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Original Article

Bio-efficacy of iron and zinc fortified wheat flour along with bio-assessment of its hepatic and renal toxic potential

Bioeficácia da farinha de trigo fortificada com ferro e zinco e bioavaliação do seu potencial tóxico hepático e renal

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Abstract

Study was planned to assess the bio-efficiency along with toxicity of iron and zinc fortified whole wheat flour in Sprague dawley albino rats. Whole wheat flour was fortified with different dosage of sodium iron EDTA (NaFeEDTA), ferrous sulphate ($FeSO_4$), zinc oxide (ZnO) and zinc sulphate ($ZnSO_4$). The rats (n=3) in each group were fed on fortified wheat flour for 2 months. Liver biomarkers including alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST) and bilirubin were recorded from serum samples. Increased concentration of $ZnSO_4$ affected the liver biomarkers to be highest among all whereas, bilirubin levels were less than the rats fed on control diet. The above mentioned fortificants have negligible effect on renal biomarkers including creatinine and urea. Moreover, hematological parameters were also checked and reportedly, sodium iron EDTA fed rats presented highest amount of hemoglobin, iron and total iron binding capacity. Highest zinc level was observed in rats fed on whole wheat flour fortified with 60mg/Kg Zinc oxide. Microscopic observation of liver tissue depicted that rats fed on iron and zinc fortified wheat flour have more toxic effects whereas, histopathology presentation of kidney tissue has least toxic impact. It has been concluded that mandatory fortification of wheat flour with iron and zinc may cause increased serum biomarkers along with toxicity of vital organs like liver, hence fraction of wheat flour may be fortified to fulfill the requirements of deprived and vulnerable group.

Keywords: bio-efficacy, bio-assessment, Sprague Dawley albino rats, blood chemistry, histopathology.

Resumo

O estudo foi planejado para avaliar a bioeficiência juntamente com a toxicidade da farinha de trigo integral fortificada com ferro e zinco em ratos albinos Sprague dawley. A farinha de trigo integral foi fortificada com diferentes dosagens de ferro sódico EDTA (NaFeEDTA), sulfato ferroso (FeSO4), óxido de zinco (ZnO) e sulfato de zinco (ZnSO4). Os ratos (n = 3) de cada grupo foram alimentados com farinha de trigo fortificada por 2 meses. Biomarcadores hepáticos incluindo fosfatase alcalina (ALP), alanina transaminase (ALT), aspartato aminotransferase (AST) e bilirrubina foram registrados a partir de amostras de soro. O aumento da concentração de ZnSO4 afetou os biomarcadores hepáticos como sendo os mais altos entre todos, enquanto os níveis de bilirrubina foram menores do que os ratos alimentados com dieta controle. Os fortificantes mencionados acima têm efeito insignificante nos biomarcadores renais, incluindo creatinina e ureia. Além disso, os parâmetros hematológicos também foram verificados e, segundo relatos, os ratos alimentados com EDTA de sódio e ferro apresentaram maior quantidade de hemoglobina, ferro e capacidade total de ligação de ferro. O maior nível de zinco foi observado em ratos alimentados com farinha de trigo integral fortificada com 60 mg/Kg de óxido de zinco. A observação microscópica do tecido hepático mostrou que ratos alimentados com farinha de trigo fortificada com ferro e zinco têm mais efeitos tóxicos, enquanto a apresentação histopatológica do tecido renal tem menos impacto tóxico. Concluiu-se que a fortificação obrigatória da farinha de trigo com ferro e zinco pode causar aumento dos biomarcadores séricos juntamente com toxicidade de órgãos vitais como o fígado, portanto a fração da farinha de trigo pode ser fortificada para atender os requisitos do grupo carente e vulnerável.

Palavras-chave: bioeficácia, bioavaliação, ratos albinos Sprague Dawley, química do sangue, histopatologia.

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1. Introduction

Wheat flour fortification with iron, zinc and folic acid has been globally recognized as one of the most effective and low-cost micronutrient interventions. United Nations have recommended flour fortification as a preventive food-based approach to improve micronutrient status of populations (Cardoso et al., 2019). While discussing the suggested levels for flour fortification with iron, World Health Organization (WHO) has recommended that the type and quantity of an additive such as vitamins and minerals, either as a voluntary standard or a mandatory requirement, lies with national decision of each country (Dwyer et al., 2015). However, the choice of compounds and quantities should be viewed in the context of each country's situation. It has also been reviewed there that flour fortification with folic acid increases the intake of folate by women and can reduce the risk of neural tube and other congenital disabilities. At the same time, it has been studied that zinc fortified flour has deposed that it could improve zinc status in women of childbearing age (Shah et al., 2016). Moreover, it has been suggested that levels for the fortification of iron should be reviewed by experts from published efficacy and effectiveness studies with various iron fortified foods (Waller et al., 2020). Daily quantities of selected iron compounds including NaFeEDTA, ferrous sulphate, ferrous fumarate and electrolytic iron have been estimated and found to improve iron status in populations (Hurrell, 2021). It has been emphasized and reported that the excess of iron is a risk factor causing toxicity and leading to some disorders such as aging, muscle atrophy, viral replication, rosacea and pulmonary alveolar proteinosis (Rehman et al., 2021). The excessive intake of zinc leads to toxic impact with symptoms of nausea, vomiting, epi-gastric pain, lethargy and fatigue etc. Besides, excessive zinc intake will cause induced copper deficiency, anemia and neutropenia along with impaired immune functions and adverse effects on low-density lipoprotein (LDL) to high density lipoprotein (HDL) (Tatineni et al., 2020). In Pakistan, it has been endeavored to fortify maximum wheat flour (Fortificants: NaFeEDTA, ZnO and the other micronutrient includes Folic Acid) at its grinding stage in flour mills and supply to the entire population in the market. It needs to be probed if it has some repercussions on human health or it is only needed by human beings suffering from malnutrition. Further deliberations need to investigate that fortification may not be strategized as mandatory in its Policy.

It is required to find out if the entire population requires iron and zinc fortified wheat flour or only the target group or vulnerable section such as the populace with anemia, malnutrition or stunted growth needs to be focused. Adverse impact of both the micronutrients on healthy individuals has also been investigated. Impact of their overload inside body beyond its prescribed dose or approved standards has been studied and found to cause toxicity and inhibit the absorption of other minerals at tissue level. It has been observed that its overload may prohibit certain cellular functions or may disturb specific cytological mechanisms instead of supporting normal physiological functions. The objective of current study was to assess the effect of different fortification levels of iron and zinc on various hematological and histopathological parameters in experimental Sprague dawley rats.

2. Material and Methods

2.1. Animals and experimental design

Sprague dawley albino rats (n=51) were used for the experimental study to check the impact of iron and zinc salts supplementation with whole wheat purchased from local market. The study was split into 17 groups based on fortification of whole wheat flour with various salt concentrations viz., NaFeEDTA (15 mg), NaFeEDTA (25 mg), NaFeEDTA (35 mg), NaFeEDTA (40 mg), $FeSO_4$ (10 mg), $FeSO_4$ (20 mg), $FeSO_4$ (30 mg), $FeSO_4$ (40 mg), ZnO (20 mg), ZnO (30 mg), ZnO (40 mg), ZnO (60 mg), $ZnSO_4$ (25 mg), $ZnSO_4$ (35 mg), $ZnSO_4$ (45 mg), $ZnSO_4$ (60 mg) and control group with whole wheat flour without supplementation while each group contains n=03 rats. The diet of rats were prepared as described by (Akhtar et al., 2010) with minor modifications.

2.2. Sampling

All experimental animals were fed with supplemented wheat flour for 02 months (May - June 2021), followed by examination for various blood biochemistry and histopathology parameters. All blood biochemistry parameters were performed by Test Zone Clinical Laboratory and Diagnostic Centre, Lahore and Sachal Medical Services, Saadan Hospital, Lahore. Histopathology study was conducted at Fatima Memorial Medical College, Lahore and University of Veterinary and Animal Sciences, Lahore, Pakistan.

2.3. Liver function tests

Liver function test (LFT) parameters were performed by ZHEJIANG KANGTE BIO-TECH kit method by following manufacturer recommendations. In-vitro, rapid quantitative analysis of alkaline phosphate (ALP) in rat serum samples was performed. BD blood vacutainers were used for serum separation followed by centrifugation at 8000 rpm speed for 5 minutes. Sample preparation was done using kit reagents and serum in specified volume. By using blood biochemistry analyzer apparatus, distilled water was detected to probe as blank followed by suction of prepared sample from microcentrifuge tube. Alkaline phosphatase (ALP) menu was checked on apparatus and results were noted from the screen. As well, quantitative analysis of alanine transaminase (ALT), aspartate aminotransferase (AST) and total bilirubin level of all experimental rats were conducted following ZHEJIANG KANGTE BIO-TECH kit method with specified reagent kits. All result values were recorded for 16 experimental groups and 1 control group for various concentrations of NaFeEDTA, $FeSO_4$, ZnO and $ZnSO_4$ following the method of Kumar and his colleagues with minor modifications (Kumar et al., 2021).

2.4. Renal function tests

A volume of 2-3 mL of venous blood was collected from each rat for quantitative analysis of RFT parameters. Estimation of serum creatinine level and serum urea (BUN) level was performed using ZHEJIANG KANGTE BIO-TECH specified reagent kits following the method of Okpogba et al. (2021) with minor modifications and final readings were noted from blood biochemistry analyzer screen.

2.5. Blood biochemistry

Blood Biochemistry parameters such as hemoglobin (Hb), iron (Fe), zinc (Zn) and total iron binding capacity (TIBC) were estimated. Blood hemoglobin (Hb) level and TIBC were determined by blood hematology analyzer while biochemical parameters of Zn and Fe were estimated by VetTest® analyzer using specified reagents for each supplement following the method of Kari and his colleagues with minor modifications (Kari et al., 2021).

2.6. Histopathology

For histopathology analysis, 10% neutral buffered formalin were used for fixation of Sprague Dawley rats liver and kidney specimens and placed in automatic tissue processor for dehydration and impregnation followed by embedded in paraffin blocks. For each specimen, section of 4µm thickness were cut and processed for hematoxylin and eosin staining. The stained specimens were mounted and examined under light microscope for abnormalities in tissues (Zhou et al., 2011).

2.7. Statistical analysis

The data were analyzed through one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using student package for social sciences (SPSS) version 20.0. Results obtained were compared by post-hoc between groups and $P<0.05$ were considered statistically significant while $P>0.05$ denoted as statistically non-significant.

3. Results

The impact of iron and zinc overload along with their threshold level for wheat fortification was assessed in Sprague Dawley rats. Various types and quantities of both supplements have been used to see the influence of variable quality and quantity in different treatment groups. All experimental units were fed for 02 months and blood biochemistry including hematology, renal function test and liver function test along with histopathology of liver and kidney were studied as mentioned below.

3.1. Liver function tests

The highest level of alkaline phosphatase (ALP) (384.34±18.48 U/L) was observed in rats supplemented with 60 mg/Kg of $ZnSO_4$ while lowest level of ALP (201.34±24.11 U/L) was observed in rats supplemented with 15 mg/Kg of NaFeEDTA. The ALP in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are more than group of rats fed on control diet. Significant differences were observed among the experimental units.

The highest level of alanine transaminase (ALT) (63.33±13.01 U/L) was recorded in rats supplemented with 60 mg/Kg of $ZnSO_4$ while lowest ALT (28.33±7.50 U/L) was recorded in rats supplemented with 20 mg/Kg ZnO. The ALT in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are in relation and close range to control group. Non-significant differences were observed among all experimental units.

The highest level of aspartate aminotransferase (AST) (194.67±56.88 U/L) was observed in rats supplemented with 60 mg/Kg $ZnSO_4$ whereas, lowest AST (102.67±76.78 U/L) was observed in rats supplemented with 40mg/Kg of $FeSO_4$. The AST in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are more than group of rats fed on control diet. Significant differences were observed among the experimental units.

The highest level of bilirubin (0.36±0.28 mg/dL) was recorded in rats supplemented with 60 mg/Kg of ZnO whereas; lowest bilirubin (0.20±0.00 mg/dL) was recorded in rats supplemented with 10 mg/Kg of $FeSO_4$. The bilirubin in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are less than groups of rats fed on control diet. Significant differences were observed among the experimental units (Table 1).

3.2. Renal function tests

The highest level of creatinine (0.51±0.02 mg/dL) was recorded in rats supplemented with 40 mg/Kg of $FeSO_4$ while lowest creatinine (0.33±0.05 mg/dL) was recorded in rats supplemented with 25 mg/Kg of $ZnSO_4$. The creatinine in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are in relation and close range to control group. Non-significant differences were observed among all experimental units.

The highest level of urea (39.13±12.52 mg/dL) was recorded in rats supplemented with 40 mg/Kg of NaFeEDTA while lowest urea (19.00±1.00 mg/dL) was recorded in rats supplemented with 25 mg/Kg of $ZnSO_4$. The urea in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are more than group of rats fed on control diet. Significant differences were observed among the experimental units (Table 2).

3.3. Hematology

The highest hemoglobin (Hb) level (14.77±1.30 g/dL) was observed in rats supplemented with 40 mg/Kg of NaFeEDTA whereas lowest Hb level (10.30±4.40 g/dL) was observed in rats supplemented with 60 mg/Kg of ZnO. The hemoglobin level in these rats differed significantly with each other and among other groups of rats. The hemoglobin levels in rats fed on various fortification levels of NaFeEDTA, ZnO, $FeSO_4$ and $ZnSO_4$ are in relation and close range to control group. Furthermore, non-significant differences were observed among these experimental units.

The highest iron level (210.67±51.86 µg/dL) was recorded in rats supplemented with 40 mg/Kg of NaFeEDTA whereas lowest iron level (117.33±43.66 µg/dL) was recorded in

Table 1. Liver function parameters under the effect of varied iron and zinc supplementation in fortified wheat flour.

Group Name	Fortification Level (mg/Kg)	Alkaline Phosphatase (U/L)	Alanine Transaminase (U/L)	Aspartate Aminotransferase (U/L)	Bilirubin (mg/dL)
Control Diet	---	113.67±33.86^{a}	34.67±1.52^{a}	100.33±00.57^{a}	0.60±0.00^{b}
NaFeEDTA	15mg	201.34±24.11^{ab}	55.00±20.95^{a}	166.67±49.94^{ab}	0.30±0.10^{ab}
NaFeEDTA	25mg	249.67±39.68^{ab}	49.67±15.63^{a}	172.33±47.07^{ab}	0.33±0.23^{ab}
NaFeEDTA	35mg	280.34±75.98^{ab}	41.67±17.78^{a}	144.33±41.95^{ab}	0.33±0.23^{ab}
NaFeEDTA	40mg	276.34±122.69^{ab}	48.67±13.6^{a}	162.00±40.95^{ab}	0.33±0.23^{ab}
$FeSO_4$	10mg	248.34±48.08^{ab}	39.00±9.84^{a}	161.67±35.07^{ab}	0.20±0.00^{a}
$FeSO_4$	20mg	347.34±86.75^{b}	46.00±13.11^{a}	158.67±27.59^{ab}	0.33±0.23^{ab}
$FeSO_4$	30mg	272.00±70.00^{ab}	38.00±17.24^{a}	142.00±39.96^{ab}	0.35±0.27^{ab}
$FeSO_4$	40mg	237.34±89.14^{ab}	34.00±13.22^{a}	102.67±76.78^{a}	0.34±0.21^{ab}
ZnO	20mg	326.34±66.59^{b}	30.00±3.60^{a}	176.33±37.68^{ab}	0.36±0.28^{ab}
ZnO	30 mg	339.34±102.65^{b}	58.00±43.58^{a}	184.67±65.12^{ab}	0.23±0.05^{ab}
ZnO	40 mg	366.34±75.03^{b}	38.67±19.39^{a}	164.00±32.23^{ab}	0.33±0.23^{ab}
ZnO	60 mg	342.00±187.66^{b}	32.33±6.65^{a}	169.33±38.08^{ab}	0.36±0.28^{ab}
$ZnSO_4$	25 mg	238.34±11.01^{ab}	33.67±6.80^{a}	116.67±06.80^{ab}	0.23±0.05^{ab}
$ZnSO_4$	35mg	354.67±114.84^{b}	30.33±3.21^{a}	130.00±14.00^{ab}	0.23±0.05^{ab}
$ZnSO_4$	45mg	282.00±55.57^{ab}	38.33±7.50^{a}	140.67±51.32^{ab}	0.24±0.04^{ab}
$ZnSO_4$	60mg	384.34±18.48^{b}	63.33±13.01^{a}	194.67±56.88^{b}	0.24±0.02^{ab}

Value with different superscripts (a, b) differ significantly (P<0.05), with similar differ non-significantly (P>0.05) and vice versa.

Table 2. Renal function parameters under the effect of varied iron and zinc supplementation in fortified wheat flour.

Group Name	Fortification Level (mg/Kg)	Creatinine (mg/dL)	Urea (mg/dL)
Control Diet	---	0.47±0.11^{a}	16.67±01.57^{a}
NaFeEDTA	15mg	0.47±0.05^{a}	29.33±13.65^{ab}
NaFeEDTA	25mg	0.47±0.05^{a}	36.67±04.93^{ab}
NaFeEDTA	35mg	0.47±0.05^{a}	29.33±15.30^{ab}
NaFeEDTA	40mg	0.40±0.10^{a}	39.13±12.52^{b}
$FeSO_4$	10mg	0.50±0.10^{a}	28.00±15.39^{ab}
$FeSO_4$	20mg	0.40±0.10^{a}	35.33±17.09^{ab}
$FeSO_4$	30mg	0.43±0.12^{a}	33.33±16.16^{ab}
$FeSO_4$	40mg	0.51±0.02^{a}	31.33±16.19^{ab}
ZnO	20mg	0.43±0.11^{a}	24.67±09.86^{ab}
ZnO	30mg	0.47±0.05^{a}	19.33±04.04^{ab}
ZnO	40mg	0.50±0.17^{a}	26.33±11.93^{ab}
ZnO	60mg	0.37±0.05^{a}	29.67±11.59^{ab}
$ZnSO_4$	25mg	0.33±0.05^{a}	19.00±01.00^{ab}
$ZnSO_4$	35mg	0.40±0.00^{a}	19.00±02.00^{ab}
$ZnSO_4$	45mg	0.37±0.05^{a}	24.67±06.65^{ab}
$ZnSO_4$	60mg	0.40±0.00^{a}	23.00±03.60^{ab}

Value with different superscripts (a, b) differ significantly (P<0.05), with similar differ non-significantly (P>0.05) and vice versa.

rats supplemented with 60 mg/Kg of ZnO. The iron level in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are in relation and close range to control group. Non-significant differences were observed among all experimental units.

The highest level of zinc (269.27±15.32 µg/dL) was observed in rats supplemented with 60 mg/Kg ZnO while lowest zinc level (116.47±51.96 µg/dL) was observed in rats supplemented with 15 mg/Kg of NaFeEDTA. The zinc level in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$

and ZnO are more than group of rats fed on control diet. Non-significant differences were observed among all experimental units.

The highest level of total iron binding capacity (TIBC) (566.67±10.79 µg/dL) was observed in rats supplemented with 40 mg/Kg of NaFeEDTA while lowest TIBC (315.80±75.07 µg/dL) was observed in rats supplemented with 60 mg/Kg of $ZnSO_4$. The TIBC in rats fed on various levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are in relation and close range to control group. Significant differences were observed among the experimental units (Table 3).

3.4. Histopathology

The highest microscopic liver lesion score (3.00±0.00) was observed in rats supplemented with 60 mg/Kg of $FeSO_4$ while least microscopic liver lesion score (1.33±0.57) was observed in rats supplemented with 40 mg/Kg of NaFeEDTA. The microscopic liver lesion score in rats fed on different levels of NaFeEDTA, $FeSO_4$, $ZnSO_4$ and ZnO are more than group of rats fed on control diet. Non-significant differences were observed among experimental units.

The highest microscopic kidney lesion score (1.00±0.00) was observed in rats supplemented with 35 mg/Kg of NaFeEDTA whereas, least microscopic kidney lesion score (0.00±0.00) was observed for most of the experimental units including, 15 mg/Kg of NaFeEDTA, 25 mg/Kg of NaFeEDTA, 35 mg/Kg of NaFeEDTA, 10 mg/Kg of $FeSO_4$, 20 mg/Kg of $FeSO_4$, 30 mg/Kg of $FeSO_4$, 20 mg/Kg of ZnO, 30 mg/Kg of ZnO, 25 mg/Kg of $ZnSO_4$, 35 mg/Kg of $ZnSO_4$, 45 mg/Kg of $ZnSO_4$ and 60 mg/Kg of $ZnSO_4$. Significant differences were observed among the experimental units (Table 4) (Figures 1-2).

4. Discussion

Wheat flour is fortified deliberately to increase the contents of an essential micronutrients especially minerals including trace elements. Supplementation of industrially processed wheat flour is a simple, inexpensive and effective strategy to provide essential minerals for a large segment of population (Borrill et al., 2014). Decision regarding which nutrient to be added and use of its appropriate amount is based upon the nutritional needs and deficiencies of the population. For this purpose, government authorities need to assess the fortification qualitatively and quantitatively (Balk et al., 2019). In current study, sprague dawley albino rats were used in experimental animal model to identify the effect of iron and zinc fortification on hematology, liver function parameters, renal function parameters along with lesion score of liver and kidney. Whole wheat flour and the body of rats already might be having minerals according to their body requirement and beyond that limit physiological functions inside body got disturbed so the vital body organs caught toxicity. Prolonged exposure to fortified meals has further accentuated toxicity and the vital body organs have got inflamed.

In present study, increased fortification level of zinc, increased the serum alkaline phosphatase (ALP). These finding are in line to the observations of Tang and his colleagues who reported increased alkaline phosphatase

Table 3. Hematological parameters under the effect of varied iron and zinc supplementation in fortified wheat flour.

Group Name	Fortification Level (mg/Kg)	Hemoglobin (g/dL)	Iron (µg/dL)	Zinc (µg/dL)	Total Iron Binding Capacity (µg/dL)
Control Diet	---	13.73±00.51^{ab}	204.33±26.95^{a}	85.33±08.32^{a}	360.33±10.20^{ab}
NaFeEDTA	15mg	12.87±01.91^{ab}	171.00±05.29^{a}	116.47±51.96^{a}	387.47±22.24^{abc}
NaFeEDTA	25mg	14.23±00.90^{ab}	172.00±49.00^{a}	157.83±92.56^{a}	430.13±24.13^{abc}
NaFeEDTA	35mg	14.10±00.96^{ab}	181.00±61.65^{a}	161.33±72.67^{a}	467.97±47.34^{abc}
NaFeEDTA	40mg	14.77±01.30^{b}	210.67±51.86^{a}	231.57±20.52^{a}	566.67±10.79^{ab}
$FeSO_4$	10mg	11.30±02.16^{ab}	132.33±38.08^{a}	229.43±19.37^{a}	351.00±46.49^{ab}
$FeSO_4$	20mg	13.30±00.95^{ab}	146.00±80.21^{a}	238.60±25.30^{a}	372.50±27.40^{ab}
$FeSO_4$	30mg	13.73±01.92^{ab}	172.33±52.00^{a}	195.03±36.38^{a}	360.03±13.41^{ab}
$FeSO_4$	40mg	14.07±01.45^{ab}	179.00±71.25^{a}	211.93±16.14^{a}	525.50±41.93^{bc}
ZnO	20mg	11.67±02.20^{ab}	159.00±29.81^{a}	138.17±35.88^{a}	382.33±37.47^{abc}
ZnO	30 mg	13.67±01.19^{ab}	154.33±75.51^{a}	236.57±42.42^{a}	356.73±38.84^{ab}
ZnO	40 mg	13.57±00.89^{ab}	148.00±63.66^{a}	247.90±19.57^{a}	385.80±79.58^{abc}
ZnO	60 mg	10.30±04.40^{a}	117.33±43.66^{a}	269.27±15.32^{a}	498.43±33.57^{abc}
$ZnSO_4$	25 mg	11.50±03.79^{ab}	166.67±27.42^{a}	143.63±23.10^{a}	411.90±3.73^{abc}
$ZnSO_4$	35mg	13.10±01.93^{ab}	201.67±18.17^{a}	208.27±39.58^{a}	388.07±15.29^{abc}
$ZnSO_4$	45mg	12.10±02.16^{ab}	185.33±33.24^{a}	245.87±57.66^{a}	412.30±54.45^{abc}
$ZnSO_4$	60mg	13.30±01.96^{ab}	179.33±38.37^{a}	267.17±20.45^{a}	315.80±75.07^{a}

Value with different superscripts (a, b, c) differ significantly (P<0.05), with similar differ non-significantly (P>0.05) and vice versa.

Table 4. Histopathology: liver and kidney lesion scores under the effect of varied iron and zinc supplementation in fortified wheat flour.

Group Name	Fortification Level	Liver Lesion Score*	Kidney Lesion Score**
Control Diet	---	0.00±0.00^{a}	0.00±0.00^{a}
NaFeEDTA	15mg	1.33±0.57^{b}	0.00±0.00^{a}
NaFeEDTA	25mg	1.67±0.57^{b}	0.00±0.00^{a}
NaFeEDTA	35mg	1.67±0.15^{b}	0.00±0.00^{a}
NaFeEDTA	40mg	1.33±0.57^{b}	1.00±0.00^{b}
$FeSO_4$	10mg	2.67±0.57^{b}	0.00±0.00^{a}
$FeSO_4$	20mg	2.00±1.00^{b}	0.00±0.00^{a}
$FeSO_4$	30mg	2.00±1.00^{b}	0.00±0.00^{a}
$FeSO_4$	40mg	3.00±0.00^{b}	0.67±0.57^{ab}
ZnO	20mg	2.33±1.15^{b}	0.00±0.00^{a}
ZnO	30mg	2.67±0.57^{b}	0.00±0.00^{a}
ZnO	40mg	2.67±0.57^{b}	0.33±0.57^{ab}
ZnO	60mg	2.67±0.57^{b}	0.67±0.57^{ab}
$ZnSO_4$	25mg	2.00±0.00^{b}	0.00±0.00^{a}
$ZnSO_4$	35mg	2.33±0.57^{b}	0.00±0.00^{a}
$ZnSO_4$	45mg	2.67±0.57^{b}	0.00±0.00^{a}
$ZnSO_4$	60mg	1.67±0.57^{b}	0.00±0.00^{a}

*Normal, no tissue changes = 0; cellular swelling in hepatocytes = 1; microvascular changes in hepatocytes = 2; marked cellular swelling and necrosis of hepatocytes = 3. **Normal, no tissue changes seen = 0; peritubular congestion is seen = 1. Value with different superscripts (a, b) differ significantly (P<0.05), with similar differ non-significantly (P>0.05) and vice versa.

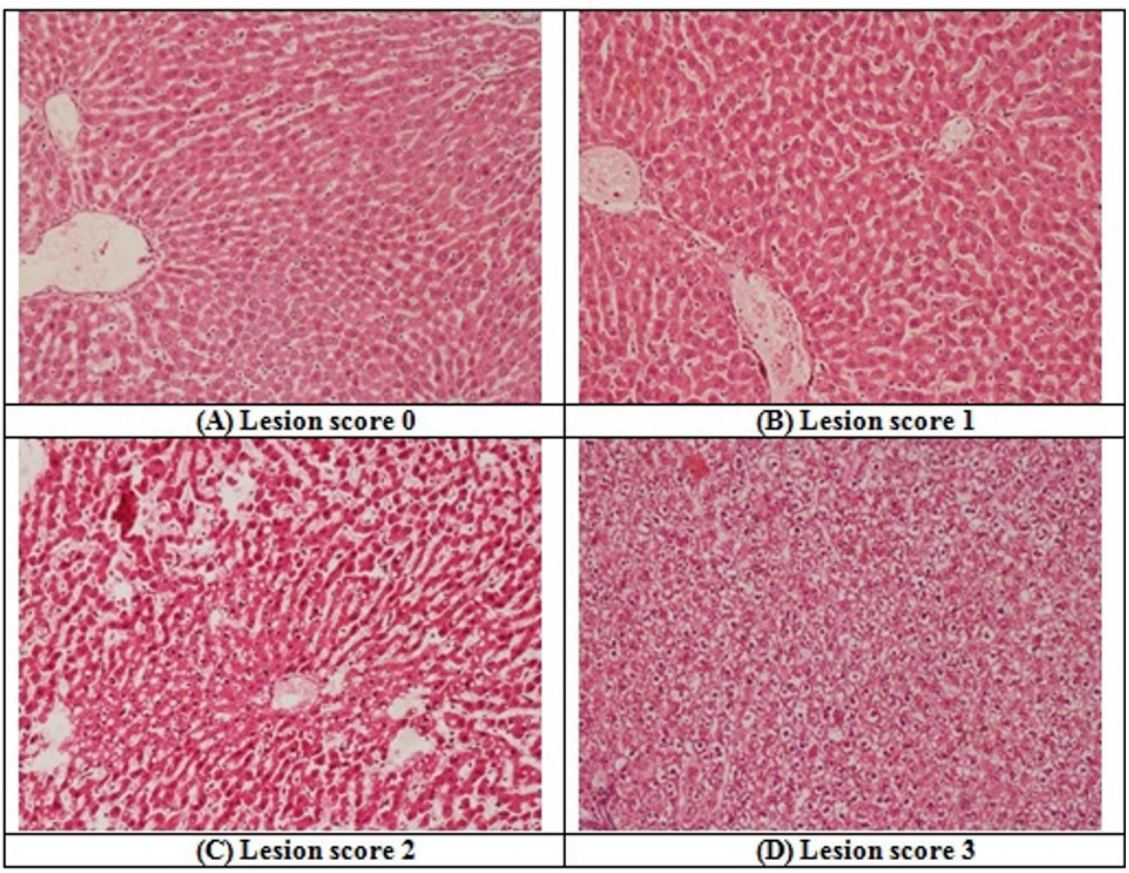

Figure 1. Microscopic morphology of representative liver tissues under the effect of varied iron and zinc supplementation in fortified wheat flour (100X; magnification). (A) Normal, no tissue changes = 0, (B) cellular swelling in hepatocytes = 1, (C) microvascular changes in hepatocytes = 2, (D) marked cellular swelling and necrosis of hepatocytes = 3.

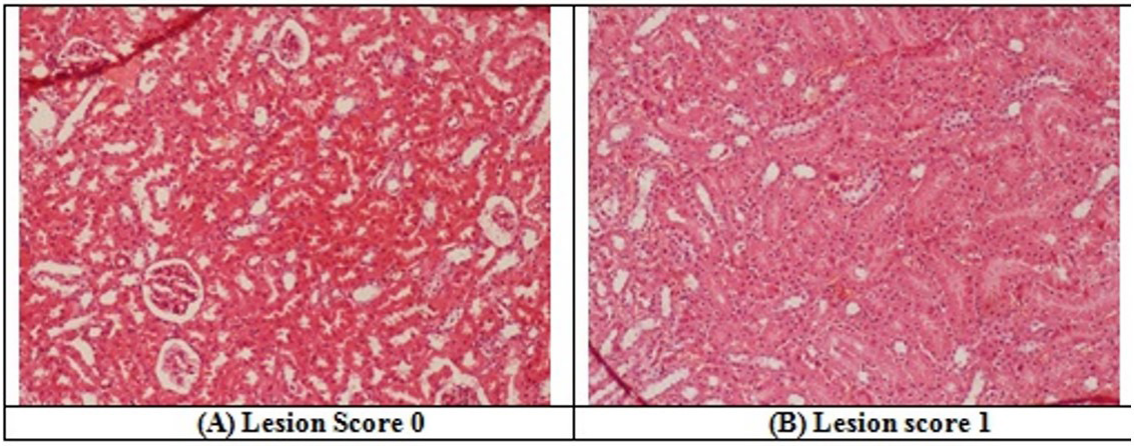

Figure 2. Microscopic morphology of representative kidney tissues under the effect of varied iron and zinc supplementation in fortified wheat flour (100X; magnification). (A) Normal, no tissue changes seen = 0, (B) peritubular congestion is seen=1.

with increased level of zinc supplementation (Tang et al., 2016). Whereas, the finding of Sidhu and coworkers are in contrast to present study as supplementation of zinc sulfate decreased the alkaline phosphatase possibly due to protein deficiency (Sidhu et al., 2005). The fortification of wheat flour with ferric sodium EDTA decreased the alkaline phosphatase which is coherent with recommendations of the European food safety authority (EFSA Panel on Food Additives and Nutrient Sources Added to Food, 2010). Similarly, the highest level of alanine aminotransferase (ALT) was recorded in the group of rats fed on zinc sulfate fortified wheat flour which is relation to the findings of Sidhu and team members who reported increased alanine aminotransferase level in rats after supplementation with zinc sulfate (Sidhu et al., 2005). The current findings regarding alanine aminotransferase illustrates that least ALT was produced by zinc oxide supplementation which is contrast to the work by Tang and his colleagues as zinc oxide nanoparticle supplemented diet increased the ALT in albino rats (Tang et al., 2016). Sidhu and his coworkers reported increased level of aspartate aminotransferase (AST) with the passage of time after continuous supplementation of zinc sulfate which is in accordance to the findings of the current study as there is increased level AST with zinc sulfate fortified wheat flour (Sidhu et al., 2005). The bilirubin level of rats fed on fortified wheat flour are reported as decreased in current study which has similarity with the trials conducted by Abtahi and his co-workers (Abtahi et al., 2014).

The zinc and iron fortification of wheat flour has very negligible effect on the renal function test including creatinine and bilirubin. Similar results were reported by Adejuwon and coworkers as albino rats fed on formulated wheat flour diets depicted creatinine and urea level with in normal range. As healthy kidneys remove excessive amount of blood urea and creatinine which indicate that fortified wheat flour has no negative effect on the kidney function (Adejuwon et al., 2021). In a similar type of study the creatinine and urea level in blood are decreased in diabetic albino rats fed on fortified wheat flour diet (Erukainure et al., 2013).

We have identified that the increased fortification of wheat flour with iron has increased hemoglobulin, serum iron and total iron binding capacity in the rats. Similarly, Hong and his teammates have compared iron bioavailability and bio-efficacy of $FeSO_4$, ferrous gluconate and AOS-iron groups by measuring haemoglobin (Hb), Red Blood Cells (RBC), serum iron (SI) and total iron binding capacity (TIBC) etc. It has been found that $FeSO_4$ group and LD AOS-iron group increased RBC count in anaemic group to normal whereas values for RBCs were higher in all iron supplementation groups than IDA groups. Iron deficiency leads to a decline in Hb content, i.e. RBCs in anaemic groups whereas iron supplementation has improved bioavailability. The serum iron (SI) concentration is total amount of iron in serum and total iron binding capacity (TIBC) indicates maximum amount of iron needed to bind to all transferrin in serum. Usually, SI and TIBC reflect the status of iron during circulation in blood (He et al., 2019). It is worth mentioning that citing the safety of oral iron, it has been reported that oral iron is often poorly tolerated noting gastrointestinal issues and intravenous iron is being used more frequently to replete iron stores. Ferric compounds are perhaps better tolerated than ferrous compounds, although the former have been shown to be inferior to the latter in effectiveness of iron replacement. It has been reported by Dijkhuizen and coworkers that a combination of iron and zinc is not more effective than iron alone on growth and development. Furthermore, it has been proved that zinc deficiency in patients with iron deficiency is only treated with ferrous sulfate and there is no need for zinc supplementation. Results of our research have been found consistent with the reported findings (Dijkhuizen et al., 2001).

The research data of Engwa and his colleagues suggest that iron and zinc depletion in body may cause its deficiency, diseases or syndromes but excessive supply may lead to acute or chronic toxicities. These metals, often in minute quantity, accumulate and get deposited in tissues or cells

or nucleic acid and disturb their functions, besides damage blood, lungs, liver, kidneys and other vital body organs. In addition, its repeated and long-term accumulation may disrupt endocrine and reproductive system, kidney and liver function failure and eventually cancer. It has been added that in their ionic species, these metals form very stable bio-toxic compounds with proteins and enzymes and are difficult to be disassociated. Free radicals generated inside the cell by these metals selectively activates transcription factors and suggest cell proliferation or cell death, thus inducing carcinogenesis (Engwa et al., 2019). These findings are in accordance to present study as increased dosage of zinc and iron fortificants have more toxicity as observed in histopathological slide of liver. These fortificant have least effect on the kidney which is in accordance with decreased levels of creatinine and urea in serum.

Biochemical reports and Histopathological study corroborate with the same facts that how vital body organs have got malfunctioned upon higher levels of iron and zinc. Overdose or excess of minerals has been found to damage vital body organs or will hamper its physiological or biological functions. Besides, the concentration and type of fortificant has been found to be a decisive factor for evading its toxic effects. It is observed that liver has got inflamed and caught toxicity when received more than required quantities of iron and zinc in meals. It is pertinent to mention here that the liver along with serum chemistry of rats receiving the salts containing sulphate have shown toxicity whereas the ones receiving zinc oxides or iron in EDTA form have been found to be lesser reactive.

5. Conclusion

Instant research and scientific studies held by many other researchers have established to avoid iron and zinc overload leading to infections or inflammations inside body. Foregoing researches in view, it is inferred that the wheat flour industry needs to abstain from adopting mandatory or 100% iron and zinc fortification of entire stock and a fraction of crops may be fortified to address the sole need of vulnerable group.

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