Plant Archives Volume 20 No. 2, 2020 pp. 7359-7366 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

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

Sarah Abdulsalam Mohamed* and Majida A.J. Al-Qayim

Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Baghdad, Iraq.

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 $CuSO_4$ (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 malondialdyhyde 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 & Hazegh-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 is a multi-factorial incidence, it could be caused by hereditary or acquired (Myint *et al.,* 2018a). Copper deficiency anemia is the result of impaired iron transportation with transferrin where it does carry ferric iron (Fe^{3+}) oxidized from ferrous (Fe^{2+}) 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

***Author for correspondence :** E-mail : sarasalamm92@gmail.com

(Andreyeva *et al.,* 2019). 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 cooper 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 AGAPPE (Switzerland).Serum Total bilirubin (µmol/L) estimated by colorimetric method using a commercial kit and following the company instruction BIOSYSTEM reagent and instrument.

Oxidant –Antioxidant balance

Determine of the serum malondialdyhyde (MDA) (nmol/ml serum) estimated by colorimetric method (Guidet & Shah, 1989) T- AOC (mmol/L) measured by Eliza 593nm. This kit is used to measure the total antioxidant capacity in serum.

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)

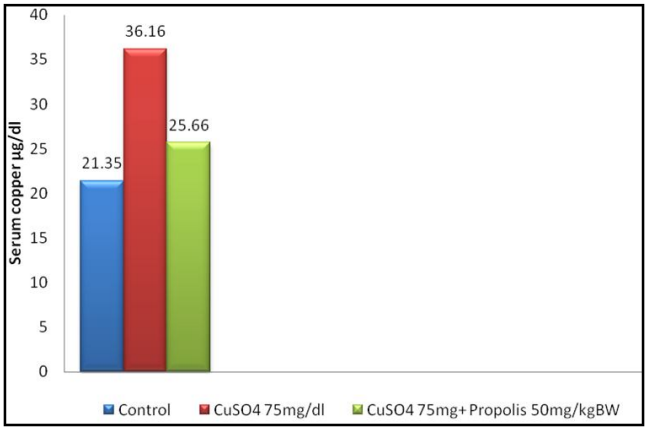

Fig. 1: Serum Cu (microgram/dl) in male rats received Copper sulfate supplement (75mg/kg. B.w) alone or combined with propolis 50mg/kg for 60 days.

0.726±0.05).

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 (0.03660.002), than in controlled group (0.20780.118).

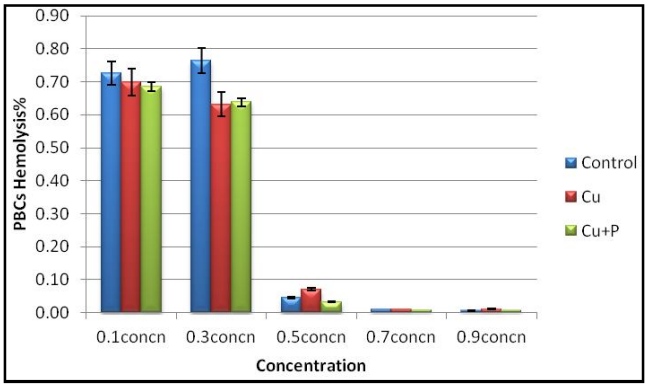

Fig. 2: Erythrocyte hemolysis percentage (%) in different NaCl concentration in male rats received Copper sulfate supplement (75mg/kg. B.W) alone or combine with propolis 50mg/kg for 60days.

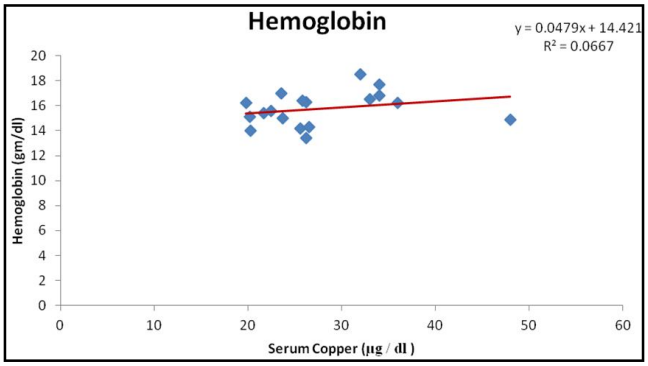

Fig. 3: Relationship between Total serum copper and hemoglobin in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days. The R^2 value show significant regression of variable of y on variation of x values.

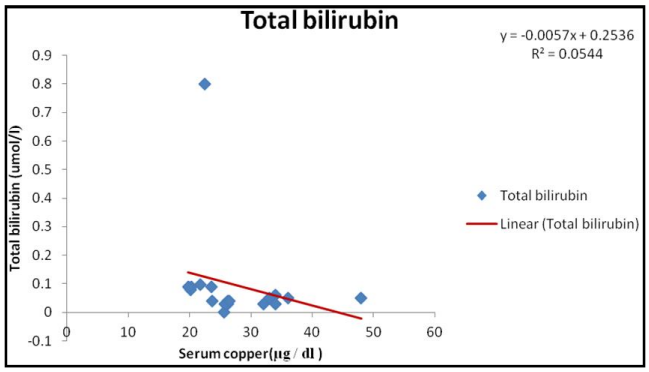

Fig. 4: Relationship between Total serum copper and total bilirubin in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days. The R2 value show significant regression of variable of y on variation of x values.

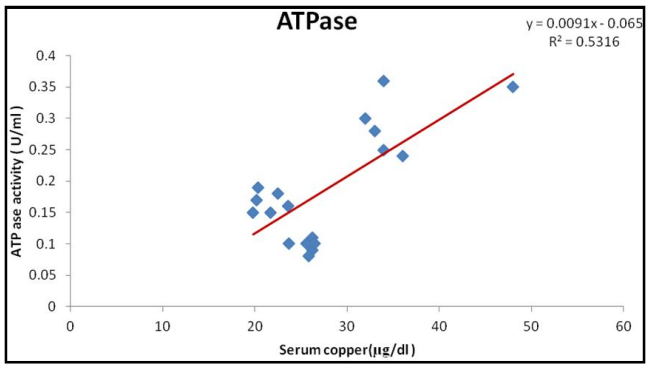

Fig. 5: Relationship between Total serum copper, ATPase activity, in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days. The R^2 value show significant regression of variable of y on variation of x values.

In regard to the relationship between serum copper level and hemoglobin concentration, results revealed a positive refashion ship, hemoglobin increased with serum Cu increase (R2 = 0.0667) as shown in Fig. 3. ATPase activity recorded a positive relationship with increase of serum copper level with R2= 0.5316 Fig. 4. On the contrary total bilirubin showed inverse relationship with serum copper level with significantly R2 = 0.0544 in Fig. 5.

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 50 mg/kgBW for 60days. Means±SE.

Group	Hb (gm/dl)	ATPase (U/ml)	Bilirubin (µmol/L)
Control	15.550^{AB}±0.41	0.1660^{B}±0.006	0.2078^{A}±0.11
Cu ($Cuso_4$ 75mg/kg B.W)	16.766^{A}±0.50	0.2966^{A}±0.02	0.0450^{A}±0.005
CuP ($Cuso_4$ 75 mg + propolis 50 mg/kg B.W)	14.933^{B}±0.49	0.2166^{A}±0.11	0.0366^{A}±0.002
LSD	1.4288	0.2065	0.2064

Capital lettrs denotes significant differences between groups. N = 6.

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;

23.7001.023) when compared with control group (27.80), The result of the total antioxidant capacity (TAOC) increased significantly in treated group, TAOC showed the highest value in CuP group (1.2833) followed by Cu group (0.73330.066) in compare with control group (0.666).

The relationship between the serum copper level and oxidant and anti-oxidant markers studied in the present study were represented in Fig. 7. Results revealed that there were no effects for copper on anti -oxidant markers production (R2= 0.008), but on the contrary there were reverse effects on oxidant marker (MDA) with R2= 0.054.

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 (Cholewiñska *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 appositive 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.

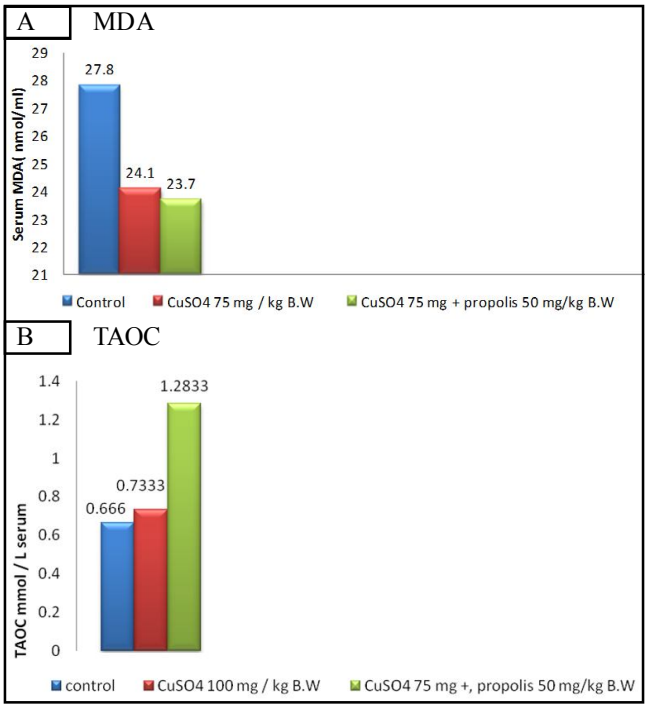

Fig. 6: Changes in MDA (nmol/ml serum) and TAOC (mmol/L) in male rats received Copper sulfate (75 mg/kg B.W.) alone or combined with propolis (50 mg.kg B.W) for 60 days Means ± SE N = 6.

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

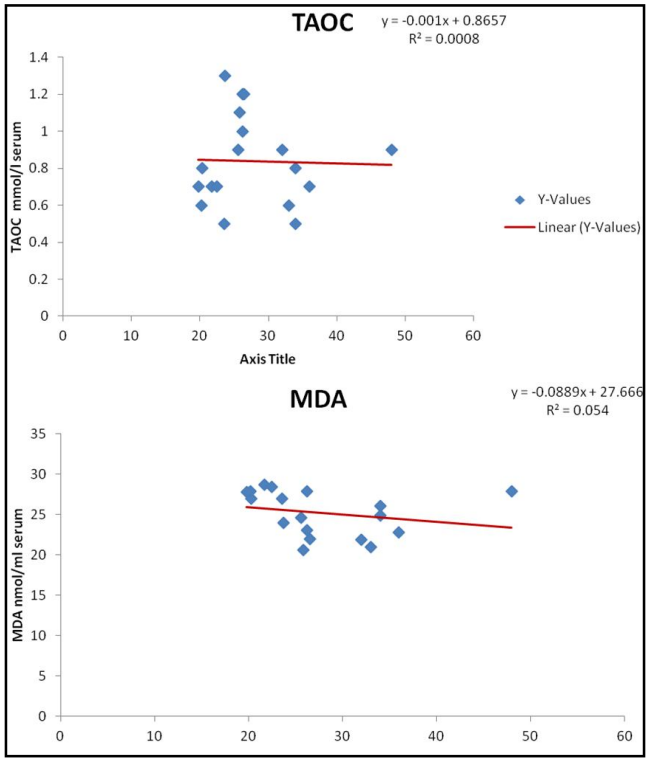

Fig. 7: Relationship between Total serum copper and TAOC and MDA in male rats received 75mg/kg Copper sulfate alone or combined with propolis 50mg/kg for 60 days.. The R^2 value show significant regression of variable of y on variation of x values.

(Azab *et al.,* 2019). The protective mechanism of propolis against side effect on red blood cells membrane against hypotonic hemolysis could be attributed to many properties. The antioxidants effects of propolis have been instrumental in avoiding oxidative effects of mineral (*e.g.* Aluminum) on RBCs membrane and thus maintain the other hematological parameters within normal limits (Farooqui & A Farooqui, 2010). The present results was agree with results of (Hamed & Al-Qayim, 2009; Yousef & Salama, 2009), who reported that propolis important antioxidant & propolis has beneficial influence and could be able to antagonize the effect of iron overload. Propolis is heterogeneous substance with a large number of elements and many vitamins includes; B1, B2, B6, E and vitamins C and several amino acid (Abubakar *et al.,* 2014), the protective role of each of vitamins E & C in reducing the effects of minerals like iron on blood (Aziz & Zabut, 2011). To minimize the effect of these ROS and the result ant oxidative stress, (Mohanty *et al.,* 2014). Copper involved in essential metabolic processes depending on it is capability in electron transfer for it is actively and freely redox exchange form. This make it work as a prosthetic elements for many enzymes and required for cellular signal, metabolic function, cells homeostasis growth and development (Cruces-Sande *et al.,* 2019).

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 decrease 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 erythrogensis.

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 stabile 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 (Surai, 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 Cu^{2+} (Gaetke & Chow, 2003; Ralph & McArdle, 2001) Activity of antioxidant enzymes which reflected by TAOC in the present study in 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 cupper 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 ($CuSO_4.5H_2O$) 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.

References

Abubakar, M.B., W.Z. Abdullah, S.A. Sulaiman and B.S. Ang (2014). Polyphenols as key players for the antileukaemic effects of propolis. *Evidence-Based Complementary and Alternative Medicine*, 2014.

Ahmed, R., E.M. Tanvir, M. Hossen, R. Afroz, I. Ahmmed, N.E. Rumpa, S. Paul, S.H. Gan, S.A. Sulaiman and M. Khalil (2017). Antioxidant properties and cardioprotective mechanism of Malaysian propolis in rats. *Evidence-Based Complementary and Alternative Medicine*, 2017.

Al-Hejuje, M.M., N.A. Hussain and H.T. Al-Saad (2017). Applied Heavy Metals Pollution Index (HPI) as a Water Pollution Indicator of Shatt Al-Arab River, Basrah-Iraq. *International Journal of Marine Science*, **7(35):** 353–360. https://doi.org/10.5376/ijms.2017.07.0035.

Al-Qayim, A.J.M., L. Ghali and T. Al-Azwai (2014). Comparative effects of propolis and malic acid on hematological parameters of aluminum exposed male rats. *Glob. J. Biosci. Biotechnol.*, **3(1):** 6–11.

Ali, R. (2018). Physiological and Antioxidant Responses of Japanese Quail to Dietary Copper Supplementation. *Journal of Animal and Poultry Production*, **9(12):** 489–495. https://doi.org/10.21608/jappmu.2018.41165.

Anderson, G.J., D.M. Frazer, A.T. McKie and C.D. Vulpe (2002). The ceruloplasmin homolog hephaestin and the control of intestinal iron absorption. *Blood Cells, Molecules & Diseases*, **29(3):** 367–375. https://doi.org/10.1006/bcmd.2002.0576.

Andreyeva, A.Y., A.A. Soldatov, A.I. Krivchenko, I.V. Mindukshev and S. Gambaryan (2019). Hemoglobin deoxygenation and methemoglobinemia prevent regulatory volume decrease in crucian carp (*Carassius carassius*) red blood cells. *Fish Physiology and Biochemistry*, **45(6):** 1933–1940.

Arnal, N., L. Dominici, M.J.T. de Tacconi and C.A. Marra (2014). Copper-induced alterations in rat brain depends on route of overload and basal copper levels. *Nutrition*, **30(1):** 96-106.

Azab, A.E., A.A. Adwas, A.S.I. Elsayed and F.A. Quwaydir (2019). Oxidative stress and antioxidant mechanisms in human body. *Journal of Applied Biotechnology & Bioengineering*, **6(6):**.

Aziz, I.I.A. and B.M. Zabut (2011). Determination of blood indices of albino rats treated with aluminum chloride and investigation of antioxidant effects of vitamin E and C. *Egyptian Journal of Biology*, **13(1):** 1–7.

Cholewiñska, E., J. Jucœkiewicz and K. Ognik (2018). Comparison of the effect of dietary copper nanoparticles and one copper (II) salt on the metabolic and immune status in a rat model. *Journal of Trace Elements in Medicine and Biology*, **48:** 111–117.

Cruces-Sande, A., A.I. Rodríguez-Pérez, P. Herbello-Hermelo, P. Bermejo-Barrera, E. Méndez-Álvarez, J.L. Labandeira-García and R. Soto-Otero (2019). Copper increases brain oxidative stress and enhances the ability of 6-hydroxydopamine to cause dopaminergic degeneration in a rat model of Parkinson's disease. *Molecular Neurobiology*, **56(4):** 2845–2854.

Dameron, C.T. and E.D. Harris (1987). Regulation of aortic CuZn-superoxide dismutase with copper. Effects *in vivo*. *Biochemical Journal*, **248(3):** 663–668.

Farias, M.G. (2017). Advances in laboratory diagnosis of hereditary spherocytosis. *Clinical Chemistry and Laboratory Medicine (CCLM)*, **55(7):** 944–948.

Farombi, E.O., M. Hansen, G. Ravn-Haren, P. Møller and L.O. Dragsted (2004). Commonly consumed and naturally occurring dietary substances affect biomarkers of oxidative

stress and DNA damage in healthy rats. *Food and Chemical Toxicology*, **42(8):** 1315–1322.

Farooqui, T. and A. Farooqui (2010). Molecular mechanism underlying the therapeutic activities of propolis: a critical review. *Current Nutrition & Food Science*, **6(3):** 186–199.

Fedorova, E.Y., V.I. Maksimov and O.V. Smolenkova (2019). ATPase Activity in Red-Blood and Muscle Tissue Cells in Sheep. *Russian Agricultural Sciences*, **45(3):** 300–303.

Gaetke, L.M. and C.K. Chow (2003). Copper toxicity, oxidative stress and antioxidant nutrients. *Toxicology*, **189(1–2):** 147–163.

Gopinivas, G.R. and J.C. Unni (2008). Q. Is the drug combination of ivermectin and albendazole a rational combination. If yes, what is the dose and what is the safety profile? *Indian Pediatrics*, **45:** 157.

Guidet, B. and S.V. Shah (1989). Enhanced in vivo H_2O_2 generation by rat kidney in glycerol-induced renal failure. *American Journal of Physiology-Renal Physiology*, **257(3):** F440–F445.

Hamed, S. and M.A.J. Al-Qayim (2009). *Protective Role of Propolis against Iron Overload Induced Genotoxicity and Oxidant/Antioxidant Status*.

Hansen, T.W.R., R.J. Wong and D.K. Stevenson (2020). Molecular Physiology and Pathophysiology of Bilirubin Handling by the Blood, Liver, Intestine and Brain in the Newborn. *Physiological Reviews*, **100(3):** 1291–1346.

Hendi, N.K.K., H.S. Naher and A.H. Al-Charrakh (2011). In vitro antibacterial and antifungal activity of Iraqi propolis. *Journal of Medicinal Plants Research*, **5(20):** 5058–5066.

Hordyjewska, A., L. Popiolek and J. Kocot (2014). The many "faces" of copper in medicine and treatment. *Biometals*, **27(4):** 611–621.

Jarosz, L.S., A. Marek, Z. Gradzki, M. Kwiecień and B. Kaczmarek (2018). The effect of feed supplementation with a copper glycine chelate and copper sulphate on selected humoral and cell mediated immune parameters, plasma superoxide dismutase activity, ceruloplasmin and cytokine concentration in broiler chickens. *Journal of Animal Physiology and Animal Nutrition*, **102(1):** e326–e336.

Jensen, E.L., A.M. Gonzalez-Ibanez, P. Mendoza, L.M. Ruiz, C.A. Riedel, F. Simon, J.J. Schuringa and A.A. Elorza (2019). Copper deficiency-induced anemia is caused by a mitochondrial metabolic reprograming in erythropoietic cells. *Metallomics*, **11(2):** 282–290.

Kalia, P., N.R. Kumar and K. Harjai (2016). Studies on the therapeutic effect of propolis along with standard antibacterial drug in *Salmonella enterica* serovar Typhimurium infected BALB/c mice. *BMC Complementary and Alternative Medicine*, **16(1):** 485.

Karim, N. (2018). Copper and Human Health- A Review. *Journal of Bahria University Medical and Dental College*, **8(2):** 117–122. https://jbumdc.bahria.edu.pk/wp-content/uploads/2018/06/12-2.pdf.

Kato, J., A.A. Ruram, S.S. Singh, S.B. Devi, T.I. Devi and W.G. Singh (2007). Lipid peroxidation and antioxidant vitamins in urolithasis. *Indian Journal of Clinical Biochemistry*, **22(1):** 128–130.

Kherd, A.A., N. Helmi, K.S. Balamash, T.A. Kumosani, S.A. AL-Ghamdi, M. Qari, E.A. Huwait, S.S. Yaghmoor, A. Nabil and M.A. AL-Ghamdi (2017). Changes in erythrocyte ATPase activity under different pathological conditions. *African Health Sciences*, **17(4):** 1204–1210.

Kim, J., H. Lee and S. Shin (2015). Advances in the measurement of red blood cell deformability: A brief review. *Journal of Cellular Biotechnology*, **1(1):** 63–79.

Kumara, P., A. Biswasb, V.K. Bhartia and R.B. Srivastavaa (n.d.). *The Journal of Veterinary Science*.

Lee, I.C., J.W. Ko, S.H. Park, J.O. Lim, I.S. Shin, C. Moon, S.H. Kim, J.D. Heo and J.C. Kim (2016). Comparative toxicity and biodistribution of copper nanoparticles and cupric ions in rats. *International Journal of Nanomedicine*, **11:** 2883–2900. https://doi.org/10.2147/IJN.S106346.

Lee, I.C., J.W. Ko, S.H. Park, N.R. Shin, I.S. Shin, C. Moon, J.H. Kim, H.C. Kim and J.C. Kim (2016). Comparative toxicity and biodistribution assessments in rats following subchronic oral exposure to copper nanoparticles and microparticles. *Particle and Fibre Toxicology*, **13(1):** 1–16. https://doi.org/10.1186/s12989-016-0169-x.

Lew, V.L. and T. Tiffert (2017). On the mechanism of human red blood cell longevity: roles of calcium, the sodium pump, PIEZO1 and gardos channels. *Frontiers in Physiology*, **8:** 977.

Linder, M.C. and M. Hazegh-Azam (1996). Copper biochemistry and molecular biology. *The American Journal of Clinical Nutrition*, **63(5):** 797S-811S.

Lodish, H., X. Gao an H.Y. Lee (2019). *Efficient generation of human red blood cell via enriching peripheral blood erythroid progenitors*. Google Patents.

Majewski, M., K. Ognik and J. Jucękiewicz (2019a). Copper nanoparticles modify the blood plasma antioxidant status and modulate the vascular mechanisms with nitric oxide and prostanoids involved in Wistar rats. *Pharmacological Reports*, **71(3):** 509–516. https://doi.org/10.1016/j.pharep.2019.02.007.

Majewski, M., K. Ognik and J. Jucękiewicz (2019b). The interaction between resveratrol and two forms of copper as carbonate and nanoparticles on antioxidant mechanisms and vascular function in Wistar rats. *Pharmacological Reports*, **71(5):** 862–869.

Mohanty, J., E. Nagababu and J.M. Rifkind (2014). Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. *Frontiers in Physiology*, **5:** 84.

Myint, Z.W., T.H. Oo, K.Z. Thein, A.M. Tun and H. Saeed (2018a). Copper deficiency anemia: review article. *Annals of Hematology*, **97(9):** 1527–1534. https://doi.org/10.1007/

s00277-018-3407-5.

Myint, Z.W., T.H. Oo, K.Z. Thein, A.M. Tun and H. Saeed (2018b). Copper deficiency anemia. *Annals of Hematology*, **97(9):** 1527–1534.

Ralph, A. and H. McArdle (2001). Copper metabolism and copper requirements in the pregnant mother, her fetus and children. *Copper in the Environment and Health*.

Ranganathan, P.N., Y. Lu, L. Jiang, C. Kim and J.F. Collins (2011). Serum ceruloplasmin protein expression and activity increases in iron-deficient rats and is further enhanced by higher dietary copper intake. *Blood*, **118(11):** 3146–3153. https://doi.org/10.1182/blood-2011-05-352112.

Schultze, M.O. (1941). The relation of copper to cytochrome oxidase and hematopoietic activity of the bone marrow of rats. *Journal of Biological Chemistry*, **138:** 219–224.

Singh, Y., A. Chowdhury, C. Mukherjee, R. Dasgupta and S.K. Majumder (2019). Simultaneous photoreduction and Raman spectroscopy of red blood cells to investigate the effects of organophosphate exposure. *Journal of Biophotonics*, **12(5):** e201800246.

Song, D. and J.L. Dunaief (2013). Retinal iron homeostasis in health and disease. *Frontiers in Aging Neuroscience*, **5:** 24.

Song, M., X. Li, X. Zhang, H. Shi, M.B. Vos, X. Wei, Y. Wang, H. Gao, E.C. Rouchka, X. Yin, Z. Zhou, R.A. Prough, M.C. Cave and C.J. McClain (2018). Dietary copper-fructose interactions alter gut microbial activity in male rats. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, **314(1):** G119–G130. https://doi.org/10.1152/ajpgi.00378.2016.

Surai, P.F. (2016). Antioxidant systems in poultry biology: superoxide dismutase. *Journal of Animal Research and Nutrition*, **1(1):** 8.

Uriu-Adams, J.Y. and C.L. Keen (2005). Copper, oxidative stress and human health. *Molecular Aspects of Medicine*, **26(4-5):** 268–298. https://doi.org/10.1016/j.mam.2005.07.015.

Vashchenko, G. and R.T.A. MacGillivray (2013). Multi-copper oxidases and human iron metabolism. *Nutrients*, **5(7):** 2289–2313.

Venkataraman, P., M. Sridhar, S. Dhanammal, M.R. Vijayababu, N. Srinivasan and J. Arunakaran (2004). Antioxidant role of zinc in PCB (Aroclor 1254) exposed ventral prostate of albino rats. *The Journal of Nutritional Biochemistry*, **15(10):** 608–613.

Wapnir, R.A. (1998). Copper absorption and bioavailability. *The American Journal of Clinical Nutrition*, **67(5):** 1054S-1060S.

Williams, D.M. (2019). Clinical Syndromes of Copper Deficiency. *Nutritional Anemia: Scientific Principles, Clinical Practice and Public Health*, 111.

Wu, T., M. Song and X. Shen (2020). Seasonal dynamics of copper deficiency in Wumeng semi-fine wool sheep. *Biological Trace Element Research*, **1-8:**.

Yousef, M.I. and A.F. Salama (2009). Propolis protection from reproductive toxicity caused by aluminium chloride in male rats. *Food and Chemical Toxicology*, **47(6):** 1168–1175.

Zabaiou, N., A. Fouache, A. Trousson, S. Baron, A. Zellagui, M. Lahouel and J.M.A. Lobaccaro (2017). Biological properties of propolis extracts: Something new from an ancient product. *Chemistry and Physics of Lipids*, **207:** 214–222.