Mechanisms of Pituitary Ovarian Axis Dysfunction and the Role of Vitamin E in Chronic Iron Over Load Rat Model

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Abstract

**Background:** Iron has a critical role in mammal’s physiological processes. However, its deposition in tissue has been reported to induce primary or secondary physiology dysfunction. Studies that evaluate the effect of chronic iron overload (CIO) on hypothalamic-pituitary-ovarian axis functions are particularly sparse. Therefore, the present study was designed to clarify the mechanism of CIO in deteriorating the pituitary-ovarian axis and the role of vitamin E as a protective agent.

**Material and Methods:** Thirty adult female albino rats weighing (180-210g) were divided into three equal groups: group I (intra-peritoneal injected (ip) with saline), group II (IP treated with ferric hydroxide polymaltose as a single dose of 100mg/kg body weight every other day for six weeks), and group III (IP received the same dose of ferric hydroxide polymaltose in group II, co-administrated with \(\alpha\)-tocopherol \(10mg/100g\) body weight” every other day for six weeks). Serum was analyzed for iron, ferritin, glucose, insulin, some pituitary and ovarian hormones, activity of some oxidant and antioxidant enzymes. Histo-pathological examination of pituitary gland and ovary were done.

**Results:** In CIO (group II) serum iron and ferritin levels were significantly increased, while vitamin E (group III) revealed non-significantly decreased in these parameters. Comparing both CIO and vitamin E treated groups with control group revealed significant decrease in levels of FSH, LH, estrogen, progesterone, prolactin, T3, T4, TSH, superoxide dismutase (SOD), and catalase (CAT) activities and significant increase in glucose, HOMA-IR and malonaldehyde (MDA) activity levels. However, levels of FSH, LH, estrogen, progesterone, prolactin, T3, T4, TSH, SOD, and CAT activities were significantly increased and glucose, HOMA-IR and MDA activity levels were significantly decreased in group III in comparison to group II.

**Conclusion:** CIO caused iron deposition in pituitary and ovarian tissues accompanied by hyperglycaemia, hyperthyroidism, hypogonadism, insulin resistance. The antioxidant \(\alpha\)-tocopherol had a partial protective effect against iron overload.

**Key Words:** Iron overload – Pituitary ovarian axis – Antioxidant – Sex hormone.

Introduction

IRON is an element of crucial importance to living cells; it plays critical physiological roles in mammals, such as being an essential component of heme in hemoglobin, myoglobin, cytochromes as well as iron-sulfur complexes of the electron transport chain. Iron is also required for activity of ribonucleoside reductase, the rate-limiting enzyme of the first metabolic reaction committed to DNA synthesis. So, iron has an important role in metabolic processes including O2 transport, electron transport, oxidative phosphorylation and energy production, cell growth, DNA synthesis, apoptosis, gene regulation and inflammation [1-3]. Therefore deficiency of iron can result in myriad disorders. Even mild iron deficiency can adversely affect cognitive performance, behavior, physical growth of infants and work performance of adults [4,5].

Physiological iron homeostasis results in exchange of only 1-2mg of iron per day between the body and the environment [6]. Disruption of this homeostasis quickly overloads the body with iron [7]. While iron overload is often undiagnosed or misdiagnosed; iron toxicity is associated with primary or secondary physiology dysfunction with substantial morbidity and increased mortality [8,9].

Genetic disorder in the iron regulatory system induces primary iron overload, as the hereditary hemochromatosis which characterized by abnormal iron metabolism with excessive iron absorption and store in the body [10,11]. In addition, iron overload can also occur with prolonged intake of iron-containing supplements, with chronic liver disease and multiple blood transfusions when regular transfusion is the only available therapy as in thalassemia major, myelodysplasia (including sideroblastic anemia), aplastic anemia, and Sickle
cell disease. Patients receiving regular RBC transfusions unavoidably and invariably develop cumulative iron overload [12,13].

Subsequently free iron is deposited primarily in parenchymal cells of the liver, heart, endocrine tissues, bone marrow, muscles, testes and ovaries [14-16]. Excessive iron deposition in liver will lead to further injury such as hepatocellular necrosis, inflammation, fibrosis, and even carcinoma [17]. In the heart, free iron in extremely low concentrations is toxic to cardiomyocytes leading to congestive cardiomyopathy [18].

Excess free iron, unbound to protective molecules such as transferrin or ferritin, catalyzes reactions that generate free radicals. If these radicals allowed running free in the body, it will be involved in oxidative stress, pathogenesis of different organs and cell death as a result of membrane lipid peroxidation [19,20].

Chronic iron deposit on the hypothalamic-pituitary-gonadal axis, may explain the impairment of reproductive function in female β-thalassemia patients [21], pituitary gland are the most vulnerable to the harmful effects of iron [22], excessive pituitary iron deposits, often develop hypogonadism [23]. Despite the advance in using the iron overload models [24], the toxicology effect of iron overload in reproductive function is not fully understood. Therefore, the present work aimed to investigate the effect of chronic iron overload on the PG axis of female rats and to identify the possible mechanism/s that could contribute to this effect. In addition, the study was carried out to examine the potential role of vitamin E as an antioxidant in modulating the chronic iron overload effects on pituitary ovarian axis.

**Material and Methods**

**Experimental animals:**

The study was conducted on Thirty adult female albino rats age and weight matched (weighing 180-210g), obtained from Faculty of Veterinary Medicine, Zagazig University. During the acclimatization period (one week) and throughout the study period, rats were kept in the animal unit of the Faculty of Medicine, Zagazig University. Rats were housed in stainless steel rodent cages under environmentally controlled conditions (comfortable temperature "20±2°C" with a 12 hours dark/light cycle) and fed the commercial rodent chow with free access to water. All investigations were conducted in accordance with the guiding principles for the care and use of research animals and were approved by the Institutional Research Board (IRB), Faculty of Medicine, Zagazig University.

**Experimental design:**

The experimental work (from animal grouping to serum collection and histopathological examination) was done in the period from 10th of November 2015 to 27th of December 2015. One, week after acclimatization, rats were randomly divided into three (3) equal groups (n=10).

**First group (control group):** Rats were injected intraperitoneal (i.p) with 0.2ml of saline (ADWIC Laboratory Chemicals, Egypt) only as a single dose every other day (3/week) for six weeks.

**Second group (CIO) group:** Rats were injected (i.p) with ferric hydroxide polymaltose (Amriya Pharm. Ind. Alexandria, Egypt) as a single dose (100mg/kg body weight) every other day (3/week) for six weeks [17,25].

**Third group:** Rats were injected (i.p) with ferric hydroxide polymaltose as a single dose (100mg/kg body weight) accompanied by vitamin E (α-tocopherol) (10mg/100g body weight) (sigma chemicals co. (Aldrich, St. Louis, Mo) every other day (3/week) for six weeks [26,27].

**Determination of the estrous phases:**

Vaginal secretion was collected with a plastic pipette filled with 10mL of normal saline by inserting the tip into as the rat vagina. Vaginal fluid was placed on glass slides. Unstained material was observed under a light microscope, with 10 and 40 x objective lenses. Vaginal smear shows: A predominance of nucleated epithelial cells with smooth margins in the proestrus phase, large anucleated cornfield (keratinized) cells with irregular margins in the estrus phase, many cornified cells plus infiltration of leukocytes in the met estrus phase. Finally, absence of the cornified cells and presence of small leukocytes is shown in the diestrus phase [28,29].

**Blood sampling:**

At the end of the experimental period estrus stage was checked then, after overnight fasting. Blood samples (about 5ml/rat) were collected from retro orbital vessels in clean plastic centrifuge tubes and allowed to clot. Serum was separated by centrifugation of blood at 3000 rpm for 15 minutes. The supernatant serum was pipetted off and stored frozen at −20°C until used.
Tissue sampling:

After blood sampling, rats were sacrificed by cervical dislocation the ovaries were excised and then kept in 10% formalin for histological examinations, dehydrated, cleared in zylol, and embedded in parablast. Paraffin sections were cut serially at 5 µm thickness. Pituitary glands washed with normal saline then were kept in 10% formalin for 48 hours. The samples washed up by tap water for two hours, then the tissue was prepared for the light microscope study which include: (Dehydration, clearing, infiltration, embedding, sectioning, staining, mounting, drying, cleaning and labeling respectively) [30].

Sections were processed on slide and stained with Haematoxylin and eosin (H & E) stain [30] to study general microscopic characters of the ovary and pituitary gland and with Perl's Prussian blue stain to record iron deposition [32].

The serum was analyzed for:

- Iron using commercial kit (spinreact, co., spain) according to the method described by Burits and Ashwood [33].
- Ferritin (FE) using commercial FE Enzyme-linked immunosorbent assay (ELISA) kit (mybiosource, Catalog Number: MBS722921) applies the quantitative sandwich enzyme immunoassay technique according to the method described by Linpisarn et al. [34].
- Follicle stimulating hormone (FSH), Luteinizing hormone (LH), prolactin (PRL), Estradiol (E2), and Progesterone (PROG) using ELISA rat kits: BC-1029, BC-1031, BC-1111, BC-1113 and BC-1115, respectively, BioCheck Inc 323 Vintage Park Dr. Foster City, CA 94404, according to the method of Tietz [38].
- Catalase activity, Superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels using Biodiagnostic kit method (Biodiagnostic company, Dokki, Giza, Egypt), according to the method of Aebi [36], Nishikimi et al., [37] and Satoh [38] respectively.
- Thyroid stimulating hormone (TSH), Triiodothyronine (T3) and thyroxine (T4) using ELISA kits provided from mybiosource, catalog Number; MBS013061, MBS283344, MBS843386 respectively, according, to the method of Soos and Siddle [39], Boscato and Stuart [40], Schuurs and Van Weeman [41] respectively.
- Glucose using commercial kit provided by spinreact, co., spain. according to method described by Trinder [42].
- Insulin: Using commercial ELISA Kit provided from mybiosource, catalog number MBS724709 according to the method described by Temple et al. [43].
- HOMA-IR index was calculated to estimate insulin resistance from the equation of Mathew et al. [44].

\[
\text{IR} = \frac{\text{Insulin (µU/ml x Glucose (mg/dl)}}{405}
\]

Statistical analysis:

Results were presented as mean ± standard deviation. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 20.0 (SPSS Inc., Chicago, IL, United States). Repeated measures of analysis of variance (ANOVA) were applied followed by the Student-least significant differentiation (LSD), post hoc test to compare means of each two different groups. Pearson's correlation analysis was performed to screen potential relations between serum ferritin levels and all parameters. For all statistical tests done, \( p \)-value <0.05 was considered to be statistically significant.

Results

There were significant increase in both serum iron and ferritin levels of group II (CIO) and group III (CIO+Vit. E) (\( p <0.001 \)) when compared with that of group I (control). However, no significant deference detected between group II and Group III (\( p >0.05 \)) (Table 1). As regards pituitary and ovarian sex hormones, the mean values of serum levels of FSH, LH, prolactin, estrogen and progesterone were significantly decreased in both of group II (\( p <0.001 \)) and group III (\( p <0.05, p <0.01, p <0.05, p <0.01 \& p <0.05 \) respectively) when compared to that of group I. In addition, the levels of these hormones were significantly higher in group III when compared to that of group II (\( p <0.01, p <0.05, p <0.05, p <0.001 \& p <0.01 \) respectively). Pearson's correlation analysis between serum ferritin levels and pituitary and ovarian hormones revealed significant negative correlation with FSH, LH, prolactin, estrogen and progesterone in group II (\( p <0.01, p <0.01, p <0.01, p <0.01 \) and \( p <0.001 \) respectively) and group III (\( p <0.01, p <0.01, p <0.01, p <0.001 \) and \( p <0.01 \) respectively). However, in group I serum ferritin levels showed significant negative correlation only with progesterone (\( p <0.01 \)) (Table 2).

Regarding serum levels of some oxidant and antioxidant activity (Table 3), in group II and group III, there was significant increase in levels of MDA

\[
\text{HOMA-IR} = \frac{\text{Insulin (µU/ml x Glucose (mg/dl)}}{405}
\]

\[
\text{HOMA-IR} = \frac{\text{Insulin (µU/ml x Glucose (mg/dl)}}{405}
\]
activity \((p<0.001)\), simultaneously with significant decrease in the levels of both SOD and CAT activity \((p<0.001 & p<0.05)\) in comparison to that of group I. Comparing group III with group II resulted in significant decrease in MDA levels \((p<0.001)\) and significant increase in both of SOD and CAT levels \((p<0.001)\). While serum ferritin levels significantly positive correlated with MDA levels in the three studied groups \((p<0.001)\). It negatively correlated with SOD and CAT only in group II and group III \((p<0.01 & p<0.001 \text{ respectively})\).

Concerning glucose, metabolism (Table 4), there were significant increase in serum glucose \((p<0.001, p<0.01)\) and calculated HOMA-IR levels \((p<0.001)\) in both of group II and group III when compared with that of group I and positively correlated with serum ferritin levels in group II \((p<0.05 \& p<0.01)\) and group III \((p<0.001)\). Serum insulin levels showed non-significant increase among the three groups and significantly positive correlated with ferritin levels in group II and group III \((p<0.001)\).

Regarding thyroid hormones (Table 5), there were significant decrease in the mean values of TSH \((p<0.001)\), T3 \((p<0.001 \& p<0.01)\) and T4 \((p<0.001 \& p<0.01)\) in group II and group III respectively when compared with that of group I. moreover, in group III these hormones were significantly higher than that of group II \((p<0.01, p<0.05 \& p<0.001 \text{ respectively})\). While TSH negatively correlated with serum ferritin levels, in all groups \((p<0.01, p<0.001 \& p<0.001 \text{ respectively})\), T3 negatively correlated in group II \((p<0.001)\) and group III \((p<0.01)\), finally T4 correlated negatively in both of group II and group III \((p<0.01)\).

The vaginal smears obtained from rats of CIO group showed persistence of the estrus phase of estrus cycle which indicates the absence of ovulation.

### Table 1: Serum iron (µ/dl) and ferritin (ng/mL) levels in the three studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Mean ± SD</th>
<th>(p) VS Group I</th>
<th>(p) VS Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron (µ/dl)</td>
<td>Group I</td>
<td>229.0±14.18</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>3496.8±497.1</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>3287.9±379.3</td>
<td>0.001</td>
<td>0.05</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>Group I</td>
<td>57.6±6.48</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>240.0±19.66</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>227.25±20.76</td>
<td>0.001</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Table 2: Serum levels of FSH (mIU/ml), LH (mIU/ml), Prolactin (ng/ml), Estradiol (pg/ml), and progesterone (ng/ml) in the three studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Mean ± SD</th>
<th>(p) VS Group I</th>
<th>(p) VS Group II</th>
<th>(r) with ferritin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (mIU/ml)</td>
<td>Group I</td>
<td>5.30±0.97</td>
<td>0.001</td>
<td></td>
<td>-0.30 ((p&lt;0.05))</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>3.00±1.01</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.88 ((p&lt;0.01))</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>4.26±0.83</td>
<td>0.05</td>
<td>0.01</td>
<td>-0.85 ((p&lt;0.01))</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>Group I</td>
<td>0.85±0.22</td>
<td>0.001</td>
<td></td>
<td>-0.87 ((p&lt;0.01))</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>0.43±0.10</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.84 ((p&lt;0.01))</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>0.59±0.09</td>
<td>0.01</td>
<td>0.05</td>
<td>-0.82 ((p&lt;0.01))</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>Group I</td>
<td>10.96±1.81</td>
<td>0.001</td>
<td></td>
<td>-0.23 ((p&lt;0.05))</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>6.95±1.62</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.82 ((p&lt;0.01))</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>8.86±1.75</td>
<td>0.05</td>
<td>0.05</td>
<td>-0.87 ((p&lt;0.01))</td>
</tr>
<tr>
<td>Estrogen (pg/ml)</td>
<td>Group I</td>
<td>39.89±3.70</td>
<td>0.001</td>
<td></td>
<td>-0.51 ((p&lt;0.05))</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>28.74±2.79</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.84 ((p&lt;0.01))</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>35.5±3.52</td>
<td>0.001</td>
<td>0.001</td>
<td>-0.90 ((p&lt;0.001))</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>Group I</td>
<td>34.55±2.68</td>
<td>0.001</td>
<td></td>
<td>-0.80 ((p&lt;0.01))</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>25.14±3.18</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.89 ((p&lt;0.001))</td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>30.34±4.16</td>
<td>0.05</td>
<td>0.01</td>
<td>-0.88 ((p&lt;0.01))</td>
</tr>
</tbody>
</table>
Table (3): Serum levels of MDA (nmol/ml), SOD activity and CAT activity (mmol/l) in the three studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Mean ± SD</th>
<th>p VS Group I</th>
<th>p VS Group II</th>
<th>r with ferritin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/ml)</td>
<td>Group I</td>
<td>37.18±5.14</td>
<td>p&lt;0.001</td>
<td>+0.90 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>62.84±8.59</td>
<td>p&lt;0.001</td>
<td>+0.91 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>54.95±7.96</td>
<td>p&lt;0.05</td>
<td>+0.89 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>SOD (u/l)</td>
<td>Group I</td>
<td>48.27±5.12</td>
<td>p&lt;0.001</td>
<td>-0.55 (p&gt;0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>37.15±4.56</td>
<td>p&lt;0.001</td>
<td>-0.87 (p&lt;0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>43.03±4.99</td>
<td>p&lt;0.01</td>
<td>-0.93 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>CAT (mmol/l)</td>
<td>Group I</td>
<td>51.18±6.54</td>
<td>p&lt;0.001</td>
<td>-0.31 (p&gt;0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>32.94±6.11</td>
<td>p&lt;0.001</td>
<td>-0.86 (p&lt;0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>44.90±4.58</td>
<td>p&lt;0.001</td>
<td>-0.95 (p&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

Table (4): Serum levels of glucose (mg/dl) and Insulin (µIU/ml) with calculated HOMA-IR in the three studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Mean ± SD</th>
<th>p VS Group I</th>
<th>p VS Group II</th>
<th>r with ferritin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>Group I</td>
<td>97.8±6.08</td>
<td>p&lt;0.001</td>
<td>+0.45 (p&gt;0.05)</td>
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<tr>
<td></td>
<td>Group II</td>
<td>248.20±31.02</td>
<td>p&lt;0.001</td>
<td>+0.76 (p&lt;0.05)</td>
<td></td>
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<tr>
<td></td>
<td>Group III</td>
<td>158.10±14.63</td>
<td>p&lt;0.001</td>
<td>+0.90 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Insulin (µIU/ml)</td>
<td>Group I</td>
<td>9.08±1.05</td>
<td>p&gt;0.05</td>
<td>+0.42 (p&gt;0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>10.02±1.08</td>
<td>p&gt;0.05</td>
<td>+0.93 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>9.57±1.06</td>
<td>p&gt;0.05</td>
<td>+0.91 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Group I</td>
<td>2.20±0.38</td>
<td>p&lt;0.001</td>
<td>+0.42 (p&gt;0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>6.23±1.42</td>
<td>p&lt;0.001</td>
<td>+0.89 (p&lt;0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>3.77±0.75</td>
<td>p&lt;0.001</td>
<td>+0.91 (p&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

Table (5): Serum levels of TSH (µIU/ml), T3 (ng/ml) and T4 (µg/dl) in the three studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Mean ± SD</th>
<th>p VS Group I</th>
<th>p VS Group II</th>
<th>r with ferritin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH (µIU/ml)</td>
<td>Group I</td>
<td>0.54±0.13</td>
<td>p&lt;0.001</td>
<td>-0.78 (p&lt;0.01)</td>
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<tr>
<td></td>
<td>Group II</td>
<td>0.17±0.08</td>
<td>p&lt;0.001</td>
<td>-0.92 (p&lt;0.001)</td>
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<td></td>
<td>Group III</td>
<td>0.32±0.09</td>
<td>p&lt;0.001</td>
<td>-0.88 (p&lt;0.01)</td>
<td></td>
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<tr>
<td>T3 (ng/ml)</td>
<td>Group I</td>
<td>1.32±0.20</td>
<td>p&lt;0.001</td>
<td>-0.58 (p&gt;0.05)</td>
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</tr>
<tr>
<td></td>
<td>Group II</td>
<td>0.72±0.23</td>
<td>p&lt;0.001</td>
<td>-0.96 (p&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>0.99±0.27</td>
<td>p&lt;0.05</td>
<td>-0.86 (p&lt;0.01)</td>
<td></td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>Group I</td>
<td>6.55±1.58</td>
<td>p&lt;0.001</td>
<td>-0.61 (p&gt;0.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>2.68±0.69</td>
<td>p&lt;0.001</td>
<td>-0.83 (p&lt;0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III</td>
<td>4.94±0.93</td>
<td>p&lt;0.001</td>
<td>-0.82 (p&lt;0.01)</td>
<td></td>
</tr>
</tbody>
</table>
Histo-pathological examination:

Fig. (1): Photomicrograph of normal ovary showing growing Graffian follicle with central ovum surrounded by normal ovarian stroma (Hematoxylin & Eosin x200).

Fig. (2): Photomicrograph of normal ovary stained with Prussian blue showing absence of iron granules in the graffian follicle and ovarian stroma (Prussian blue x200).

Fig. (3): Photo micrograph of ovary of iron injected rat showing pyknotic nuclei and deeply stained cytoplasm with indistinct cell borders of ovarian cell (T) (Hematoxylin & Eosin x200).

Fig. (4): Photomicrograph of ovary of iron injected rat showing numerous small bluish iron granules in the ovarian tissue (T) (Prussian blue x200).

Fig. (5): Photomicrograph of iron-injected rat treated with vitamin E showing return of the ovarian tissue to its normal state with numerous graffian follicles at various stages of development (T) (Hematoxylin & Eosin x200).

Fig. (6): Photomicrograph of iron injected rat after treatment with vitamin E showing decrease of iron granules from the ovarian tissue. (Prussian blue x200).
Fig. (7): Photomicrograph of normal pituitary gland showing papillary structures lined by normal small round uniform hormone producing cells (T) (Hematoxyline & Eosin x200).

Fig. (8): Photomicrograph of normal pituitary gland showing absence of iron granules in the pituitary gland cells (Prussian blue x200).

Fig. (9): Photomicrograph of pituitary gland of rat injected with iron showing pyknotic nuclei of the pituitary cells (T) (Hematoxyline & Eosin x200).

Fig. (10): Photomicrograph of pituitary gland of rat after iron injection showing numerous small blue iron dots in the cells (T) (Prussian blue x200).

Fig. (11): Photomicrograph of iron injected rat treated with vitamin E showing normal tubular and papillary structures of pituitary gland (Hematoxyline & Eosin x200).

Fig. (12): Photomicrograph of iron injected rat treated with vitamin E showing decrease of blue iron granules in the cells of pituitary (T) (Prussian blue x200).

**Discussion**

The hypothalamo-pituitary-ovarian (HPO) axis is a major modulator of female reproductive function as it has the golden role in regulating the fundamental hormonal pathways for normal fertility [45]. Although iron is an important metal for HPO axis control, such as ovarian follicular development [17,46], its deposition in body tissues poses endocrine abnormalities [47].

Acute iron overload accumulated in hypothalamus has induced malfunctioning of the reproductive organs [48,49] and exhibited an abnormal pitu-
itary function [50] as inadequate pituitary responsiveness to the GnRH test [51].

Previous studies that employed chronic iron overload (CIO) have demonstrated that iron accumulation can impair HPO axis function [52,53], however, the precise mechanism/s of this impairment is not clearly understood. Therefore, the present study was designed to clarify some of the possible mechanisms of CIO effect on HPO axis using a rat model.

The present work revealed that CIO induced by i.p injection of ferric hydroxide polymaltose given as a single dose of 100mg/kg body weight every other day for six weeks resulted in a significant increase in serum iron and ferritin levels which are accompanied with iron deposition in the pituitary and ovarian tissues. There was also a significant decrease in serum levels of estrogen, progesterone and thyroid hormones. Beside this significant decay in steroidogenesis, iron deposition in ovarian tissue showed histopathological impairment in oogenesis.

These results regarding the increase in circulating and tissue iron in CIO were proved by Neves et al. [54] who reported that when rats were treated ip with iron-dextran developed an iron overload that is similar to that observed as a secondary effect to iron-loading anemia (thalassemia) and excessive blood transfusions [55]. In addition, Rossi et al. [49] proved that iron deposition in the pituitary and ovary even in acute iron overload rat model was prominent and exhibited a positive correlation with the serum iron levels.

In the present work, the significant pituitary and ovarian dysfunction induced by CIO resemble that occurring in b thalassemia major women, (probably due to chronic iron accumulation), leads to a decrease in serum prolactin level and impairment of ovarian granulose cell with abnormal estrogen production [52,56,57]. Piperno et al. [56] also found that prolactin increments after THR stimulation were significantly reduced in hemochromatosis patients. This PRL behavior did not significantly change after iron depletion, suggesting that the damage was not reversible, possibly due to lactotrophs destruction or reduction of their hormone content. In addition, β thalassemic patients showed low-surge serum LH and FSH levels that lead to impairment in the induction of ovulation [58]. Moreover, in acute iron overload the ovary iron levels exhibited a positive correlation with the number of atretic ovarian follicles and negative correlation with the number of corpus luteum cells. The degree of abnormal ovarian follicular development was accompanied by a reduction in the serum E2 levels [49].

Since the antioxidant capacity serves a decisive role in the normal development of ovarian follicles and early embryos [59]. Our study concerned with oxidative stress (OS) parameters revealed that CIO was associated with significant changes in serum levels of many of the indicators of oxidative stress as an increase in serum levels of MDA together with a decrease in serum levels of SOD and catalase. Serum ferritin levels significantly correlated positively with MDA and negatively with both of SOD and catalase, this finding can provide evidence that iron toxicity is responsible to some extend for OS condition. These results are supported by the animal studies of acute iron overload that revealed an imbalance in the antioxidant status as an increase in plasmatic MDA levels which positively correlated with the serum iron levels [49,60].

Chronic iron overload as a result of multiple blood transfusions in patients with beta-thalassemia major also developed OS [61]. The iron toxicity associated with several organ dysfunctions [13,52] is attributed to its ability to produce cellular OS and reactive oxygen species (ROS), which could deteriorate lipid membranes, proteins, and nucleic acids [62] leading to changes in pituitary and ovarian morphology and function [49]. In contrast, other investigators reported that patients with moderate-to-severe pituitary iron overload retained normal pituitary function [50]. Increase in OS and lower enzymatic antioxidant defense mechanisms accelerate ovarian follicle aging [63].

The impaired function of HPO-axis encountered in the present study could be explained by many other possibilities.

The first possibility is that CIO may be associated with hyperglycemia and insulin resistance (i.e Diabetes mellitus). This possibility was proved in the present study as we found a significant increase in both glucose levels and insulin resistance, while there was insignificant increase in basal insulin levels in CIO group. In the same context, Fairbanks and Beutler [64] reported that patients with hemochromatosis developed glucose intolerance, β cell dysfunction and insulin resistance secondary to iron accumulation in the pancreas. Lu et. al. [65] documented the binding of iron with specific transferrin receptors on beta cell of iron over loaded rats. Adewoya et al. [66] observed that CIO in rats
caused hyperglycemia, insulin resistance and pancreatic beta cell dysfunction thus predisposing to type-2 diabetes. Moreover, Suvarna et al. [67] mentioned that insulin resistance was higher in patients not on chelation therapy, and correlated significantly with parameters of iron overload, i.e., total units of blood transfused, splenomegaly and serum ferritin in thalassemia major patients.

Iron deposition induced hyperglycemia through interfering with the hepatic insulin extraction and insulin’s ability to increase hepatic glucose uptake and insulin’s ability to decrease hepatic glucose output, besides decreasing muscle glucose uptake [68]. This Hyperglycemia causes oxidative stress that triggers the release of several inflammatory markers (such as IL6 and TNF $\alpha$), inducing harmful effects on pancreas, liver and muscle [69]. The inflammatory process in the pancreas is characterised by the presence of immune cell infiltration, apoptotic cells, fat deposits, amyloid deposits and eventually fibrosis [70] resulting in pancreatic beta cell dysfunction and reduction of $\beta$ cell mass [66], so the insulin levels in the CIO group of the present study were not significantly increased and this is at variance to significant increased insulin levels in hereditary and high fat diet induced type-2 diabetes.

Many studies demonstrated that diabetes especially type-2 induces alternations of gonadotropins and sex steroids and negatively affected the serum FSH, LH, E2 and progesterone levels [71]. In addition, other studies showed that the diabetic state is associated with anovulation, estral cycle alteration, exogenous gonadotrophin insensitivity, steroidogenesis depression and ovarian atrophy [72]. Moreover, a significant increase in atretic follicles, disjunction of granulosa cells, and appearance of picnotic nucleus in granulosa cell layer are noticeable in cases of type-2 diabetes together with a decrease in ovarian weight [73].

Diabetes has marked effects on normal cellular processes, resulting in disturbance of mitochondrial free radical production and imbalance between ROS generation and antioxidant defenses [74]. This OS has been implicated in the progression of long-term diabetes complications including microvascular and macrovascular dysfunction [75], leading to DNA damage, inhibited protein ATP production and diminished gonadotrophin [76]. Thus the diminished levels of gonadotrophins (LH and FSH) and increased mitogen-activated protein kinase (MAPK) in the ovary of diabetic rats which contribute to the decrease in progesterone secretion [77] may be attributed to increased ROS generation [78].

Thus, regarding the results of the present study, it can be claimed that the disturbance in HPO axis indicated by the presence of altered levels of FSH, LH, E2 and progesterone in CIO female rats could be attributed at least in part to the diabetic state observed in these rats.

The second possible explanation for the impaired function of HPO axis observed in the present study may be the presence of CIO induced hypothyroidism. The present study revealed a significant decrease in serum levels of T3&T4 together with a significant decrease in serum levels of TSH, in CIO rats. These results are in accordance with those of many investigators who showed that iron deposition within the thyroid gland that may occur due to iron overload induced by multiple transfusions could be associated with primary or secondary hypothyroidism [79].

Many researchers reported that deposition of iron in the thyroid gland causes fibrosis of the glandular parenchyma, that can lead to progressive thyroid dysfunction with different degrees of severity from normal function, primary, compensated hypothyroidism or mild hypothyroidism up to overt hypothyroidism [80,81]. In addition, iron deposition progressively damages the pituitary gland and hypothalamus leading to either secondary and/or tertiary hypothyroidism in patients with $\beta$ thalassemia [22,82]. Hypothyroidism is also seen in patients with hereditary hemochromatosis (HHC) which may reflect increased iron burden in thyroid tissue, with associated fibrosis and inflammation [83]. HHC patients, who had hypothyroidism due to impaired thyrotroph function, also had hypogonadotrophic hypogonadism [84].

It is well known that hypothyroidism impairs reproductive function both in humans and experimental animals. In several species induction of hypothyroidism resulted in prolonged irregular estrous cycles [85], decreased number of primordial, antral and Graffian follicles [86], and produced ovarian atrophy [87]. Moreover, Armada-Dias et al. [88] reported that hypothyroidism was associated with dysfunction in the pituitary-ovarian axis that impaired folliculogenesis and development of corpora lutea. In women, hypothyroidism is associated with delayed onset of puberty [89], anovulation [90], menstrual irregularity and infertility [91].

In 2013, Ajayi et al. [92] suggested that impaired reproductive function seen in altered thyroid states is due to low LH and oestradiol levels, and alterations in female organs cytoarchitecture. Also, hypothyroidism was associated with reduced go-
n antidrophins levels and impaired pre-ovulatory luteinizing hormone surge leading to suppression of ovulation [93,94].

Treesh and Khair [95] observed thick tunica albuginea and excessive amount of collagen fibers in the ovarian stroma of hypothyroid rats. This is due to the decrease in collagen degradation by diminished lysosomal enzymes in hypothyroid rats [96]. Also, hyper cellular ovarian stroma with luteinization of stromal cells that appear with a vacuolated cytoplasm were detected by many investigators [95,97].

The third possible explanation for the impairment of HPO axis function encountered in the present study may be the presence of iron overload-induced hypoparathyroidism. This possibility is supported by findings of Hagag et al. [98] who reported that bone mineral density and parathyroid functions are significantly lower in thalassemic patients, and observed a significant negative correlation between levels of parathormone and serum ferritin. In addition, many investigators reported that frequent blood transfusion therapy could result in iron deposition in cells of the parathyroid gland free radical formation that causes mitochondrial and lysosomal membrane damage leading to glandular tissue damage and fibrosis [99,100]. Therefore, serum ionized calcium and vitamin D levels were found to be significantly lower while serum phosphorus and alkaline phosphatase level were found to be significantly higher than normal in case of CIO due to decreased circulatory levels of parathormone [101,102].

Vitamin-D deficiency induced by hypoparathyroidism is associated with reduced fertility rates, hypogonadism, and decreased aromatase and 5 α-reductase activities in the ovary, impaired corpus luteum formation, uterine hypoplasia, impaired folliculogenesis and histological abnormalities of the ovary [103,104].

In the present study, it was found that the administration of vitamin E simultaneously to iron loading (group III) significantly ameliorated the effect of CIO on PO axis. The mean values of FSH, LH, estrogen, progesterone were significantly higher than that of CIO group. However, they were still significantly lower in comparison to control group. Moreover, the antioxidant effect of vitamin E was revealed by the presence of a significant decrease in MDA together with a significant increase of both SOD and catalase enzymes. Furthermore pancreatic and thyroid functions were found to be significantly improved after administration of vitamin E as indicated by the observation of a significant decrease in HOMA-IR plus a significant increase in T3 and T4 in comparison to CIO group.

These results are in line with that of Hamed et al. [105] who concluded that, administration of vitamin E concurrently with lead or after lead exposure obviously increased the plasma levels of FSH, LH, estradiol and progesterone in adult female rats. In the parallel context Hamadouche et al. [106] demonstrated a great increase in the plasma levels of LH, FSH and testosterone with oral administration of vitamin E after lead exposure in adult male rats.

It is well known that, natural Vitamin E as well as synthetic is a lipid-soluble antioxidant [107], and a potent scavenger of peroxyl radicals, so vitamin E is the primary inhibitor of the free radical-mediated chain reaction of lipid peroxidation in mammals including humans [108]. Thus, the mechanism by which vitamin E induced its effect is partially through its antioxidant capacity as proved in this work (reduced MAD with increase in both of catalase and SOD). In addition, vitamin E was found to be a potentially useful therapeutic agent in the treatment of several disorders associated with heavy metals induced oxidative damage [109]. Sharma and Bhattacharya [110] suggested that cotreatment with vitamin E has a protective role against lead induced ovarian damage in mice.

A part from antioxidant, Karanth et al. [111] reported that vitamin E has a neurotransmitter effect in the hypothalamus as it activated the formation of nitric oxide. This nitric oxide increases FSH and LH secretion from the anterior pituitary gland through activation of cyclic guanosine monophosphate as a second messenger in the GnRH neurons. Furthermore, Khan et al. [112] reported a significant increase in the size and area of FSH and LH gonadotropes with vitamin E administration. Hamed et al. [105] observed that vitamin E treatment induced an engorgement in gonadotrophic cells with secretory granules, euchromatic nuclei and numerous mitochondria which could explain the increased plasma levels of LH and FSH. Increased plasma levels of estradiol and progesterone could be a result of FSH and LH increase or direct effect on ovaries as Cicek et al. [113] demonstrated that vitamin E had an anticoagulant effect resulting in adequate blood supply to ovarian follicles.

In conclusion:

The present study revealed that experimentally induced CIO in an adult female rat model is accompanied with impairment of PO axis function
that could result in infertility. This dysfunction of the axis may be attributed to CIO-induced oxidative stress together with some endocrine disorders like diabetes mellitus (IR plus hyperglycemia) and hypothyroidism. Moreover, the present study showed that co-administration of vitamin E plays a significant role in amelioration of this CIO-induced dysfunction of PO axis.

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الملخص العربي

عنصر الحديد له دوراً أساسياً في الوظائف الحيوية في جسم الإنسان، وقد يؤدي زيادة تخزين الحديد داخل أعضاء الجسم إلى خلل أوبCHA على النحو واحد من الأعراض المشتركة بين مرض الورم الدم ومرض السكري. وقد تتعلق إلى آليات أخرى في هذا الخلل. لذلك قد صمم هذا البحث لاستكشاف الأثر التهابي لل الحديد المكمل على موت الدم الحيوية والمسامع في آلية اليدين والقدمان. وقد أثبتت الدراسة على عدد من كاسيات الحديد القابضة والتسمية إلى ثلاث مجموعة متنوعة (3 جرعة في كل مجموعة) كما يلي:

المجموعة الأولى: كميجرو ضارب. وقد حققت عن طريق الفضلات البروتيك بحمى متجسدة. المجموعة الثانية: متالزي بالديد، وقد حققت عن طريق الفضلات البروتيك بجرعة من أكاسيات الحديد قدرها (0.1 ملغ/جرام لكل 10 كجم من وزن الجسم) ثلاث جرعات يومياً بعد يوم كل أسبوع.

المجموعة الثالثة: معلبة بجرعة من الحديد قدرها (الكازا في المجموعة الثانية) بالإضافة إلى جرعة فيتمين (0.6 ملغ/جرام لكل 10 كجم). وقد تم حقنها عن طريق الفضلات البروتيك يومياً بعد يوم ثلاث جرعات في الأسبوع لمدة 6 أسابيع. تم تحديد نزاعات ثقب في المجموعات الثلاثة وذلك عن طريق تقييم مسحة من الاتصالات المذهلة وتمييز الخلايا الموجودة داخل الغدة باستخدام الميكروسكوب الضوئي وتم الحصول على عينات الدم البروتيكية عند المرحلة الثالثة من الفحص ثم فصل الدلب وحفظه عند (20) درجة مئوية إلى حين استعماله. وقد أعطى عينة من هذه الخلايا عينة賽ناسيسي ونسخة المبيض وذلك لاستكمال الدراسة بالعربية معلومات النموذجية ودراسة التغييرات النسيجية.

النهاية عن ترتيب الحديد.

وقد أظهرت النتائج ما يلي:

• زيادة ذات إذا كانت في مستوى كم من الحديد والقروتين في مصل العظام في المجموعة الثانية مقارنة بالمجموعة الضاربة ونقصاً في المجموعة الثالثة مقارنة بالمجموعة الثانية.

• زيادة ذات إذا كانت في مستوى كم من السكر والدهون في المجموعة الثانية مقارنة بالمجموعة الضاربة ونقصاً في المجموعة الثالثة مقارنة بالمجموعة الثانية.

• انخفاضاً في مستوى إلا في المجموعة المنخفضة للفحص، وفي المجموعات الأخرى، ونسبة ممن البروتيك ورومون المحمول للقهوة الدريبي واستروجين والبروجسترون، ونسبة ممن الحديد السريع في المجموعة الثانية مقارنة بالمجموعة الضاربة.

• بينما حدث زيادة ذات إذا كانت في المجموعة الثالثة مقارنة بالمجموعة الثانية.

• أجبر على الفحص الميكروسكوبية في المجموعة الثانية لتسنج مقاومة الدم الحيوية: عند وجود تغيرات مرضية نسبية تمتلئ في ترتيب الحديد في الخلايا مما يدلي إلى احتمالات وفرة الأوكسوجين يدتها، عن وجود تغييرات مرضية نسبية تمتلئ في ترتيب الحديد في الخلايا مما يدلي إلى ضعف الدم الحيوية ونسبة ممن الحديد السريع في اختبار بعض هذه التغييرات مع نفس في ترتيب الحديد في المجموعة الثالثة (في يوم فيتمين).

على الرغم من النتائج السابقة يمكن استنتاج أن الحديد المكمل اللازم قد يؤدي إلى خلل في وظيفة مجرى الدم الحيوية والمسامع لما قد يؤدي إلى عوز الحبيبان في ذلك الامراض التاكسدي جنبًا إلى جانب مع بعض الأعراض في القوة الصمام التي أدت إلى داء السكري واضطرابات القدرة القهوة التي كان انخفاضاً من أفراد الدم الحيوية. أظهرت الدراسة أيضاً أن (فيتمين) الميضاد للأكسدة يلعب دوراً في تحسين ذلك الخلل الحادث في وظائف مجرى الدم المقياس والمضار.