Correlation between Trace Element Levels in Iraqi Breast Cancer Patients
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Abstract

Trace elements (TEs) such as copper (CU), zinc (ZN), and iron (FE) play critical roles in biological and metabolic effects such as enzymatic reaction activation or inhibition, reactive oxygen species (ROS), competition for binding positions between trace elements and metal proteins, and changes in the permeability of cellular membranes that affect cancer events. This study used blood samples to assess the trace element concentrations in benign breast disease, breast cancer without metastasis, breast cancer with metastasis, and healthy adults as controls. The serum was separated and then used to measure the ZN, CU, and FE using a simple colorimetric assay. The statistically significant association reveals that the p values are larger than 0.05, so the data is considered statistically significant. The levels of trace elements (ZN, CU, and FE) differ between the four groups; the control group had the highest median of ZN and CU compared to the other groups; and the benign group had the highest median of Fe compared to the other groups.

Keywords: Breast cancer, Trace elements(TEs) ,Colorimetric assay. Oxidative stress (OxS), Reactive oxygen species (ROS).

العلاقة بين العناصر النزرة في عينة الدم لمرضى سرطان الثدي العراقيين
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الخلاصة

تلعب العناصر النزرة (TEs) مثل النحاس (CU) والزنك (ZN) والحديد (FE) أدوارًا حاسمة في التأثيرات البيولوجية والأيضية مثل تنشيط التفاعل الإنزيمي أو تثبيطه، وأنواع الأوكسجين التفاعلية (ROS) ، والتنافس على مواقع الربط بين العناصر النزرة والبروتينات المعدنية، والتغيرات في النفاذية استخدمت هذه الدراسة عينات الدم لتقييم تركيزات العناصر النزرة في عينة دم الاشخاص المصابين وغير المصابين وذلك بسحب ٥ مل عينة من الدم الوريدي من الاشخاص المصابين وغير المصابين. تم فصل المصل ثم استخدامه لقياس ZN و CU و FE باستخدام اختبار قياس الألوان البسيط. يكشف الارتباط ذو الأهمية الإحصائية أن قيم p أكبر من ٠٫٠٥، وبالتالي تعتبر البيانات ذات دلالة إحصائية. تختلف مستويات العناصر النزرة (ZN) و CU و (FE بين المجموعات الأربع ؛ وكان لدى المجموعة الضابطة أعلى متوسط من ZN و CU مقارنة بالمجموعات الأخرى ؛ وكان لدى المجموعة الحميدة أعلى متوسط من Fe مقارنة بالمجموعات الأخرى.

الكلمات المفتاحية: سرطان الثدي , العناصر النزرة, الفحص اللوني الملائم ,الاجهاد التأكسدي ,مركبات الاوكسجين التفاعلية

Introduction

Inflammation or chronic infection has been considered as a vital risk factor for gaining malignant properties in normal cells [1, 2]. The active oxygen species created in the tissues that are inflamed, like superoxide anion, hydrogen peroxide, and hydroxyl radical, destroy the construction and function of specific tissues by causing protein denaturation and damage of DNA, and enhance the development of tumors [3, 4]. The TEs, like zinc (ZN), copper (CU) and iron (FE), have a vital role in several biological processes by catalyzing or reticence the reactions of the many enzymes, competing with other metalloproteins and elements on sites of binding, also affecting the permeability of the cell membrane or by extra processes. The trace elements are able to cooperate in peroxidation of lipid to form free radicals are also able to play a role in electron transport and catalyze free radical-chain reactions, which may lead to disturbances in blood constituents [5-7]. ZN has a key role in human body processes because it can enchain more than 2700 enzymes and proteins, such as hydrolases, oxidoreductases, transferases, isomerases, ligases, and lyases. Zinc containing proteins consists of about 10% of human proteins and can preserve both the content and the shape of other proteins [8]. In human body tissues, copper and iron are important redox active metals that can initiate the creation of ROS and are able to oxidize constituents of the cells, like unsaturated lipid bonds in the lipid bilayer of the cellular membrane [8]. Zinc can compete with copper and/or iron for other metals with negative charges in lipid bilayers. So, ZN has the ability to protect the membrane of cells from oxidative damage caused by oxidation of lipid and can bypass oxidative stress [8]. If TEs in the human tissues miss this equilibrium, they become able to cause unsaturated bond reactions in the lipid bilayer of the cellular membrane, causing denaturation of protein, also able to cause destruction of nucleic acids, and cause cellular oxidative damage, resulting in oxidative stress.

Numerous performances have demonstrated that these TEs can participate in cancer events. Variation in the allocation and the concentration of TEs inside the tissues or in the blood of the human body has been documented in patients with many types of cancers.

Materials and Methods

Specimens collection and preparation
Venous blood of about 5ml was drawn from all patients and control (healthy persons), then relocated into a gel tube, remain at room temperature for about 30 minutes to be clot, then serum was gained by centrifuging at 1000 rpm for 10 minutes (which kept frozen until analysis), the serum was separated to be used for measuring the ZN, CU and FE.

Subjects selection
A case-control study was conducted at Al-Karama Teaching Hospital in Wasit, Iraq from October 2021 to February 2022. Patients with breast cancer in various stages were enrolled in this study (I-IV). The participants were split into four groups:
Group 1 consists of 30 healthy females who serve as controls.
Group 2 consists of 30 females with benign breast disease.
Group 3 consists of 30 females with non-metastatic breast cancer.
Group 4 consists of 30 patients with metastatic breast cancer.

Inclusion criteria
1-Adult female (18–60) years old.
2-Patients with histopathological diagnosis of breast cancer
3-Willing to participate
4-Female with benign breast disease

Exclusion criteria
1-Male
2-History of chronic renal failure
3-History of hepatic diseases
4-History of autoimmune diseases
5-History or newly diagnosed diabetes mellitus and hypertension.

Methods
Assessment of the plasma levels of Zinc
BioVision's (Zinc Assay Kit) is a convenient colorimetric assay [9-11] in which zinc binds to a ligand with the development of absorbance at 560nm.

Table 1. Zinc assay kit

Components	100 assays	Cap code	Part number
Zinc reagent 1	16ml	WM	K387-100-1
Zinc reagent 2	4ml	amber	K387-100-2
7% TCA	5ml	clear	K387-100-3
Zinc standard (50 mM)	0.1ml	yellow	K387-100-4

Assay procedure
1.Adding four parts of ZN reagent 1 to one part of reagent 2.
2.To prepare the standard curve, the ZN standard is diluted to 0.5 mM by mixing 10 l of the 50 mM ZN standard with 990 l of dH2O. In a succession of wells, 0, 2, 4, 6, 8, 10 l are added. To create 0, 1, 2, 3, 4, 5 nmol/well of Zinc Standard, the volume is adjusted to 50 l/well using dH2O.
3.200 l of zinc reaction mix is added to each standard and sample, and incubated at room temperature for 10 minutes.
4.Using a microplate reader, the OD is measured at 560 nm.

Assessment of the plasma level of Iron
The Iron Assay Kit [12,13] from Abcam is a simple colorimetric assay. Before use, all materials and reagents are equilibrated to the correct temperature.

Reagent preparation
• The small vials are centrifuged at low speed before opening.
• Standard and Reducer of Iron : Ready to use as supplied. Before. Equilibrated to room temperature prior to use and stored at - 20°C.
• Iron Assay Buffer : Ready to use as supplied. Before. Equilibrated to room temperature prior to use and stored at - 20°C.
• Iron Probe: Ready to use as supplied. It is saved in ice during the assay. The probe is aliquoted in enough volume to perform the assay. -20 °C storage the probe is used at intervals of two months after thawing.

Standard preparation
• Set of standards should be freshly is prepared for every utilize
• the working standard solutions is discarded after use.
• Standard of 1 Mm is prepared by diluting 10 μl of Iron Standard in 990 μl of dH2O
• 1Mm standard is used.

Table 2. Preparation of standard and buffer of Iron

Standard (d)	Volume of iron 1mM standard	Assay Buffer(µl)	Final volume standard in well (µl)	End Conc. Iron in well (nmol/well)
1	0	300	100	0
2	6	294	100	2
3	12	288	100	4
4	18	282	100	6
5	24	276	100	8
6	30	270	100	10

Sample preparation

Many dilutions of the sample are performed to be sure the readings are within the standard value range.

Total Fe (II + III) ion detection in samples.

1. Building the reaction wells

* Standard dilutions of 100 l are added to all standard wells.

*2 - 50 l of samples are added to sample wells (the volume is adjusted to 100 l/well with Iron Assay Buffer).

2. Fill each standard well with 5 l of iron reducer.

3. 5 liters of test A buffer is added to all samples for the iron (II) assay.

4. 5 l of Fe Reducer is added to each sample for total iron (II+III) analysis.

5. Standards and samples are combined and incubated at 37°C for 30 minutes.

6. Fill 100 l of iron probe into the wells containing the iron standard and test samples.

7. Next, incubated for 60 minutes at 37°C, sheltered from light.

8.Finally, a colorimetric microplate reader is used to directly measure OD at 593 nm.

Assessment of the plasma levels of copper

My BioSource Copper Assay Kit is a colorimetric assay [14,15].

Preparation of reagents

To make the working solution, R1 and R2 are combined in a 4:1 ratio (R1 is acetate buffer (pH 5.0) and sodium deoxycholate (SDC), while R2 is ascorbic acid and 3,5-dibromo-PAESA).

Method of operation

1-to 15 l of sample with 300 l of working solution (R1 and R2) is added.

2-mixed and incubated at 37°C for 5 minutes. The absorbance is set to 359nm and auto-zero with a blank well.

Table 3. Copper kit

Reagent	Ingredient	Final concentration
Reagent (R1)	-Acetate buffer -sod.deoxycholate	$0.2mol\backslash L$ $2 \times 10\text{-}2mol/L$
Reagent (R2)	-Ascorbic acid -3,5dibromo-PAESA	$5 \times 10\text{-}2mol/L$ $2 \times 10\text{-}5mol/L$

Results

Descriptive data

All data collected is analyzed using the SPSS statistical package for social science (SPSS version 25). The data were presented as the median, Statistical analysis involves descriptive statistics, Tables, and Figures. The k-independent test is used to check the uniform distribution of data. In this study, the statistically significant association was determined. The p values are based on 2-sided tests and p 0.05, so the data was considered statistically significant. (not normal distribution).The level of ZN in the 4 groups has a statistical difference (p-value is.000) , the highest median was reported in the control group and the lowest median was reported in the metastasized breast cancer group as shown in Table (4) and Figure (1), In Copper the difference in the 4 groups has a statistical difference (p-value is.000) and the highest median was reported in the control group and the lowest median was reported in the breast cancer free from metastasis group as shown in Table (4) and Figure (2). In Iron, the difference in the 4 groups has a statistical difference (p-value is.000) the highest median was reported in the benign group and the lowest median was reported in the metastasized breast cancer group as shown in Table (4) and Figure (3).

Table 4. of Descriptive Data of 4 groups patients of (TEs)

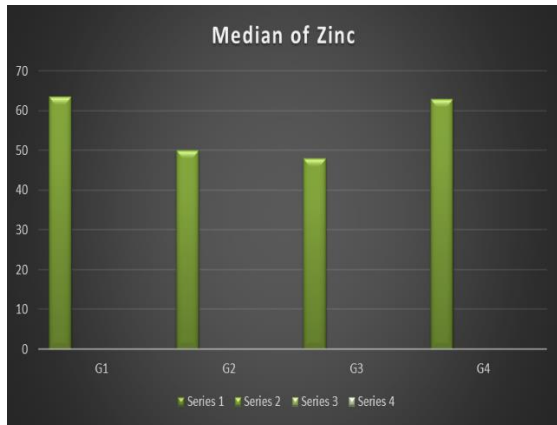|		control	Benign	Met.B.C	B.C without Met
ZN	Median	63.500	50.000	48.000	63.000
	Std.deviation	11.092	15.441	9.021	23.338
CU	Median	90.000	78.000	66.500	35.500
	Std.deviation	4.143	11.724	11.708	12.290
FE	Median	65.000	72.500	48.500	54.855
	Std.deviation	4.491	15.398	11.941	12.478

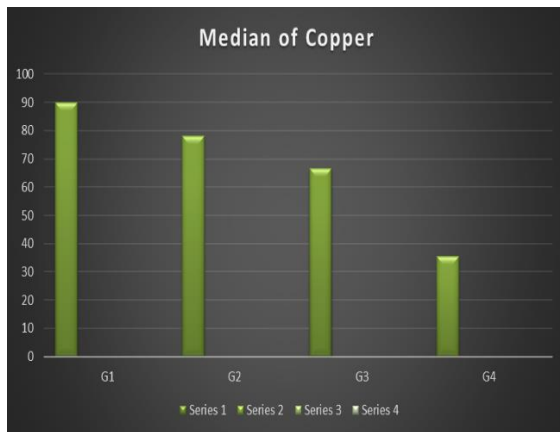**Figure1. Assessment of Zn in studied groups**

Figure 2. Assessment of Cu in studied groups
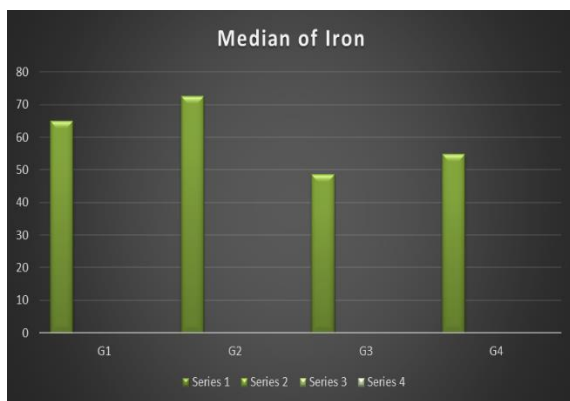

Figure 3. Assessment of FE in studied groups

Discussion

In this research, the levels of three trace TEs in human serum was calculated by using a convenient colorimetric assay in female patients suffering from breast cancer and healthy people as controls in Iraq. Many performances have alluded to the fact that some environmental compounds like phytoestrogens, xenoestrogens, and metalloestrogens exhibit estrogen-like properties, including the capability to induce several types of cancer like BC [16]. Also, TEs are important constituents of antioxidants and may have protective effects on cells [17]

In this study, in breast cancer suffering group , the ZN levels were lower than in controls, which is consistent with the outcome of past performance in Nigeria [18,19], China [20,21], and Kuwait[22].

The low level of CU in the patients with BC group compared to the normal healthy control group could not be attributed to malnutrition or certain diseases since the medical history and physical examination did not reveal any short-term or long-term illnesses.

Variation in TEs levels in different patients could follow race, personal culture, pollution, and also variation in human body structure and genetics [23]. Genetic loci that can affect blood copper and zinc concentration in Australian and British people were identified in a genome-wide linked study [24].

Study design variation, how many participants, criteria of patients suffering from BC (such as types of cancers with either histopathological and/or molecular/genetical variations), the moment of collecting the sample (before-treatment or after-treatment), or methods that are used for analyzing (X-ray fluorescence spectroscopy, AAS, ICP-MS, etc.) could also affect the variety of outcomes [24]. Many studies conducted in China [21], Taiwan [23], India [27], and Nigeria [28,20] described greater levels of copper in patients with BC than in healthy ones.

At the same time, the adverse studies (greater copper levels in healthy people than in patients with BC) in this study, which agrees with previous Kuwait [22], and Korea [28], and there is no variation reported in a study achieved in Canada [29] or in India [27].Because CU is an important constituent in several necessary enzymes, copper is important for the three characteristic phenomena related to cancer progression: starting from the proliferation step, then the angiogenesis step, and finally the metastasis [30]. CU suggested a possible target for breast cancer diagnosis, prognosis, and treatment [31]. Nonetheless, there is inadequate information to help physicians and patients on the usage of antioxidant supplements like CU throughout the treatment of BC [32].

Iron is essential in the activation or reticence of various proteins and enzymes involved in a variety of biological processes [33,34].

Most performances have measured the serum levels of FE by calculating iron-bound proteins (circulating levels), for example, transferrin and ferritin [35-37]. However, this method might include errors in reflecting the true iron level, and some studies have measured the FE concentration directly [35].

In this study, there is no measurement of the levels of transferrin or ferritin but measured free serum iron level to represent the iron level in the body. Most patients who are suffering from cancer are experience anemia due to cachexia or therapeutic drugs which cause a low serum iron level. This phenomenon has been investigated in several types of cancers, one of them being the BC [33,38]. These studies agree with this study, in which the iron serum level in the patients with BC is lower than in control .

Generally, this study has several limitations. Knowledge about food intake was not evaluated in this analysis. Also, ceruloplasmin was not calculated (copper-carrying protein) or other proteins included in antioxidant pathways, which might change human body trace element levels [19].

Conclusion

The current study focused on the correlation between trace element levels in Iraq Breast Cancer patients which can be summarized as follows :

 the levels of trace element that include Zinc, Copper and Iron were significantly correlated with cancer incidence as they reduced significantly in breast cancer patients with / without metastasis in comparing with healthy control and patients with benign breast disease .

Funding

Conflicts of Interest

The authors declare that there is no conflict of interest

Ethics Statements

This work was performed according to the Ethics Committee of the College of Pharmacy in University of Baghdad.

Author Contribution

First authors contribution: Collected the data, performed the analysis and writing of the manuscript. Second author contribution: Have made a substantial contribution to the concept and the design of article ,Conceived and designed the analysis ,revised it critically and approved the version to be published.

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