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Biochemical effects of Iron and folate overdose on the female mice

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ABSTRACT:

Background: Iron and Folic acid are essential micronutrients for reproductive health. Although dietary supplements are common during pregnancy, excessive intake can impair liver and kidney function. **Aim:** To study the effect of Iron and Folic acid supplements and overdoses on the hematopoietic function, liver functions and renal functions of female mice. **Methodology:** The animals were raised and provided with appropriate conditions for reproduction in an animal house at the Faculty of Veterinary Medicine, University of Tripoli, until the required number for the experiment was obtained. The doses were given to 36 female mice only, which were divided into 6 groups: the control group and 5 other groups were divided as follows: Group I (control), Group II (Fe 15mg/kg), Group III (FA 8mg/kg), and Group IV (FA 4mg/kg). Group V (Fe with FA 8mg/kg) and Group VI (Fe with FA 4mg/kg). Every other day intraperitoneal injections of Fe were administered, and oral FA supplements were provided daily for a week, mice were monitored and weighed weekly and fed daily for 3 weeks. Liver and kidney enzyme levels were measured, and blood component levels were assessed. Data were analysed using analysis of variance (ANOVA). **Results:** The effect of treatments on liver and kidney functions was analysed using one-way analysis of variance (ANOVA). The results showed that Iron and Folic acid supplementation had an impact on liver and kidney functions. Liver enzymes in female rats supplemented with Iron showed a significant increase ($p < 0.05$) compared to female rats supplemented with Folic

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acid alone or with Iron, which led to a significant increase in liver enzymes and serum creatinine levels, and worsened kidney function. Although liver enzymes increased after supplementing with Iron alone (group II), Folic acid alone (group III), but after treatment with both (group V and VI), the increase in liver enzymes was significantly lower.

Conclusion: Excess Iron and Folic acid negatively affect female health, with high Iron doses potentially increase the risk of the liver and kidney. This research highlights the importance of screening of Iron levels before supplementation and raises awareness about the risks of unnecessary Iron intake.

KEYWORDS: Folic acid, Iron toxicity, anemia, supplementation, Liver, Kidney.

التأثيرات الكيميائية الحيوية لجرعة زائدة من الحديد وحمض الفوليك على إناث الفئران

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الملخص:

يُعدّ الحديد وحمض الفوليك من المغذيات الدقيقة الأساسية للصحة الإنجابية. على الرغم من شيوع تناول المكملات الغذائية أثناء الحمل، إلا أن الإفراط في تناولها قد يُضعف وظائف الكبد والكلى. **الهدف:** دراسة تأثير مكملات الحديد وحمض الفوليك والجرعات الزائدة منها على وظائف الدم والكبد والكلى لدى إناث الفئران. **المواد وطرق العمل:** رُبيت الحيوانات ووُفِّرت لها ظروف مناسبة للتكاثر في حظيرة حيوانات بكلية الطب البيطري، جامعة طرابلس، حتى تم الحصول على العدد المطلوب للتجربة. أُعطيت الجرعات لـ 36 فأرة أنثى فقط، قُسِّمت إلى 6 مجموعات: المجموعة الضابطة و5 مجموعات أخرى، كما

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يلي: المجموعة الأولى (الضابطة)، المجموعة الثانية (حديد 15 ملغ/كغ)، المجموعة الثالثة (حمض الفوليك 8 ملغ/كغ)، والمجموعة الرابعة (حمض الفوليك 4 ملغ/كغ). المجموعة الخامسة (حديد مع حمض الفوليك 8 ملغ/كغ)، والمجموعة السادسة (حديد مع حمض الفوليك 4 ملغ/كغ). أُعطيت حقن حديد داخل الصفاق كل يومين، وأُعطيت مكملات حمض الفوليك عن طريق الفم يوميًا لمدة أسبوع. رُصدت الفئران ووزنت أسبوعيًا، وأُطعمت يوميًا لمدة ثلاثة أسابيع. قيست مستويات إنزيمات الكبد والكلى، وقُيّمت مستويات مكونات الدم. حُللت البيانات باستخدام تحليل التباين (ANOVA). **النتائج:** تم تحليل تأثير العلاجات على وظائف الكبد والكلى باستخدام تحليل التباين أحادي الاتجاه (ANOVA). أظهرت النتائج أن مكملات الحديد وحمض الفوليك كان لها تأثير على وظائف الكبد والكلى. أظهرت إنزيمات الكبد لدى إناث الفئران التي تناولت مكملات الحديد زيادة ملحوظة ($p < 0.05$) مقارنةً بإناث الفئران التي تناولت مكملات حمض الفوليك وحده أو مع الحديد، مما أدى إلى زيادة ملحوظة في إنزيمات الكبد ومستويات الكرياتينين في المصل، وتدهور وظائف الكلى. على الرغم من أن إنزيمات الكبد زادت بعد تناول مكملات الحديد وحده (المجموعة الثانية)، وحمض الفوليك وحده (المجموعة الثالثة)، إلا أن الزيادة في إنزيمات الكبد كانت أقل بكثير بعد العلاج بكليهما (المجموعتين الخامسة والسادسة). **الخاتمة:** يؤثر فرط الحديد وحمض الفوليك سلبًا على صحة المرأة، حيث قد تزيد جرعات الحديد العالية من خطر الإصابة بأمراض الكبد والكلى. يُسلّط هذا البحث الضوء على أهمية فحص مستويات الحديد قبل تناول المكملات الغذائية، ويزيد الوعي بمخاطر تناول الحديد غير الضروري.

الكلمات المفتاحية: حمض الفوليك، سمية الحديد، فقر الدم، المكملات الغذائية، الكبد، الكلى.

INTRODUCTION

Micronutrients like Fe (Fe) and FA (FA) are vital for pregnancy and fetal development. Many women of reproductive age fail to meet the recommended intake levels and are at high risk of anemia, including gestational anemia, often caused by Fe and/or FA deficiencies. Globally, anemia affects 38.2% of pregnant women, with the highest rates in South-East Asia (48.7%), Africa (46.3%), and the Eastern Mediterranean Region (38.9%) (WHO, 2021). Despite guidance from gynecologists and nutritionists, the effectiveness of FA and Fe supplementation is influenced by factors

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such as food matrix, bioavailability, gut microbiota, and genetic variations (Billah *et al*, 2022) (Suliburska *et al*, 2020). According to the 2020 Global Nutrition Report, none of the 194 countries are on track to achieve the 2025 goal of halving anemia rates among women of reproductive age (Chu *et al*, 2019) (Mousa *et al*, 2019). FA is crucial for DNA and protein synthesis, cell division, and metabolic processes important for reproduction (Pertiwi *et al*, 2022). Fe is essential for mitochondrial respiration, cell growth, DNA synthesis, and oxygen transport as part of hemoglobin; however, excess Fe can produce reactive oxygen species that disrupt hormonal balance and damage tissues (Wald, 2022).

The aim of this study was to investigate the effects of Fe and FA supplementation and the impact of overdose on the hematopoietic function, liver functions and renal functions.

METHODOLOGY

Study Setting

The study was conducted at the Faculty of Veterinary Medicine, University of Tripoli. The conditions were maintained at a temperature of $20 \pm 5°C$ with a 12-hour light-dark cycle. Mice had free access to food and water (Di Bona *et al*, 2014).

Animal Model

Albino mice were used due to their common use in biomedical research.

Pilot Study

A pilot study was performed to identify toxic doses of FA (FA) and Fe (Fe). Sixteen female mice were randomly assigned to two groups ($n = 8$ each) receiving different doses of FA or Fe for seven days. Feed intake and body weight were monitored. In the Fe group (30 mg/kg IP), 75% mortality occurred within 3–5 days, identifying this dose as lethal (Qin *et al*, 2021).

Study Design

An experimental study, completely randomized design (CRD) was applied. Thirty-six female mice (12–14 weeks old) were used.

Group Allocation and Treatment

Mice were randomly assigned to six groups of six mice each. Five groups received treatments while one served as the control. Two groups were administered FA (Prefolic® 50 mg/3 ml, ZAMBON Italia) orally at doses of 8 mg/kg/day and 4 mg/kg/day for seven

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consecutive days. One group received Fe (Endofer® 100 mg/ml, FATRO S.P.A.) alone via intraperitoneal injection at a dose of 15 mg/kg every other day for one week. Two additional groups received combined treatments: one group received Fe 15 mg/kg plus FA 4 mg/kg/day, and the other received Fe 15 mg/kg plus FA 8 mg/kg/day. The control group received no treatment.

Blood Collection and Biochemical Analysis

a) Complete Blood Count (CBC)

A 3ml syringe was used to draw blood directly from the heart. It was placed into EDTA tubes. The samples were taken directly to the lab and placed in a (Mindray®) recycling machine. And then the samples were placed into the rat CBC analyzer machine.

b) Biochemical analyses

A 3 mL syringe was used to draw blood directly from the heart. It was placed in white tubes (free from anticoagulants). Serum was separated from whole blood after 30 minutes of being collected by centrifugation at 3000 rpm. The serum was used in a number of tests, and the tubes were inserted into a biochemical testing device (Mindray®).

•Liver functions test (LFT).

•Renal functions test (RFT).

Ethical Approval

All aspects of the study protocol have been reviewed and authorized by the Animal Ethics Committee of the Tripoli Biotechnology Research Center (BTRC). The animal procedures were conducted in strict compliance with the regulations of the committee.

Statistical Analysis

Data were analyzed using SPSS version 30. One-way ANOVA p-values < 0.05 were considered statistically significant.

RESULTS

- **The impact of various treatments on female mice liver function**

The effect of treatments on liver and kidney functions was analysed using one-way analysis of variance (ANOVA). The results showed that Fe and FA supplementation had an impact on liver and kidney functions. Liver enzymes in female mice supplemented with Fe

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(group II) showed a significant increase ($p < 0.05$) compared to female rats supplemented with FA alone (group III, IV), also observed a decrease in enzyme levels in mice that received a combination of FA and Fe (groups V and VI) **(Table1).**

The iron-only group (Fe) showed the highest increase in all liver enzymes, indicating significant liver stress. FA alone at 8 mg/kg caused a moderate increase in enzyme levels, more than the 4 mg/kg dose, suggesting a dose-dependent effect.

Combining FA with iron helped reduce enzyme levels compared to iron alone:

The FA 4 mg/kg + Fe group showed lower levels of GPT and GOT than the FA 8 mg/kg + Fe group, indicating better protection at the lower dose. However, both combination groups still had higher enzyme levels than the control and FA-alone groups.

Iron supplementation significantly increased liver enzymes, indicating liver stress. Folic acid helped reduce this effect, especially at the lower dose (4 mg/kg), suggesting it may have a protective role against iron-induced liver toxicity.

Table1. Comparison of liver function between various treatment groups

Parameters	Control	Fe	FA 8mg/kg	FA 4mg/kg	FA 8mg/kg + Fe	FA 4mg/kg + Fe	*p-value*
LFT							
ALP	90.50	489.166	173.00	150.00	215.166	168.833	< 0.001
GPT	27.66	178.833	61.166	70.666	166.33	110.00	0.005
GOT	24.16	287.00	122.66	112.83	279.50	157.66	< 0.001

One-way ANOVA; significance at $p < 0.05$. Comparison of liver function between various treatment groups

•The impact of various treatments on female mice Renal function

The study investigated the effect of Fe and low dose and high dose of FA on the kidney function. The result of the current study showed an effect of Fe on the renal function. This result showed a significant ($p < 0.05$) effect on creatinine levels on animals supplemented with Fe (group II) and FA over dose (group III) comparing to control group (group I) **(Table2).**

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• (Iron only) Group: Fe supplementation led to an increase in both creatinine and urea, indicating potential kidney stress. (FA 8 mg/kg): FA alone caused a notable increase in creatinine, but urea levels remained similar to the control group. (FA 4 mg/kg): This lower dose of FA caused a moderate increase in creatinine, while urea levels were slightly lower than the control. (FA 8 mg/kg + Fe): The combination of folic acid (8 mg/kg) and iron reduced both creatinine and urea levels compared to the iron-only group, showing some protective effect on kidney function. (FA 4 mg/kg + Fe): This combination, especially at the lower folic acid dose (4 mg/kg), showed the best improvement, reducing both creatinine and urea levels to near control levels.

• Fe supplementation alone increased kidney markers, indicating kidney stress. FA had a slight protective effect, with the 4 mg/kg + Fe combination showing the best results in reducing kidney stress.

Table 2. Comparison of renal function between vari ous treatment groups.

Parameters	Control	Fe	FA 8mg/kg	FA 4mg/kg	FA 8mg/kg + Fe	FA 4mg/kg + Fe	*p-value*
RFT							
Creatinine	**0.7698**	**3.0712**	**1.8225**	**3.5988**	**1.6522**	**1.2987**	**0.007**
Urea	**36.833**	**39.166**	**38.833**	**36.833**	**35.33**	**33.833**	**0.581**

One-way ANOVA; significance at $p < 0.05$. Comparison of renal function between various treatment groups

•The impact of various treatments on maternal CBC

The results show the effects of FA at different doses group alone (III, IV), or in combination with Fe (groups V and VI), on various hematological parameters compared to control and a Fe -only group (groups I, II). Statistically significant differences ($p < 0.05$) were observed in platelet count, RDW, MCH, MCV, HCT, and hemoglobin levels, indicating that these parameters were notably influenced by the treatments, especially when FA was combined with Fe Groups (V and VI).

The combination of FA and Fe generally improved red blood cell indices and hemoglobin concentration, suggesting enhanced erythropoiesis. However, no significant changes were found in WBC, RBC, and MCHC, implying limited impact on white blood cell count and hemoglobin concentration per cell. Overall, folic

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acid, particularly in combination with iron, had a positive and significant hematological effect **(Table3).**

The Fe group showed significant changes, including reduced platelets, hemoglobin, and RBC counts, indicating potential negative effects on blood parameters. (FA) alone showed mild changes, while the FA + Fe combinations helped moderate the negative effects of iron, particularly with FA 4 mg/kg + Fe, which showed improvements in several blood parameters.

Table3. Comparison of complete blood count between various treatment groups.

Parameters	Control	Fe	FA 8mg/kg	FA 4mg/kg	FA 8mg/kg + Fe	FA 4mg/kg + Fe	*p*-value
CBC							
WBC	14.816	17.016	13.066	12.450	12.183	18.100	0.132
Platelet	1361.83	988.00	1113.50	1880.33	924.17	1233.83	< 0.001
RDW	14.555	14.683	14.766	18.866	14.300	14.350	< 0.001
MCHC	25.666	26.533	26.533	26.166	26.433	26.650	0.057
MCH	12.200	13.000	13.133	11.666	12.900	12.966	0.007
MCV	46.583	48.683	47.433	45.633	49.583	49.650	0.025
HCT	48.183	53.333	58.683	48.983	54.966	55.950	0.005
HGB	11.266	14.750	14.966	12.700	15.816	15.133	< 0.001
RBC	10.291	11.801	12.283	10.938	11.350	11.595	0.086

One-way ANOVA; significance at $p < 0.05$. Comparison of complete blood count between various treatment groups.

DISCUSSION

Our findings are consistent with those of a study conducted in Bangladesh on female rats, which found that Fe deposition in the liver is associated with hepatic oxidative stress, inflammation, and fibrosis (Reza *et al*, 2015).

Although liver enzymes increased after supplementing with Fe alone, FA alone, and also after treatment with both, the increase in liver enzymes was significantly lower in rats treated with a combination of both, this may be attributed to the fact that FA can help reverse inflammation and slow the progression of non-alcoholic fatty liver disease. FA may have this beneficial effect because it regulates the activity of liver Fe transporters and the transcription of genes associated with oxidative stress and liver fibrosis (Xu *et al*, 2024). This finding also demonstrated a significant effect on creatinine levels in animals supplemented with Fe compared to the control groups.

These findings are consistent with a study conducted in Japan that investigated the side effects of high doses of FA. The study found

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that FA at low doses is highly beneficial for the kidneys and against oxidative stress, but that at high doses, FA is highly toxic (Doi *et al.*, 2006). Consistent with these findings, a study conducted in India also showed that high levels of FA in the kidneys can impair cellular antioxidant systems that require NADPH, exacerbating redox imbalances and oxidative stress in the kidneys(Gupta *et al*, 2012) . Regarding Fe and the kidneys, our findings are consistent with those of a study conducted in the Netherlands, which suggested a role for Fe in impaired kidney function, as Fe particles can directly damage renal tubular cells(Martines *et al*, 2013). The results of this study are also consistent with those of a previous study in Brazil, which suggested that the liver and spleen may regularly mobilize Fe, causing kidney failure (Sousa *et al*, 2020).

Furthermore, the notable rise in hemoglobin concentration without a significant change in red blood cell (RBC) counts suggests a potential increase in reticulocytes. Additionally, analysis of morphological parameters indicated a significant impact of Fe and folate supplementation on mean corpuscular volume (MCV) and red cell distribution width (RDW).

CONCLUSION

The findings regarding the effects of high-dose FA and Fe supplementation on liver and kidney function have important clinical implications. Health care providers should carefully consider the potential risks and benefits of FA and Fe supplements, especially in individuals with pre-existing kidney disease, liver disease, or risk factors for liver-kidney dysfunction. More research is needed to better understand the mechanisms behind the observed effects of FA and Fe supplements on liver and kidney function.

These findings highlight the risks of excessive Fe supplementation without medical supervision and emphasize the importance of proper dosing of micronutrient supplements. Screening and individualized supplementation strategies should be implemented to avoid complications.

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