OSMOREGULATION AND ANTIOXIDANT EFFECTS OF COPPER SULFATE AND PROPOLIS IN RED BLOOD CELLS OF MALE RATS

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Abstract
This experiments was conducted to elucidate the effect of copper supplementation on performance, RBCs bioactive markers, ATPase activity and antioxidant responses. Twenty one adult Wister rats were randomly distributed into four equal groups; first group was a control group (C group); animals of 2nd group (Cu) received copper (75 mg/kg body weight), animals of 3rd group (CuP) received Copper sulfate (75mg /kg body weight) + propolis (50 mg/kg body), for 60 days. Results revealed that the dose of CuSO₄ (75 mg/Kg) did not affect significantly the animals performances. Serum copper level of Cu group (36.16µg/dl) produce improvement in red blood cell membrane hypotonic osmotic fragility, hemoglobin concentration as well as the ATPase enzyme activity. Moreover, Cu improved significantly total antioxidant capacity (TAOC) and reduces the malondialdehyde acid (MDA). There was no significant role for the combination of propolis with copper sulfate on different parameters with exception of TAOC & MDA. In conclusion, the dose of 75mg/kg was a safe and it was convincing to improve the level of copper in the blood and showed its importance in maintaining the size and membrane integrity of red cells, with no any side effects.

Key words: Copper, Osmotic fragility, ATPase, Antioxidant, Propolis

Introduction
Copper is an important element broadly distributed in nature with an atomic number 29 and atomic weight 63.546 and has two forms of oxidation (Linder & Hazeqh-Azam, 1996). Copper has a very important biological role come from being a protein catalytic cofactor for variable essential enzymes and oxidation reduction reaction, (Hordyjewska et al., 2014). It is required for cytochrome C oxidase as an electron transporter in the mitochondria for ATP production (Karim, 2018). Copper has an essential role in oxidant-antioxidant stability with its ability as an electron donor or acceptor (Ralph & McArdle, 2001) make it a good player in scavenging free radical (Uriu-Adams & Keen, 2005) and activating the antioxidant enzymes like superoxide dismutase (Vashchenko & Mac Gillivray, 2013). Serum copper level is a risk factor that should be fine regulated since copper storage is very limited process in the body. Excess copper excreted through biliary excretion in addition to unabsorbable copper or copper from desquamated mucosal cell (Karim, 2018). Copper deficiency anemia is the result of impaired iron transportation with transferrin where it does carry ferrie iron (Fe³⁺) oxidized from ferrous (Fe²⁺) by cereloplasmin sufficient cereloplasmin the protein responsible for carrying serum copper (Fedorova et al., 2019; D. Song & Dunaief, 2013). Healthy red blood cells process their own oxidant – antioxidant balance with the cellular antioxidant system to avoid membrane oxidative damage (Singh et al., 2019). Red blood cells have the ability to change their shape (Deformability) for the purpose of facilitating its passage through the microvascular system to deliver oxygen to tissues. This deformability is very important process it is cell volume and osmotic pressure dependent (Kim et al., 2015). The regulation of volume of red blood cells is a very important task for maintenance the cell, this fine and critical task depends on cell membrane functional structures. The Na-K ATPase known as a crucial enzyme in maintaining red blood cells intracellular homeostasis (Radosinka et al., 2016), which required low cell membrane permeability to cation and ATP consumption (Lew & Tiffert, 2017). Regulatory volume mechanism showed an correlation between hemoglobin and red blood cells osmoregulation...
Increased oxidative stress induced by reactive oxygen species affect the cells deformability (Diederich et al., 2018). Propolis is material used to close holes in the honeycomb, adhesive, smell like resin Its color of depend on the area and the plant source (Ahmed et al., 2017) taken from bee wax it has an pharmacological and biological properties, propolis composition is flavonoid which is used as antifungal antiviral, antioxidant, antibacterial (Kalia et al., 2016). And prevent lipid peroxidation in red blood cells of human (Zabaiou et al., 2017).

In Iraq (Al-Hejuje et al., 2017) indicated that level of copper ion in soil and river’s water is below the permissible limits of drinking water according to the WHO (2011). In an attempt to explain the correlation between serum copper level with red blood cells membrane integrity and osmoregulation by ATPase enzyme activity favoring cells deformability, the present study was designed.

**Material and methods**

**Experimental design**

Propolis was prepared by putting 50 gm of it in 500 ml of tap water for 3 days on a hot plat magnetic stirrer and kept away from light exposure (Hendi et al., 2011). 21 Wistar rats were divided into 3 groups; the1st group as a control group the 2nd group orally administered with copper sulfate 75 mg/kg bw and the 3rd group treated with propolis 50 mg/kg b.w and copper sulfate 75 mg/kg bw all by oral gavage. Blood Samples were collected from anesthetized rats (IM injection of ketamine 90mg/kg B.W and xylazine 40mg/kg B.W by cardiac puncture. Red blood cells osmotic fragility and blood ATPase were determined in fresh anticoagulant blood sample. And serum was isolated for antioxidant evaluation (MDA, TAOC) copper serum as well as the bilirubin.

**Serum copper (µg/dl)**

Serum copper level (microgram/dl) measured by Phoenix - Model 986 AAS Series - Flame Autosampler the Phoenix-986 AA is an easy to use atomic absorption spectrophotometer with a graphite furnace attachment for flameless analysis.

**Red blood cells Bioactivity markers**

Red blood cell osmotic fragility test(RBC %) applied according to (Farias, 2017) to measure the hypotonic resistance of red blood cells membrane. Whole blood ATPase estimated by colorimetric method using commercial kit provided by ELABSCIENCE (USA) Following instruction of the company. Hemoglobin (gm/dl) measured by colorimetric method by using a commercial kit and following the company instruction

**Results**

**Serum copper**

The result showed that serum copper (microgram/dl) was significantly increased (P<0.05) in Cu group (36.16 ± 2.427) and non-significant in CuP group (25.660.414) when compare with control group (21.350.614) Fig. 1.

**Red blood cells bioactivity markers**

The results obtained from osmotic fragility indicated for RBC hemolysis (%) in different hypotonic concentration of NaCl are represented in Fig. 2. Results revealed that the highest red blood cells hemolytic were at NaCl concentration 0.01 and 0.03 for all experimental groups, as results indicated that percentage of red blood cells hemolysis increased significantly (P<0.05) proportional with hypotonic solutions of NaCl to reach the maximum values for control only (0.763±0.06) and (0.632±0.01., 0.638±0.02) for Cu and CuP groups in 0.3%, In between groups the significant differences for osmotic fragility test appeared at 0.1% of NaCl concentration. As shown in figure both treated groups (Cu & CuP) showed significant decrease (P<0.05) in the percentage of red blood cells hemolytic (0.699±0.02., 0.685±0.02) respectively, when compare with control group)

**Fig. 1:** Serum Cu (microgram/dl) in male rats received Copper sulfate supplement (75mg/kg. B.w) alone or combined with propolis 50mg/kg for 60 days.
Osmoregulation and antioxidant effects of copper sulfate and propolis in red blood cells of male rats

Table 1 represent the results for hemoglobin (gm/dL), ATPase (U/ml) and Total bilirubin (µmol/L). Results revealed that there were significant increase (P<0.05) in hemoglobin of Cu group (16.766 ± 0.508) and significant decrease (P<0.05) in CuP group (14.933 ± 0.493) when compare with control (15.550 ± 0.414). ATPase activity (U/ml) showed significant increase (P<0.05) in Cu and CuP groups (0.2966 ± 0.020; 0.2166 ± 0.116), respectively, when compare with control group (0.166 ± 0.006). Total serum bilirubin (µmol/L) showed marker decrease in copper sulfate receiving group (0.0450 ± 0.005) and the CuP group (0.0366 ± 0.002), than in controlled group (0.2078 ± 0.118).

Fig. 3: Relationship between Total serum copper and hemoglobin in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days. The R² value show significant regression of variable of y on variation of x values.

Fig. 4: Relationship between Total serum copper and total bilirubin in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days. The R² value show significant regression of variable of y on variation of x values.

Oxidant/Anti-oxidant status

Fig. 6A and B shows the result of Oxidant/anti-oxidant status, Oxidant marker Malonyaldehyde acid (MDA) decreased significantly (P<0.05) in treated groups. Mean value of MDA in Cu and CuP groups was (24.100 ± 1.083; 0.726±0.05).

Table 1: The change in hemoglobin(gm/dl), ATPase (U/ml) and bilirubin (micromole/l) in male rat receiving copper sulfate (75 mg/kg B.W) alone or combined with propolis 50mg/kg for 60days. Means±SE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (gm/dl)</th>
<th>ATPase (U/ml)</th>
<th>Bilirubin (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.550±0.41</td>
<td>0.1660±0.006</td>
<td>0.2078±0.11</td>
</tr>
<tr>
<td>Cu (Cuso₄ 75mg/kg B.W)</td>
<td>16.766±0.50</td>
<td>0.2966±0.02</td>
<td>0.0450±0.005</td>
</tr>
<tr>
<td>CuP (Cuso₄ 75 mg + propolis 50 mg/kg B.W)</td>
<td>14.933±0.49</td>
<td>0.2166±0.11</td>
<td>0.0366±0.002</td>
</tr>
<tr>
<td>LSD</td>
<td>1.4288</td>
<td>0.2065</td>
<td>0.2064</td>
</tr>
</tbody>
</table>

Capital letter denote significant differences between groups. N = 6.
copper sulfate had lower Cu digestibility than those with no supplement and greater copper excretion by liver and kidney (Cholewińska et al., 2018). The copper excretion rate is in a constant with copper intake, increase copper in diet increased copper excretion in feces (Arnal et al., 2014). At the same meaning copper deficit diet did not cause excessive fecal excretion of copper (Lee, Ko, Park, Lim, et al., 2016). There are appositely correlation between copper intake and urine excretion in a dose response curve (Lee, Ko, Park, Shin, et al., 2016) Many physiological factors affect the rate of copper absorption mainly the gastric acidity and duodenum pH (Wapnir, 1998). In the present study this might be affected by the time of feeding was after copper gavage resulting in less acidity of gastrointestinal tract consequently reducing ion absorption.

**Discussion**

**Serum copper**

The present results confirmed that the dose (75 mg/kg) received by the copper supplemented rats covered the dietary Cu requirement with no excess. The serum level of copper received group in the present experiment was with the normal limit of copper level in rats. The present results are in agree with results obtained by (Ranganathan et al., 2011; M. Song et al., 2018) and little differed than (Majewski et al., 2019a). The increase in copper intake might be excreted more in rats with copper supplement to keep constant normal serum copper level. Rats supplemented with copper nanoparticles or copper sulfate had lower Cu digestibility than those with no supplement and greater copper excretion by liver and kidney (Cholewińska et al., 2018). The copper excretion rate is in a constant with copper intake, increase copper in diet increased copper excretion in feces (Arnal et al., 2014). At the same meaning copper deficit diet did not cause excessive fecal excretion of copper (Lee, Ko, Park, Lim, et al., 2016). There are appositely correlation between copper intake and urine excretion in a dose response curve (Lee, Ko, Park, Shin, et al., 2016) Many physiological factors affect the rate of copper absorption mainly the gastric acidity and duodenum pH (Wapnir, 1998). In the present study this might be affected by the time of feeding was after copper gavage resulting in less acidity of gastrointestinal tract consequently reducing ion absorption.

**Red blood cells bioactive markers**

Fragility osmotic test evaluate the integrity of red blood cells membrane to resist hypotonic effects. Results of the present study indicated that red blood cells membrane integrity of experimental animals were improved by copper and propolis supplement. Copper as a prosthetic group for antioxidant enzymes strength the ROS scavenging ability of red blood cells membrane.

![Fig. 6](image_url) Changes in MDA (nmol/ml serum) and TAOC (mmol/L) in male rats received Copper sulfate (75 mg/kg B.W.) alone or combined with propolis (50 mg/kg B.W.) for 60 days Means ± SE N = 6.

![Fig. 7](image_url) Relationship between Total serum copper and TAOC and MDA in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days. The R² value show significant regression of variable of y on variation of x values.
Disorders of red cells and erythropoiesis include abnormality in red blood cells production and viability. These abnormalities are not seen in red blood cells from healthy organism (Lodish et al., 2019). The inability of RBCs to control their volume is known to impair their function. The significant difference observed between the experimental and control rats with a lower hemolysis in experimental rats could be attributed to many factors. Hemoglobin concentration was ameliorated in a dose response relationship with serum copper level. The present results indicated a efficient role of Cu\(^{2+}\) in hemoglobin synthesis could be resulted from different biochemical mechanisms. Copper is important elements in transporting of iron from storage tissues to bone marrow were hemoglobin Synthesis (Anderson et al., 2002) because copper is responsible for the conversion of ferrous to ferric which transport iron to the tissue. Other mechanism attributed to the role of copper in enhancement of hematopoiesis of bone marrow for red blood cells production, significantly this decreased with increase in serum copper (Myint et al., 2018b; Williams, 2019). Furthermore, copper is an important cofactor sustention of mitochondrial respiration of hematopoietic stem cells, promoting heopoiesis cells proliferation and differentiation, particularly erythroid cells (Jensen et al., 2019). In the present study the copper supplemented to rats served as a promoting factor for erythropoiesis.

ATPase enzyme in whole blood sample represent enzyme positioned in the RBCs membrane and circulated from other cells. In the present results, obviously copper induced ATPase activity, however this increase whether from RBCs ATPase or from circulated it is not investigated in the present study. ATPase copper dependent enzyme regulated activity by serum copper level (Myint et al., 2018a). In the present study the administrated dose of copper covered the normal requirements of the animals need for this ion and for best basolateral transforming of copper ATPase must be available. it keeps the RBCs size swallowed in case the Na-k ATPase is inhibited. ATPase act as a pump it ejects 3 Na ion out of cell and 2 k intra cellular against concentration gradient (Gopinivas & Unni, 2008) therefore this enzyme keeps the cell hemostasis and it conceders as an indicator for red blood cells disorders (Kherd et al., 2017). In the present study, serum copper level was within normal limit as noticed by normal enzyme activity. There were less ATPase activity in copper plus propolis group, this attributed to the chelating activity of propolis (Al-Qayim et al., 2014) and results in less ATPase production. Total Bilirubin in serum is a biological marker for hemoglobin catabolism and for hepatocytes activity in bilirubin metabolism (Hansen et al., 2020). Once again the present reduction in total serum bilirubin in copper supplemented rats indicated that the dose was safe and in addition it could be considered a complementary dose to copper deficient rats used in the present study. The more stable red blood cells the less hemolytic and less bilirubin formation. The results of bilirubin are based on the results of RBCs osmotic fragility and the ATPase enzyme results as well as the results of hemoglobin. From here we can be certain the level of copper in the blood has an inverse relationship with the indicators of the decomposition of red blood cells, the most important of which is bilirubin.

Antioxidants response

The lipid peroxidation (MDA) significantly reduced in the present results agreed with those reported by (Jarosz et al., 2018) who reported that SOD activity increased by feeding broiler chickens on diets rich in copper sulfate. It is well-known that Cu has the ability to regulate SOD activity in the tissues and body fluids of both growing and aged animals (Sura, 2016). Thus, excess of dietary copper might be the main reason of high SOD activity in quails fed Cu supplemented diets. Moreover, Cu deficiency was reported by many investigators to reduce both Cu and Zn-SOD forms activity without inhibiting the biosynthesis or storage of these
elements in the tissues (Dameron & Harris, 1987). The reduction observed of MDA levels in rats administered Cu-supplemented diets is consistent with the findings of (Kumara et al., n.d.) the higher levels of MDA may be a diagnostic criteria for the antioxidant defense system damage due to oxidative stress (Farombi et al., 2004; Kato et al., 2007; Venkataraman et al., 2004). Copper have an essential role in oxidant-antioxidant stability it have the ability as an electron donor or exceptor, its oxidation state change between cuprous Cu+ and cupric form Cu2+ (Gaetke & Chow, 2003; Ralph & McArdle, 2001) Activity of antioxidant enzymes which reflected by TAOC in the present study copper supplemented rats were more efficient. Copper deficiency seriously affected the activity of antioxidant enzymes and reduced the function of the antioxidant (Wu et al., 2020) There is a certain relationship between plasma copper level and total antioxidant activity in rats (Majewski et al., 2019b)

**Conclusion**

Copper deficient rat it is an essential component for RBC survival, hemoglobin production and red blood cell integrity and ATPase enzyme activity. A 75 mg/kg bw of copper sulfate by oral intake for five days a week conceder as complementary dose in agree with the dietary supplementation with cupric sulfate pentahydrate (CuSO4·5H2O) at level of 50 or 100 mg/kg by (Ali, 2018), were improve productive performance, immunity and lowering blood cholesterol content of growing Japanese quail. Propolis played a role as a chelating agent increase the copper deficiency effects in parameters studied in the present study, with the exception of it is role as antioxidant against.

It is important to note the methodological limitations of this study, we used one single dose of cupper and propolis, however different dosages should be implemented.

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