 [a] ±5.63
Dose	95.2 [b] ±3.65
Dose + Antidote	188 [c] ±3.92
Antidote	185.78 [d] ±2.34

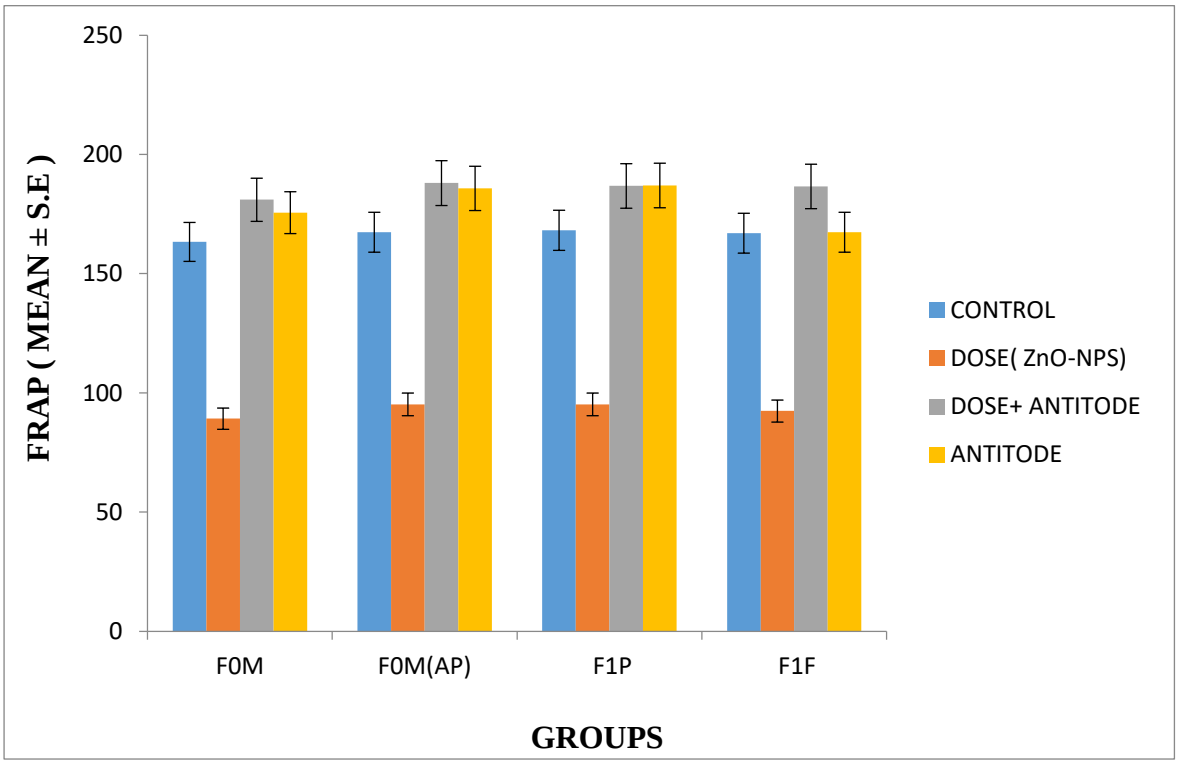

Fig 14: Antioxidant Capacity of Lungs Recovered from Mothers of all groups

Table 7: FRAP and TPC capacities in fresh orange juice

Sample	Parameters	Antioxidant capacity
Orange juice	FRAP	265.10±0.06 µM Ascorbic acid
Orange juice	TPC	3.101±0.0041 µg gallic

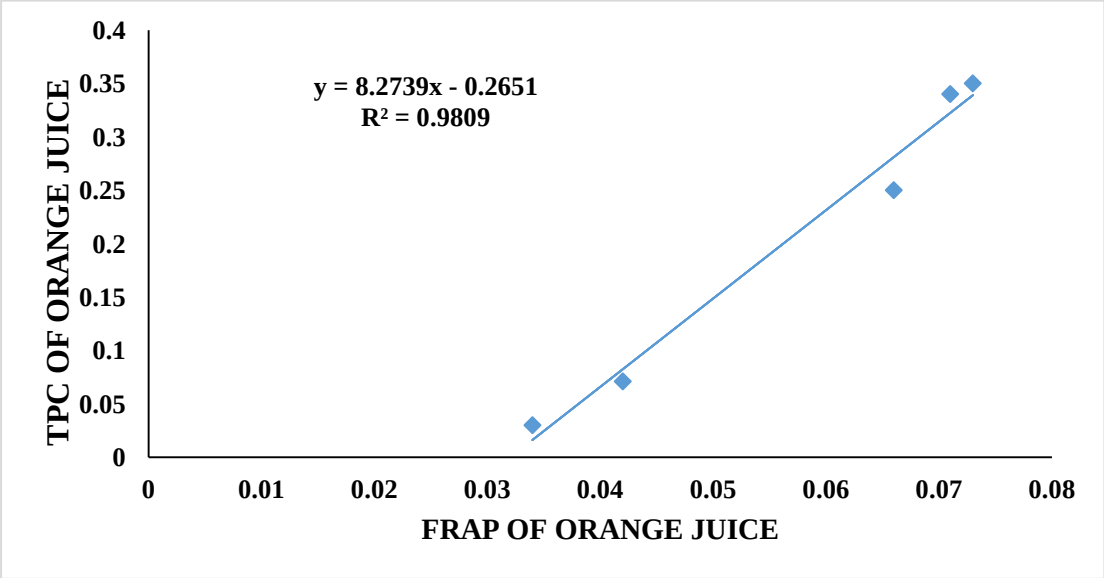

Fig.15: Relation of T.P.C AND F.R.A.P 0F FRESH JUICE

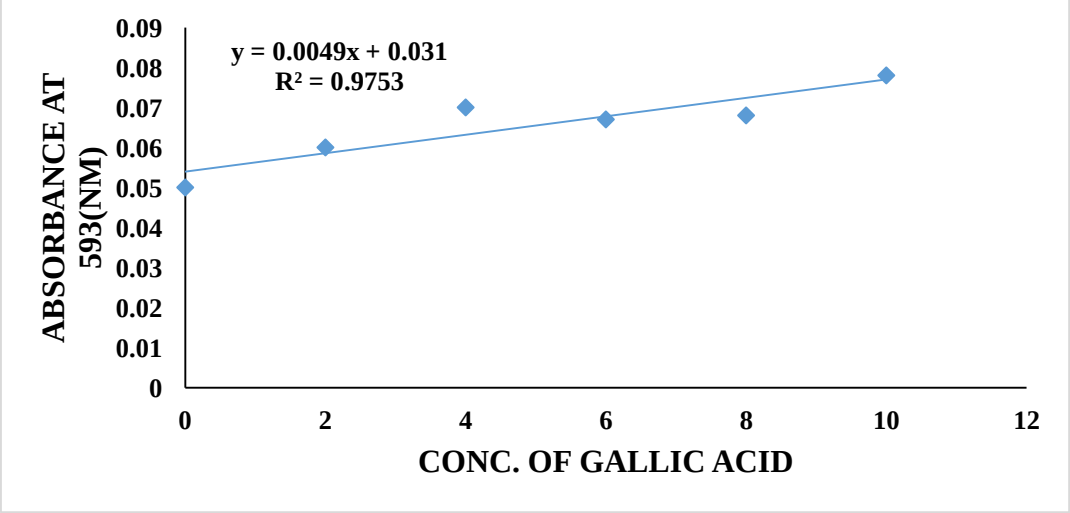

Fig 16: Plotted standard curve shows the absorbance and concentration of Gallic acid

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Histopathological analysis

Histology of lungs tissues was performed under standard conditions by using histological stains. The histology of lungs tissues was examined under 40X of Compound microscope. Figure 17,18,19 And 20 shows lungs tissues histology

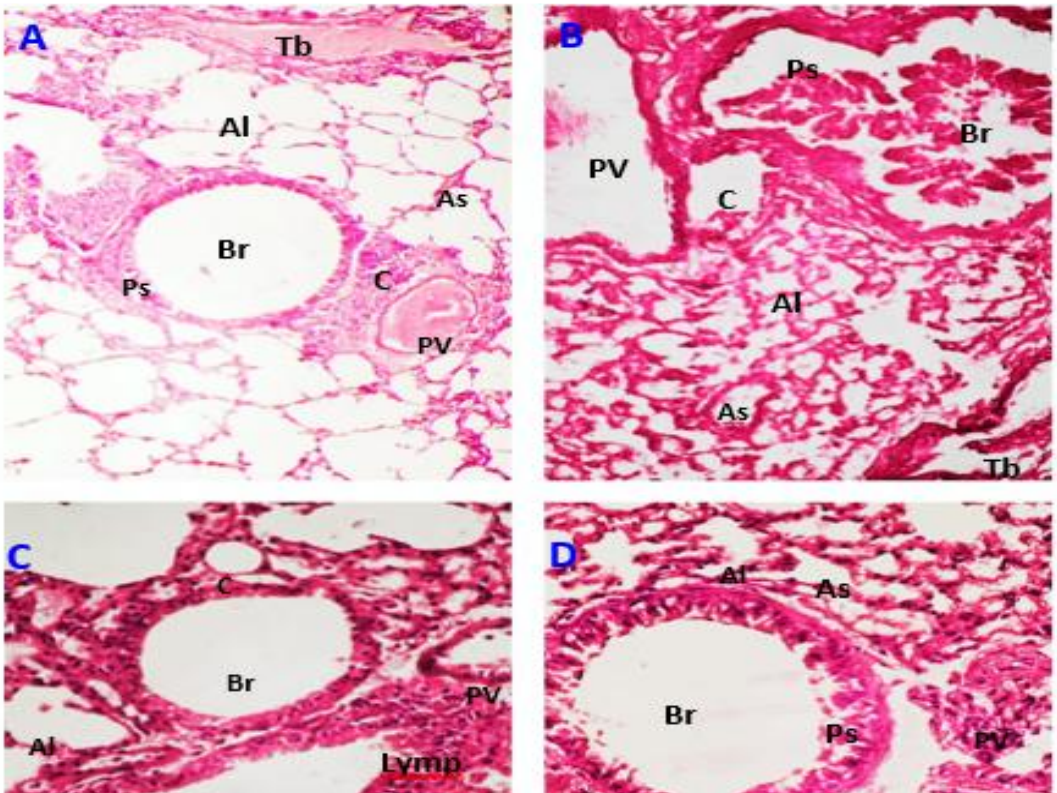
Histopathological analysis of lungs tissues of Mothers of control group showed normal structure of bronchi, cartilage, pulmonary vessel, alveolus, alveolar septum, alveolar spaces, terminal bronchioles and small amount of mononuclear inflammatory cells in the peribronchiolar space (Fig 17,18,19 And 20 **A**).

From ZnO-NPs treated mother, recovered lungs tissues indicated the distorted structure of pulmonary bronchi, peribronchiolar interstitial infiltration, congestion of pulmonary vessel, perivascular infiltration, irregular cartilage, thickening the alveolar walls that lead to reduction in alveolar space and diffused terminal bronchiole (Fig 17,18,19 And 20 **B**).

From ZnO-NPs and fresh orange juice treated mother's lung tissues showed defined structure of bronchi with less peribronchiolar interstitial infiltration, regular shape of pulmonary vessel with little mononuclear inflammatory cells, reduced alveolar structure with defined alveolar septum and alveolar spaces, normal terminal bronchiole with short length (Fig 17,18,19,20 **C)**.

Histopathological sections of lungs recovered from orange juice treated mother which revealed normal structure of bronchi with less accumulation of leukocytes, peribronchiolar space with less differentiated infiltration, congested pulmonary vessels with perivascular infiltration, normal cartilage, alveolar structure with normal septum and reduced alveolar spaces and normal terminal bronchioles (Fig 17,18,19 And 20 **D**).

Histopathological analysis of lungs of F1 mother dissected on GD-18



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Histopathological Analysis of Fetus Lungs of Mother (dissected on 18th day of gestation period) from All Group

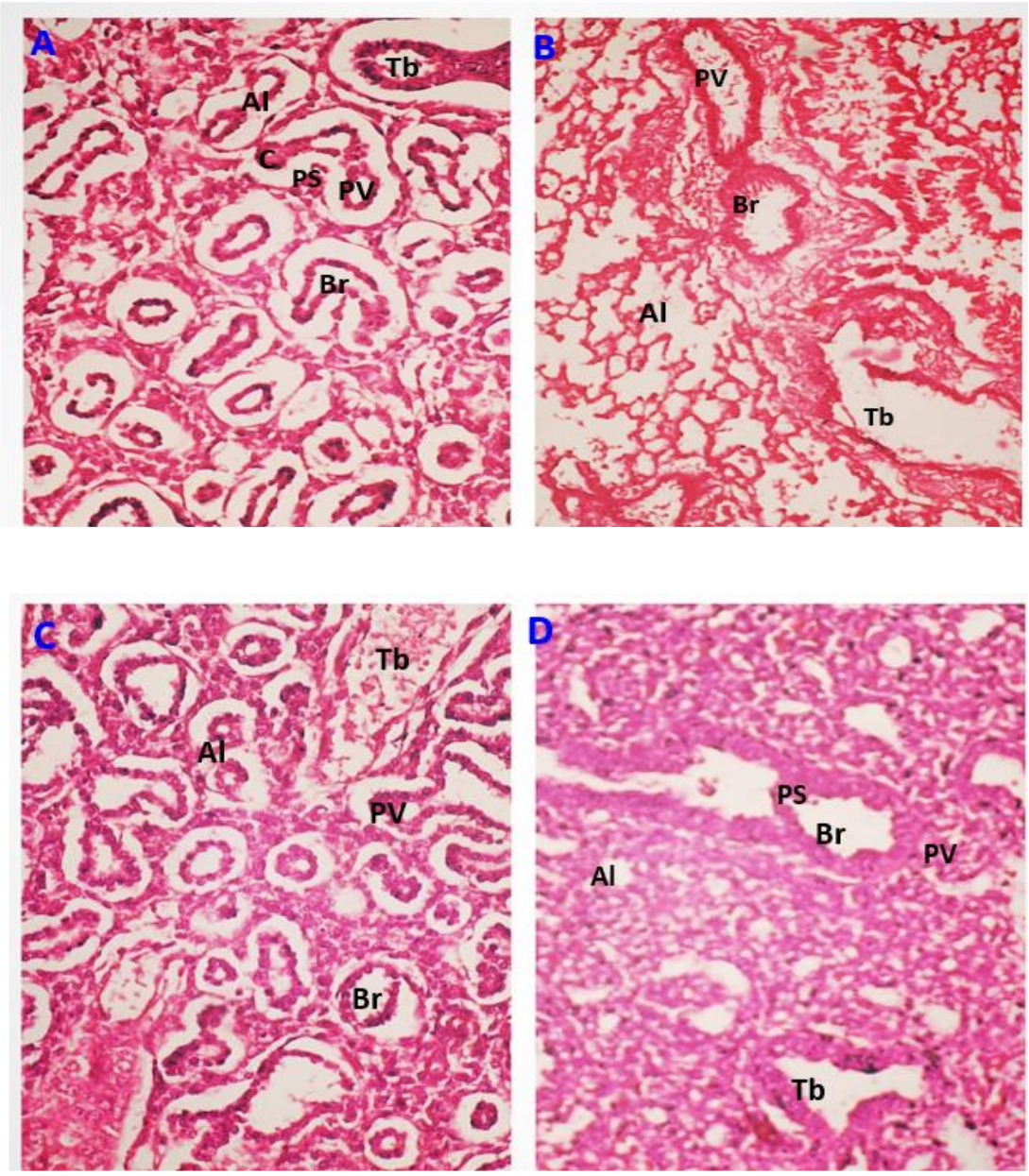

Fig 18: (A) Pups with control(40X) (B,) Pups with Dose (C)Pups Dose+Antidote (D) Pups with Antidote Bronchi (Br) Alveolus(Al) Alveolar septum,(As) Peribrochiolar space (Ps) Pulmonary vessel (PV) Tb=Terminal bronchiole

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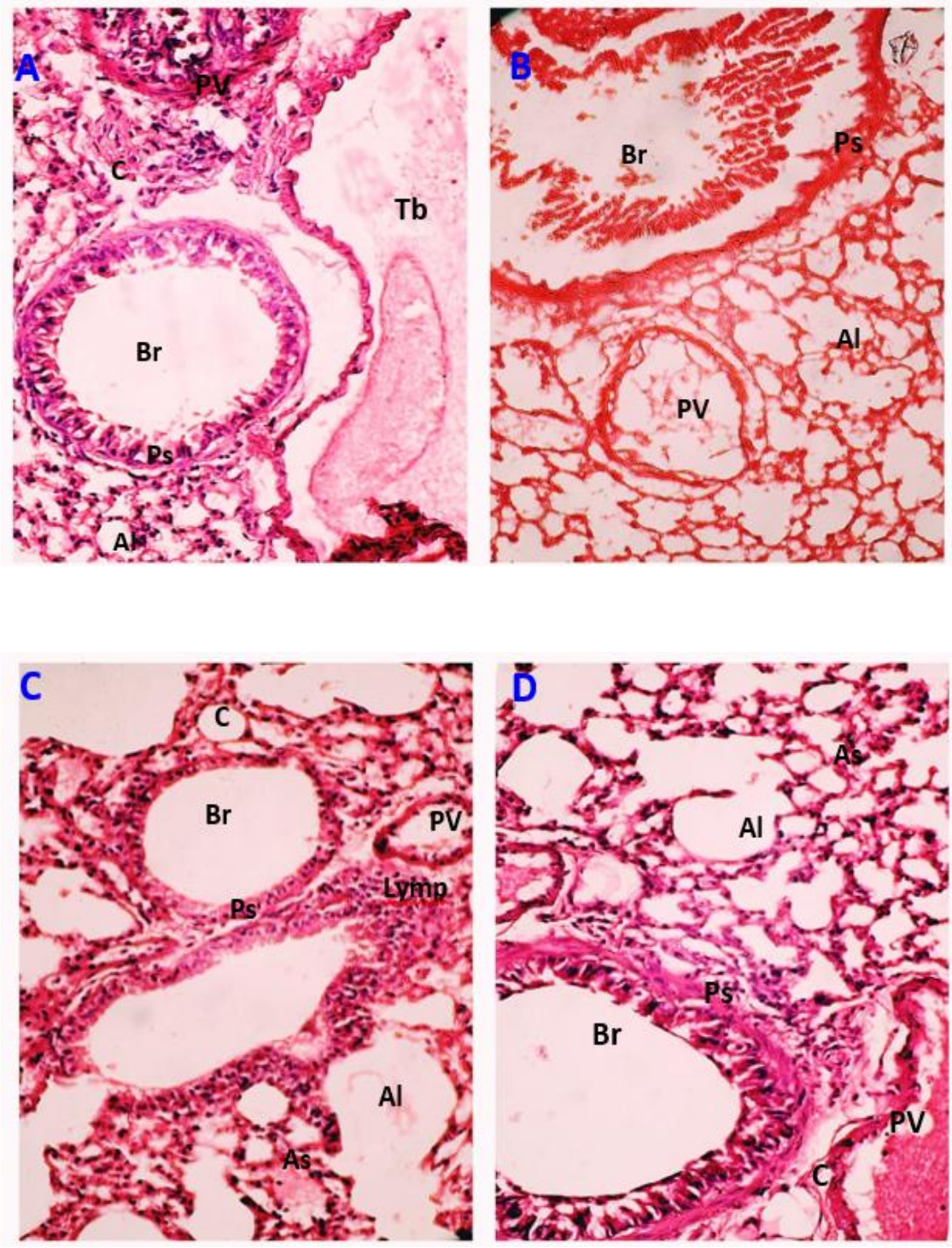
Histopathological Analysis of Lungs of F0-M (AP) Mother sacrifice after parturition from All Groups



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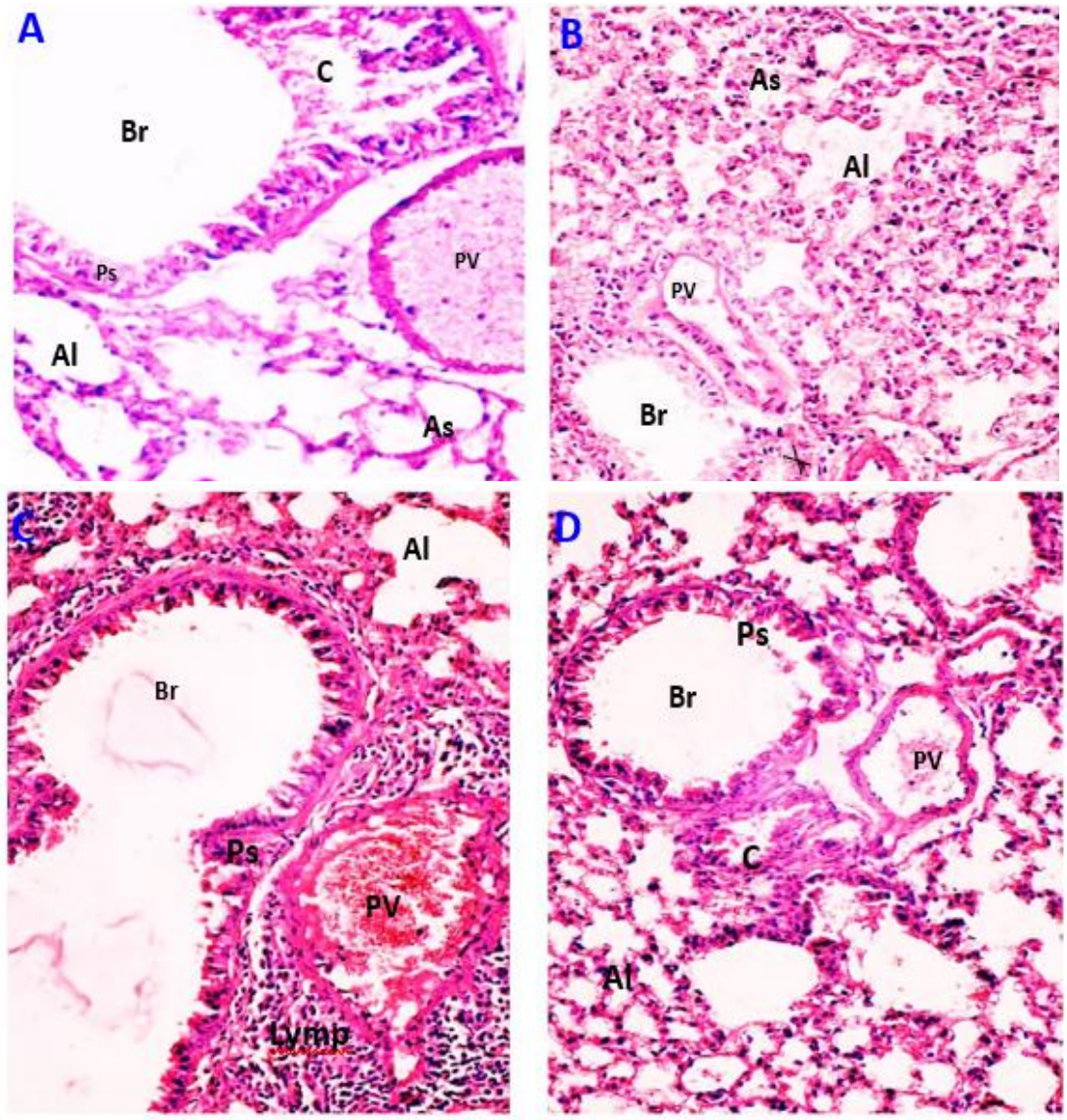
Histopathological Analysis of Pups Lungs of F0-M (AP) Mother sacrifice after parturition from All Groups



C=Cartilage Lymp=Lymphocytes

DISCUSSION

Nanoparticles are small particles (less than 100 nm) exhibiting specific characteristics when comparison to their bulk counter parts (Kabir *et al.*, 2018). In medicine, environmental remediation, pharmacology fields, electronics, veterinary, and the renewable energy are just a few of the applications for nanoparticles (NPs) (Schirmer & Auffan, 2015). Because of their vast production and uncontrolled discharge, NPs have become important in obtaining a complete and mechanistic

understanding of ecosystems (Ke & Lamm, 2011). ZnO nanoparticles toxic effects are as membrane damage, inflammation, damage of DNA, apoptosis, and also other consequences such as complicated cell to cell and the cellular matrix of interactions, along with alteration in specific hormones, have all been demonstrated to be hazardous to mammalian cells during the in vitro and in vivo investigations (Osman *et al.*, 2010). Whereas zinc oxide nanoparticles can pass through body via the GI tract or the respiratory systems, it is not known if they can reach the bloodstream or organ systems such as the liver (Hussain *et al.*, 2001; Bilal, 2021)

ZnO nanoparticles suppress the ability of females to reproduce by causing damage to their genital system. They also have an influence over the development of the fetus before it is born (Kulvietis *et al.* 2011). Toxicology during development process has been identified as a critical component of all studies, according to the scientific community. According to the authors, ZnO-NPs are effective in overcoming the placental barrier because of their nano size (Zhao *et al*.2016). Body mass is crucial in determining the toxic effects of a chemical, and the body weight of Dose treated female mice (F0-M sacrificed on the GD-18 and F0-M (AP) sacrificed after parturition) showed a significant decrease when contrast to the sampler and OJ groups, but the results showed a non-significant comparison when treated with Dose+Antidote respectively, when we made comparison to the control and antidote. The accumulation of ZnONPs in the female reproductive tract has been shown recently, resulting in reproductive harm, which is an agonist to the research questions posed by (Zhai *et al*.,2018) The most critical period of pregnancy is the organogenesis stage, during which organ development begins. It has been shown that ZnONPs exposure to pregnant females results in a reduction in body mass (Lee *et al*.,2016), that is consistent with my observations. Other research has shown that pregnant females that are exposed to ZnONPs had lower body weights than their offspring, according to another study (Jo *et al*.,2013). The weight of pregnant female mice was shown to be lowered following oral administration of ZnO NPs therapy, as demonstrated in my research findings. The findings of Wang *et al*., 2017 detail the treatment of mice with ZnO NPs for a period of three weeks, which resulted in a reduction in body mass in the mice.

The weight of organ in mice shows great value in short term exposure of toxic chemicals. The ZnO NPs exposure changes the organ weight, which was measured by their mode of action, metabolism and toxicokinetic. My study the morphometrics analysis of lungs of treatment groups showed increase in size and body mass of organ. The present study reported the increase in weight of organs in ZnO NPs treated groups and no change in size and in Antidote treated groups as related to the control. Findings of my study are agonist with study of Choi *et al*. Co-relate effects of ZnO NPs and $ZnSO_4$ with equal amounts and justify that both cause embryological re-tardations. The lungs weight of F0-M and F1-P was increased

significantly by maternal exposure to ZnO NPs. These results matched sufficiently with early study that ZnO NPs increased the lungs weight (Sharma *et al*.,2012).

Fetuses recovered from ZnONPs female mice sacrificed on GD-18 showed change in morphology distortion as contrast to sampler and OJ treated groups. There is no disturbance in structure and morphology shown by Control and Antidote groups. My study suggests that ZnO NPs could produce toxic effects on pup's lungs. (Filippi *et al*., 2015).

There were no standard fluctuations in body weight between the F0-M(AP) mothers of all three groups, however there had been a substantial uptake in lungs mass removed from dosed F0-M Mothers antagonist to the normal and antidote groups. Previous research has described the method of ZnONPs caused toxic effect into the upcoming generation, i.e. the productions (ROS), and ZnONPs have been referred to be teratogens in recent studies because they produce pulmonary Toxic effects (Zhao *et al*.,2016). It was shown that ZnONPs may readily pass the placenta by generating ROS and causing harm to the next generation of children and adults (Chen *et al*., 2020).

The biochemical analysis of lungs includes Lactate dehydrogenase level and complete blood count analysis. It showed that L.D.H level increased in ZnO NPs treated groups indicate high level of infections in the mice body because of more absorption of ions by toxicity induced organs. L.D.H levels remained almost same in Fresh Orange juice treated group.

The levels of hemoglobin, HCT, MCV, lymphocytes, monocyte and eosinophils significantly decreased in ZnO NPs treated groups as compared to Fresh orange juice treated groups and the control, showed degree of toxicity and range of infections with in the body due to low level of immunity .There is high level of MCH, MCHC, WBC, platelets and neutrophils in dose treated groups showing the infection and toxicity as compared to other groups. High blood count of WBC describes the infiltration and toxic effect of ZnO NPs in pregnant female mice. My study behaving agonist to the recent study showing pulmonary macrophages clear ZnO NPs in alveoli (Madl and Pinkerton *et al*.,2009). High production of leucocytes (WBC and neutrophils) cause production of granuloma, pro-inflammatory cytokines, ROS and chemokines which co-related with study of granuloma development in lungs and peritoneal cavity by Khan et al. (2022).

Histopathology of lungs of ZnO NPs treated mothers showed a significant distortion and disturbance in lungs structure especially pulmonary bronchi, peribronchiolar interstitial infiltration, pulmonary vessel, alveolar septum, alveoli, alveolar spaces and terminal bronchiole. My results are compared to the study of potential pulmonary pathogenicity consequences by (Pauluhn, 2014). ZnO NPs are reached to be throughout the lungs deposit, reaching towards alveolar parts in lungs (Hoet *et al.,* 2004). Infiltration of peribronchiolar wall and perivascular wall with

mononuclear phagocytic cells show resemblance with study that showed insoluble ZnO NPs are difficult to dispose of have been connected to the emergence of dangerous effects (Sattar et al., 2024). Reduction in alveolar spaces, distortion of alveolar septum in ZnO NPs treated females' mothers justified by study of inhalation of ZnO NPs cause mucociliary clearance, surface integration, macrophage engulfment, and the penetration through to the tissues of lungs, translocate or remove materials that collect on airway surface (Oberdorster *et al*., 2005).

The fruit extract orange juice is widely utilized all over the globe (Meléndez-Martínez *et al.,* 2007), and it is particularly popular in Europe. Taking fresh natural juices has been shown to minimize oxidative stress while also improving blood cholesterol levels, which helps to prevent platelets from clumping together in the bloodstream (Hallfrisch *et al.,* 1994). Fresh juices are increasingly being used to manage oxidative stress in the body (Tonin *et al.,* 2015). Because of the presence of bioactive components with antioxidant properties, such as vitamin C, this protection is provided against oxidative stress. Vitamin C is the most abundant water-soluble antioxidant in the body and helps to antioxidant defense against oxidative stress. This fact is associated with its capacity to operate as a reducing agent as well as an oxygen scavenger in a variety of situations (Kitts, 1997). Citric acid, gallic acid, ferulic acid, vitamin A and C, naringenin, hesperidin's, and folic acid are all found in fresh orange juice, along with other nutrients (Arabi *et al.,* 2017). When tested for antioxidant capacity using the FRAP and TPC assays, fresh orange juice was found to be a powerful antioxidant. Similar findings were reported by Stella *et al*. (2011) who casually refer to the relationship between antioxidant capacity and the amount phenolic content in Fresh OJ.

Present study exhibited that administration of zinc oxide nanoparticles to the pregnant females at the time of organogenesis induced teratogenicity and transgenerational pulmonary toxicity in F0-M Mothers and also in their offspring's which was recovered on 18th day of GP and 21st day of GP. Biochemical analysis showed elevated levels L.D.H in blood serum described as toxicity indicator enzymes. Histopathological analysis of lungs tissues recovered from F0-M, F0-M(AP) and F1-P showed congestion and distortion in lungs structure and showed protective potential against zinc oxide nanoparticles toxicity. So, it is concluded that for pregnant females, when Antidote (Fresh orange juice) administrated along with ZnO NPs, it compensate toxic effects of zinc oxide nanoparticles and exhibited protective effects against zinc oxide nanoparticles induced toxicity. So, for the pregnant females, it is highly recommended to take fresh orange juice during 1st to 8th week of pregnancy to prevent infant from micro exposure to zinc oxide nanoparticles. Ferric reducing antioxidant power assay and Total phenolic content Assays devised that the orange juice contains strong antioxidant agents as it has sufficient ratio of Ascorbic acid and Phenolic residues that might have reduced the toxicant production of oxidative stress.

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