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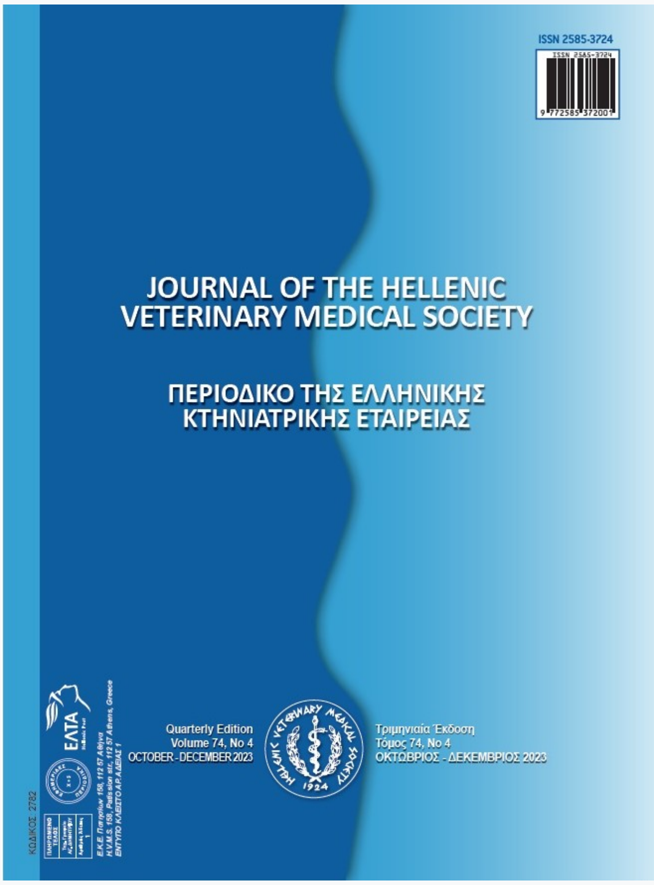

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Evaluation of serum ceruloplasmin to copper ratio in repeat breeder Holstein dairy cows

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ABSTRACT: Repeat breeder (RB) syndrome is one of the most important problems that affect reproductive performance in different parts of the world and has caused severe economic damage in the dairy industry. Primary and secondary copper deficiency is also a nutritional problem in some tropical regions that may be associated with an impaired reproductive function of dairy cows. This study aimed to evaluate the serum copper, ceruloplasmin (CP) and zinc values, ceruloplasmin to the copper ratio (CP/Cu), and superoxide dismutase (SOD) activity in RBs and their relationship with some reproductive markers in the studied cows. For this purpose, fifty RB and twenty healthy dairy cows were recruited. After blood sampling, serum copper, zinc, and CP concentrations, and SOD activity were determined. The number of open days, BCS, distance to the first insemination, and milk production were also assessed in all tested cows. No significant difference was observed between serum copper values in RB and healthy cows ($p>0.05$). Although the serum CP value and SOD activity in the RB group were higher than in the healthy group, these differences were not insignificant. There was no significant difference in the CP/Cu ratio in the RB and healthy groups ($p>0.05$). The reproductive markers were not significantly different between the groups. It does not seem that copper and zinc deficiencies play a role in the occurrence of RB syndrome. A slight increase in serum CP value and SOD activity in the RB compared with the healthy cows suggests that non-reproductive inflammatory factors, especially in high-yielding cows, may play a role in the pathogenesis of this syndrome.

Keywords: Cow;Repeat breeder; ceruloplasmin; CP/Cu ratio.

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INTRODUCTION

Copper, one of the essential micronutrients, plays a critical role in maintaining normal organ function in animals. More than 20 types of metalloenzymes, cofactors, and metalloproteins rely on the crucial activity of this element. These enzymes and proteins are associated with the destruction of free radicals, the synthesis of connective tissues, the formation of myelin and bones, pigmentation, and the formation of hair and wool(Sousa, Hamad Minervino et al. 2012). In certain tropical regions, copper deficiency is a significant nutritional issue that can be caused by either low levels of copper in the soil and animal feed (known as the primary deficiency) or the existence of high levels of copper antagonist elements such as sulfur, iron, or molybdenum.(Marques, Riet-Correa et al. 2003). The normal level of copper in the blood serum of dairy cows is 0.7-2.1 µg/mL(Radostits, Gay et al. 2006) and serum ceruloplasmin is about 14 mg/dL (Kincaid, Gay et al. 1986).

There is evidence that the molybdates are associated with impaired fertility and milk production after induction of toxic effects of molybdenum on the activity of copper-dependent metalloenzymes in ruminants(Telfer, Kendall et al. 2004). There are conflicting reports on the relationship between serum copper and fertility. For example, Rowlands et al.1977 have shown that there is no correlation between blood copper and fertility, While Telfer et al.2003 shown that infertility and depigmentation are the most common symptoms of copper deficiency(Telfer, Kendall et al. 2004). The release of ceruloplasmin in the blood closely reflects the release of plasma copper, butbecause ceruloplasmin levels may increase during inflammation and infection as an acute phase protein, measuring ceruloplasmin alone is not sufficient to assess blood copper(Suttle 2002). The normal ratio of ceruloplasmin to plasma copper in cattle has been determined to be more than 1.9 and its limit value has been reported between 1.6-1.8(Telfer, Kendall et al. 2004).

The superoxide dismutase (SOD) enzyme acts as a defense mechanism against free radicals. Superoxide dismutase is a copper-dependent enzyme and its major intracellular form is copper-zinc superoxide dismutase in the cytoplasm and nucleus(Abd Ellah, Okada et al. 2009).

One of the most important problems affecting the reproductive performance of dairy cows is repeat breeding cow syndrome (RB)which has caused severe economic damage to the dairy industry(Bartlett, Kirk et al. 1986, Yusuf, Nakao et al. 2010).A RB cow returns to estrus without any visible pathological problem after at least 3 services(Ahuja and Parmar 2017, Noakes, Parkinson et al. 2018). It should be noted that copper deficiency or high soil and forage molybdenum can be a geographical problem. On the other hand, the analysis of soil elements such as copper and related antagonists in soil and plants have not been well studied in most parts of IRAN, including Khuzestan Province. Few studies have shown that the soil in many parts of IRAN is deficient in copper or that the soil contains copper antagonists. Although the impact of copper deficiency or excess molybdenum on cows' fertility is well-documented, there have been no studies conducted in the Iran, particularly in Khuzestan province, to evaluate their potential correlation with infertility. Because the growth and ovulation of ovarian follicles are affected by pituitary gonadotropins and due to the negative effects of low ceruloplasmin to serum copper ratio on the secretion of pituitary hormones, the present study was designed. In the present study, the effect of copper, ceruloplasmin, ceruloplasmin to copper ratio, and activity of SOD enzyme, and zinc on fertility was investigated. Due to the role of zinc in infertility and its role along with copper in SOD activity(Ahuja and Parmar 2017), the evaluation of zinc and the activity of this enzyme in RB cows has also been considered in the present study.

MATERIALS AND METHOD

The study was carried out from September 2020 to February 2021in a commercial dairy herd in the Khuzestan province of Iran. Cows were housed in free stall barns and milked three times a day at an approximate 8-hr interval. The average milk production in fresh cows was 40 kg. The voluntary waiting period was 55 days on this farm. Cows were housed in sand bedding and were fed twice a day with a total mixed ration (TMR) formulated to meet or exceed dietary nutritional requirements for lactating dairy cows (Council 2001). All components of the diet (corn silage, alfalfa, straw, and wheat bran) were prepared from forage grown in Khuzestan. This study was performed on 70 Holstein cows with a body condition score (BCS) of 2.5 to 3.5.BCS of the cows was rated from 1 to 5 by two skilled technicians based on the method presented in the Edmonson study(Edmonson, Lean et al. 1989). After reproductive examination by rectal palpation and sonography method (Ahmadi, Makki et al.

2019), 50 cows were selected from the cows that had more than 3 artificial insemination (AI) services for pregnancy but did not conceive and also did not have a specific reproductive disease at the time of sampling, as the patient group. Also, 20 cows that were conceived in the first or second service were considered as a control group.To reduce the possible effect of pregnancy on this group, no more than 2 months had passed since their pregnancy. Samples were taken from cows that had not received any copper supplements in their history and had not had as many other postpartum problems as possible. It should be noted that the AI of all cows was performed by an insemination technician. All cows in the RB group had undergone at least three AI and days in milk (DIM) was over 150 at the time of sampling.

From the RB and healthy groups, blood was drawn once from the tail vein and away from stress using a disposable syringe and transferred to tubes containing clot-activating gel. Blood samples were centrifuged at 3000 g for 10 min and serum was isolated. The serums were transferred to microtubesand transferred to the clinical pathology laboratory of the Faculty of Veterinary Medicine of the Shahid Chamran University of Ahvaz by using sample containers and a cold chain. Samples were stored at -80 °C until testing.

CP was measured by the ELISA method and using a ZellbioGmbhkit) made in Germany (and an ELISA reader (model DANA-DA3200). Kit's work is based on sandwich ELISA technology using biotin dual antibodies to measure CP. Finally, the concentration of CP was calculated according to the calibrator curve based on mg/L.

Serum SOD activity was measured by the calorimetric method using a RANSOD kit (RANDOX, England) in the clinical pathology laboratory.

The standard atomic absorption method is commonly used to measure blood copper concentrations, but in this study, due to limitations, optical spectrophotometry was used. This measurement was performed by using the laboratory kit(Paadco company, Spain) and photometer measuring device of clinic II of the measuring equipment company of Iran.

The standard atomic absorption method is commonly used to measure blood zinc concentrations, but in this study, due to limitations Serum zinc was measured by calorimetric method, using the laboratory kit (QCA company, Spain) and by the photometer of Clinic II of Iran Measurement Equipment Company.

Statistical analysis

Reproductive performance outcomes were characterized by days to first AI (days from parturition to the first service) and Days open (days from parturition to insemination leads to pregnancy).Data were analyzed using SPSS software (Version 21, SPSS Inc., Munich, Germany). Data analysis was performed using a t-test for two independent samples (control and RB group). Pearson correlation test was also used to examine the relationship between serum indices in the patient group. For allstatistical analyses, the significance level was set at $p< 0.05$.

RESULT

The mean serum values of the mentioned indicators in the studied cows are shown in Table 1.

The mean values of indicators related to production and reproduction including open days (Open Days), body condition score (BCS), calving to the first insemination, and milk production (Milk Record) in the studied cows are shown in Table 2.

Pearson correlation between the values of serum markers mentioned in RB cows is shown in Table 3.

Pearson correlation between reproductive index including open days, BCS, calving to first insemina-

Table 1. Comparison of mean values± standard deviation of serum copper, ceruloplasmin, ceruloplasmin to copper, zinc, and superoxide dismutase ratio Repeat Breeder and control cows.

Index	RB Group	Control Group	P value
Cu (μg/dL)	109.5 ± 21.4	98.1 ± 7.5	0.03
CP (mg/L)	90.1 ± 47	79.8 ± 44	0.42
CP (mg/L)/Cu (μg/dL)	0.4± 0.99	1 ± 0.2	0.9
CP (mg/dL)/Cu (μmol/L)	0.55 ± 0.3	0.53 ± 0.3	0.81
Zn (μg/dL)	99.4 ± 21.7	103.2 ± 16.5	0.49
SOD (IU/mL)	2.53 ± 1.4	2 ± 1.8	0.2

CP: Ceruloplasmin, SOD: Soperoxiddesmotase.

tion, and milk production with serum indices mentioned in RB cows are shown in Table 4.

As shown in Table 4-4, Pearson correlation coefficient between the reproductive index and serum levels of copper, ceruloplasmin, CP / Cu, zinc, and serum superoxide dismutase activity showed that there was no significant correlation between open days, calving to first service and BCS with serum levels of copper, ceruloplasmin, and ceruloplasmin to copper ratio as well as with zinc and superoxide dismutase.

DISCUSSION

The results of the present study showed that although serum copper (mg/L) levels in RB(0.59± 0.04) and anestrous cows (0.65± 0.03)were lower than in control cows(0.75 ± 0.06),but there was no significant difference between the control and patient groups (p<0.05).These results were consistent with Das et al. 2009,Barui et al. 2015,Ahmed et al. 2017 and Ceylan et al. 2008 studies (Ceylan, Serin et al. 2008, Das, Dutta et al. 2009, Barui, Batabyal et al. 2015, Mohamed, Faisal et al. 2017). They stated that although there is no significant difference between serum copper levels in cows with reproductive disease and healthy cows, even a slight decrease in copper concentration can lead to a high incidence of fetal mortality in cows. Ahmed et al. 2010 and Akhtar et al. 2014 reported

Table 2. Comparison of mean± standard deviation of parities, open days, BCS, first service, and milk production in Repeat Breeder and control cows.

Index	RB Group	Control Group	P value
Parities	2.87 ± 0.87	2.66 ± 0.76	p > 0.05
Open Days	85 ± 267	24 ± 82.2	p < 0.001
BCS	0.3± 3.1	0.3 ± 3.2	p > 0.05
Day to first service	16 ± 69.7	17.6± 76	p > 0.05
Milk production Mean (Liter)	7 ± 25.7	7 ± 28.6	p > 0.05

Table 3. Calculation of Pearson Correlation Coefficient Between Serum Levels of Copper, Ceruloplasmin, CP / Cu, Zinc And Superoxide Dismutase In Repeat Breeder Cows.

Index		Cu	CP	CP (mg/L)/Cu (µg/dL)	Zn	SOD
Cu	PCC*	1	0.035	-0.526***	0.024	0.046
	Sig. (2-tailed)**	-	0.828	0.001	0.876	0.786
CP	PCC	0.035	1	**0.720	-0.149	-0.026
	Sig. (2-tailed)	0.828	-	0.000	0.357	0.887
CP (mg/l)/ Cu (µg/dl)	PCC	-0.526***	0.720***	1	-0.099	0.024
	Sig. (2-tailed)	0.001	0.000	-	0.555	0.899
Zn	PCC	0.024	-0.149	-0.099	1	0.125
	Sig. (2-tailed)	0.876	0.357	0.555	-	0.460
SOD	PCC	0.046	-0.026	0.024	0.125	1
	Sig. (2-tailed)	0.786	0.887	0.899	0.460	-

*Pearson Correlation Coefficient
** 2-Taild Test
*** Correlation at the level of 0.01 is bilaterally significant

Table 4. Calculation of Pearson correlation coefficient (PCC) between the values of reproductive indices with serum indices of copper, ceruloplasmin, ceruloplasmin/copper, zinc, and superoxide dismutase ratio in Repeat Breeder cows.

Index		Cu	CP	CP (mg/l)/ Cu (µg/dl)	Zn	SOD
Open Days	PCC	-0.043	-0.269	-0.144	-0.070	-0.102
	Sig. (2-tailed)	0.791	0.107	0.408	0.662	0.556
BCS	PCC	-0.072	-0.293	-0.137	0.121	-0.053
	Sig. (2-tailed)	0.641	0.063	0.406	0.428	0.749
Days to first AI	PCC	-0.078	-0.023	0.080	-0.087	0.002
	Sig. (2-tailed)	0.589	0.892	0.647	0.590	0.993
Milk Record Mean	PCC	0.042	0.117	0.008	-0.064	0.114
	Sig. (2-tailed)	0.794	0.490	0.962	0.692	0.506

that serum copper levels in RB buffaloes were significantly lower than in healthy buffaloes, according to our study this may be due to physiological differences between cattle and buffaloes(Akhtar, AsimFarooq et al. 2014, Mohamed, Faisal et al. 2017).

The serum concentrations of CP in the present study in RB and healthy cows were 90.1 ± 47 and 79.8 ± 44 mg/L, respectively. Based on studies by Radostits et al.2007 and Kaya et al.In 2016, serum levels of this enzyme were reported to be 45 to 100 and 135.2 ± 3.2 mg/L, respectively, which is consistent with the results of our study. These results show that although the concentration of CP in the RB group is higher than in the control, but its values in both groups are in the normal range and there is no significant difference between them. Also, no significant correlation was found between serum levels of CP and copper.

Laven et al. 2007 and Underwood & Suttle, 1999 showed that if the purpose of the diagnostic test is to determine the normal or borderline status of copper, the serum and plasma activity of CP can be considered as a reliable alternative to plasma copper concentrations(Suttle 2002, Laven, Lawrence et al. 2007).However, Suttle, 2002 stated that the use of serum levels of CP in determining the status of blood copper is problematic because, in the process of clotting, the activity of the enzyme CP may be reduced by up to 20%(Suttle 2002, Laven, Lawrence et al. 2007), which seems to justify samples below the normal range of serum CP(14.3% in the patient group and 22.2% in the control group) in the present study. Constable et al. 2017also said that the evaluation of CP in measuring the copper status has a disadvantage, because since CP is an acute phase protein, the levels of this enzyme in the blood increase in inflammatory processes and infections, making it difficult to diagnose copper deficiency (Radostits, Gay et al. 2006).A non-significant increase in CP (mg/L) in RB cows in the present study (90.1±47) compared with healthy cows (79.8±44) may also be affected by possible inflammatory responses due to subclinical endometritis in this group(Salasel, Mokhtari et al. 2010, Pothmann, Prunner et al. 2015).

In the present study, the ratio of CP to copper was calculated based on the serum concentration of CP (mg/L) to the serum concentration of copper (μg/dL) and the results showed that this ratio was not significantly different in both patient and control groups. However, measuring liver copper reserves may help determine the etiology of the syndrome.

Suttle, 1983 stated that the activity of the SOD enzyme can be used as an indicator to determine the status of copper in the blood (Suttle 1983).Mulryan& Mason, 1992 stated that SOD activity is mainly useful in severe copper deficiency in cattle and is not an ideal primary indicator for borderline or copper deficiency due to molybdenum overdose (Mulryan and Mason 1992).The normal amount of erythrocyte superoxide dismutase activity in cattle is reported to be between 2 and 5 IU per mg of hemoglobin, and less than 2 is considered copper deficiency(Constable, Hinchcliff et al. 2016).Ahmed et al. 2017 reported that the serum levels of SOD in RB buffaloes were not significantly different from those in healthy buffaloes(Mohamed, Faisal et al. 2017), so it was in our study. However, an insignificant increase in the activity of this enzyme in infertile cows compared to control cows could indicate a possible inflammatory response in line with an insignificant increase in serum CP.

The results of serum zinc concentration (μg / dl) in RB cows (99.4 ± 21.7) and control cows (103.2 ± 16.5) were not significantly different from each other, which was consistent with the Ceylan et al 2008 study. But the results of Barui et al 2015 and Ahmed et al 2017 studies showed that plasma zinc levels in reversible and infertile cows were significantly lower than in healthy cows(Barui, Batabyal et al. 2015, Mohamed, Faisal et al. 2017).Although in most studies, the concentration of zinc in the blood of infertile cows was significantly lower than that of healthy cows, in the present study, zinc deficiency did not play a role in the etiology of RB syndrome. Because there is a very broad or multifactorial etiology of infectious and non-infectious agents, including zinc deficiency, for RB cow syndrome, zinc deficiency may be both a primary cause and an incident with non-causal association in infertile RB cows.

Based on the results of the present study, the mean BCS in RB and control cows was 3.1 ± 0.3 and 3.2 ± 0.3, respectively, indicating that there was no significant difference between the two groups, Yusuf et al. 2012 have reported similar results(Yusuf, Rahim et al. 2012).But Purohit, 2008 reported that poor postpartum BCS in cows have a significant effect on the likelihood of early embryonic death(Purohit 2008). Therefore, although it has been suggested that inappropriate BCS can be one of the causes of low fertility; But this may not be very effective in RB cows on dairy farms because RB cows by definition must have at least three unsuccessful AI. Given that the voluntary waiting period in dairy farms is about 60 days, after three unsuccessful AI the cows enter the middle lactation period, which gradually decreases production and increases appetite and as a result the BCS at AI is probably in the normal range. Due to the use of synchronization programs in most industrial herds to control voluntary waiting period and insemination of cows in a certain period after calving, the average interval between calving to first AI was not significantly different between RB and control cows and this was consistent with other studies(Moss, Lean et al. 2002, Yusuf, Nakao et al. 2010).

In conclusion, given the above, a few points are important; since copper and CP levels may decrease during the clotting process, the use of serum copper and CP concentrations alone may not be reliable in determining blood copper status in cows.

Due to the fact that a significant increase in serum CPlevel and SOD activity was found in RB compared to healthy cows, it is possible that non-reproductive inflammatory factors, especially in high-producing cows, may play a role in the pathogenesis of this syndrome.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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