

Pro-Oxidant/ Antioxidant Balance Correlates with Red Blood Cell Indices and Anemia Severity in the Anemic Patients

Soheil Mohammad Shafiee¹, Fatemeh Amiri*²,
Faezeh Yousefi¹, Negar Akbari Nasab¹

Abstract

Background: The oxidant/ antioxidant balance is disrupted in anemia. Antioxidant capacity depends on antioxidant enzyme activity and some trace elements. This study aimed to evaluate oxidant/ antioxidant status and its correlation with red blood cell indices and anemia severity in anemic patients.

Methods: Blood samples were taken from 90 anemic patients and 95 healthy people. Circulatory miR-122 was assayed by real-time PCR. Malondialdehyde (MDA), pro-oxidant/ antioxidant balance (PAB), super oxide dismutase (SOD), glutathione peroxidase (GPxs) activity, total antioxidant capacity (TAC), and zinc were measured by colorimetric method. Selenium was also determined using atomic absorption.

Results: Selenium and zinc decreased significantly in the case group (**P=0.004 and ***P=0.000). The amount of miR-122 up-regulated in the anemia (**P=0.003). MDA was significantly raised in the case vs control (***P=0.0002). PAB was higher in the case group (**P=0.005). SOD and GPxs activity was decreased along with TAC in anemic patients (*P=0.02, **P=0.008, *P=0.038). Zinc and PAB levels correlated with some red blood cell indices. PAB was associated with anemia severity.

Conclusion: Increased PAB and decreased zinc/selenium increased oxidant levels in anemic patients. RBC indices and anemia severity were correlated with oxidant/ antioxidant somewhere.

Keywords: Anemia, Antioxidants, MIRN122, Oxidants, Selenium, Zinc.

Introduction

Anemia is a prevalent blood disorder characterized by a decrease in hemoglobin, the number and volume of red blood cells (RBCs) (1,2). There are various types of anemia, including iron-deficiency (ID), hemolytic, and chronic disease-induced variants. (3). Iron-deficiency anemia (IDA) is the most prevalent among them (1, 3). Anemia severity and symptoms are affected by antioxidant/ oxidant status, especially in the case of IDA (4, 5).

Antioxidants are substances that react with oxidants by giving extra electrons and neutralizing them without producing other

radical species. Those are divided into enzymatic groups, like glutathione peroxidase (GPxs) and super oxide dismutase (SOD), and non-enzymatic groups. Micro-particles, such as beta-carotene, free radical chelators, and trace elements, are more important non-enzymatic antioxidants (6, 7). The total antioxidant capacity (TAC) is the molar amount of these molecules that oxidant neutralizes. In healthy individuals, there is a balance between the production of free radicals and the antioxidant defense system (8-10). But in pathological conditions, including anemia this balance might be

1: Student Research Committee, Hamadan University of Medical Sciences, Hamadan, Iran.

2: Department of Medical Laboratory Sciences, School of Paramedicine, Hamadan University of Medical Sciences, Hamadan, Iran.

*Corresponding author: Fatemeh Amiri; Tel: +81 38380109; E-mail: f.amiri@umsha.ac.ir.

Received: 20 Feb, 2024; Accepted: 20 Apr, 2024

disrupted. Pro-oxidant/antioxidant balance (PAB) is the disruption of oxidant/ antioxidant balance in favor of oxidant and oxygen reactive species (ROS). Then, measurement of PAB with a standard method might help clarify the ethology and pathogenesis of different diseases (9, 11).

Selenium is crucial in the body's antioxidant defense system, particularly within the GPxs structure. Additionally, selenium acts as both an oxidation and reduction agent. Selenium deficiency can lead to decreased immune function and inflammation due to the accumulation of free radicals (7, 12). As an affecting protein stability ion, zinc is another essential trace element involved in DNA synthesis, tissue repair, cell proliferation, body development, and immune system function. It also exhibits significant antioxidant properties and protects cells from damage caused by free radicals. Zinc deficiency results in various health problems such as mental retardation and immune system failure (13, 14).

Several studies have been conducted to establish a correlation between selenium deficiency and anemia (7, 15, 16). Seleno-proteins protect red blood cell (RBC) membranes from oxidant damage (17). Moreover, selenium deficiency is even associated with IDA (18). Variation in zinc levels contribute to anemia, particularly IDA (19). An independent association between selenium and/ or zinc levels and hemoglobin concentrations in old and/ or young ages was reported in the literatures (18-21).

Anemic patients have a high oxidant level, necessitating an increase in TAC to prevent serious tissue damage (22). Selenium exhibits a direct correlation with TAC; an increased selenium level results in a subsequent increase in TAC level. Additionally, there is a relationship between GPxs, selenium, and TAC levels, whereby increasing GPxs level leads to increased TAC (23, 24). Zinc can potentially increase GPxs, TAC, and cell membrane strength (25, 26).

According to the literature review, and to evaluate antioxidant status in anemia, this study was conducted to assay different

oxidant/ antioxidant-related parameters in anemic patients. The correlation between selected trace elements with TAC and PAB was determined. Zinc, selenium, and PAB were also investigated across RBC indices and anemia severity.

Materials and Methods

Subjects and Sampling

Ninety anemic patients, as the case group, and 95 healthy people, as the control group, participated in this study after filling out a consent form. All steps of the study were performed after confirmation by the ethical committee of Hamadan University of Medical Sciences (ethics code IR.UMSHA.REC.1401.155). All confirmed anemia patients referred to Shahid Beheshti Hospital were included in the study. Patients with a history of any acute and chronic diseases, people who used drugs/ supplements, smoking, pregnant women, and people with a history of blood transfusion were excluded from the study. Blood samples were taken from case and control groups with and without anticoagulants. Serum samples were collected by centrifugation and then kept at -70 °C.

RBC Indices Measurement and Anemia Severity Assessment

Complete blood count (CBC) was performed (SYSMEX XP-300, Japan) to measure hemoglobin (Hb), hematocrit (Hct), MCV (Mean corpuscular volume), MCH (Mean corpuscular hemoglobin), and MCHC (Mean corpuscular hemoglobin concentration). The anemia cases were defined based on the Hb concentration. The cut off/ levels of Hb for anemia detection were recommended by world health organization (WHO) guideline (1, 2). Then the severity of anemia was categorized for more investigation (1).

Measurement of Zinc

According to the kit protocol (BIOREX FARS, Iran), zinc was measured by colorimetric method. Briefly, 1000 µl of working solution was added to 50 µl of the serum samples. Then, test tubes were incubated for 10 minutes at

room temperature. Absorption was optically measured relative to the blank at 546 nm.

Measurement of Selenium

Selenium was measured by the atomic absorption method (Varian AA240, USA) with a flame burner head and correction system. Briefly, samples were analyzed at 196 nm with an aperture width of 0.7 nm. The selenium concentration was determined utilizing the standard curve. (27).

Circulatory MIRN122 micro RNA (miR-122) Extraction and Expression Analysis

The circulatory miR-122, accession number NR_029667, was extracted from plasma by GeneAll RiboEx™ LS Kit (GeneAll Biotech, South Korea) based on the manufacturer's instruction. cDNA was synthesized using Stem-loop primer (Metabion, Germany) and ExcelRT™ Reverse Transcriptase kit (SMOBio, South Korea). Real time PCR reaction was performed using SYBR green master mix (Takara, Japan) in Corbett Rotor-Gene 600 thermocycler (Germany). miR-16 and SNORD47 geometric mean cycle threshold (CT) were used to calculate fold change using $2^{-\Delta\Delta Ct}$ method by considering melting curve analysis.

Evaluation of Oxidant/ Antioxidant Status

Malondialdehyde (MDA), as an oxidant marker resulting from lipid peroxidation, was assayed in the subjected serums by colorimetric assay (28). To evaluate PAB, chemical reagents were provided from the Iranian blood transfusion organization as a gift. The assessment method was described by Ghasemi et al. previously (11). Briefly, standard, tetramethylbenzidine, and horseradish peroxidase solutions were prepared. Ten μ l of standard solutions and serum samples were mixed with 200 μ l of working solution in each well of 96-well plates. After 15 minutes of incubation in the dark place, the reaction was stopped using 2N HCl, and optical density was measured at 450 nm. The pro-oxidant/antioxidant balance (PAB) of samples was determined in arbitrary

HK units via the standard curve. The activity of SOD and GPxs enzymes was assessed using kits (ZellBio, Germany) following the manufacturer's protocols. Serum TAC levels were measured with the TAC assay kit (NAVAND, Iran) according to the recommended procedure, employing a single electron transfer mechanism based on divalent iron reduction ability. Optical absorption was recorded at 593 nm using a microplate reader.

Microvesicles (MVs) Isolation and Confirmation

Total MVs isolation was carried out by ultracentrifugation from subjected plasma as described previously (29). Phosphate buffer solution-diluted plasmas were subjected to centrifuge in various steps in different times and centrifugation force (28). The size of isolated MVs was measured by dynamic light scatter (DLS) (HORIBA Scientific, Japan). The scanning electron microscopy (SEM) technique was utilized for the morphology characterization of MVs. CD235a and CD47 markers were evaluated by flowcytometry (antibodies: Dako, Denmark, instrument: Life Technologies Attune Nxtm, USA) to estimate and confirm RBC-MVs.

Data Analysis

SPSS version 16 was utilized to analyze the data. Continuous variables were presented as mean \pm standard deviation (SD). Independent t-test was used to compare different parameters between case and control groups. Pearson correlation coefficient was used to determine the relationship between zinc and selenium with TAC and PAB as well as a correlation between zinc, selenium, PAB, and RBC indices. Kruskal-Wallis test/ ANOVA was performed to compare the severity of anemia across selenium, zinc, and PAB. P-value <0.05 was considered for significant difference.

Results

Demographic, RBC Indices, and Trace Elements of Subjects

For this study, 90 anemic patients, 44 (49%) male and 46 (51%) female, and 95 healthy

individuals, 48 (50.5%) male and 47 (49.5%) female were selected. Control and case's age mean, and SD was 37±10 and 34±9 years, respectively. There is no significant difference between the two groups regarding age (P=0.12) and gender (P=0.208). Anemic cases were determined according to the WHO definition for anemia regarding gender (1, 2).

Among anemic patients, 85 (94%) cases were confirmed as IDA, and the others were non-IDA non-hemolytic. The levels of Hct, Hb, MCV, MCH, and MCHC showed a significant decrease in the anemia group. Selenium (**P=0.004) and zinc (***P=0.000) had a significant decrease in the anemic group compared to the control group (Table 1).

Table 1. Comparison of red blood cell indices, ferritin, Iron, Zinc, and selenium of study subjects.

Parameters	Control (n=95)	Anemia (n=90; IDA:85)	P-value
Hemoglobin (g/dL)	14.20± 1.10	8.90± 2.02	***<0.001
Hematocrit	40.50± 1.80	29.10± 1.80	***<0.001
MCV (fL)	82.42± 3.10	63.95± 4.2	***<0.001
MCH (pg)	29.70± 1.50	24.35± 2.00	***<0.001
MCHC (g/dL)	31.45± 1.25	28.90± 1.33	**0.008
Ferritin (ng/mL)	55.80± 11.50	6.50± 3.1	***<0.001
Iron (mg/dL)	122.46± 5.89	21.98± 5.23	***<0.001
Zinc (µg/dl)	75.56± 6.35	53.90± 3.90	***<0.001
Selenium (µg/l)	57.50± 3.59	40.81± 4.00	**0.004

MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration. **P-value < 0.01, ***P-value < 0.001.

Antioxidants Reduced in Anemic Patients

Circulatory miR-122 was analyzed in the plasma of study subjects by real-time PCR. The expression level miR-122 was up-regulated in anemia (**P=0.003) (Fig. 1A). The amount of MDA was significantly raised in the cases vs the controls (***P=0.0002) (Fig. 1B). More increased PAB level was determined in anemic patients (**P=0.005) (Fig. 1C). The activity of SOD and GPxs enzymes was decreased in anemia in comparison to control (*P=0.02 and **P=0.008, respectively) (Figs. 1D & 1E). Lower levels of TAC were detected in the anemia group (*P=0.038) (Fig. 1F).

More RBC-MVs Shed from RBC Membrane in Anemic Patients

Dynamic light scattering (DLS) determined the MV size, an average of 190 nm (Fig. 2A), and SEM confirmed the MVs' morphology along with the estimated size (Fig. 2B). Flowcytometry analysis verified the expression of CD235a and CD47 as RBC markers on MVs (Fig. 2C). More expression of CD235a was detected on isolated RBC-MVs of anemic patients (10.3%) compared with those isolated from control (8.23%). RBC-MVs isolated from cases and control expressed CD47 1.35% and 0.81%, respectively (Fig. 2C). CD235a expression was meaningfully different between the two groups (*P=0.041) (Fig. 2D).

Oxidant/ Antioxidant Status in Anemia

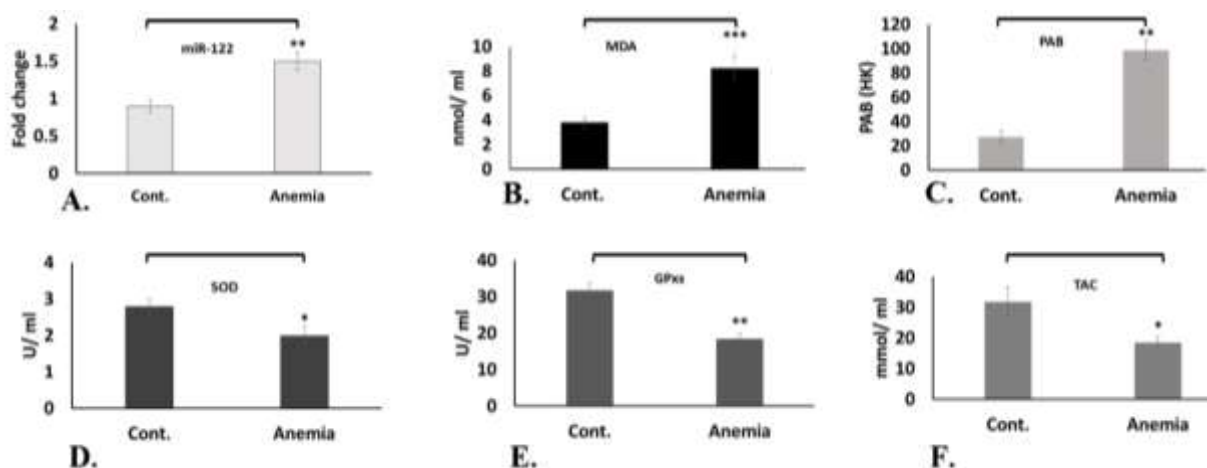


Fig. 1. Analysis of oxidant/ antioxidant status. Different oxidant/ antioxidant markers were analyzed. **A.** Circulatory miR-122 expression level. Significantly higher level of miR-122 was detected in anemia group. **B.** MDA measurement in different groups. MDA level was higher in anemia group. **C.** PAB of control and anemia groups. Anemia had more PAB compared to control. **D.** SOD activity. Low activity of SOD was determined in anemia with compared to control. **E.** GPxs activity of different groups. GSH-Px activity decreased in anemia group. **F.** TAC measurement. TAC level reduced in anemia when compared to control. Data was shown as mean± SD. *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001. MDA: Malondialdehyde, PAB: Pro-oxidant/antioxidant balance, SOD: Superoxide dismutase, GPxs: Glutathione peroxidase, TAC: Total antioxidant capacity.

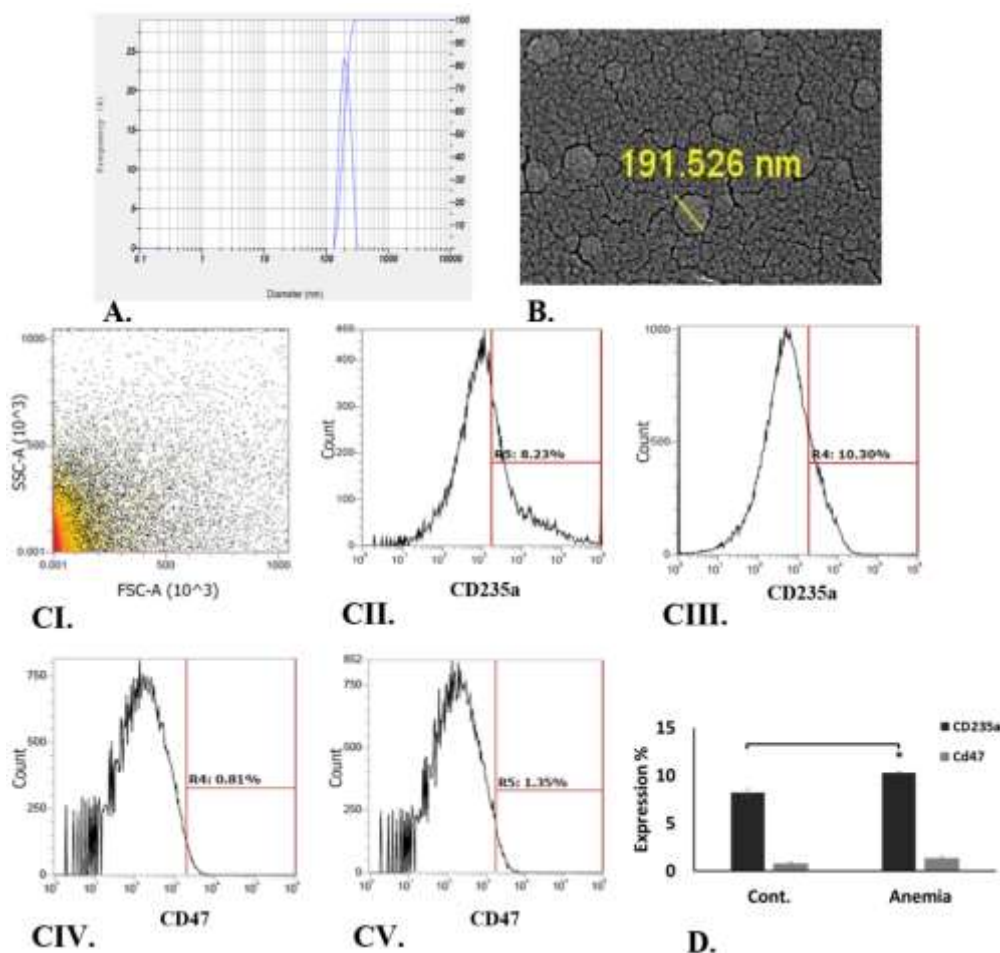


Fig. 2. Microvesicles (MVs) isolation and characterization. MVs was isolated from plasma. **A.** Dynamic light scatter (DLS) analysis. The average size of MVs was 190 nm. **B.** Image of isolated MVs by scanning electron microscope. **CI-CV.** Flowcytometry assay of red blood cell-MVs (RBC-MVs). **CI.** Gating, **CII.** Expression of CD235a in control, **CIII.** expression of CD235a in anemia, **CIV.** expression of CD47 in control, **CV.** Expression of CD47 in anemia. **D.** Quantification of flowcytometry results. Expression of CD235a was higher in anemic patients significantly. More RBC-MVs were

There was a negative correlation between Zinc with PAB in anemia patients

There was no significant relationship between selenium and zinc with TAC. Regarding zinc-

PAB cross association in the anemia group, these parameters had a meaningful correlation, and decreased zinc affected PAB level negatively (*P=0.03) (Table 2).

Table 2. Relationship between selenium and zinc with TAC and PAB.

Parameters	Group (n)	Pearson Coefficient	P-value
Selenium- TAC	All (185)	0.23	0.80
	Control (95)	0.31	0.15
	Anemia (90)	0.15	0.14
Zinc-TAC	All (185)	0.22	0.52
	Control (95)	0.20	0.40
	Anemia (90)	0.13	0.31
Selenium-PAB	All (185)	-0.25	0.75
	Control (95)	-0.26	0.55
	Anemia (90)	-0.19	0.07
Zinc-PAB	All (185)	-0.25	0.34
	Control (95)	-0.20	0.35
	Anemia (90)	-0.49	*0.03

TAC; Total Antioxidant Capacity, PAB; Pro-oxidant/ antioxidant Balance. *P-value < 0.05.

Zinc and PAB were associated with anemic patients' Hb, MCH, MCHC, and MCV values.

The difference in selenium did not affect the RBC indices. But the amount of zinc was found to be significantly related to the amount of Hb (*P=0.04), MCH (*P=0.03), and MCHC

(**P=0.001). The increase in zinc was directly correlated with the increase of these three indices in anemic patients. PAB was also correlated with Hb (**P=0.004), MCV (*P=0.02), MCH (**P=0.009), and MCHC (*P=0.041) (Table 3).

Table 3. Relationship between selenium, zinc, and PAB with RBC indices in anemic patients (n=90).

Parameter		Hct	Hb	MCV	MCH	MCHC
Zinc	Pearson correlation	0.032	0.349	0.203	0.310	0.477
	P-value	0.825	*0.04	0.161	*0.03	**0.001
Selenium	Pearson correlation	0.041	0.073	0.024	0.147	0.112
	P-value	0.777	0.617	0.869	0.315	0.444
PAB	Pearson correlation	-0.100	-0.572	-0.444	-0.503	-0.385
	P-value	0.551	**0.004	0.020*	**0.009	*0.041

Hb; hemoglobin, Hct; hematocrit, MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, PAB; Pro-oxidant/ antioxidant Balance. *P-value < 0.05, **P-value < 0.01.

Anemia severity was affected by PAB

No significant relationship between the severity of anemia with selenium ($P=0.194$) and zinc ($P=0.485$) was found. Although, a slight decrease in the average amount of

selenium and zinc were seen among them. The PAB was significantly different among mild, moderate, and severe anemia ($*P=0.041$) (Table 4).

Table 4. The amount of zinc, selenium, and PAB in different anemia severity.

Parameter	Severity			P-value
	Mild (n=23)	Moderate (n=47)	Severe (n=20)	
Se ($\mu\text{g/l}$)	49.23 \pm 4.10	47.00 \pm 5.07	42.78 \pm 4.40	0.194
Zn ($\mu\text{g/dl}$)	59.50 \pm 3.53	55.91 \pm 3.80	52.00 \pm 3.40	0.485
PAB (Arbitrary HK)	78.98 \pm 10.53	90.45 \pm 11.99	110.50 \pm 14.89	*0.041

PAB; Pro-oxidant/ antioxidant Balance. *P-value < 0.05.

Discussion

The current study revealed elevated levels of pro-oxidant/oxidant markers, including MDA, PAB, and mir-122, in anemic patients. Conversely, zinc, selenium, SOD activity, GPxs activity, and TAC were decreased in these patients, leading to increased production of RBC-MVs. Statistical analysis demonstrated a negative correlation between zinc levels and PAB, as well as a correlation between PAB and the severity of anemia. Zinc levels were significantly associated with RBC indices. Additionally, PAB correlated with Hb, MCV, MCH, and MCHC. The underlying mechanisms of these findings and a comparison with different publications are discussed below. In a situation of increased PAB, disruption in the balance between pro-oxidants and antioxidants induces elevated pro-oxidants and ROS (9, 11), resulting in increased MDA production in tissue and serum. TAC reflects the overall antioxidant defense system, including GPxs and SOD enzyme levels (4, 7, 10). Several studies have reported alterations in these parameters in anemia. Parvizi et al. (2023) defined PAB in thalassemia and reported increasing PAB at different concentrations (9). Yadav et al. (2024) reported increased MDA levels in the serum of IDA children, along with decreased GPxs and SOD activity (6). Muthiah (2013) analyzed the antioxidant status of IDA,

reporting that oxidative stress not only leads to increased MDA but also results in decreased glutathione and glutathione-S-transferase (30). Akca et al. (2013) found that despite increased oxidant activity, TAC did not significantly increase in IDA patients (22). In contrast, Bay et al. (2013) showed decreased TAC levels in IDA patients, which was concerning due to the increase in oxidant levels (31). TAC is important for maintaining RBC and its related indices and may potentially alleviate anemia symptoms (9, 22). These variations in reported results could be explained by different study designs, study subjects, and the sensitivity/specificity of methods/kits.

The results of the current study revealed an up-regulation of circulatory miR-122 in anemia. Al-Rawaf et al. (2023) evaluated miR-146a, miR-125b, and miR-122 in ID and IDA, reporting consistent findings with our study, indicating up-regulation of miR-122 in ID and IDA (32). MiR-122 is involved in various processes, including inflammation, oxidative stress, and tissue regeneration (33, 34), which could potentially explain this result.

Furthermore, the RBCs of anemic patients produced more MVs compared to healthy individuals. The involvement mechanism of RBC-MVs has been defined in both pathological and physiological conditions (35,

36). Factors such as iron metabolism status, oxidative stress, and environmental parameters may contribute to increased RBC-MV production and subsequently affect RBC lifespan, aging, and death (36).

Conversely, zinc influences the stability of proteins, including antioxidants, and demonstrates direct antioxidant potential (13, 14). Consequently, a decrease in zinc levels may result in an increase in oxidants and PAB. Zinc levels were significantly associated with RBC indices such as Hb, MCH, and MCHC. Additionally, PAB correlated with Hb, MCV, MCH, and MCHC. An independent association of zinc and selenium with Hb was reported as an age-related issue (19, 21). In contrast to our results, Zhou *et al.* (2021) reported a relationship between selenium and MCHC and hemoglobin. They suggested that increasing selenium reduced the potentiality of anemia (3). Different settings of the study and control might explain this discrepancy in results. Zinc and selenium antioxidant activity could interfere with RBC membrane integrity and iron metabolism, resulting in variations in RBC indices (17, 19). By increasing PAB, RBCs face harsh oxidative conditions, which might intensify the adverse effects on RBC metabolism, membrane integrity, and Hb content (39). Parvizi *et al.* (2023) determined a correlation between different levels of PAB, gender, and RBC indices. According to their results, PAB had a significant correlation with female gender, but there was no association between PAB and Hb and MCV (9). Oxidants contribute to the progression and severity of anemia (20, 21). Yadav *et al.* (2024) assessed the oxidant/ antioxidant status across mild,

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moderate, and severe anemia in IDA children. They concluded that the levels of MDA and protein carbonyl, as oxidant markers, and antioxidant enzymes were not associated with anemia severity (6). They evaluated each oxidant and antioxidant marker separately using different methods. In the current study, a simpler assay, PAB, was performed to provide more comprehensive information about the oxidant/antioxidant balance.

In conclusion, this study showed that the antioxidant levels of anemic patients were lower than those of the healthy group of the same gender and age due to decreased zinc and selenium along with increased PAB. This alteration affected the value of RBC indices in anemia. Pro-oxidants were found to be related to the severity of anemia. Further research is needed to determine the mechanisms involved in the oxidant/antioxidant status in anemia.

Financial Support

This study was funded by Hamadan University of Medical Sciences, research number and grant code: 140104072434.

Acknowledgments

We express our gratitude to the colleagues of the central laboratory of Hamadan pharmacy faculty and Beheshti Hospital. Special thanks to Dr. Ghasemi for their technical support and for providing the PAB assay reagents.

Conflict of Interests

All authors declare that there is no conflict of interest regarding the publication of this paper.

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