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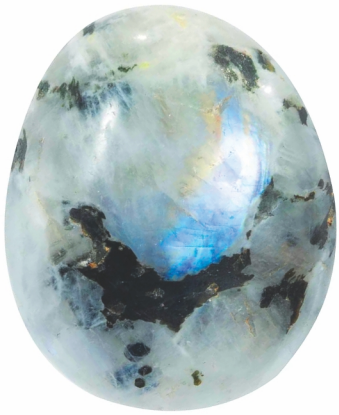

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Low Protein S Antigen in COVID-19 Patients

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Abstract

Introduction. The coronavirus disease 2019 (COVID-19) pandemic has spread globally with high morbidity and mortality. Coagulation abnormality in COVID-19 forming a spectrum from only abnormal laboratory parameters to micro-thrombosis of small blood vessels to disseminated intravascular coagulation being a major contributing factor in disease complications and mortality. Protein S is a single-chain vitamin-K-dependent glycoprotein chiefly synthesized in the liver by endothelial cells.
Purpose. To evaluate the level of protein S (protein C, antiphospholipid ab) in COVID 19 patients, correlate protein S with other hematological and coagulations test (PT, PTT, D-dimer, Fibrinogene) and correlate protein S with inflammatory markers (CRP, ESR, Ferritin).
Materials and methods. This is cross sectional study that will take sixty COVID-19 patients and twenty control adult. Left over blood samples will be taken for evaluation of protein S by clotting assay (STA-Staclot Protein S). Other lab and clinical data will be taken from patients medical records.
Results. The coagulation parameters assessment demonstrated normal median platelet count in about three-quarters of patients. However, D-dimer levels were particularly elevated (median 1.66 mg/ml) with 68% of patients above the normal range (<0.5 mg/mL). On the other side; the natural coagulation inhibitor, free Protein S test results showed lower than normal levels range(normal range 60–130%) in 64% of patients with median level of about 50%.
Conclusion. Mild protein S deficiency was detected in more than half of patients with COVID-19 infection. Nevertheless no positive correlation was found between protein S and studied hematological and inflammatory parameters.
Keywords: COVID-19, protein S, inflammatory markers, hypercoagulability, ferritin

■ INTRODUCTION

According to WHO designation, COVID-19 is a human infectious disease caused by infection with a coronavirus called SARS-CoV-2 [1]. And since the time the pandemic began, it spread rapidly through the whole globe affecting up to 771.407.825 till time of writing at 18th. October, 2023 [2].

Wide clinical spectrum for this disease is observed, ranging from asymptomatic cases, mild upper respiratory tract disease, to severe cases of pneumonia with associated acute respiratory distress syndrome [3, 4]. In addition, progression to death is a recognized complication in large number of patients. Frequent studies on patients with COVID-19 have implicated the systemic cytokine effects of infection; known as a cytokine storm with an elevation of associated hyper-inflammatory clinical markers and a pro-coagulant, thrombotic milieu as pathophysiological factors [5].

This is accompanied or preceded by, a hyper-coagulable inflammatory state, which is characterized by microangiopathic pulmonary changes [6, 7].

Clinically hypercoagulability has wide range of manifestation; from venous thromboembolism to arterial events [8, 9].

Complementary factor to hypercoagulability state in COVID-19 is coagulation dysfunction [10]. That's the consequence of the dyshomeostasis of a regulator, protein S [11].

Protein S, is a single-chain vitamin-K-dependent glycoprotein, mainly being synthesized by endothelial cells of the liver and in addition to its well-defined anticoagulant function as a cofactor for the activated protein C, its own another extremely important function, which is the activation of the immunosuppressive TAM receptors, with their essential role in preventing a status of hyperinflammation, as the one seen in acute lung injury [12, 13].

In COVID-19 infection, the levels of protein C and protein S in comparison to the normal population were reduced, as well its levels served as a marker for disease severity and mortality, this suggests that protein S plays a role in the thromboembolism associated with COVID-19 infections [14].

■ PURPOSE OF THE STUDY

To evaluate the level of protein S (protein C, antiphospholipid ab) in COVID 19 patients, correlate protein S with other hematological and coagulations test (PT, PTT, D-dimer, Fibrinogene) and correlate protein S with inflammatory markers (CRP, ESR, Ferritin).

■ MATERIALS AND METHODS

A cross sectional study was conducted at multiple hospital in Baghdad on sixty hospitalized patients infected with COVID-19 and twenty blood sample were collected as control from non-infected people at time of sample collection between October 2020 to March 2021.

According to the Iraq health government COVID-19 protocol, blood samples were obtained daily from every hospitalized patient with COVID-19, including samples to perform the basic coagulation test (PT, PTT and D-dimer).

Inclusion criteria were blood samples from adult patients with COVID-19, aged 18 years and older hospitalized in multiple hospitals, COVID-19 diagnosis was documented by positive PCR results in nasopharyngeal swab or by radiological findings.

To avoid interference with coagulation test results, patients on medications with vitamin K antagonists for at least 10 days prior to the blood sample withdrawal were excluded.

Left over samples from routine basic coagulation tests was used to estimate protein S level by clotting assay (STA-Staclot Protein S) Cat. NR. 00746 while other laboratory tests results including a basic coagulation test results as well as routine laboratory tests that include complete blood count, biochemical and acute phase reactant proteins such as C-reactive protein, ferritin and lactate dehydrogenase protein are collected from the hospital medical records of each patient.

In addition to the demographic and clinical data; results are all collected in sync with time of coagulation test and protein S level assessment.

■ RESULTS

Complete blood count: the hematological parameters are assessed in those patients and showed a normal hemoglobin value, neutrophil count with low lymphocytes count;

Table 1
Complete blood count parameters in patients with COVID-19

Parameter		Results
Hb (g/dl) Reference range (Men: 13–17, women: 12–15)	Mean±SD	13.12+2.44
	Median	13.5
	Range	6.7–19.4
	Anemia n (%)	18 (36.0)
	Normal n (%)	29 (58.0)
	Polycythemia n (%)	3 (6.0)
Neutrophils ($\times10^9$/l) Reference range (2–7)	Mean±SD	9.91+5.86
	Median	8.7
	Range	2.46–27.2
	Neutropenia n (%)	0
	Normal n (%)	19 (38.0)
	Neutrophilia n (%)	31 (62.0)
Lymphocytes ($\times10^9$/l) Reference range (1–3)	Mean±SD	1.02+0.68
	Median	0.87
	Range	0.2–3.2
	Lymphopenia n (%)	28 (56.0)
	Normal n (%)	20 (40.0)
	Lymphophilia n (%)	2 (4.0)
Neutrophils/lymphocytes ratio Reference range (1–3)	Mean±SD	13.39+10.36
	Median	10.2
	Range	1.4-49
	Low n (%)	0 (0.0)
	Normal n (%)	4 (8.0)
	High n (%)	46 (92.0)
Eosinophils ($\times10^9$/l) Reference range (0.02–0.5)	Mean±SD	0.03+0.05
	Median	0.01
	Range	0.0–0.26
	Eosinopenia n (%)	29 (58.0)
	Normal n (%)	21 (42.0)
	Eosinophilia n (%)	0

Notes: Hb – hemoglobin, SD – standard deviation, n – number of cases.

Table 2
Inflammatory parameters in patients with COVID-19

Parameter		Results
ESR (mm/hr) Reference range (for men: 17–50 yr ≤10, 51–60 yr ≤12, 61–70 yr ≤14, > 70 yr ≤30, for women: 17–50 yr ≤12, 51–60 yr ≤19, 61–70 yr ≤20, >70 yr ≤35)	Mean±SD	55.56+26.34
	Median	52.5
	Range	5–100
	Normal n (%)	4 (8.0)
	High n (%)	46 (92.0)
CPR (mg/l) Reference range (<10)	Mean±SD	74.22+61.9
	Median	59.65
	Range	2.3-260.8
	Normal n (%)	2 (8.0)
	High n (%)	48 (92.0)
S. Ferritin (ng/ml) Reference range (for men: 15–300, for women: 15–200)	Mean±SD	679.6+405.06
	Median	603
	Range	20–1650
	Low n (%)	0 (0.0)
	Normal n (%)	4 (8.0)
	High n (%)	46 (92.0)
LDH (U/l) Reference range (120–246)	Mean±SD	483.57+213.56
	Median	419
	Range	152–900
	Low n (%)	0 (0.0)
	Normal n (%)	4 (8.0)
	High n (%)	46 (92.0)

Notes: ESR – erythrocytes' sedimentation rate, CPR – C-reactive protein, S – serum, LDH – lactate dehydrogenase, SD – standard deviation, n – number of cases.

resulting in higher neutrophils/lymphocytes ratio with reduced esosinphils below the reference range in around 60% as shown in table 1.

Inflammatory markers: the assessed inflammatory markers show marked elevation in almost all patients and this is demonstrated in table 2 that studies the level of these markers.

Coagulation parameters

The coagulation parameters assessment demonstrated normal median platelet count in about three-quarters of patients. However, D-dimer levels were particularly elevated (median 1.66 mg/ml) with 68% of patients above the normal range (<0.5 mg/mL). On the other side; the natural coagulation inhibitor, free Protein S test results showed lower than normal levels range (normal range 60–130%) in 64% of patient s with median level of about 50%. All are demonstrated in table 3.

The correlation of protein S with clinical and inflammatory markers

Table 4 highlights the correlation between the level of protein S and clinical parameter (age of the patients), haematological findings (Hb level, neutrophil, lymphocytes, and eosinophils counts, platelet count) and inflammatory markers (ESR, CRP, ferritin, LDH and D-dimer).

Table 3
Coagulation parameters in patients with COVID-19

Parameter		Results
Platelets (×10⁹/l) Reference range (150–410)	Mean±SD	281.24+120.27
	Median	259.5
	Range	23-668
	Thrombocytopenia n (%)	5 (10.0)
	Normal n (%)	39 (78.0)
	Thrombocytosis n (%)	6 (12.0)
D-dimer (mg/ml) Reference range (<0.5)	Mean±SD	3.01+3.9
	Median	1.22
	Range	0.1–16
	Normal n (%)	16 (32.0)
	High n (%)	34 (68.0)
Protein S (%) Reference range (60–130)	Mean±SD	55.12+16.95
	Median	50.0
	Range	26–91
	Low n (%)	32 (64.0)
	Normal n (%)	18 (36.0)
	High n (%)	0 (0.0)

Notes: APTT – activated partial thromboplastin clotting, PT – prothrombin time, SD – standard deviation, n – number of cases.

Table 4
Correlation of protein S with other parameters

Parameter	Protein S	
	R	P
Age (yr)	–0.053	0.716
Hb (g/dl)	–0.034	0.814
Neutrophils (×10⁹/l)	0.139	0.337
Lymphocytes (×10⁹/l)	0.111	0.443
Neutrophils/lymphocytes ratio	0.058	0.691
Eosinophils (×10⁹/l)	0.142	0.326
Platelets (×10⁹/l)	0.081	0.577
ESR (mm/hr)	–0.104	0.471
CPR (mg/l)	0.005	0.973
S. ferritin (ng/ml)	0.052	0.719
LDH (U/l)	0.140	0.333
D-dimer (mg/ml)	0.120	0.406

Notes: ESR – erythrocytes' sedimentation rate, CPR – C-reactive protein, S – serum, LDH – lactate dehydrogenase, SD – standard deviation, n – number of cases.

Comparison of coagulation parameters between male and female patients with COVID-19

The differences in coagulation parameters between male and female patients are illustrated in this table 5 were no significant difference is seen.

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Table 5
Comparison of coagulation parameters between male and female patients with COVID-19

Parameter	Male n=34			Female n=16			P value
	Median	Mean±SD	Range	Median	Mean±SD	Range	
Platelets (×10⁹/l)	245.00	271.41+ 119.56	23–668	276.50	302.13+ 122.95	125–498	0.355**
D-dimer (mg/ml)	0.69	3.2+ 4.34	0.1–16	1.41	2.62+ 2.83	0.1–9.21	0.787**
Protein S (%)	50.00	53.21+ 16.12	26–90	58.50	59.19+ 18.46	26–91	0.248*

Notes: ESR – erythrocytes' sedimentation rate, CPR – C-reactive protein, S – serum, LDH – lactate dehydrogenase, SD – standard deviation, n – number of cases, * Unpaired t-test, ** Mann – Whitney test.

Comparison of complete blood count parameters and protein S in patients with COVID-19

The differences in complete blood count parameters according to the level of protein S (Low protein S <60%) or (high protein S >60%) is shown in this table 6. Were the majority of patients with low protein S are anaemic, lymphopenic and eosinopenic. Though no significant statistical difference is seen.

Table 6
Comparison of complete blood count parameters according to protein S level in patients with COVID-19

Parameter		<60% n=32 n (%)	≥60% n=18 n (%)	P value
Hb (g/dl) Reference range (Men: 13–17, women: 12–15)	Anemia	13	5	0.815**
	Normal	18	12	
	Polycythemia	1	1	
Neutrophils (×10⁹/l) Reference range (2–7)	Neutropenia	0	0	0.552*
	Normal	11	21	
	Neutrophilia	8	10	
Lymphocytes (×10⁹/l) Reference range (1–3)	Lymphopenia	20	8	0.651**
	Normal	11	9	
	Lymphophilia	1	1	
Neutrophils/lymphocytes ratio Reference range (1–3)	Low	0	0	0.612*
	Normal	2	2	
	High	30	16	
Eosinophils (×10⁹/l) Reference range (0.02–0.5)	Eosinopenia	22	7	0.072*
	Normal	10	11	
	Eosinophilia	0	0	

Notes: Hb – hemoglobin, n – number of cases, * Fisher exact test, ** Yates chi square test.

Comparison of inflammatory parameters and protein S in patients with COVID-19

The differences in level of inflammatory markers according to the level of protein S (Low protein S <60%) or (high protein S >60%) is shown in this table 7. Were the majority of patients with low protein S have higher ESR, CRP and LDH apart from ferritin is lower. Though no significant statistical difference is seen.

Table 7
Comparison of inflammatory parameters according to protein S level in patients with COVID-19

Parameter	<60% n=32			≥60% n=18			P value
	Median	Mean±SD	Range	Median	Mean±SD	Range	
ESR (mm/hr)	53.50	57.97+ 27.43	5–100	51.50	51.28+ 24.44	10–100	0.394*
CPR (mg/l)	62.75	81.4+ 68.38	2.3–260.8	49.98	61.47+ 47.42	10–201.2	0.430**
S. Ferritin (ng/ml)	595.20	729.46+ 451.3	20–1650	641.00	590.96+ 297.52	135.2– 1310	0.571**
LDH (U/l)	421.20	473.93+ 193.68	152–894	394.00	500.71+ 250.14	228–900	0.968**

Notes: ESR – erythrocytes' sedimentation rate, CPR – C-reactive protein, S – serum, LDH – lactate dehydrogenase, SD – standard deviation, n – number of cases, * Unpaired t-test, ** Mann Whitney test.

Comparison of coagulation parameters and protein S in patients with COVID-19

The differences in level of coagulation parameters according to the level of protein S (Low protein S <60%) or (high protein S >60%) is shown in this table 8. Were no difference in platelet count neither D-dimer level according to the level of protein S.

Table 8
Comparison of coagulation parameters according to protein S level in patients with COVID-19

Parameter	<60% n=32			≥60% n=18			P value
	Median	Mean±SD	Range	Median	Mean±SD	Range	
Platelets ($\times 10^9$/l)	256.00	272.06+ 112.07	23–542	269.00	297.56+ 135.46	125–668	0.657
D-dimer (mg/ml)	1.48	3.44+ 4.29	0.1–16	0.80	2.26+ 3.05	0.1–10	0.454

■ DISCUSSION

Many reports have been published discussing different topics in COVID-19 infection since its appearance, particularly coagulopathy. The combination of inflammation and thrombosis cause a positive feedback loop between these two pathways. Thrombocytopenia had been observed only in 10% of our sample patients and this is less than what is observed in meta-analysis data for patient infected with COVID-19; probably due to the difference in sample size [15].

D-dimer is high in most of the sample patients in about 68% which is comparable to data analysis seen of seven studies [16, 17]. And this is a consistent finding in multiple studies despite the fact that the exact cause for such elevation is poorly understood. This elevation could be associate with disseminated intravascular coagulation, as a reflection of fibrin by product degradation accumulating within the lung parenchyma and the alveoli and thus causing lung damage.

Likewise, a mild protein S deficiency was found in our study in more than half of the patients and that is comparable to the result of three previous studies [18].

Protein S itself may directly regulate inflammatory responses. Activated protein or protein S alone and combined activated protein C + protein S therapy decreased inflammatory markers and cytokines; so it may have a protective role possibly by direct

inhibition of the local expression of inflammatory cytokines without affecting coagulation [19]. This the expected theory behind absence of the positive relationship between coagulation markers and systemic inflammatory response in our study data.

While acquired thrombophilia, as an expected squeal with COVID-19. May be explained by multifactorial events and involves endothelial dysfunction, the release of pro-inflammatory cytokines, enhanced platelet activity, an imbalance of coagulation factors, and complement activation [20].

■ CONCLUSION

Mild protein S deficiency was detected in more than half of patients with COVID-19 infection. Nevertheless no positive correlation was found between protein S and studied hematological and inflammatory parameters.

■ REFERENCES

1. Walls AC, Park YJ, Tortorici MA, et al. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell*. 2020;181:281–292. Available at: https://doi.org/10.1016/j.cell.2020 02.058
2. WHO. Coronavirus disease 2019 (COVID-19) Situation Dashboard [Internet]. Available at: https://covid19.who.int/. Accessed: October, 22, 2023.
3. Carsana L, Sonzogni A, Nasr A, et al. Pulmonary post-mortem findings in a series of COVID-19 cases from northern Italy: a two-centre descriptive study [published online ahead of print, 2020 Jun 8]. *Lancet Infect Dis*. 2020;S1473–3099(20):30434–5. doi: 10.1016/S1473-3099(20)30434-5
4. Li MY, Li L, Zhang Y, Wang XS. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty*. 2020;9:45. Available at: https://doi.org/10.1186/s40249-020-00662-x
5. Wang T, Chen R, Liu C, et al. Attention should be paid to venous thromboembolism prophylaxis in the management of COVID-19. *Lancet Haematol*. 2020;7(5):e362–e363.
6. Panigada M, Bottino N, Tagliabue P, et al. Hypercoagulability of COVID-19 patients in intensive care unit. a report of thromboelastography findings and other parameters of hemostasis. *J Thromb Haemost*. 2020;18(7):1738–1742.
7. Oudkerk M, Büller HR, Kuijpers D, et al. Diagnosis, prevention, and treatment of thromboembolic complications in COVID-19: report of the national institute for public health of the Netherlands. *Radiology*. 2020;201629.
8. Bilaloglu S, Aphinyanaphongs Y, Jones S, et al. Thrombosis in Hospitalized Patients with COVID-19 in a New York City Health System [published online ahead of print, 2020 Jul 20]. *JAMA*. 2020;e2013372. doi: 10.1001/jama.2020.13372
9. Middeldorp S, Coppens M, van Haaps TF, et al. Incidence of venous thromboembolism in hospitalized patients with COVID-19 [published online ahead of print, 2020 May 5]. *J Thromb Haemost*. 2020. doi: 10.1111/jth.14888. doi:10.1111/jth.14888
10. Xiong M, Liang X. Changes in blood coagulation in patients with severe coronavirus disease 2019 (COVID-19): a meta-analysis. *Br J Haematol*. 2020;189(6):1050–1052. Available at: https://doi.org/10.1111/bjh.16725
11. LEMKE G, SILVERMAN GJ. Blood clots and TAM receptor signalling in COVID-19 pathogenesis. *Nat Rev Immunol*. 2020;20(7):395–396.
12. Sriram K, Insel PA. Inflammation and thrombosis in COVID-19 pathophysiology: proteinase-activated and purinergic receptors as drivers and candidate therapeutic targets. *Physiol Rev*. 2021 Apr 1;101(2):545–567.
13. Keith Gomez, John H McVey. *Normal haemostasis*. In Postgraduate Haematology. A Victor Hoffbrand, Douglas R Higgs, David M Keeling, Atul B Mehta (eds.). JohnWiley & Sons Ltd, Lodonn, UK. 2016; 696 p.
14. Elshafie A, Foda E, Yousef MMG, El-Naby KAA. Evaluation of protein C and S levels in patients with COVID-19 infection and their relation to disease severity. *Egypt J Intern Med*. 2023;35(1):14. doi: 10.1186/s43162-023-00195-3
15. Guan W, Ni Z, Hu Y, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. 2020;382:1708–1720.
16. Wang L, He W, Yu X, et al. Coronavirus disease 2019 in elderly patients: characteristics and prognostic factors based on 4-week followup. *J Infect*. 2020;80(6):639–645. Available at: https://doi.org/10.1016/j.jinf.2020.03.019
17. Sun YJ, Feng YJ, Chen J, et al. Clinical Features of Fatalities in Patients with COVID-19. *Disaster Med Public Health Prep*. 2021 Apr;15(2):e9–e11.
18. Ruan Q, Yang K, Wang W, et al. Clinical predictors of mortality due to COVID-19based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med*. 2020;46(5):846–848. Available at: https://doi.org/10.1007/s00134-020-05991-x
19. Tu WJ, Cao J, Yu L, et al. Clinicolaboratory study of 25 fatal cases of COVID-19 in Wuhan. *Intensive Care Med*. 2020;46(6):1117–1120. Available at: https://doi.org/10.1007/s00134-020-06023-4
20. Takagi T, Taguchi O, Aoki S, et al. Direct effects of protein S in ameliorating acute lung injury. *J Thromb Haemost*. 2009;7:2053–2063.

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Correlation between Ocular Anatomy and Chlamydial Infection

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Abstract

Introduction. C. trachomatis is the causative agent of many diseases cause as blindness and is the most infection common sexually transmitted pathogen.
Purpose. To determine the relationship between ocular anatomy and chlamydial infections.
Materials and methods. A 100 swabs were taken from patients whom suffering from an eye infection disease. The patients who attended the government hospitals in Basrah province from Feb to Jul 2022. DNA was extracted from swabs by a Bacall swab of DNA extraction kit and then molecular methods. C. Trachomatis isolated from patients who suffer from conjunctivitis, blepharitis, Lacrium cystitis, and keratitis.
Results. The infection rate of Chlamydia was 12%. Males were infected more than females. This study diagnosed C. Trachomatis from eye infections for the first time in Iraq. PCR assay showed high sensitivity as determined for the presence of C. Trachomatis among patients. In this study, the patient infected with C. Trachomatis was distributed in seven age groups.
Conclusion. C. Trachomatis is a causative agent of eye infections diseases in Basrah province – south of Iraq. This study diagnosed C. Trachomatis from eye infections for the first time in Iraq. Young men are more effected than women.
Keywords: C. Trachomatis, eye infection diseases, ocular anatomy, blepharitis, keratitis

■ INTRODUCTION

C. Trachomatis is a bacterium that causes many diseases including trachoma, lymph granuloma, pelvic, and non-gonococcal urethritis, this can make it (in women) difficult or impossible to get pregnant in the future [1, 2]. The life cycle of Chlamydia is characterized by two stages, the Elementary Body (EB), which adheres to and invades the eukaryotic cell

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in a larger (0.3 to 0.6 Mm in diameter) and the vegetative form (0.6 to 1.5 Mm in diameter), the Reticulate Body (RB) [3, 4]. Neonatal conjunctivitis is inflammation occurring within the first 30 days of life. C. trachomatis (most common) causative agent is typically contracted during vaginal delivery from exposure to bacteria from the birth canal [5].

Several layers make up the eyeball, it consists of a dense and elastic imperfect of supporting tissue which are opaque sclera and transparent cornea. The outermost layer of the eyes includes the sclera, conjunctiva, and the cornea [6, 7].

The cornea is a central transparent front part (outer layer) of the eye (watch glass) and consists of several layers including Bowman's membrane, Epithelium, Dua's layer (pre-Descemet's layer), Stroma (substantia propria), Endothelium, and Descemet's membrane. It is avascular with no blood vessels except the limbus [6].

The sclera is the 'dense white tissue' supporting wall and it is continuous with the clear cornea. The outer surface is covered by the conjunctiva, under which is the episclera and the innermost layer consists of elastic lamina fusca fibers [6]. The anterior chamber is a space filled with aqueous humour, and bounded anteriorly by the cornea, and posteriorly by the iris and pupil [6]. The angle of the anterior chamber is a peripheral recess, bounded posteriorly by the iris root and the ciliary body and anteriorly by the corneo-sclera [6, 8]. Lens is a biconvex mass of peculiarly differentiated epithelium, consisting of three main parts. The uveal tract consists of three parts (two posterior [choroid and ciliary body], while anterior form a free circular diaphragm [iris]) [6].

Iris is thinnest when attached to the ciliary body. It is composed of a stroma have branched connective tissue cells, largely un-pigmented in blue irides with little pigmented, with a rich supply of blood vessels [8].

Ciliary body is situated in the antero-posterior section and is shaped roughly like an isosceles triangle, with the base forwards. The chief mass of the ciliary body is composed of ciliary muscle. The inner surface is divided into two portions (the anterior part (pars plicata) and the posterior part (pars plana)) [6]. The choroid is an extremely vascular membrane in contact everywhere with the sclera. On the inner side, the choroid is covered by lamina vitrea or membrane of Bruch as a thin elastic. The blood vessels of the choroid increase in size from within outwards. The posterior chamber and vitreous humour are triangular spaces between the posterior aspect of the iris and the anterior surface of the lens. The vitreous chamber lies behind to lens, containing vitreous humour [6, 8].

The retina corresponds in lines extent to the choroid, it is continued anteriorly as a double layer of epithelium as pupillary margin. It consists of several layers formed by three strata of cells and their synapses, which are visual cells (externally), bipolar cells (intermedially), and ganglion cells (internally), the axons of which run into the central nervous system [6]. The layers of the retina are (outer to inner): rods and cones, retinal pigment epithelium, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer, and cells running centrally into the optic nerve [8].

The tear film end products are released by a lacrimal functional unit which consists of the lacrimal and Meibomian glands, ocular surface, and interconnecting innervation [9].

The conjunctiva is a mucosal surface that extends from the corneoscleral limbus to the eyelid margin and covers the sclera up to the limbus [8]. It divides into three distinct regions (bulbar, fornix, and palpebral) [9].

■ PURPOSE OF THE STUDY

To determine the relationship between ocular anatomy and chlamydial infections.

■ MATERIALS AND METHODS

Study population

A 100 swabs were taken from patients who suffering from eye infection disease. Cases who attended the government hospitals in Basrah province from Feb to Jul 2022.

Sample collection

C. Trachomatis isolated from patients who suffered from conjunctivitis, blepharitis, Lacrium cystitis, and keratitis by swabing.

DNA extraction

DNA was extracted from swabs by a Bacall swab DNA extraction kit and then molecular methods [10], as shown in Tables 1 and 2. All sample swabs entering all steps of PCR amplification including denaturation, annealing and extension. Then gel electrophoresis used to visualized the findings.

Table 1
PCR reaction setup

Chemicals	Volume (µl)
Master mix	12.5
Premier Forward	1
Premier Reverse	1
DNA	5
H2O	5.5
Total	25

Table 2
PCR amplification setup

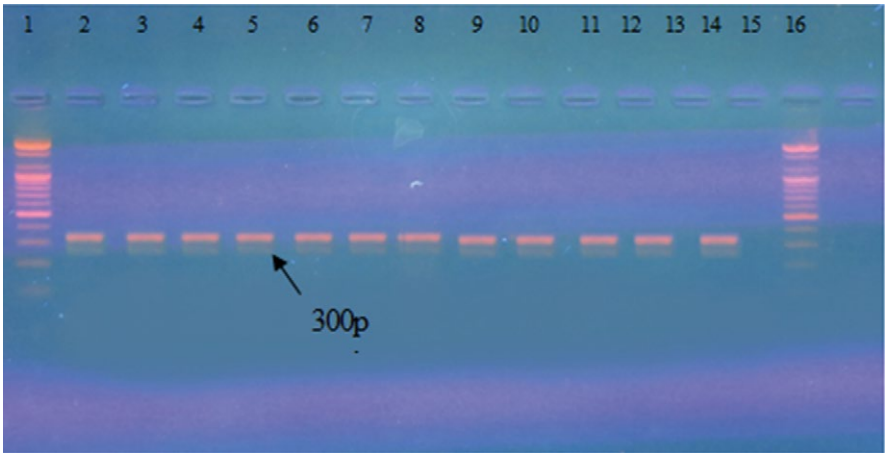
No. of Stage	Steps	Temperature (° C)	Time (min)	No. of Cycle
1	Denaturation 1	94	3	1
2	Denaturation 2	94	1	30
3	Annealing	64	1	
4	Extension 1	72	1	
5	Extension 2	72	5	1

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 24 was used. Qualitative data were presented as frequencies and percentages. Chi-square (X^2) was used to compare between groups. P-value <0.05 was considered statistically significant.

■ RESULTS

C. Trachomatis isolated from patients who suffer from conjunctivitis, blepharitis, Lacrium cystitis, and keratitis. The infection rate of Chlamydia was 12%. Males were infected more than females 66% : 34%. PCR assay showed high sensitivity as determined for the presence of C. Trachomatis among patients, as shown in Figure. In this study, the



Results of 16 SrRNA gene amplification of C. trachomatis with a size of (300 bp): 1, 16 = Ladder, 2–14 = Positive samples, 15 = Negative samples

patient infected with C. Trachomatis was distributed in seven age groups, as shown in Table 3.

Table 3
Distribution of patients by age groups

Age groups (years)	No.	C. trachomatis
1–10	25	12
11–20	26	8
21–30	20	20
31–40	10	10
41–50	6	0
51–60	15	12
61–70	1	0
X^2=21.021; p<0.01		

■ DISCUSSION

Trachoma is one of the different blinding diseases in the world. It is the common infection cause of blindness. The causative agent (Chlamydia trachomatis) infection usually begins in childhood. The WHO estimates 84 million people suffer from trachoma and up to 1.3 million are thought to be blind due to the eye disease [11]. In this study, the infection rate was 12%.

C. trachomatis can pass from one person to another within a few minutes [11]. The present study found males were infected more the females, trachoma is a public health problem in developing countries where living conditions are crowded and general hygiene is poor [12]. Diagnosis of C. trachomatis from eye infections for the first time in Iraq. Diagnoses of Chlamydia Trachomatis eye infection are generally unsuits factory by standard laboratory methods. PCR (this type of test detects the genetic material, DNA, or RNA of C. Trachomatis) has been a successful test for the diagnosis of C. Trachomatis

[13, 14]. The younger patients (1–30 years) were more infected by C. Trachomatis, with a similar result recorded by [15, 16].

■ CONCLUSION

C. Trachomatis is a causative agent of eye infections diseases in Basrah province – south of Iraq. This study diagnosed C. Trachomatis from eye infections for the first time in Iraq. Young men are more effected than women.

■ REFERENCES

1. Parte AC. LPSN – List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *Int J Syst Evol Microbiol.* 2018;68(6): 1825–1829.
2. Elwell C, Mirrashidi K, Engel J. Chlamydia cell biology and pathogenesis. *Nat Rev Microbiol.* 2016;14(6):385–400.
3. Kuo CC, Stephens RS, Baroil PM, Kaltenboeck B. *Chlamydia.* In Whitman WB (ed.). Bergy's manual of systematics of Archaea and bacteria. John Willy & sons. 2015; 1–28.
4. Darville T. Chlamydia trachomatis infections in neonates and young children. *Semin Pediatr Infect Dis.* 2005;16(4):235–44.
5. Tan AK. Ophthalmia Neonatorum. *N Engl J Med.* 2019;380(2):e2.
6. Sihota R, Tandon R. *Anatomy and Physiology*. In: Parsons' Diseases of the Eye (22nd edition). Elsevier India Private Limited, ND, India. 2015; 3–189. Available at: https://books.google.iq/books/about/Parson_s_Diseases_of_the_Eye_E_Book.html?id=JTJTBwAAQBAJ&redir_esc=y
7. Joossen C. Dry eye syndrome: The establishment of an optimized animal model and the evaluation of novel treatment options. PhD Thesis. Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen Departement Farmaceutische Wetenschappen. University of Antwerp, 2017. Available at: https://repository.uantwerpen.be/docman/irua/b9b91e/12448.pdf
8. Willoughby CE, Ponzin D, Ferrari S, et al. Anatomy and physiology of the human eye: effects of mucopolysaccharidoses disease on structure and function – a review. *Clinical and experimental ophthalmology.* 2010;38:2–11.
9. Martin HR, Corrales HRM. Ocular surface anatomy and physiology, disorders and therapeutic care, (1st edition). CRC press, Taylor & Francis Group. London, UK. 2013; 24–39. Available at: https://www.routledge.com/Ocular-Surface-Anatomy-and-Physiology-Disorders-and-Therapeutic-Care/Herranz-CorralesHerran/p/book/9780367380779?srsltid=AfmBOorgq-9JV9vxGj8IYoBdlcfctLegt_mt3YDlYLIf5GxOb-Ks5iTI
10. Sambrook J, Rusell D. *Molecular cloning: A laboratory manual (4th edition).* Cold spring laboratory press, NY, USA. 2001;512–524. Available at: https://www.cshlpress.com/pdf/sample/2013/MC4/MC4FM.pdf
11. Jahnke R, Matthiesen S, Zaeck LM, et al. Chlamydia trachomatis Cell-to-Cell Spread through Tunneling Nanotubes. *Microbiol Spectr.* 2022;10(6):e0281722.
12. World Health Organization. Factsheet. Trachoma, 2024. Available at: https://www.who.int/news-room/fact-sheets/detail/trachoma
13. Bobo L, Munoz B, Viscidi R, et al. Diagnosis of Chlamydia trachomatis eye infection in Tanzania by polymerase chain reaction/enzyme immunoassay. *Lancet*. 1991;338(8771):847–50.
14. Van Der Pol B, Kraft CS, Williams JA. Use of an adaptation of a commercially available PCR assay aimed at diagnosis of chlamydia and gonorrhea to detect Trichomonas vaginalis in urogenital specimens. *J Clin Microbiol.* 2006;44(2):366–73.
15. Heymann D. *Chlamydial infections*. In: Control of Communicable diseases manual, (20th edition). American public health association, Washington, USA. 2015; 99–101.
16. Batteiger B, Tan MC. *Trachomatis.* In: Mandell, Doglas and Bennett (eds.) principles and practice of infection diseases (8th edition). Elsevier, Philadelphia, USA. 2015; 456–478.

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Molecular Detection of Methicillin-Resistant Staphylococcus Aureus (MRSA) According to by mecA Gene from Clinical Specimens

Conflict of interest: nothing to declare.
Authors' contribution: Alaa Khattar Mousa – conceptualization, data curation, resources, software, validation, visualization, writing – original draft and writing – review & editing; Hanaa Khaleel Ibraheim – conceptualization, investigation, methodology, project administration, resources, visualization, writing – original draft and writing – review & editing.
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Abstract

Introduction. Methicillin-resistant Staphylococcus aureus is one of the more prevalent diseases related to an increase in resistance to antibiotics.
Purpose. To isolate and identify the source of MRSA in a hospital setting using molecular techniques.
Materials and methods. Collected 80 patients' nasal and wound swabs were from Al-Fayhaa Hospital and Al Basrah Teaching Hospital (40 wounds and 40 nasal swabs from operation rooms and patients' rooms). Isolates bacteria using Bacteriological analysis and validated by the use of mannitol salt agar injection and overnight incubation at 37 °C and were examined using colony morphology, Gram staining and biochemically tested for catalase, oxidase activity and we used to confirm MRSA identified The thermal cycling procedure for PCR was created using by detection of the mecA gene.
Results. Staphylococcus aureus was discovered in 63 (78.75%) samples, which were isolated using standard microbiological methods. The rate of S. aureus isolates was 35 (87.5%) out of 40 wound swab isolates and 28 (70%) out of 40 nose swab isolates. MRSA isolates, this rate isolation 43 (68.25%), including results 25 (71.42%) out of 35 isolates nasal swabs, while 18 (64.28%) out of the 28 wound swabs samples were positive for this mecA gene**.**
Conclusion. It was found that an elevated incidence of MRSA in all of the species. The bacteria leading to the infection of the wound could originate from self-infection or spread from one person to another. The mecA genome was identified in MRSA isolates, confirming the effectiveness of using the mecA gene as a genetic indicator of methicillin resistance to identify MRSA.
Keywords: methicillin-resistant Staphylococcus aureus, mecA gene, PCR, Staphylococcus aureus, isolates bacteria

■ INTRODUCTION

Methicillin-resistant Staphylococcus aureus is one of the more prevalent diseases related to an increase in resistance to antibiotics [1]. MRSA infections have grown in the past few years, resulting in more than 90 percent of the overall human S. aureus infections [2]. MRSA is frequently resistant to a wide range of antibiotics, including macrolides, aminoglycosides, lincosamide, and all-lactams, making treatment challenging [3]. MRSA colonization is usually asymptomatic in healthy people [4]. The distribution and prevalence of MRSA in hospitals varies considerably between regions as well as within a single country [2]. MRSA has piqued the interest of medical researchers and practitioners because to the increasing difficulties in managing and treating it [5]. Hospital-acquired MRSA infections are prevalent, harm patient outcomes, and raise healthcare expenditures due to increased hospital stay, antibiotic use, and mortality. MRSA infections are prevalent and significant causes sources of nosocomial infections and medical facilities when patients have weakened immune systems [6]. HA-MRSA infections cause morbidity and mortality globally [7]. MRSA is typically transmitted in hospitals by patients, who serve as significant reservoirs, healthcare workers (HCWs], environmental surfaces, and, on rare cases, the air [8]. S. aureus was the third most frequent pathogen in most Iraqi cities, according to a survey on the percentage of bacterial contamination in operating rooms. From January to June of this year [9]. The Staphylococcus chromosomes include the mecA gene. It boosts S. aureus virulence by causing methicillin resistance in the Staphylococcus cassette chromosome (SCCmec) region of the genome, which many MRSA molecular tests developed to identify amplification of the mecA gene in Staph aureus [10]. The virulence gene mecA is encoded within the bacterial chromosome; the total length of these genes in MRSA is around 720 bp. It is encoded by a protein chain that is about 239 amino acids long by NCBI, 2019. PBP2a is a movable intrinsic genomic component transported on a chromosomal islands (SCCmec) that is produced by the mecA gene [5]. PBP-2a is less affine to -lactams than PBP-2, which is produced by methicillin-sensitive Staph aureus (MSSA), because it hinders the active site from binding-lactams [2]. Staph aureus, an opportunistic infection that is found all over the world and may invade the nostrils and skin without causing any symptoms [11]. MRSA carriers (chronic or intermittent carriage) account for around 20–40% of the total human population [6]. Staphylococcus colonization was an elevated element that led to the spread of Staph aureus infection by transmission directly or contact with contaminated surfaces [12]. Staphylococcus infection is more common in young people than in adults [13]. Molecular-typing methods are an efficient and vital tool to swiftly identify and monitor common pathogen strains, and they also restrict their dispersion mechanism and hence regulate the diseases they cause [14].

■ PURPOSE OF THE STUDY

To isolate and identify the source of MRSA in a hospital setting using molecular techniques.

■ MATERIALS AND METHODS

Collection of Samples

80 Nasal and Wound swabs were collected from Al-Fayhaa Hospital and Al Basrah Teaching Hospital including (40 wound and 40 nasal swabs from operation rooms and

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patients' rooms). A doctor supervised the collection of the samples. A questionnaire paper was produced and added to the appendix to collect and record all patient information.

Isolation and identification of S. aureus

The isolate was discovered using routine laboratory tests. Staphylococcus aureus is a kind of bacteria. Bacteriological analysis was identified and validated by the use of mannitol salt agar injection and overnight incubation at 37 °C under aerobic conditions. Primary cultures were examined using colony morphology and Gram staining. The suspect colonies have been established using mannitol salts agar and biochemically tested for catalase and oxidase activity [15].

Molecular detection of isolates

Extraction of genomic DNA

S. aureus isolates' genomic DNA has been extracted using a DNA kit (Geneaid, USA) and according to the manufacturer's procedure.

MRSA genotypic detection

Detection of the mecA gene, with primer sequences shown in table 1, was used to confirm MRSA isolates. The thermal cycling procedure for PCR was created using [16].

Table 1
The sequence of mecA gene primers for amplification the genes

Genes	Sequence		Amplico Size, bp	Refe.
mecA	F	5´- GTGAAGATATACCAAGTGATT-3′	147	([16]
	R	5´- ATGCGCTATAGATTGAAAGGAT-3′		

Using previously described primers, all S. aureus MRSA isolates were screened for the presence of mecA genes, mixed forward and reverse primers for PCR were used to provide a stock solution concentration of (100 pmol/l), and the negative control was distilled water. Table 2 shows that the total volume of the polymerase chain reaction mixture is 25 µL.

Table 2
PCR mixture for detection S. aureus MRSA isolates

PCR mix		Volume (µL)
Promega green master mix		12.5
DNA template		2.5
Primer	Forward primer	1.5
	Reverse primer	1.5
Nuclease free water		7
Total		25

The program was utilized in the polymerase chain reaction described by [16], with a few changes to the reaction parameters. Table 3 illustrates this.

Table 3
PCR Amplification Programs for mecA gene

Gene	Temperature (° C)/Time					Cycle No.
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
mecA	94/5 min	95/30 sec	50/45 sec	72/1 min	72/7 min	30

Statistical analysis

Obtained data were documented and tabled in the Microsoft Office Software version 2016 (Microsoft, USA); while statistically, t-test and One Way ANOVA in the Graph Pad Prism Soft ware version 6.0.1 (Graph Pad Soft ware, Inc., USA) were served to estimate significant differences between study specimen at $P<0.05$.

■ RESULTS

Isolation of S. aureus and characterization

On Mannitol agar media, S. aureus metabolizes menthol sugar, changes its color from red to yellow, and develops aerobically. The cells emerged as Gram-positive cocci (irregular clusters) after Gram staining and further purification using biochemical analysis (with a positive result for Catalase but negative findings for Slide Coagulase, Coagulase test, and Oxidase). The percentage of isolation showed that 63 (78.75%) of 40 wound swab isolates and 28 (70%) of 40 nasal swabs of suspected S. aureus have been identified utilizing established microbiology techniques.

DNA extraction

All positive samples were extracted with the Genomic DNA Mini Kit (Geneaid, Korea) according to the manufacturer's instructions. At an optical density of 260/280 nm, the Nanodrop System (Thermo-Scientific, UK) was used to assess the concentration and purity of isolated DNAs.

MecA gene detections

S. aureus isolates carrying the mecA gene were identified using PCR. The current study's findings revealed that some MRSA isolates having mecA gene, mecA gene molecular weight 147 bp, figure, indicated 25 (71.42%) out of 35 isolates. Table 4 shows that nasal swabs were positive for this gene, whereas 18 (64.28%) of the 28 wound swab samples were positive, with significant differences ($P<0.05$).

Table 4
MRSA identification using genotypic methods

Type of specimen	No. of S. aureus isolates	mecA gene	
		Positive	Negative
Nasal swabs	35	25 (71.42%)	10 (28.57%)
Wound swabs	28	18 (64.28%)	10 (35.71%)
Total	63	43 (68.25%)	20 (31.74%)

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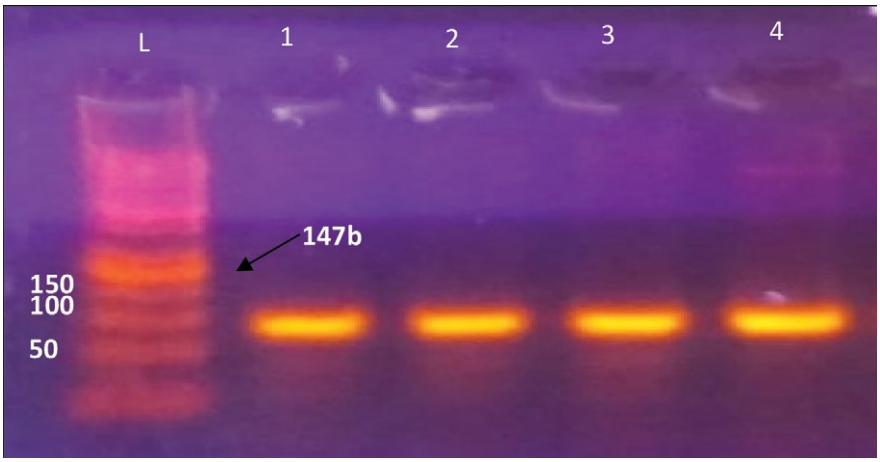

MecA gene gel electrophoresis amplified products of PCR from Staph aureus isolated by the PCR technique. 1.5% agarose gel M: DNA ladder (50–1000 bp)

■ DISCUSSION

The current study found that MRSA isolated from the hospital environment (operating rooms and patients' rooms) can contaminate wounds and cause wound infection; therefore, tight sanitary measures can prevent wound infection during or after an operation. The germs that cause wound infection may be transmitted from one patient to another during bandage changes, especially if the patient is hospitalized for an extended period of time [17]. Infection of the wound can occur throughout the surgical process or once a patient is released from a hospital. MRSA isolation rate (68.25%) in this study mecA gene. This finding coincided with numerous earlier investigations that found mecA genes in all MRSA isolates. Alternative study has shown that Methicillin resistance can develop without the presence of mec A and that MRSA might have alternative mechanisms of resistance, such as a modified target site or reduced treatment storage [18]. The absence of the mce A gene could also be due to a technological error during detection. There is growing concern about MRSA contamination and infections in hospitals, particularly in post-operative wounds [18]. In this study, isolates from wounds swabs revealed a high prevalence of MRSA containing mecA gene 18/28 (64.28%), while nasal swabs from patients revealed a rate of MRSA containing mecA gene 25/35 (71.42%). The identification of an elevated rate of MRSA isn't unexpected, and it is also in line with previous Iraqi research [19]. MRSA frequency was 53% [20] in a 2015 study conducted in Iraq's Kurdistan area, whereas the rate was 69% [21] in another investigation performed between Iran and India, the proportion was considerably lower 16.6% [22]. MRSA frequency from S. aureus obtained from blood and nasal colonization was 51.4% in a Korean hospital [23]. MRSA was widespread in Asian countries in general [2]. MRSA prevalence decreased in the German research [6]. Significant rates of Staph aureus resistance to penicillin and ampicillin were observed in Turkey in 2017. In 2018, a study in Isfahan, Iran, discovered MRSA nose infection in 51.9% of patients and 16% of health professionals [24].

■ CONCLUSION

It was found that an elevated incidence of MRSA in all of the research samples, and there were potential sources of MRSA wound infection. The bacteria leading to the infection of the wound could originate from self-infection or spread to one person to another throughout bandage changing, especially if the patient is admitted to the hospital for an extended period of time. The mecA genome was identified in all MRSA-isolated, confirming the effectiveness of using the mecA gene as a genetic indicator of methicillin resistance to identify MRSA.

■ REFERENCES

1. Suhaili Z, Rafee P', Mat Azis N, et al. Characterization of resistance to selected antibiotics and Panton-Valentine leukocidin-positive Staphylococcus aureus in a healthy student population at a Malaysian University. *Germs.* 2018;8(1):21–30.
2. Hussain MS, Naqvi A, Sharaz M. Methicillin-resistant *Staphylococcus aureus* (MRSA). *The Professional Medical Journal*. 2019;26(01):122–127.
3. Mahmoudi S, Mamishi, S, Mohammadi M, et al. Phenotypic and genotypic determinants of mupirocin resistance among Staphylococcus aureus isolates recovered from clinical samples of children: an Iranian hospital-based study. *Infection and Drug Resistance*, 2019;12:137–143.
4. Yang D, Ho YX, Cowell LM, et al. A Genome-Wide Screen Identifies Factors Involved in S. aureus-Induced Human Neutrophil Cell Death and Pathogenesis. *Front Immunol.* 2019;10:45. doi: 10.3389/fimmu.2019.00045
5. Al-Kharash KR, Rehman S, Alkhamis F, et al. Comparative and molecular analysis of MRSA isolation from infection sites and carrier colonization sites. *Annals of clinical microbiology and Antimicrobials.* 2018;17(1):1–11.
6. Schubert M, Kämpf D, Wahl M, et al. MRSA Point Prevalence among Health Care Workers in German Rehabilitation Centers: A Multi-Center, Cross-Sectional Study in a Non-Outbreak Setting. *Int J Environ Res Public Health*. 2019;16(9):1660.
7. Yarbrough ML, Warren DK, Allen K, et al. Multicenter evaluation of the Xpert MRSA NxG assay for detection of methicillin-resistant *Staphylococcus aureus* in nasal swabs. *Journal of Clinical Microbiology*, 2018;56(1):e01381–17.
8. Al-Zaidi JR. Methicillin Staphylococcus aureus (MRSA) nasal carriage among health care workers in Intensive Care units. *Medical Journal* of *Babylon*. 2014;11(3):749–757.
9. Merdaw MA, Abdul-kareem J. Microbial contamination in the operating theatres in Iraq, *Iraqi New Medical Journal*. 2018;5(9):5–8.
10. Gittens-St Hilaire MV, Chase E, Alleyne D. Prevalence, molecular characteristics and antimicrobial susceptibility patterns of MRSA in hospitalized and nonhospitalized patients in Barbados. *New Microbes New Infect.* 2020;35:100659. doi: 10.1016/j.nmni.2020.100659
11. Afshari SG, Sepahi AA, Goudarzi H, et al. Distribution of SCCmec types in methicillin-resistant Staphylococcus aureus isolated from burn patients. *Archives of Clinical Infectious Diseases*. 2017;12(2):e62760.
12. Dalman M, Bhatta S, Nagajothi N, et al. Characterizing the molecular epidemiology of Staphylococcus aureus across and within fitness facility types. *BMC Infect Dis.* 2019;19(1):69.
13. Pan HH, Huang YC, Chen CJ, et al. Prevalence of and risk factors for nasal methicillin-resistant Staphylococcus aureus colonization among children in central Taiwan. *J Microbiol Immunol Infect.* 2019;52(1):45–53.
14. Rezai S, Valadan R, Ahangarkani F, Rezai MS. The Spa Typing and Characterization of Nasal Carriage Methicillin-resistant Staphylococcus Aureus Isolates From Healthy Children. *J. Pediatr. Rev.* 2020;8(1):59–64.
15. Reddy PN, Srirama K, Dirisala VR. An Update on Clinical Burden, Diagnostic Tools, and Therapeutic Options of Staphylococcus aureus. *Infect Dis (Auckl)*. 2017;10:1179916117703999. doi: 10.1177/1179916117703999
16. Zhang K, Mcclure J, Elsayed S, et al. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec Types I to V in methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.*, 2005;43(10):5026–5033.
17. Al-Abbas AKA. Aerobic bacteria their antimicrobial sensitivity pattern in Karbala city, Iraq. *Iraq Medical Journal.* 2017;1(4):94–98.
18. Yang F, Zhang S, Shang X, et al. Short communication: Detection and molecular characterization of methicillin-resistant Staphylococcus aureus isolated from subclinical bovine mastitis cases in China. *J Dairy Sci.* 2020;103(1):840–845.
19. Al-azawi N, Ali KM, Alwan A, et al. Antibiotic resistance pattern of HAMRSA strains isolated from leukemia patients in Baghdad, Iraq. *International Journal of Infectious Diseases*. 2016;45:81–82.
20. Hussein NR, Assafi MS, Ijaz T. Methicillin resistant *Staphylococcus aureus* nasal colonisation amongst healthcare workers in Kurdistan Region, Iraq. *Journal of Global Antimicrobial Resistance*. 2017;9:78–81.
21. Jahanshahi A, Zeighami H, Haghi F. Molecular characterization of methicillin and vancomycin resistant *Staphylococcus aureus* strains isolated from hospitalized patients. *Microbial Drug Resistance*. 2018;24(10):1529–1536.
22. Karmakar A, Dua P, Ghosh C. Biochemical and Molecular Analysis of Staphylococcus aureus Clinical Isolates from Hospitalized Patients. *Can J Infect Dis Med Microbiol*. 2016;2016:9041636. doi: 10.1155/2016/9041636
23. Mussa AA, Al-mathkhury HJF. Incidence of Ciprofloxacin-Resistant of Methicillin Resistant Staphylococcus aureus isolated from Iraqi patients. *Iraqi Journal of Science*. 2018;59(3A):1225–1230.
24. Yılmaz EŞ, Aslantaş Ö. Antimicrobial resistance and underlying mechanisms in *Staphylococcus aureus* isolates. *Asian Pacific Journal of Tropical Medicine*. 2017;10(11):1059–1064.

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Serological Assessment of Toxoplasma Gondii Infection in Marriage Applicants and Blood Donors

Conflict of interest: nothing to declare.
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Abstract

Introduction. Identification and serological study were conducted for blood donors and applicants for marriage to investigate the infection with Toxoplasma gondii.
Purpose. To study Toxoplasma gondii infection in blood donors and potential spouses without symptoms in the main blood and applicants for the Thi-Qar marriage bank, employing the ELISA technique, as well as to investigate of the impact of several parameters, including age, blood type, and sex and location of residence.
Materials and methods. For both sexes during the period from on October 2023 until the end of January 2024, the ELISA technique was used for 140 serum samples from blood donors and 160 serum sample from applicants for marriage, according to Serological criterion (sex, age group, area region, blood group).
Results. The total toxoplasma gondii positive rate was 32% using The ELISA antibody IgG and (3%) using the antibody for blood donors. The positive rate for antibody IgM was (22%) for blood donors and the positive rate for antibody IgG was 3%for applicants for marriage. The result showed that the prevalence of toxoplasma gondii was slightly higher in rural areas compared to cities.
Conclusion. Infection frequency was in males then females for blood donors and higher females than males for applicants for marriage in all age groups and blood donors. Generally, people over 36 old are more vulnerable to infection.
Keywords: toxoplasma gondii, ElSIA technique, blood donors, marriage applicants, antibody IgM

■ INTRODUCTION

Obedient intracellular parasitic protozoa, T. gondii, is the source of the zoonotic disease toxoplasma, which causes encephalitis, mental retardation, chorioretinitis, loss of vision in congenitally infected individuals, and miscarriage in livestock [1–3]. The infection

is present all over the planet. This parasite has exposed around one-third of humankind, yet the seropre valence varies widely between population groups and countries (from less than 10% to more than 90%) [4]. People who are infected may not show any symptoms for the rest of their lives unless they experience immunosuppression [5]. The only animals that pass oocysts in their feces are felidae, or cats, who are both intermediate and definitive hosts. All other mammals and birds are considered intermediate hosts. Goat and sheep meats contain significant diseases. Toxoplasmosis sources [6]. Typically, diagnosis produced using molecular methods and/or immunological tests, or by combining these methods [7]. Indirect ELISA is one type of serological test that can be used to demonstrate IgM or IgG. It is a quick, easy, and reliable procedure [8, 9].

■ PURPOSE OF THE STUDY

To study Toxoplasma gondii infection in blood donors and potential spouses without symptoms in the main blood and applicants for the Thi-Qar marriage bank, employing the ELISA technique, as well as to investigate of the impact of several parameters, including age, blood type, and sex and location of residence.

■ MATERIALS AND METHODS

Three hundred blood samples, ranging in age from 18 to 57, were collected from both healthy blood donors and potential spouses. Samples were taken from candidates for the Thi-Qar marriage facility and blood donors. Each subject had four milliliters of venous blood drawn from the radial vein. The serum was then pipetted into five Eppendrof tubing using a micropipette and kept for later use at –20 °C.

Anti-T. gondii antibody (IgG) and (IgM) detection using ELISA technique: IgG and IgM antibody measurements were carried out and analyzed in accordance with the manufacturer's instructions.

Serological testing

Two kits from HMG, Germany were used for this assay, according to Laboratories, Inc. One was for the detection of IgG, while the other was for the detection of antibodies specific to IgM. T. gondii in the serum for humans.

Serological testing (ELISA)

This assay was performed by using two kits (HMG, Germany) Laboratories, Inc. One was for detection of IgG and another for detection of IgM specific antibodies agains. T. gondii in the people serum.

■ RESULTS

Table 1 present on blood donor and marriage applicants were tested for various factors. The results showed that in urban areas, 73.5%of blood donors were negative, while 22.4%were IgG positive. In rural areas, 71.4% of marriage applicants were negative, with 25.3% testing IgG positive. Overall, the total percentage of negative results was 80%, while 18.1% were IgG positive. No significant differences were found between urban and rural areas.

Table 1
The prevalence of toxoplasma gondii antibodies by residence area

Applicants for marriage				Blood donor		
Residence	**IgG (+)**	**IgM (+)**	**(–)**	**IgG (+)**	**IgM (+)**	**(–)**
	No. (%)					
Urban	11 (22.4)	2 (4.1)	36 (73.5)	9 (14.8)	1 (1.6)	51 (83.6)
Rural	23 (25.3)	3 (3.3)	65 (71.4)	20 (20.2)	2 (2)	77 (77.8)
p-value	0.9			0.66		

Table 2 present data on blood donors male 70 total Negative 61 (87.1) IgM positive (1.4%) IgG positive 8 (11.4) female 90 total Negative 72 (80%) IgM positive 4 (4.4%) IgG positive 14 (15.6%). Applicants for marriage male 132 total Negative 98 (74.2%) IgM positive 2 (1.5%) IgG positive 32 (24.2%) female 8 total Negative 2 (25%) IgG positive 6 (75%). Total Male 160 negative 133 (83.1%) Igm positive 5 (3.1%) IgG 22 (13.8%) female 140 total negative 100 (71.4%) IgM positive (71.4%) IgG positive38 (27.1%).

Table 2
The prevalence of toxoplasma gondii antibodies by sex

Applicants for marriage				Blood donor		
Gender	**IgG (+)**	**IgM (+)**	**(–)**	**IgG (+)**	**IgM (+)**	**(–)**
	No. (%)					
Male	32 (24.2)	2 (1.5)	98 (74.2)	8 (11.4)	1 (1.4)	61 (87.1)
Female	6 (75)	0	2 (25)	14 (15.6)	4 (4.4)	72 (80)
p-value	0.001			0.39		

On blood donors and applicants for marriage, categorized by age groups and antibody test results. The results show varying percentages of positive and negative cases for IgM and antibodies in different age ranges. The data indicates a higher percentage of positive IgG cases in older age groups while a decrease in positive IgM cases as age increases. The total number of cases analyzed is 160 with a breakdown of antibody test results provided for age group. The data highlights the importance of age in antibody test results among blood donors and marriage applicants. Focusing on blood group and antibodies. It includes counts and percentage for blood groups A, B, AB, O, as well as IgM and IgG antibodies. The total count and percentage for all blood groups are provide. The analysis includes the p-value, degrees of freedom, and chi-square value. The result indicates the distribution of blood types.

■ DISCUSSION

This research is the first to look into the prevalence of Toxoplasma gondii infection in the blood donors and applicants for marriage from Thi-Qar province, Blood Donors :We used ELISA to test 140 blood donors and found no significant difference in infection rates between urban and rural residents (76.6% vs. 68.3% for IgG and IgM, respectively. These findings align with previous studies by Hamza [10] and Al-Deen et al. [11], who also reported no significant association between residence and Toxoplasma antibody presence.

For marriage applicants (n=160), the present study found no significant difference in IgG prevalence between urban (22.4%) and rural (25.3%) areas. This aligns with Jassam's, 2010 [12]. study on schizophrenic patients, which showed no significant difference in IgG prevalence based on residence.

While Igm among candidates for marriage in the rural region was (3%) higher than in Urban (2%) coincided with the study of Salibay et al. [13], in the Philippines, where They revealed that prevalence was higher in suburban patients than urban inhabitants. When Al-saadii was researched in males and females, it was found that 111 (91.73%) of the cases were in rural regions compared to 10 (8.16%) in urban areas [14]. The variation in the reading of Toxoplasma gondii in a living environment may be caused by inadequate treatment, direct contact with cats and other animals as they give birth to animals in home parks, and a lack of health education in certain rural areas.Another factor could be that living in a more crowded area increases the risk of infection during pregnancy and childbirth. Conditions in urban areas [15] may differ from research conducted in rural and urban locations with regard to hygiene and sample sizes; also, people in rural areas may eat more prepared foods and frequent restaurants in metropolitan areas. It is also crucial to highlight in this study that the main mode of transmission in Iraq is induced by oocyst ingestion.

The current study's ELISA test-based infection percentage revealed that, among marriage-seeking applicants, males and women had seropositive rates of 24.2% and 75.0%, respectively, based on their gender. The results matched those of Al-Maamuri [16], who discovered that 76.8% of females and 82.43% of males were seropositive. While Saleh, [18], discovered 17.83% of females and 9.1% of males were seropositive, Mahmood et al. [17] identified 13.3% of males and 43.3% of females to be seropositive. However, compared to females (26.19%), men (31.26%) in Kirkuk City's study by Salman and Mustafa [19] showed a greater seropositive rate.

While ELISA-IgM findings were recorded, male values were 1.5% higher than female results (0.0%). was, and the current investigation supported a previous Walle et al. study [20] was found to be 4.0% in men and 2.0% in women. The research conducted by Al-Ghargholi [21] revealed different results, with Toxoplasma seroprevalence of 52.3% in men and 50.3% in women (IgG and (IgM)). 5.6% of men and 18.7% of women, respectively. Male blood donors with chronic infections had an IgG level of 24.3%, which was greater than the 2.14% level for acute infections. This difference could be the result of either male or female applicants for marriage being exposed to the T. gondii infection depending on where they live or work, or it could be the result of different size specimens that were exclusively taken from male blood donors with female.

Whereas the age group of (32–36) years old had the highest proportion of Toxoplasma gondii infection by ELISA for IgG in blood donors, with 75% In agreement with Abdulla et al. [22], who discovered that 35% of people in the age group (31–40) had IgG. The ELISA (IgG) examined candidates for marriage who were 42 years of age or older, with a prevalence of 33.3%. This is consistent with [18], who discovered that the prevalence was 30% in the 40–49 year age group, and [16], who discovered that the prevalence was 85.39% in the 41–50 year age group. This, however, was not the case for Al-mosawi [23], who reported different results, with Toxoplasma seroprevalence of 66.6% for IgG and 26.4% for IgM in individuals aged 25 to 29. 45 and higher IgM had years percentage of 26.4%, which was in line with both IgG IN in the 45+ age range. The findings confirms the 49.2% prevalence

of both IgG and IgM in the 20–24 age range. While ELISA-IgM recorded inconsistent results, they were in agreement with the presence of the highest percentage of blood donors and marriage applicants in the age category of 22–26–26 years (5.6%) and 6.9%, respectively [14]. which has observed that 30% of the age group (18–25) and [18] of 19–29 years Was 10.8%, while Mahmood et al. [24] reported comparable results with Toxoplasma in individuals aged 18 to 25. Was 30% with IgM.The current findings may be related to the varying numbers of afflicted individuals in each group. Additionally, the individuals may be encountering toxoplasma Cat-related soil exposure during childhood has led to the accumulation of anti-Toxoplasma antibodies in humans at varying percentages, which can cause the chronic infection toxoplasmosis, Saplding et al. [25]. The variations in the specificity and sensitivity of the approach utilized to diagnose each host's response could be the cause of these discrepancies between the preceding result and the present result. To the parasitic strain John, Suzuki, Y, and k [26].

It is noteworthy that the current study found a relationship between the blood group system and toxoplasma infection, with samples tested by ELISA IgG with blood group AB+ 4 (20.0%) having the highest prevalence and samples of group O+ 15 (37.5%) having the lowest prevalence. This relationship was significant (p-value = 0.46).However, the ELISA IgM antibodies test revealed that the blood group had the lowest proportion of toxoplasma (10.0%) and the highest percentage of toxoplasma (75.3%). According to a study conducted in Russia among blood donors, the seroprevalence of toxoplasmosis was twice as high in subjects with blood group AB than in subjects with blood group O (54% versus 27%, respectively) Henery, [27]. Nevertheless, Al-Kaysi and Ali concur on this outcome [28]. They demonstrated that

For the O+ and AB blood groups, toxoplasma infection was more preventable, with seropre valences of 35.8% and 38%, respectively. Percentage of infection by ELISA test: The current study's results are broken down by blood group, with the blood group AB+ 5 applicants having the highest prevalence of IgG infection (20.0%) and the blood group O+ 17 applicants having the lowest prevalence (38.6%), with a significant difference between the two (p-value: 0.21). However, the ELISA IgM antibodies test revealed that the blood group had the lowest percentage of 6 (15.0%) and the highest percentage of 2 (10.0%) of toxoplasma. The presence or lack of the A and B carbohydrate antigens on the surface of red blood cells determines the A, B, and O blood group system Hakomori [29]. Their findings are contradicting findings from four investigations that link this parasite to the Band AB blood group [30].

■ CONCLUSION

Infection frequency was in males then females for blood donors and higher females than males for applicants for marriage in all age groups and blood donors. Generally, people over 36 old are more vulnerable to infection.

■ REFERENCES

1. Shaapan RM. The common zoonotic protozoal diseases causing abortion. *Journal of parasitic diseases.* 2016;40:1116–29. Available at: https://doi.org/10.1007%2Fs12639-015-0661-5
2. Khudair Khalaf A. Sero-prevalence of Toxoplasma gondii among Diabetic patients in Thi-Qar province/South of Iraq. *University of Thi-Qar Journal of Science.* 2022;9(2). Available at: http://dx.doi.org/10.32792/utq/utjsci/v9i2.907

3. Abdallah MC, Kamel M, Karima B, et al. Cross-sectional survey on Toxoplasma gondii infection in cattle, sheep, and goats in Algeria: seroprevalence and risk factors. *Veterinary sciences.* 2019;6(3):63. Available at: https://doi.org/10.3390/vetsci6030063
4. Rosso F, Les JT, Agudelo A, et al. Prevalence of infection with Toxoplasma gondii among pregnant women in Cali, Colombia, South America. *American Journal of Tropical Medicine and Hygiene.* 2008;78(3):504–8. Available at: https://doi.org/10.4269/ajtmh.2008.78.504
5. Herrmann DC, Pantchev N, Vrhovec MG, et al. Atypical Toxoplasma gondii genotypes identified in oocysts shed by cats in Germany. *International journal for parasitology.* 2010;40(3):285–92. Available at: https://doi.org/10.1016/j.ijpara.2009.08.001
6. Sevglİl M, Babür C, Nalbantoğlu S, et al. Determination of seropositivity for Toxoplasma gondii in sheep from Şanlıurfa Province. *Turkish Journal of Veterinary & Animal Sciences.* 2005;29(1):107–111.
7. Sukthana Y. Toxoplasmosis: beyond animals to humans. *TRENDS in Parasitology.* 2006;22(3):137–42. Available at: https://doi.org/10.1016/j.pt.2006.01.007
8. Yaseen AN, Shareef SO, Qader AH. Using of Serological and Molecular Technique for Toxoplasma Gondii Diagnosis in Goat and Cow in Some Regions of Baghdad Governorate. *HIV Nursing.* 2022;22(2):1466–72. Available at: https://www.hivnursing.net/index.php/hiv/article/view/582/679
9. Wallander C. *Toxoplasma gondii in wild boars and domestic pigs in Sweden.* Acta Universitatis Agriculturae Sueciae. PhD Thesis. 2016. Available at: https://pub.epsilon.slu.se/13081/1/wallander_c_160219.pdf
10. Hieran A, Albadry BJ. A Human CCL3L1 gene expression in blood donors infected with HIV-1. *University of Thi-Qar Journal of Science.* 2023;10(1):181–184. Available at: http://dx.doi.org/10.32792/utq/utjsci/v10i1.1060
11. Al-Deen FMN, Kadir MA, Al-Sugmiany RZM. Diagnostic study for Toxoplasmosis by Serological test and PCR technique in Kirkuk province. *Tikrit Journal of Pure Science.* 2018;23(1):19–25.
12. Hussien MR, Al-Saeed AT, Eassa SH. Toxoplasmosis seropositivity and male sex hormones. *J. Immunol Infect Dis.* 2018;5(1):1–8. Available at: https://www.annexpublishers.co/full-text/JIID/5101/Toxoplasmosis-Seropositivity-and-Male-Sex-Hormones.php
13. Salibay CC, Dungca JZ, Claveria FG. Serological survey of Toxoplasma gondii infection among urban (Manila) and suburban (Dasmariñas, Cavite) residents, Philippines. *The journal of protozoology research.* 2008;18(1):26–33. Available at: https://doi.org/10.32268/jprotozoolres.18.1_26
14. Al-Saadii SH. The effect of toxoplasmosis on the level of some male sex hormones In Samples from National Blood Transfusion Center/Baghdad. PhD Thesis. College of Science. University of Baghdad. Available at: https://repository.uobaghdad.edu.iq/publication/2hZGIIsBVTCNdQwCKMcU
15. Mortensen PB, Pedersen CB, Westergaard T, et al. Effects of family history and place and season of birth on the risk of schizophrenia. *New England Journal of Medicine.* 1999;340(8):603–8. Available at: https://doi.org/10.1056/nejm199902253400803
16. Al-Maamuri SD. Seroprevalence of Toxoplasmosis in a sample of Iraqi schizophrenic patients. *Sc. Thesis. Sci. Univ. Baghdad.* 2014:118. Available at: https://orcid.org/0000-0002-5337-6904
17. Mahmood AE, Etawi ZM, Ahmed MA. Toxoplasma seroprevalence in healthy voluntary blood donors from blood bank of Baghdad. *Al Mustansiriyah Journal of Pharmaceutical Sciences.* 2014;14(1):9–13. Available at: https://doi.org/10.32947/ajps.v14i1.120
18. Saleh MA. Determination of antibodies (IgG, IgM) against Toxoplasma gondii in some Iraqi individuals by using ELISA technique. *Baghdad Science Journal.* 2011;4:940–6. Available at: https://doi.org/10.21123/bsj.2011.8.4.940-946
19. Salman YJ, Mustafa WG. Correlation between Toxoplasma gondii and Thyroid function hormone levels in sera of patients Attending private clinics and laboratories in Kirkuk City. *Int. J. Curr. Res. Biosci. Plant. Biol.* 2014;1(4):27–34. Available at: https://doi.org/10.1186%2Fs12879-019-4450-0
20. Van de Walle A, Tiwary P, De Jong M, et al. Efficient stochastic generation of special quasirandom structures. *Calphad.* 2013;42:13–8. Available at: https://doi.org/10.1016/j.calphad.2013.06.006
21. Al-Gharrgholi NA. *Seroepidemiological and Molecular Study for Toxoplasma gondii Among Diabetic Mellitus Patientes in Babylon Province*. M. Sc. PhD Thesis. College of Science. University of Babylon. Available at: https://iqdr.iq/search?view=56c0a6b9a7c30f452e5d2fe96caf5617
22. Abdulla HE, Al-bashier NM, Al-kawaz U, et al. Cross-sectional study of infertile males with toxoplasmosis in Baghdad province. *International Journal of Scientific & Engineering Research.* 2015;6(1):254. Available at: https://www.ijser.org/paper/Cross-sectional-study-of-infertile-males-with-toxoplasmosis.html
23. Al-Mosawi AM. *Immunoepidemiological study of toxoplasmosis among women in Babylon Province.* PhD Thesis. College of Science. University of Babylon. Available at: https://pharmacy.uobasrah.edu.iq/images/resalet_majester_dr_atruha/resalet_majester/MSC_muntah_abdal_hameed/muntah%20abal%20hameed%20MSC%20en.pdf
24. Mahmood S, Ban AQ, Zghair K. Prevalence of toxoplasmosis of males blood donors in Baghdad. *Iraqi Journal of Science.* 2013;54(4):832–41. Available at: https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/12340
25. Spalding SM, Amendoeira MR, Klein CH, Ribeiro LC. Serological screening and toxoplasmosis exposure factors among pregnant women in South of Brazil. *Revista da Sociedade Brasileira de Medicina Tropical.* 2005;38:173–7. Available at: https://doi.org/10.1056/nejm199902253400803
26. Suzuki Y, Joh K. Effect of the strain of Toxoplasma gondii on the development of toxoplasmic encephalitis in mice treated with antibody to interferon-gamma. *Parasitology research.* 1994;80:125–30. Available at: https://doi.org/10.1007/bf00933779
27. Henery SM. Molecular diversity in the biosynthesis of GI tract. Glycoconjugates. Receptors. *Transt. clin. Biol.* 2001;8(1):226–30. Available at: https://doi.org/10.1007/bf00933779
28. Al-Kaysi AM, Ali N. Serological and biochemical study of HBV, HCV, HIV and toxoplasmosis infection among blood donors in Iraq. *Egypt J Comp Path Clin Path.* 2010;23:1–9. Available at: https://www.semanticscholar.org/paper/f812debab7931ee97e75303df963fd4bf17daf5d
29. Hakomori Sl. Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer. *Biochimica et Biophysica Acta (BBA)-General Subjects.* 1999;1473(1):247–66. Available at: https://doi.org/10.1016/s0304-4165 (99)00183-x
30. Midtvedt T, Vaage L. Relationship between Toxoplasma gondii antibodies and blood group. *European journal of clinical microbiology & infectious diseases*. 1989;8(6):575–576. Available at: https://doi.org/10.1007/bf01967490

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Rosemary's (Rosmarinus Officinalis) Antifungal Activities Against Tinea Capitis

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Abstract

Introduction. A fungal infection of the scalp hair is called tinea capitis. Microsporum and Trichophyton are the two main dermatophyte species that cause it. The outer root sheath of the hair follicle can be penetrated by the fungi, which may then infiltrate the hair shaft.
Purpose. To investigate the antifungal properties of rosemary in relation to the species that cause tinea capitis.
Materials and methods. Between November 2020 and April 2023, 150 hair samples from the Al-Hussein Teaching Hospital in the province of Muthanna were examined under the supervision of experts. The tinea capitis case included samples from 76 ectothrix, 52 endothrix, and 22 Favus.
Results. Based on the species isolates, the most common fungi in ectothrix were Microsporum canis 17 (19.1%), Microsporum gypseum 14 (15.7%), Trichophyton equinum 9 (10%), and Trichophyton verrucosum 7 (7.8%). The two most common fungi in endothrix, however, were Trichophyton tonsurans 19 (22%), and Trichophyton violaceum 14 (15.7%). T. schoenleinii 8 (10%) was the only species of the favus that was found.
Conclusion. Rosemary leaves have anti-tinea capitis properties. Ointment, cream, or lotion should be made with these fractions at the recommended percent and tested in vivo to determine whether they are effective against trichomonia capitis, given the increased effect of the N-hexane fraction in decantation.
Keywords: rosmarinus, anti-dermatophyte, microsporum, trichophyton, rosemary, tinea capitis

■ INTRODUCTION

Tinea capitis is a condition that mainly affects hair shafts and follicles and is caused by a superficial fungal infection of the skin of the scalp, eyebrows, and eyelashes. The condition is believed to be a form of superficial mycosis or dermatophytosis [1].

The cutaneous layer of both human and cattle skin contains pathogenic keratinolytic fungi called dermatophytes, which cause dermatophytosis, a disease that is found all over the world. are separated into three groups: kinds of Trichophyton, Microsporum, and Epidermophyton. The skin lesions of infected animals have a thick, gray-white crust that rises above the skin and is at least 3 cm in diameter. Lesions are concentrated in the back, head, and perineum; otherwise, they are found throughout the body, with severe cases clumping together. The highest frequency of Trichophyton verrucosum is found in cows [2]. Numerous studies have also examined a novel herbal extract that has been used as a treatment alternative for fungus activity (fungicide or fungistatic) in a number of filamentous and non-filamentous fungal species commonly found in veterinary clinical practice [3].

Infections with tinea capitis have progressed to advanced, atypical lesions in individuals with severe illnesses and immunological deficiencies in recent years. Because dermatophytosis infections are so widespread, ringworm is present in every region of the world. Nowadays, in many countries, zoophilic dermatophytes like Microsporum canis are believed to be the main causes of human dermatophyte illnesses (tinea capitis). Since herbal compounds are currently used to treat a variety of infectious diseases brought on by bacteria, fungi, protozoa, and viruses, the use of natural herbal compounds against organisms that cause dermatology has been considered. Rosemary is a fragrant perennial evergreen with aromatic leaves. It has medicinal uses for its leaves and flowering branches [4]. Camphor, cineol, and borneol are three components of essential oils that are present in rosemary plants. The antifungal properties of these essential oils derived from rosemary are strong. Additionally, rosemary juice has antibacterial qualities and encourages quicker blood circulation in the skin. due to this. In addition to its essential oils, rosemary contains a variety of chemicals that have antidermytophytosis properties. These substances consist of rosmanol, ursolic acid, caffeic acid, and rosmeridiphenol [5]. R. officinalis hydroalcoholic extract was found to be effective against two dermatophytes, M. gypseum and T. rubrum, in another study. At a concentration of 10%, the fungal growth was inhibited to the extent of 86% [6].

■ PURPOSE OF THE STUDY

To investigate the antifungal properties of rosemary in relation to the species that cause tinea capitis.

■ MATERIALS AND METHODS

Samples collection

Between November 2020 and April 2023, 150 hair samples from the Muthanna province's Al-Hussein Teaching Hospital were analyzed under the supervision of experts. Samples from 22 Favus, 52 Endothrix, and 76 Ectothrix were used in the tinea capitis case. In every case, the clinical diagnosis was confirmed by direct examination with KOH and culture in Sabouraud-chloramphenicol, both with and without cycloheximide. For two weeks, the sample was cultivated at 27 °C. Cultures that did not exhibit fungal development were kept for three more weeks before being considered negative. The colonies' macro- and micromorphological characteristics were analyzed, and specialized tests were employed if required to aid in the mycological diagnosis.

Extracts from the leaves of rosemary

The leaves of plants were gathered, allowed to dry in the shade, and then ground in a mill to a fine powder. Dried leaf powder was used to create the extracts [6].

Brewing to prepare an aqueous extract

Plant dust was boiled for one hour at a ratio of 1:10 (one gram of powder for every milliliter of solvent) in order to create an aqueous extract. After being filtered (using filter sheets), the extracted material was rotated to dry it at 40 °C [6].

Making a hydro-alcoholic extraction

To make a hydro-alcoholic extract, the crushed plant was mixed with 80% methanol at a ratio of 1 gram of powder per milliliter of solvent, or 1:10. The mixture was then stirred with a magnet stirrer and allowed to sit at room temperature for 12 to 24 hours. The resultant extract was rotated to dry at 40 °C after filtering (using filter sheets) [7].

Calculating MIC and MFC with the agar dilution method

The plates were inspected in order to record any findings and ascertain whether the fungi were growing. To stop fungal growth until the end of the second week, the target fungus's MIC was determined. The extract may inhibit fungal growth but not completely eradicate it if it continued for two weeks after the MIC of growth was noted. In contrast, if the fungus did not begin to grow until the end of the fourth week following cultivation, it would destroy itself; in this case, the MFC would be the minimum concentration of the extract with this property [8].

Decantation: making fractions from the most effective extracts with liquid-liquid extraction

The best extract was discovered in the previous step and it was appropriately prepared. After adding the extract one to five times with distilled water, the mixture was passed through solvents like butanol, hexane, chloroform, and ethyl sulfonate. One extraction solvent at a time was decanted until the extraction solvent had lost its color. All of the aqueous and organic phases were rotary dried at a temperature lower than 50 °C following decantation. The dried fractions were stored in capped containers at –20 °C until it was time to investigate their fungal effects [8].

Examining the antifungal properties of decanted fractions for sensitive fungi

Comparable in methodology to the study of extracts' antifungal effects, this phase's approach differed from that of the study because the former examined the extracts' antifungal effects while the latter examined the effects of fractions prepared by decantation on the fungus that had previously been identified as sensitive. The fraction that proceeded to the next phase was the one that proved most effective in killing the most susceptible fungus after this phase ended. Step 2 examined the antifungal effects of fractions prepared by decantation on extract-sensitive fungi, in a manner akin to the "Agar Dilution of Extracts", as opposed to the effects of diluted extracts. In order to do this, different solutions at different concentrations were extracted from fractions. Consequently, all fractions' solutions, such as aqueous fraction, N-Hexane, Chloroform, Ethyl Stat, and N-Butanol, were obtained in culture medium at concentrations of 2%, 1%,

0.5%, 0.25%, and 0.125%. Also prepared were the plates holding the Terbinafine blank, positive control, and extract blanks. After the phase pertaining to the extracts' antifungal properties, the remaining steps were carried out. The fraction that worked best on the fungi that were extract-sensitive [8].

Examination of Keratinase

Submerged cultivation was used as the cultivation technique for the enzyme analysis. The mineral liquid medium, which contained all of the nutrients found in the solid medium agar excluded was made in one liter of distilled water, along with MgSO4.7H2O (0.5 g), KH2PO4 (0.1 g), FeSO4.7H2O (0.01 g), ZnSO4.7H2O (0.005 g), NaH2PO4 (3.86 g), Na2HPO4 (3.97 g), Cycloheximide (0.5 g), and Chloramphenicol (0.05 g). The liquid medium was then autoclaved, or sterilized, for fifteen minutes. Test tubes were filled with 10 milliliters of culture medium, and various concentrations of the targeted fraction (0.5%, 0.25%, 0.125%, 0.0625%, and 0.03125%) were added one at a time. The culture medium was then supplemented with a minimal fungous mixture of 106 cells/mL. Infected tubes were incubated for fifteen minutes at 28 °C. One potential control solution was PBS, or phosphate-buffered saline. The experiment was conducted three times, and the refined culture keratinase's kinetic activity was measured using a spectrophotometric technique [9]. That is, two grams of human hair, weighing between one and two millimeters, were cut with scissors, cleaned in seventy milliliters of 50% methanol chloroform, treated with soap particles at 42 °C for twelve hours in a shaking incubator, repeatedly washed with water, and finally filtered and dried. With a magnet stirrer, 0.945 grams of KH2PO4 were dissolved in 250 ml of water to create phosphate buffer (pH 7.8, 28 mM). The mixture's pH was then measured using a pH-meter and concentrated soda solution was added. Enzyme solution was produced by combining three milliliters of buffer with three milliliters of fractions. A shaking incubator or warm water bath was used to incubate the reaction mixture at 37 °C. Subsequently, Six milliliters of the diluted enzyme solution (pH 7.8, 28 mM) were mixed with fifty milligrams of dried hair. After incubation, the remaining hairs were filtered out, and the absorbance at a wavelength of 280 nm was measured using a UV-Visible Spectrophotometer. The boiling enzyme solution served as a control (for denaturation and deactivation) [10]. As one unit of enzyme activity is equal to a 0.1 increase in absorbance, the specific enzyme activity was calculated as one enzyme unit per milligram of protein. Bradford assay was used to determine the milligrams of sample protein. The enzyme activity was assessed while the fractions were present. The Bradford assay is a highly sensitive and accurate protein measurement method with a microgram sensitivity range. This assay was developed in response to the dye Coomassie Brilliant Blue G-250's absorbance shifting from purple to blue in the presence of protein and in acidic environments. This dye, which has a negative charge, ionizes with amino proteins because they have positive charges [11]. The protein-dye binding form exhibits the highest absorbance at 595 nm in wavelength. As a result, the sample will absorb more dye and have a more intense blue color the more protein it contains. Notably, a standard curve was plotted using bovine serum albumin (BSA). This project also made use of Sigma Bradford reagent. Distilled water and BSA solution were using at a concentration of 1 mg/ml in a 1:4 ratio, the Bradford reagent (20%) was created. The wavelength of 595 nm was used to calculate the protein concentration. Blank solution, which is devoid of albumin, was utilized for calibration. After adding BSA to the Bradford solution,

it took nearly five minutes for the purple dye to turn blue. In addition to the unidentified sample, each standard sample's absorbance was measured.The standard curve was created by plotting the optical absorption values against their calculated concentrations. The final linear equation was solved, and the protein concentration in the desired samples was determined [12].

■ RESULTS

According to the data, ectothrix infections appear to have a higher proportion of positive samples than favus and endothrix infections. It is important to remember that the total number of samples varied by type, with ectothrix having the most samples overall (47, 52.8%), followed by endothrix (33, 37.1%), and favus (9, 10.1%). Additionally, as illustrated in fig. 1, the negative samples represent 53.3%, 37.7%, and 9.1% of the total.

Tinea capitis-causing species isolates from three distinct locations revealed that Microsporum canis 17 (19.1%), Microsporum gypseum 14 (15.7%), Trichophyton equinum 9 (10%), and the less common Trichophyton verrucosum 7 (7.8%) were the most prevalent fungi of the ectothrix. Trichophyton tonsurans 19 (22%) and Trichophyton violaceum 14 (15.7%) were the most prevalent fungi in endothrix, however. The only species that appears in relation to favus, however, is T. schoenleinii 10 (9%), as shown in fig. 2.

Various species responded differently to the extract. As indicated in Table 1, T. tonsurans was more susceptible to the extract than other Trichophyton and Microsporum species.

Determining the MIC and MFC of a chemical or extract against fungi is a crucial step in evaluating how well it inhibits and kills fungi. In line with Table 2, which showed the MIC and MFC values for the extracts that were used in this study.

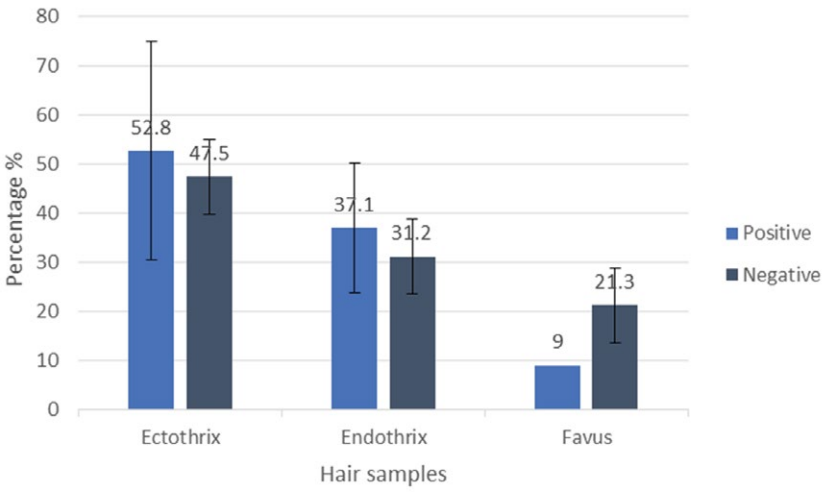

Fig. 1. Positive and negative samples (SD=22.1906 for positive and SD=13.229 for negative)

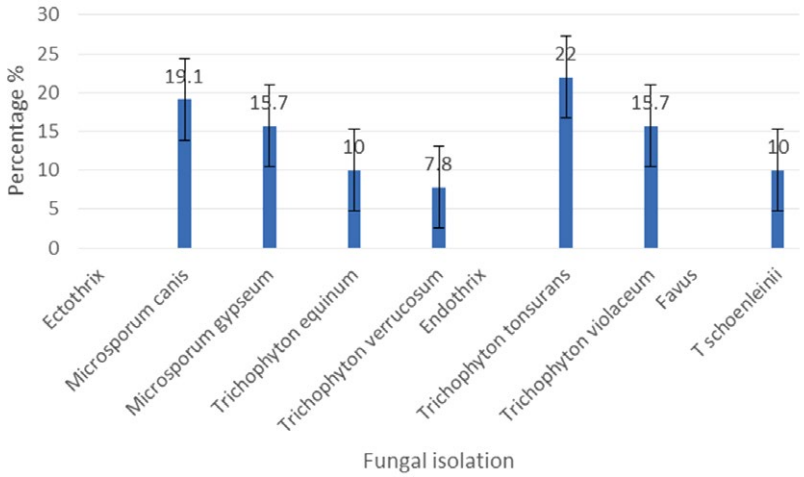

Fig. 2. Tinea capitis-causing species isolated from hair samples

Table 1
The inhibition extracts effect based on agar dilution method

Extracts fungi	Concentraction (mg/ml)	Microsporum canis	Microsporum gypseum	Trichophyton equinum	Trichophyton verrucosum	Trichophyton tonsurans	Trichophyton violaceum	T schoenleinii
Aqueous extract	10	–	–	–	–	–	–	–
	5	+	–	–	–	+	–	–
	2.5	+	+	–	–	+	–	–
	1.25	+	+	+	+	+	+	+
	0.625	+	+	+	+	+	+	+
Hydro-alcoholic	10	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	–
	2.5	–	+	–	+	–	–	+
	1.25	+	+	+	+	–	–	+
	0.625	+	+	+	+	+	+	+

Notes: the different concentrations, the not-growing (–) and growing (+) signs denote the different states of the fungus.

Table 2
Extracts' MIC and MFC in relation to tinea capitis

Extracts fungi	Microsporum canis		Microsporum gypseum		Trichophyton equinum		Trichophyton verrucosum		Trichophyton tonsurans		Trichophyton violaceum		T schoenleinii	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aqueous extract	10	10	5	5	2.5	2.5	2.5	2.5	10	10	2.5	2.5	1.25	1.25
Hydro-alcoholic	2.5	2.5	5	5	2.5	2.5	5	5	1.25	1.25	1.25	1.25	5	5

Decantation: a liquid-liquid method for preparing fractions from hydro-alcoholic extract

Different organic solvents, ranging from non-polar to polar solvents, were used to decant hydro-alcoholic extract to produce the fractions with the corresponding yields that are displayed in Table 3 below.

Table 3
Hydro-alcoholic extract fraction yields obtained through decanting

Fraction	Yield (%)
N-Hexane	19.1
Chloroform	11.5
Ethyl Stat	10.2
N-Butanol	24.9
Aqueous	32.5

Effects of hydro-alcoholic decantation fractions on anti-fungi

Table 4 shows the final results for Terbinafine against the targeted fungi and the MIC and MFC of each fraction. Although various studies have been done on the anti-dermatophyte effects of rosemary extracts.

Keratinase activity assay

Fig. 3 showed the keratinase activity assay results for tinea capitis.

■ DISCUSSION

Ectothrix seems to be the most common infection in the hair samples overall. Because there were more positive cases and a larger sample size overall, it appears that ectothrix infections are more common in hair samples. These findings were in line with the researcher's research [13].

Table 4
Comparing the hydro-alcoholic extract decantation fractions' MIC and MFC to tinea capitis

Extracts fungi	Microsporum canis		Microsporum gypseum		Trichophyton equinum		Trichophyton verrucosum		Trichophyton tonsurans		Trichophyton violaceum		T. schoenleinii	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
N-Hexane	1	1	1.5	1.5	1.25	1.25	1.25	1.25	1	1	1.5	1.5	1	1
Chloroform	20	20	10	10	20	20	5	5	10	10	10	10	5	5
Ethyl Stat	10	10	5	5	5	5	10	10	5	5	10	10	10	10
N-Butanol	20	20	5	5	10	10	10	10	20	20	5	5	5	5
Aqueous	10	10	20	20	5	5	20	20	20	20	10	10	20	20
Terbinafine	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

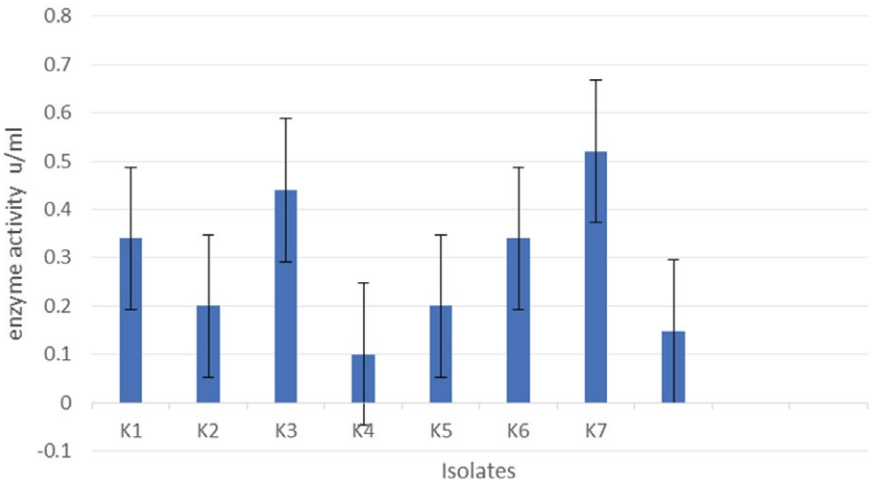

Fig. 3. Comparative analysis of the tinea capitis keratinase activity assay results in the presence of chloroform fraction, (K1 = Microsporum canis, K2 = Microsporum gypseum, K3 = Trichophyton equinum, K4 = Trichophyton verrucosum, K5 = Trichophyton tonsurans, K6 = Trichophyton violaceum, and K7 = T. schoenleinii)

These findings suggest that the distribution of fungi that cause Tinea Capitis differs between infections caused by ectothrix and endothrix. The most common species in these categories are Trichophyton tonsurans and Microsporum canis. The researchers also observed that the only species causing favus in the areas under study was Trichophyton schoenleinii. These results are helpful in determining the frequency and geographic distribution of the fungi that cause tinea capitis, which can be crucial for both diagnosis and treatment [14].

The low efficacy against the tested organism may be explained by the presence of specific molecules in crude extracts that have antagonistic actions toward other bioactive substances, according to [9]. Similarly, greater susceptibility to these organisms may be explained by T. tonsurans' slower growth, which takes 8–12 days and 7–10 days, respectively, in contrast to Microsporum spp.'s 5- to 10-day development [15, 16].

Since the fungus grew in a growth medium devoid of essence at lower concentrations of the targeted extracts, the results demonstrated that both extracts had antifungal properties. Additionally, because the hydro-alcoholic extract's (MIC) against the fungi is lower than that of the aqueous extract, it has a greater impact on the tested fungi. Thus, it was determined that rosemary's antifungal properties inhibit fungal growth in a concentration-dependent manner. Certain bacteria, including S. aureus, Proteus vulgaris, P. aeruginosa, K. pneumoniae, E. faecalis, E. coli, S. epidermidis, B. subtilis, and Candida albicans, demonstrated a relatively high sensitivity to methanol extract but were sensitive to rosemary essence, according to a study on the microbial activity of rosemary essence and methanol extract based on an Agar dilution method [16–18].

In liquid-liquid extraction, the more polar solvent yielded the highest extracted fraction yield, so the order of fraction yields from highest to lowest values would be aqueous fraction, N-butanol, N-hexane, chloroform, and ethyl stat. Each solvent's yield varies according to the kind of plant and extract utilized [18–21].

The current study had objectives beyond merely examining the extract's anti-Tinea Capitis effects. It also examined the antifungal effects of this plant cultivar on Tinea Capitis that had not previously been studied. Among these were dividing the hydro-alcoholic extract according to its polarity and assessing its anti-tinea capitis properties. Based on Table 4 results, the N-hexane fraction had the lowest MIC against the intended fungi, to the point where both its MIC and MFC were lower than the intended MIC. As a result, no growth was observed within the concentration limit. It was also demonstrated that as fractions became more polarized, their antifungal potency decreased (MIC values increased). Regarding the point that the concentration of active components in the fraction is primarily responsible for the potency of the antifungal effect, Compared to the MIC of hydro-alcoholic extracts in the first stage, the MIC of N-hexane in this stage was lower [22–24].

There are notable differences between the various concentrations of the fungi, as indicated by the effect, which besides show that the keratinase activity of a fungal isolates vary. Alternatively put, the effect is entirely dependent on fungal isolates. As such, the resulting chloroform fraction is thought to act as an enzyme activator for keratinase, strengthening its activity [25–27].

■ CONCLUSION

Once extracted and fractionated, rosemary leaves have anti-tinea capitis properties. Ointment, cream, or lotion should be made with these fractions at the recommended percent and tested in vivo to determine whether they are effective against trichomonia capitis, given the increased effect of the N-hexane fraction in decantation. In fact, the ultimate objectives of in vitro tests are the identification of antifungal factors and the prediction of clinical outcome. Tinea capitis treatments are becoming more and more specialized, requiring the strongest antifungal drugs to be identified for any fungal

agent. Therefore, the most effective medication against Tinea capitis must be found by carefully analyzing the chemical substance of rosemary and learning about its antifungal properties.

REFERENCES

1. Shihab TJ, Ibrahim ZI, Jumma QS, Khorsheed HH. Dermatophytosis in Bovine, Recent Clinical analysis, Treatment, and updated zoonotic infection: A Review Article. *Tikrit Journal of Veterinary Sciences.* 2023;23(1):120–133.
2. Grable S. *Dermatophytosis.* In: Horne K, Schwassmann M, Logas D (editors*). Small animal dermatology for technicians and nurses.* John Wiley & Sons, Inc., US. 2019; 65–84.
3. Rabieepur S, Ebrahimi M, Sadeghi E. Relationship between sexual health and contraception methods in women. *Journal of Mazandaran University of Medical Sciences.* 2015;25(130):30–39.
4. Naeini A, Khosravi AR, Chitsaz M, et al. Anti-Candida Albicans Activity of Some Iranian Plants Used in Traditional Medicine. *J. Mycol. Med.* 2009;19:168–172.
5. Yesil Celiktas O, Hames Kocabas EE, Bedir E, et al. Antimicrobial Activities of Methanol Extracts and Essential Oils of Rosmarinus Officinalis, Depending on Location and Seasonal Variations. *Food Chem.* 2007;100:553–559.
6. Yazdani D, Rezazadeh S, Amin Gh, et al. Antifungal activity of dried extracts of anise (Pimpinella Anisum L.) and star anise (Illicium verum Hook. f.) against dermatophyte and saprophyte fungi. *Journal of Medicinal Plants.* 2009;8(5):24–29.
7. Getso MI, Hashemi SJ, Ghazvini RD, et al. Determination of dermatophytes isolated from tinea capitis using conventional and ITS-based sequencing methods in Kano, Nigeria. *J Mycol Med.* 2021 Sep;31(3):101157. doi: 10.1016/j.mycmed.2021.101157
8. Mikaeili A, Modaresi M, Sozani S, Karimi I. The antifungal activities of rosemary against trichophyton tonsurans and microsporum canis. *Int. J. Pharm. Res. Allied Sci.* 2016;5:472–483.
9. Abbas LMR, Hashim AJ, Al-Hadban W. Identification local isolates of trichophyton mentagrophytes and detection of keratinase gene using PCR technique. *Iraqi Journal of Agricultural Sciences.* 2020;51(6):1534–1542.
10. Shams Ghahfarokhi M, Razafsha M, Allameh A, Razzaghi Abyaneh M. Inhibitory effects of aqueous onion and garlic extracts on growth and keratinase activity in richophyton mentagrophytes. *Iran Biomed. J.* 2003;7:113–118.
11. Uchida K, Yamaguchi H. Therapeutic efficacy of oral administration of terbinafine in guinea PIG trichophytosis model. *Jpn. Med. Mycol. J.* 1993;34:485–491.
12. Niwano Y, Tabuchi T, Kanai K, et al. Therapeutic efficacy of lanoconazole ointment in guinea pig model of tinea corporis, a comparative study with ointment and cream preparations. *Jpn J Antibiot.* 1995 Jan;48(1):150–4.
13. Ahmad I, Aqil F. In vitro efficacy of bioactive extracts of 15 medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. *Microbiological Research.* 2007;162(3):264–275.
14. Santos D, Hamdan J. Evaluation of Broth Microdilution Antifungal Susceptibility Testing Conditions for Trichophyton rubrum. *Journal of Clinical Microbiology.* 2005;43(4):1917–1920.
15. Rodríguez-Cerdeira C, Martínez-Herrera E, Szepietowski JC, et al. A systematic review of worldwide data on tinea capitis: analysis of the last 20 years. *J Eur Acad Dermatol Venereol.* 2021;35(4):844–883.
16. Mikaeili A, Modaresi M, Sozani S, Karimi I. The Antifungal Activities of Rosemary against Trichophyton Tonsurans and Microsporum Canis. *Int. J. Pharm. Res. Allied Sci.* 2016;5:472–483.
17. Hosseini N, Maleki Rad AA, Changizi Ashtiani S, Nazemi M. The Inhibitory Effects of Different Methanol Essences and Fractions of Thymus Vulgaris, Sage, Rosemary, Pennyroyal and Cinnamon on Free Radicals. *Journal of Shahid Sadoughi University of Medical Sciences, Yazd.* 2012;20(1):28–38.
18. Fazli Bazzaz BS, Izadyar A. Anti-mutagenic effects of extract fractions of S. Leriifolia. *Iran Journal of Medical Sciences.* 2001;40(4):241–250.
19. Ali-Shtayeh MS, Jamous RM, Abu-Zaitoun SY, et al. Biological properties and bioactive components of mentha spicata L. Essential oil: focus on potential benefits in the treatment of obesity, Alzheimer's disease, dermatophytosis and drug-resistant infections. *Evid Based Complement Alternat Med.* 2019 Oct 20;2019:3834265. doi: 10.1155/2019/3834265
20. Badri R, Shushizadeh MR. A new method for the oxybromination of aromatic compounds with copper (II) bromide and potassium dichromate. *Phosphorus, Sulfur and Silicon and the Related Elements.* 2005;180(2):533–536.
21. Hosseinlou A, Alinejad V, Alinejad M, Aghakhani N. The effects of fish oil capsules and vitamin B1 tablets on duration and severity of dysmenorrhea in students of high school in Urmia-Iran. Global journal of health science. *Global journal of health science.* 2014;6(7):124–129.
22. Mercer DK, CS. Stewart CS. Keratin hydrolysis by dermatophytes. *Med Mycol.* 2019;57(1):13–22.
23. Wiegand C, Mugisha P, Mulyowa GK, et al. Identification of the causative dermatophyte of tinea capitis in children attending Mbarara Regional Referral Hospital in Uganda by PCR-ELISA and comparison with conventional mycological diagnostic methods. *Med Mycol.* 2017;55(6):660–668.
24. Ghavimi H. *The antifungal effects of the shoot of a plant species of asteragalus genus on trichophyton verrucosum in culture medium and inoculated in Indian Pig's skin.* PhD Thesis. 2010; 21–22.
25. Abuzaid H, Amin E, Moawad A, et al. Liquid chromatography high-resolution mass spectrometry analysis, phytochemical and biological study of two aizoaceae plants plants: a new kaempferol derivative from Trianthema Portulacastrum L. *Pharmacognosy Research.* 2020;10:24–30.
26. Venkatachalam P, Chittibabu CV. Antifungal activity of Terminal chebula fruit extracts. *Current Botany.* 2020;11:216–220.
27. Alhajj MS, Qasem MAA, Nabi AR, Al-Mufarrej SI. In-vitro antibacterial and antifungal effects of high levels of Chinese star anise. *Brazilian Journal of Poultry Science.* 2019;21(1):001–008.

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Effects of Drenching Aqueous Extracts of Licorice Root (Glycyrrizia Galabra) and Matricaria Chamomile on Hematological Parameters of Adult Female Rabbits (Lepus Cunicolus)

Conflict of interest: nothing to declare.
Authors' contribution: Hanadi Abeudalgabar Hafth – conceptualization, data curation, investigation, methodology, project administration, software, visualization, writing – original draft and writing – review & editing; Zainab Abdulwahab Shehab Alkatrani – conceptualization, methodology, project administration, resources, software, validation, visualization, writing – original draft and writing – review & editing; Hind Abduljaleel Ahmed Alahmed – conceptualization, investigation, methodology, project administration, resources, software, visualization, writing – original draft and writing – review & editing.
Ethics statement: the scientific committee has approved this study of the Department of Physiology, Pharmacology and Chemistry, College of Veterinary Medicine, University of Basrah dated 13/9/2021, ensuring the experiment being conducted at the same time did not violate animal rights regulations.
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Abstract

Introduction. Licorice (Glycyrrhiza galabra) is a wild plant that is now used in a great variety of pharmaceutical preparations.
Purpose. To evaluate the antioxidant activity of licorice with chamomile watery extract and mix these plants on blood parameters in adult female rabbits.
Materials and methods. Forty adult female rabbits, each weighing around 1500 g and aged 4–5 months were used in the investigation. All are classified into 4 groups. The first group acted as controls. The second group received licorice water extract injections. The third group received a chamomile water extract. A combined licorice root and chamomile extract was given to the fourth group. Samples of blood were taken via the heart, and the results were evaluated (RBC, HB, PCV, WBC, MCH, MCV, and MCHC).
Results. There's a significant decrease (P≤0.05) in all blood parameters compared with a control group, but when treated animals with mixed extracted there's a significant increase in blood parameters (RBCs, WBCs, PCV, and MCV) observed. A significant increase in lymphocytes in the licorice group while there was a significant decrease (P≤0.05) in the chamomile group. There's a significant decrease (P≤0.05) in neutrophils. While the eosinophil significantly rises in the chamomile group and significantly decreases (P≤0.05) in the licorice group. Monocyte cells are significantly increased in different treated groups.
Conclusion. When the plants are mixed and fed to the animals, the animals' blood parameters considerably improve. Licorice root and chamomile eques extract causes a decrease in all blood parameters when mixed, however, its effect is still controversial when used alone.
Keywords: hematology, plants, extraction of plant, Staphaureus, Streptomutans, Mycobacterium smegmatis

■ INTRODUCTION

Licorice (Glycyrrhiza galabra) is a plant that grows wild in the Western and Eastern world and has therapeutic uses throughout the world, including the Greeks, Egyptians, and Indians. It was traditionally used in the treatment of peptic ulcers, asthma, pharyngitis, malaria, abdominal pain, insomnia, infections, and many other diseases [1–3].

The licorice plant was also valued in ancient Egypt (Tutankhamen) and was buried with a supply [4, 5]. Human studies proved its curative effect in infectious hepatitis [6], chronic hepatitis C, and subacute hepatic failure [7], hemophilia and HIV-1 infection [8], gastric and duodenal ulcers [9], and in herpes, eczema, and psoriasis. The antibacterial effect against Staphaureus, Streptomutans, Mycobacterium smegmatis and Candida albicans [10] was also documented. Accordingly, licorice is now used in a great variety of pharmaceutical preparations [11].

Licorice grows in tropical and subtropical areas [12]. Licorice was very stable in the gastrointestinal tract, and gradually absorbed Seeds, roots, and leaves are all utilized medicinally. According to Hhabra et al. [13], it is used to treat abscesses, stomatitis, conjunctivitis, and epilepsy. Used historically to treat tumors and ulcers [14]. Liquiritoside; one of the root's saponin components, has demonstrated in-vitro anti-inflammatory activity [15].

There are several uses for chamomile. Additionally used as a sedative, anxiolytic, and antispasmodic for the treatment of moderate skin inflammation and irritation. Apigenin and bisabolol are the two primary active ingredients in chamomile. Amino acids, polysaccharides, essential oils, mineral flavonoids, and other phenolic compounds are included as additional components [16].

In contemporary medicine, chamomile is used to treat gastrointestinal disorders such as flatulence, indigestion, diarrhea, motion sickness, and vomiting because of its spasmolytic, antiphlogistic, antibacterial, and multifunctional digestive properties. Matricaria chamomilla is a plant that has been used to cure cancer, diarrhea, and other illnesses like inflammation and cancer [17]. It prevents the aggregation of Helicobacter pylori and other bacterial strains [18]. When used against Trichophyton mentagrophytes, chamazulene, alpha-bisabolol, flavonoids, and umbelliferone exhibit antifungal properties [19]. The most notable antispasmodic effects appear to be produced by apigenin, alpha-bisabolol, and cisspiroethers. According to Yazdi et al. [20], flavonoids and coumarins are responsible for smooth muscle relaxation.

■ PURPOSE OF THE STUDY

To evaluate the antioxidant activity of licorice with chamomile watery extract and mix these plants on blood parameters in adult female rabbits.

■ MATERIALS AND METHODS

Study setting

An experimental study conducted the Department of Physiology, Pharmacology and Chemistry, College of Veterinary Medicine, University of Basrah at a period from 3rd of March 2022 to 2nd of April 2023.

Animals

Forty female local rabbits aged 4–5 months weighing 1500 grams were used and brought from the local market. They were purchased from a market in Basrah and housed in the veterinary school's animal house while waiting for the experiment. Animals weighing 1500 g and being between 4–5 months old. They were feed (alfa alfa) adlibtum with clean drinking water. Animals were placed into four groups of ten at random. Animals were housed in standard cages maintained under laboratory control at a temperature of 25±2 °C and 12 hrs. light/dark cycle, they were fed (alfa) ad libitum with clean drinking water. Two weeks for acclimatization in the animal house where experiments were performed. Animals were categorized into four groups: First group served as the control group and received (3 ml) of nascent salt water each day for a month. The animals in the third group received (3 ml) of chamomile extract whereas those in the second group received (3 ml) of licorice root extract for a month. The fourth group received (3 ml) of licorice and (3 ml) of chamomile for a month.

Plant materials

Plants purchased from a local market, taxonomic identification and validation through the College of Science herbarium.

Preparation of extract

After cleaning the plants and grinding them into a powder, six grams of each powder were added to 200 milliliters of distilled water, which was then heated in a reflexometer until the volume reached 100 milliliters. Stirring the extract at room temperature for one day. After that the extracts were put in centrifuge at 10 000 rpm for 15 minutes [21].

Experimental design

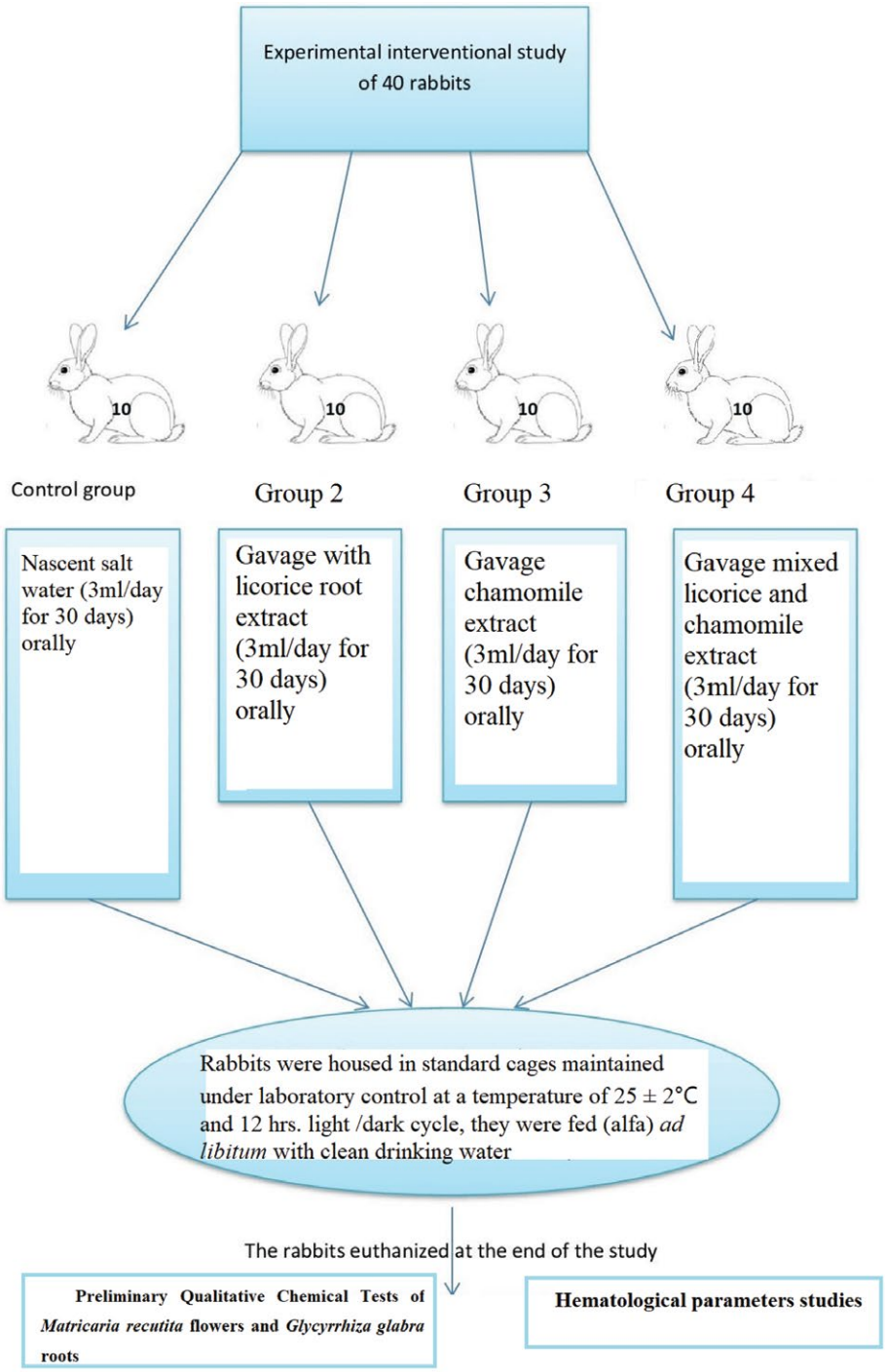
The female rabbits were randomly divided into four groups, each containing 10 rabbits. Group 1 – the control group gave nascent salt water (3 ml/day for 30 days) orally. Group 2 – gavage with licorice root extract (3 ml/day for 30 days) orally. Group 3 – gavage chamomile extract (3 ml/day for 30 days) orally and group 4 – gavage mixed licorice and chamomile extract (3 ml/day for 30 days) orally. The flow chart of the study shown in (Figure).

Preliminary qualitative chemical tests of matricaria recutita flowers and glycyrrhiza glabra roots

Chemical tests were done on alcoholic extract of Matricaria recutita flowers and glycyrrhiza glabra roots to determine its active groups, as follows: Phenolic compounds [21], Tannin [22], Flavonoids test [21], Molich test [23], Biuret test [23], Dragendroffs test [24], Saponin test [23] and Benedict test [21].

Collection of samples

The animal's heart was punctured and five ml of blood was drawn from it using a disposable syringe, blood was transferred to test tubes containing anticoagulant, to use for studying the all parameters.



Flow chart of the study

Hematological parameters studies

Total and differential leukocyte counts, packed cell volume, hemoglobin concentration and leukocyte counts, as well as blood parameters like mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were measured.

Statistics

Statistical analysis was performed using SPSS v24 (IBM Inc., Chicago, IL, USA). Quantitative data described as mean and SD. The one-way-ANOVA test for independent measures is designed to compare the means. A two-sided P-value of less than 0.05 was considered statistically significant.

■ RESULTS

This study shows a significant decrease (P≤0.05) in all blood parameters which include (RBCs, WBCs, HB, PC, MCV, MCH, and MCHC) when treated animals with both licorice root and chamomile eques extract there's as compared with a control group, but when treated animals with mixed extracted there's a significant increase (P≤0.05) in blood parameters (RBCs, WBCs, PCV and MCV). While the significant decrease in HB, MCH, and MCHC blood parameters seen.

Also, the results of this study can show a significant increase (P≤0.05) in lymphocytes in the licorice group while there's a significant decrease (P≤0.05) in the chamomile group and when mixed with the plant there's no change in parameters as compared with the control group. There's a significant decrease (P≤0.05) in neutrophils. While the eosinophil significant increase in the chamomile group and a significant decrease (P≤0.05) in the licorice group, when mixed with the plant there was no change in parameters as compared with the control group. Monocyte cells are significantly increased (P≤0.05) in different treated groups compared with the control group (Table 1).

From Table 2 can be seen there's no significance in basophile cells in the licorice and chamomile group and other deferential white blood cells when mixed with the plants as compared with the control group.

The preliminary qualitative chemical tests of alcoholic extract of Matricaria recutita flowers and Glycyrrhiza glabra roots was listed in Table 3. All findings shown a positive test (presence of the constituents) for extracts except flavonoids of Glycyrrhiza glabra for Tannin test which give negative results.

Table 1
Effect of drenching eques extract of both plants on hematological parameters of female rabbits

Parameters / Groups	RBC ×10⁴/mm³	WBC ×10³/mm³	HB (g/dl)	PCV (%)	MCV (pg)	MCH g/dl	MCHC (Fl)
	Mean ±SD						
Control	690±0.12 ^{B}	4.2 ±0.96 ^{B}	14±1.62 ^{A}	40±2.61 ^{B}	40±1.55 ^{B}	14±1.72 ^{A}	35±1.6 ^{A}
Licorice	662±0.20 ^{C}	2.8±0.84 ^{C}	12.6±1.73 ^{B}	39±2.54 ^{C}	39±1.70 ^{C}	12.4±1.64 ^{B}	31.9±2.14 ^{B}
Chamomile	660±0.30 ^{D}	2.2±0.93 ^{D}	7±2.24 ^{D}	34±1.38 ^{D}	34±1.84 ^{D}	7 ±1.83 ^{D}	20.5±1.6 ^{D}
Mixed (L.CH)	770±0.20 ^{A}	5.2±0.94 ^{A}	10.5±1.93 ^{C}	48±1.64 ^{A}	48±0.94 ^{A}	10.5±1.54 ^{C}	21.8±1.12 ^{C}

Note: the different letters refer to the significant difference among groups at a level of (P≤0.05).

Table 2
The impact of soaking extracts of licorice root and chamomile on the differential leukocyte count of female rabbits

Parameters / Groups	Lymphocyte	Neutrophil	Eosinophil	Monocytes	Basophil
	Mean ±SD				
Control	35±4.25 ^{B}	55±3.26 ^{A}	5±1.6 ^{B}	4±2.4 ^{D}	1±0.0 ^{A}
Licorice	36±1.84 ^{A}	53±2.55 ^{C}	4±1.8 ^{C}	6±1.31 ^{B}	1±0.0 ^{A}
Chamomile	34±2.21 ^{C}	52±2.81 ^{D}	6±2.10 ^{A}	7±1.10 ^{A}	1±0.0 ^{A}
Mixed (L. CH)	35±3.24 ^{B}	54±2.4 ^{B}	5±2.20 ^{B}	5±1.44 ^{C}	1±0.0 ^{A}

Note: the different letters refer to the significant difference among groups at a level of ($P \leq 0.05$).

Table 3
Preliminary Qualitative Chemical Tests of alcoholic extract of Matricaria recutita flowers and Glycyrrhiza glabra roots

Chemical constituent	Results		Chemical test
	Matricaria recutita	Glycyrrhiza glabra	
Protein	+	+	Biuret test
Carbohydrate	+	+	Molish test
Alkaloids	+	+	Dragendroffs test
Phenolic compounds	+	+	$FeCl_3$
Saponin	+	+	Saponin test
Flavonoids	+	+	Flavonoids test
Tannin	+	-	Tannin test
Glycosides	+	+	Benedict test

Notes: + – presence of the constituents; – – absence of the constituents.

■ DISCUSSION

The results of this study show that the blood parameters of female rabbits treated with licorice root (Glycyrrhiza glabra) extract alone did not significantly differ from those of the control group. In contrast, the blood parameters of rabbits treated with chamomile extract differed significantly from those of both the licorice and control groups [25].

Sapnine, a chemical extracted from the chamomile plant, may be responsible for the observed drop in blood pressure and other blood-related parameters; this substance has been linked to an increase in the hemoglobin excreted outside of red blood cells (RBCs) as a result of hemolysis, and it has also been linked to an increase in RBC production from bone marrow above the normal level, which in turn reduces RBC count [26].

Glycyrrhiza acid or extract can increase total white and red blood cell counts, although the usage of another plant (licorice) results in significant improvements in the levels of blood parameters approaching the level of parameters of the control group [27]. After 10 days of exposure, the sympathetic nervous system is activated, leading to a redistribution of peripheral blood cells and the migration of immature red blood cells from the bone marrow, both of which contribute to an increase in RBCs. At 15 and 30 days of exposure, normochromic reductions in erythrocyte and hemoglobin concentration were observed [28].

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These peripheral blood alterations during continuous vibration were likely generated by the migration of immature erythrocytes from the bone marrow into the bloodstream and did not result in a decrease in hemoglobin content [29].

Hemoglobin concentration and erythrocyte count both increased after 15 days of consuming licorice leaves, suggesting that erythropoiesis was stimulated. These results show that Glycyrrhiza glabra promotes erythroid stem cell proliferation and maturation. Glycyrrhiza glabra L.'s bioactive components, including its triterpene saponins, flavonoids, polysaccharides, pectins, amino acids, mineral salts, etc., modify metabolism and accelerate erythroid stem cell development, as evidenced by similar changes in peripheral blood red cells. Specifically, it stimulates sympathetic erythropoiesis activities, leading to an optimal erythrocyte balance [30–32].

■ CONCLUSION

When the plants are mixed and fed to the animals, the animals' blood parameters considerably improve. Licorice root and chamomile eques extract causes a decrease in all blood parameters when mixed, however, its effect is still controversial when used alone. The preliminary qualitative chemical tests of alcoholic extract of Matricaria recutita flowers and Glycyrrhiza glabra roots show a positive test (presence of the constituents) for extracts except flavonoids of Glycyrrhiza glabra for Tannin test which give negative findings.

■ REFERENCES

1. Mothibe ME, Kahler-Venter CP, Osuch E. Evaluation of the in vitro effects of commercial herbal preparations significant in African traditional medicine on platelets. *BMC Complement Altern Med.* 2019;19(1):224.
2. Chen X, F Abdallah M, Chen X, Rajkovic A. Current Knowledge of Individual and Combined Toxicities of Aflatoxin B1 and Fumonisin B1 In Vitro. *Toxins (Basel).* 2023;15(11):653.
3. Zhang Y, Sheng Z, Xiao J, et al. Advances in the roles of glycyrrhizic acid in cancer therapy. *Front Pharmacol.* 2023;14:1265172.
4. Kumari D, Gautam J, Sharma V, et al. Effect of herbal extracts and Saroglitazar on high-fat diet-induced obesity, insulin resistance, dyslipidemia, and hepatic lipidome in C57BL/6J mice. *Heliyon.* 2023;9(11):e22051.
5. Goto K, Ohtsubo T, Kitazono T. Endothelium-Dependent Hyperpolarization (EDH) in Hypertension: The Role of Endothelial Ion Channels. *Int J Mol Sci.* 2018;19(1):315.
6. Baldwin SN, Sandow SL, Mondéjar-Parreño G, et al. K_V7 Channel Expression and Function Within Rat Mesenteric Endothelial Cells. *Front Physiol.* 2020;11:598779.
7. Jang SI, Jeong S, Lee DH, et al. Safety Evaluation of Paclitaxel-Eluting Biliary Metal Stent with Sodium Caprate in Porcine Biliary Tract. *Gut Liver.* 2019;13(4):471–478.
8. Bravo V, Serrano M, Duque A, et al. Glycyrrhizinic Acid as an Antiviral and Anticancer Agent in the Treatment of Human Papillomavirus. *J Pers Med.* 2023;13(12):1639.
9. Gomez-Sanchez EP, Gomez-Sanchez CE. 11β-hydroxysteroid dehydrogenases: A growing multi-tasking family. *Mol Cell Endocrinol.* 2021;526:111210.
10. Hasan MK, Ara I, Mondal MSA, Kabir Y. Phytochemistry, pharmacological activity, and potential health benefits of Glycyrrhiza glabra. *Heliyon.* 2021;7(6):e07240.
11. Bai W, Wang Z, Liang J, Deng H. Atractylodes macrocephala-Paeonia lactiflora Class Formula for the Treatment of Irritable Bowel Syndrome: A Systematic Review With Meta-Analysis and Trial Sequential Analysis. *Cureus.* 2023;15(12):e49997.
12. Morgan AG, McAdam WA, Pacsoo C, Darnborough A. Comparison between cimetidine and Caved-S in the treatment of gastric ulceration, and subsequent maintenance therapy. *Gut.* 1982;23(6):545–51.
13. Kacholi DS. A comprehensive review of antimalarial medicinal plants used by Tanzanians. *Pharm Biol.* 2024;62(1):133–152.
14. Hadi N, Drioiche A, Bouchra EM, et al. Phytochemical Analysis and Evaluation of Antioxidant and Antimicrobial Properties of Essential Oils and Seed Extracts of Anethum graveolens from Southern Morocco: In Vitro and In Silico Approach for a Natural Alternative to Synthetic Preservatives. *Pharmaceuticals (Basel).* 2024;17(7):862.
15. Taur DJ, Patil RN, Patil RY. Antiasthmatic related properties of Abrus precatorius leaves on various models. *J Tradit Complement Med.* 2017;7(4):428–432.
16. Bashir I, Dilshad E. A comparative study of Mentha longifolia var. asiatica and Zygophyllum arabicum ZnO nanoparticles against breast cancer targeting Rab22A gene. *PLoS One.* 2024;19(8):e0308982.
17. Abosharaf HA, Farag AM, Abdel Allem AAH, et al. Chamomile Extract Reduces Cardiac Toxicity in Female Mice with Ehrlich Solid Carcinoma. *Cell Biochem Biophys.* 2024;1–7.

18. Hebel-Gerber S, García-Cancino A, Urbina A, et al. Chilean Rhubarb, Gunnera tinctoria (Molina) Mirb. (Gunneraceae): UHPLC-ESI-Orbitrap-MS Profiling of Aqueous Extract and its Anti-Helicobacter pylori Activity. *Front Pharmacol.* 2021;11:583961.
19. Fernández NJ, Damiani N, Podaza EA, et al. Laurus nobilis L. Extracts against Paenibacillus larvae: Antimicrobial activity, antioxidant capacity, hygienic behavior and colony strength. *Saudi J Biol Sci.* 2019;26(5):906–912.
20. Yazdi H, Seifi A, Changizi S, et al. Hydro-alcoholic extract of Matricaria recutita exhibited dual anti-spasmodic effect via modulation of Ca^{2+} channels, NO and PKA_2-kinase pathway in rabbit jejunum. *Avicenna J Phytomed.* 2017;7(4):334–344.
21. Bisht D, Kumar D, Kumar D, et al. Phytochemistry and pharmacological activity of the genus artemisia. *Arch Pharm Res.* 2021;44(5):439–474.
22. Rabizadeh F, Mirian MS, Doosti R, et al. Phytochemical Classification of Medicinal Plants Used in the Treatment of Kidney Disease Based on Traditional Persian Medicine. *Evid Based Complement Alternat Med*. 2022;2022:8022599.
23. Yarmolinsky L, Budovsky A, Khalfin B, et al. Medicinal Properties of Anchusa strigosa and Its Active Compounds. *Molecules*. 2022;27(23):8239.
24. Karaca N, Şener G, Demirci B, Demirci F. Synergistic antibacterial combination of Lavandula latifolia Medik. essential oil with camphor. *Z Naturforsch C J Biosci.* 2020;76(3–4):169–173.
25. Abd El-Hack ME, El-Saadony MT, Nader MM, et al. Effect of environmental factors on growth performance of Nile tilapia (Oreochromis niloticus). *Int J Biometeorol.* 2022;66(11):2183–2194.
26. Sharifi-Rad J, Quispe C, Herrera-Bravo J, et al. Glycyrrhiza Genus: Enlightening Phytochemical Components for Pharmacological and Health-Promoting Abilities. *Oxid Med Cell Longev.* 2021;2021:7571132.
27. Dai YL, Li Y, Wang Q, et al. Chamomile: A Review of Its Traditional Uses, Chemical Constituents, Pharmacological Activities and Quality Control Studies. *Molecules.* 2022;28(1):133.
28. Raeeszadeh M, Mortazavi P, Atashin-Sadafi R. The Antioxidant, Anti-Inflammatory, Pathological, and Behavioural Effects of Medicago sativa L. (Alfalfa) Extract on Brain Injury Caused by Nicotine in Male Rats. *Evid Based Complement Alternat Med.* 2021;2021:6694629.
29. Karaca N, Demirci B, Gavahian M, Demirci F. Enhanced Bioactivity of Rosemary, Sage, Lavender, and Chamomile Essential Oils by Fractionation, Combination, and Emulsification. *ACS Omega.* 2023;8(12):10941–10953.
30. Alak G, Özgeriş FB, Uçar A, et al. Effect of climate change on hematotoxicity/hepatoxicity oxidative stress, Oncorhynchus mykiss, under controlled conditions. *PLoS One.* 2023;18(11):e0294656.
31. Nazari S, Rameshrad M, Hosseinzadeh H. Toxicological Effects of Glycyrrhiza glabra (Licorice): A Review. *Phytother Res.* 2017;31(11):1635-1650.
32. Sorrenti V, Burò I, Consoli V, Vanella L. Recent Advances in Health Benefits of Bioactive Compounds from Food Wastes and By-Products: Biochemical Aspects. *Int J Mol Sci.* 2023;24(3):2019.

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Prevalence of Hypomagnesemia in Hemodialysis Patient: An Approach to Understand Risk Factors

Conflict of interest: nothing to declare.
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Abstract

Introduction. In medicine, magnesium (Mg) is termed a forgotten ion. Hypomagnesemia is characterized by a serum Mg level of less than 0.65 mmol/l. Intake of alcohol, antibiotics (pentamidine, amphotericin B, and aminoglycosides), digoxin, calcineurin, proton pump inhibitors, diuretics (thiazide and loop), and antineoplastic drugs are major risk factors for hypomagnesemia. Hypomagnesemia causes hypophosphatemia, hypokalemia, nephrocalcinosis, Gitelman syndrome, and EAST syndrome.
Purpose. To assess the factors leading to plasma magnesium deficiency and evaluating the prevalence of hypomagnesemia in CKD patients after hemodialysis.
Materials and methods. The present study examines the occurrence and possible factors influencing hypomagnesemia in CKD patients on hemodialysis. 100 participants with CKD patients under hemodialysis were recruited in the present study. A proper questionnaire and consent forms were obtained from patients. The baseline and chronic diseases (diabetes mellitus, hypertension, and ischemic heart disease) were evaluated. The blood parameters (serum Mg, parathyroid hormone, serum calcium, serum uric acid, serum phosphate, hemoglobin, and ferritin) were analyzed using a biochemical analyzer. The drugs prescribed to CKD patients were also analyzed for their effect on hypomagnesemia.
Results. Findings indicated that the dialysis duration (<1 year) influences the magnesium level. Diabetes mellitus and ischemic heart disease positively correlated with hypomagnesemia in CKD patients. The blood parameters, except serum phosphate, did not present significance with hypomagnesemia. The drug prescribed for CKD patients displayed high significance for hypomagnesemia.
Conclusion. A history of diabetes and IHD may be one of the risk factors for hypomagnesemia in individuals with CKD, and it may also operate as a mediator in the disease's progression.
Keywords: magnesium, Diabetes mellitus, CKD, hypomagnesemia, angiotensin-converting enzyme inhibitors

■ INTRODUCTION

Chronic kidney disease (CKD) is clinically characterized by a deformity in renal function or structure for more than three months, and it is an irreversible and slow process [1]. An individual adult is specified as a CKD patient if they have a glomerular filtration rate (GRF) greater or lesser than 60 ml/min/1.73 m^2 for a duration of three or more months but with damage to the structure of the kidney and filtration below 15 ml/min/1.73 m^2 is kidney failure. Approximately 850 million people worldwide are affected by CKD (1 to 5 stages) [2]. In India, the prevalence of CKD is 17%, according to the International Society of Nephrology's Kidney Disease [3]. The pathogenesis of CKD involves two possible mechanisms: an initial trigger and a perpetuating mechanism. The initial trigger or stimulus is the baseline problem caused by an immune- or inflammation-mediated reaction or toxic substance. Hypertrophy and hyperfiltration perpetuate kidney damage [4]. There are five stages in CKD based on glomerular performance: G1 (high or normal GFR), G2 (mildly decreased GFR), G3a (mildly to moderately decreased GFR), G3b (moderately to severely decreased GFR), G4 (severely decreased GFR), and G5 (kidney failure). The people display symptoms including reduced urine output, albuminuria, shortness of breath, nausea, vomiting, loss of appetite, lethargy, fatigue, and itching. However, CKD patients are usually asymptomatic in their initial stages [5]. Diabetes mellitus (DM) type-2, glomerulonephritis, hypertension, obesity, polycystic kidney disease, heart disease, acute kidney injury, alcoholism, and smoking are vital risk factors for CKD [6, 7]. The uremic syndrome was observed during the last stage of kidney disease. The screening of CKD patients includes a kidney profile test to estimate the serum creatinine and urea levels to measure GFR and albumin to creatinine ratio in urine, and imaging techniques (computerized tomography and ultrasound) are performed [8]. Diuretics, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, β-blockers, and sodium-glucose cotransporter 2 are prescribed to CKD patients in the early stages [9, 10]. Hemodialysis, peritoneal dialysis, and kidney transplantation are the renal therapies that support renal function. Among these dialysis methods, hemodialysis is an effective renal therapy that regulates the electrolyte (chloride, sodium, chloride, magnesium, potassium, and bicarbonate), fluid, acid-base balance, azotemia, and suppresses the conditions of the uremic syndrome [11].

Among the various electrolytes used in dialysate solution in hemodialysis, magnesium (Mg) levels have a crucial role because they are strongly associated with different risk factors for CKD. Mg is the second most common cation and the fourth most abundant mineral in the human body [12]. Over 300 enzymatic reactions in the body require Mg as a cofactor, synthesis of proteins, modulation of cell proliferation, reproduction, insulin and ATP metabolism, structural maintenance of DNA and RNA, blood pressure, and calcium and potassium ion transport through the transmembrane are major physiological functions controlled by Mg [13]. White and leafy green vegetables and whole grains are good sources of Mg. The optimal amount of Mg dietary intake for adults is 310–420 mg/day [14]. Bone serves as the reservoir for 60% of the Mg in the body, and the remaining is present in the soft tissue and muscles [13]. The three different forms in which serum Mg exists in the human body are: a) 5–15% – Mg attached to negative ions (anions), including sulfates, citrates, phosphates, and bicarbonates; b) 30% – Mg complexed to serum proteins (primarily albumin); and c) about 55–70% – ionized or free Mg, the active form present in total serum Mg [13]. Mg is usually engrossed mainly

in the small intestine and occasionally absorbed in the large intestine [15]. Transcellular and paracellular are two pathways in which Mg is absorbed. In the gastrointestinal tract (distal region), the transcellular absorption of Mg takes place through active transport by transient receptor channels (melastatin subtypes TRPM6 and TRPM7) [16]. 80% to 90% of Mg uptake is aided by paracellular absorption through passive transport. Primarily, the kidney regulates the filtration of Mg, and then it is reabsorbed in the thin limb of Henle's loop across the channels present in the occluding junction. The distal and proximal tubules are absorption sites for remaining Mg [17, 18].

The concentration of serum Mg levels from 0.7 to 1.1 mmol/l is normal. A serum Mg level below 0.66 mmol/l is hypomagnesemia and a serum above 1.1 mmol/l is hypermagnesemia [19]. The prevalence of magnesium deficiency (hypomagnesemia) is reported to be higher when compared to hypermagnesemia [20]. In the general population, 2.5% to 15% of the prevalence of hypomagnesemia and 12% to 20% of hypomagnesemia are reported in hospitalized patients [21]. Hypomagnesemia is associated with DM, cancer, stroke, depression, hypertension, fragility of bone, respiratory syndromes, migraine, dementia, Alzheimer's, and cardiovascular diseases [22]. An inverse relationship is found between DM type-2 and hypomagnesemia, and antidiabetic drugs (sodium-glucose cotransporter-2 inhibitors) can increase the Mg level [23].

An observational study conducted by Larsson et al. [24] in the general population has demonstrated an inverse relationship between heart failure, atrial fibrillation, ischemic stroke, cardiovascular mortality, and serum Mg concentration.

The cardiac system, muscles, and nerve membrane functions are altered due to hypomagnesemia. Hypomagnesemia may be accompanied by neuromuscular conditions (tetany, seizures, weakness, ataxia, psychosis, muscle cramps, vertigo, and depression) and cardiovascular system conditions (sensitivity to digoxin, ventricular arrhythmias, and supraventricular tachycardia) [25]. A recent study has suggested that Mg can reduce the vascular calcification mediated by phosphate, causing phosphate toxicity in the kidney. The phosphate and calcium crystallization, particularly caliprotein maturation, are deteriorated by Mg [26].

A mild elevation in plasma Mg concentration is observed during the advanced stages of renal disease because of the minimal excretion rate. The gastrointestinal absorption and concentration of Mg in dialysate determine the amount of Mg in hemodialysis patients [27]. Usually, Mg concentration in patients on hemodialysis is below the acceptable level of the treatment itself, diuretics, and proton pump inhibitors (PPI) [28–30].

The dietary intake of Mg and decreased serum Mg levels are associated with the progression of kidney disease and elevate the risk of CKD.

■ PURPOSE OF THE STUDY

To assess the factors leading to plasma magnesium deficiency and evaluating the prevalence of hypomagnesemia in CKD patients after hemodialysis.

■ MATERIALS AND METHODS

The samples were collected from 100 individuals under hemodialysis regularly after obtaining a questionnaire and consent form. The samples were collected for one year, from March 2022 to March 2023, from the Department of Nephrology, Basrah Teaching Hospital, based on the inclusion and exclusion criteria (Table 1). The questionnaire

contains information including basic details (age and gender), medical history, duration of dialysis, and drug history.

Table 1
Inclusion and exclusion criteria for sample recruitment

Inclusion criteria	Exclusion criteria
Age (18–70 years)	Salt wasting nephropathy
	Sepsis
Stable patient with euvolemic	Patients with critical illness
Patients on hemodialysis for more than three months with CKD (stage 5)	Elevated C-reactive protein
	History of kidney transplant
	Polycystic kidney disease

Treatment protocol

The machine used in the present study is the B Braun Dialog+ (2021) with a high-flow filter; the Diacap Ultra hemodialyzer filter used in our study has a surface area of 1.9 m, 1.6 m and 1.3 m. Average blood flow is 250±50 ml/min, and dialysate flow is 500–800 ml/min. The dialysis solution composition used in the present study is given in Table 2.

Table 2
Dialysate solution composition

Component	Concentration (mmol/l)
Magnesium	0.5
Sodium	138
Chloride	109
Potassium	2.0
Calcium	1.5
Bicarbonate	32
Glucose	1000
Acetate	3

Study variables

The following parameters were considered in the present study: baseline parameters (age 11–34, 35–59 and 60–84 years), gender, and duration of dialysis (>6 months and >1 year), chronic disease (DM, hypertension, and ischemic heart disease (IHD)), blood parameters (serum Mg, parathyroid hormone, serum calcium, serum uric acid, serum phosphate, hemoglobin, and ferritin), and drug history (PPI, loop diuretics, and ACE1/ARB). The results for the baseline parameters were obtained from the questionnaire. The chronic diseases were elevated using electrocardiograms and echocardiograms. The blood parameters were estimated using the Cobas C111 analyzer machine. All the blood parameters were categorized as normal, high, and low based on their normal range (1.6–2.5 mg/dl, 10–55 pg/ml, 8.5–10.5 mg/dl, 2.7–8.5 mg/dl, 2.8–4.5 mg/dl, 12.1–17.2 g/dl, and >200 ng/ml, respectively). The history of drugs involved was mostly prescribed to hemodialysis patients.

Statistical analysis

The data obtained from the present study was analyzed in SPSS version 21. The statistical significance among the various parameters was evaluated using Pearson's Chi-Square test at four different p values (P<0.05, P<0.01, P<0.005, and P<0.001).

■ RESULTS

This cross-sectional study used samples from 100 CKD patients on regular hemodialysis. The baseline parameters – gender, age, and duration of dialysis were coordinated with serum Mg levels in the patients to check whether these parameters affected the serum Mg level. Of 100 patients, 53% were male and 47% were female. CKD patients with three different age groups (11 to 34, 35 to 59, and 60 to 84 years) were considered in the present study; among these, 16% of patients fall under 11 to 34, 48% in 35 to 59, and 36% in 60 to 84 years. The results of the present study revealed that age (P=0.667) and gender (P=0.174) do not influence the serum Mg of CKD patients. The samples were divided into two groups (>6 months and <1 year) according to the duration of dialysis. The findings revealed that the duration of dialysis (<1 year) has a significant influence on serum Mg levels in CKD patients (P=0.0001). The CKD patients (63.2%) under dialysis for more than one year have displayed hypomagnesemia compared to another group.

The CKD patient's history of chronic diseases (DM, IHD, and hypertension) was correlated with serum Mg levels. Among the 100 patients, 54% had DM, 74% had high blood pressure, and 60% had IHD. The observation of the present study showed that 72.2% (P<0.0001) of CKD patients with DM had a low magnesium level. No significant difference (P=0.351) was observed between hypomagnesemia and CKD patients with hypertension. The present study's findings demonstrated significance (P=0.0001) that 85.0% of CKD patients with IHD displayed hypomagnesemia (Table 3).

Table 3
Comparison between serum magnesium and baseline parameters and chronic diseases

Baseline parameter		X^2 value	df	Total number (%)	Serum magnesium		p-value
					Normal	Low	
Gender	Male	1.846	1	53	21 (39.6%)	32 (60.4%)	0.174[f]
	Female			47	25 (53.2%)	22 (46.8%)	
Age (Year)	11–34	0.810	2	16	6 (37.5%)	10 (62.5%)	0.667[f]
	35–59			48	24 (50.0%)	24 (50.0%)	
	60–84			36	16 (44.4%)	20 (55.6%)	
Duration of Dialysis	>6 months	10.692	1	24	18 (75.0%)	6 (25.0%)	0.001[d]
	<1 year			76	28 (36.8%)	48 (63.2%)	
Diabetes mellitus	DM	15.692	1	54	15 (27.8%)	39 (72.2%)	0.0001[e]
	Non-DM			46	31 (67.4%)	15 (32.6%)	
Hypertension	Normal BP	0.871	1	26	14 (53.8%)	12 (46.2%)	0.351[f]
	High BP			74	32 (43.2%)	42 (56.8%)	
Ischemic Heart Disease	IHD	58.031	1	60	9 (15.0%)	51 (85.0%)	0.0001[e]

Notes: sample size n=100, Significance levels at P<0.05[a], P<0.01[b], P<0.005[c], P<0.001[d], P<0.0001[e], and non-significant [f], X^2 value – Chi-squared value; df – degrees of freedom; DM – diabetes mellitus; BP – blood pressure; IHD – ischemic heart disease.

The serum Mg level in CKD patients was correlated with blood parameters like parathyroid hormone, serum calcium, serum uric acid, serum phosphate, hemoglobin, and serum ferritin. The study revealed no significance between serum Mg and blood parameters except for serum phosphate (P=0.019; Table 4).

Table 4
Comparison of serum magnesium and blood parameters

Blood parameter		X^2 value	Df	Total number (%)	Serum magnesium		p-value
					Normal	Low	
Parathyroid Hormone	Normal	0.483	1	34	14 (41.2%)	20 (58.8%)	0.487[f]
	High			66	32 (48.5%)	34 (51.5%)	
Serum Calcium	Normal	0.961	1	69	34 (49.3%)	35 (50.7%)	0.327[f]
	Low			31	12 (38.7%)	19 (61.3%)	
Serum Uric Acid	Normal	0.000	1	74	34 (45.9%)	40 (54.1%)	0.985[f]
	High			26	12 (46.2%)	14 (53.8%)	
Serum Phosphate	Normal	5.487	1	43	14 (32.6%)	29 (67.4%)	0.019[b]
	High			57	32 (56.1%)	25 (43.9%)	
Hemoglobin	Normal	0.000	1	24	11 (45.8%)	13 (54.2%)	0.985[f]
	Low			76	35 (46.1%)	41 (53.9%)	
Ferritin	Normal	0.015	1	45	21 (46.7%)	24 (53.3%)	0.904[f]
	Low			55	25 (45.5%)	30 (54.5%)	

Notes: sample size n=100, Significance levels at P<0.05[a], P<0.01[b], P<0.005[c], P<0.001[d], P<0.0001[e], and non-significant[f], X^2 value – Chi-squared value; df – degrees of freedom.

The association between drugs (PPI, diuretics, and ACE1/ARB) prescribed to CKD patients on hemodialysis and hypomagnesemia was determined in the present study. The omeprazole and loop diuretics were given as PPI and diuretics, respectively. Among 100 patients, 59% were on omeprazole (20 mg to 80 mg), 67% were on loop diuretics, and 44% were on ACE1/ARB. According to the observations, 83%, 67% and 9.0% of patients were on PPI, diuretics, and ACE1/ARB drugs, respectively. The findings revealed that a higher significance (P<0.0001; Table 5) was found between the drug history of CKD patients and hypomagnesemia.

Table 5
Comparison of serum magnesium and drug history

Drug history		X^2 value	df	Total number (%)	Serum magnesium		p-value
					Normal	Low	
Proton Pump Inhibitor	On PPI	48.892	1	59	10 (16.9%)	49 (83.1%)	0.0001[e]
	Not On PPI			41	36 (87.8%)	5 (12.2%)	
Diuretics	On Diuretics	34.776	1	67	17 (25.4%)	50 (74.6%)	0.0001[e]
	Not On Diuretics			33	29 (87.9%)	4 (12.1%)	
ACE1/ARB	On ACE1	63.794	1	44	40 (90.9%)	4 (9.1%)	0.0001[e]
	Not On ACE1			56	6 (10.7%)	50 (89.3%)	

Notes: sample size n=100, Significance levels at P<0.05[a], P<0.01[b], P<0.005[c], P<0.001[d], P<0.0001[e], and non-significant[f], X^2 value – Chi-squared value; df – degrees of freedom; PPI – proton pump inhibitor.

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DISCUSSION

The present investigation uses various parameters to evaluate the causes of serum Mg insufficiency and the frequency of hypomagnesemia in patients with chronic kidney disease (CKD) following hemodialysis. The findings have revealed that a few parameters (DM, cardiovascular disease, and drugs prescribed) potentially correlate with hypomagnesemia in CKD patients. In the present study, positive inverse significance has been observed between IHD and hypomagnesemia in CKD patients. The previous study demonstrated by Dey et al. [31] showed that the low magnesium levels observed in CKD patients also positively correlated with cardiovascular risk, suggesting low magnesium levels are a marker of elevated cardiovascular risk in CKD. It is found that magnesium can increase the high-density lipoprotein and suppress the low-density lipoprotein and triglyceride. Thus, declining magnesium may have a pathogenic role in cardiovascular disease. The study has also demonstrated an association between hypertension and hypomagnesemia in CKD. However, the findings of the current study contradicted the results of Dey et al. [31].

The regulation of glucagon and insulin signaling, phosphorylation of the insulin receptor, and cellular glucose uptake require normal Mg homeostasis. It is stated that a decrease in the incidence of DM can be mediated by regular intake of Mg [32]. A study conducted by Sakaguchi et al. [33] stated that a deficiency of Mg is a novel risk for renal disease patients with DM. A total of 455 subjects were involved in the study; they were grouped as diabetic and non-diabetic samples with CKD. The findings revealed that higher significance was observed in the group with Mg deficiency than in the high Mg group in primary characteristics in CKD patients with DM. The present study also presented a strong correlation between the decline in Mg levels and the progression of CKD with DM. Pham et al. [34] stated that there is a negative correlation between the GFR and hypertension, diuretics, hemoglobin, calcium, and Mg in DM patients between hypomagnesemia and renal disease.

Two mechanisms – oral phosphate binder and simply increasing the Mg concentration – were proposed to increase the Mg concentration in dialysate fluid. First, the calcium-phosphate crystal growth may be delayed by phosphate-Mg binding, thus allowing the Mg to affect the deposition of the crystal. Secondly, Mg promotes calcification and suppresses the differentiation of vascular smooth muscle into an osteogenic phenotype [35]. Fang et al. [36] suggested that bone and mineral disease are critical complications in the advanced stages of kidney disease, the effects of hypomagnesemia in CKD patients with secondary hyperthyroidism were analyzed using parameters including parathyroid, hemoglobin, uric acid, and calcium. The results demonstrated a significant correlation between Mg disturbance and hemoglobin, uric acid, and calcium, but no significance was observed between Mg decline and parathyroid hormone.

However, Ohya et al. [37] reported that hypermagnesemia is observed in patients with low parathyroid hormones. However, in the present study, no significant correlation was found between hypomagnesemia in CKD patients and blood parameters (parathyroid hormone, calcium, uric acid, and hemoglobin). Jandaghi et al. [38] studied the effect of sevelamer and calcium carbonate on preventing hypomagnesemia in CKD patients under hemodialysis. The outcome of the study revealed that both drugs had a positive influence on serum Mg. However, calcium carbonate and sevelamer drugs did not change serum calcium and phosphate levels.

Cardiac arrhythmias, CKD, gastroesophageal reflux disease patients treated with PPI and heart disease, and hypertensive patients treated with higher doses of diuretics are prone to depletion in their Mg levels [39]. The pH of the intestine changes the administration of PPI; a rise in gastric pH leads to an elevation in the small intestine, which affects the solubility and absorption of Mg in the intestine. The PPI also can increase the colonic pH, which in turn reduces TRPM6 activity and the absorption of Mg [40, 41]. A decline in the reabsorption of paracellular Mg induced by diuretics leads to a loss of serum Mg and hypomagnesemia [42].

A recent study by Zhang et al. [40] suggests the monitored and optimal use of PPI in CKD patients because it can lead to conditions like abdominal aortic calcification, hip fracture, and hypomagnesemia. In CKD patients, hypomagnesemia results from using drugs, including PPI, calcineurin inhibitors, and diuretics. Hypomagnesemia is associated with an elevated mortality rate in CKD patients, significantly increasing cardiovascular mortality. A potential relationship has been observed between hypomagnesemia and drugs (beta-blockers, diuretics, ACE1/ARB, and PPI) prescribed to CKD patients [43]. In the present study, a higher significance is seen between the hypomagnesemia and drug history of CKD patients. In previous literature, patients with drug history, DM, hypertension, and cardiovascular disease with hypomagnesemia have been analyzed for the development of CKD. The current study has given new insight: CKD patients under hemodialysis with comorbidities including DM, hypertension, and cardiovascular disease are checked for hypomagnesemia conditions and their role in CKD progression.

■ CONCLUSION

The duration of dialysis has shown potential significance for hypomagnesemia in CKD patients. The blood parameters did not correlate with the decline in Mg. The present outcome implies that the deficiency in serum Mg is due to the loss of efficiency in the reabsorption of Mg. Monitoring PPI and diuretics drugs is essential for CKD patients. Chronic diseases, including a history of DM and IHD, are possible risk factors that induce hypomagnesemia in CKD patients and may also mediate the progression of CKD. In the future, to conform to this finding, the work has to be carried out with an increased sample size and parameters.

■ REFERENCES

1. Charles C, Ferris AH. Chronic Kidney Disease. *Prim Care.* 2020 Dec;47(4):585–595.
2. Kovesdy CP. Epidemiology of chronic kidney disease: an update 2022. *Kidney Int Suppl (2011).* 2022;12(1):7–11.
3. Varughese S, Abraham G. Chronic Kidney Disease in India: A Clarion Call for Change. *Clin J Am Soc Nephrol.* 2018;13(5):802–804.
4. Fogo AB. Mechanisms of progression of chronic kidney disease. *Pediatr Nephrol.* 2007;22(12):2011–22.
5. Webster AC, Nagler EV, Morton RL, Masson P. Chronic Kidney Disease. *Lancet*. 2017;389(10075):1238–1252.
6. Kazancioğlu R. Risk factors for chronic kidney disease: an update. *Kidney Int Suppl (2011).* 2013;3(4):368–371.
7. Naber T, Purohit S. Chronic Kidney Disease: Role of Diet for a Reduction in the Severity of the Disease. *Nutrients.* 2021;13(9):3277. doi: 10.3390/nu13093277
8. Chen TK, Knicely DH, Grams ME. Chronic Kidney Disease Diagnosis and Management: A Review. *JAMA.* 2019;322(13):1294–1304.
9. Pugh D, Gallacher PJ, Dhaun N. Management of Hypertension in Chronic Kidney Disease. *Drugs.* 2019;79(4):365–379.
10. Banerjee D, Rosano G, Herzog CA. Management of Heart Failure Patients with CKD. *Clin J Am Soc Nephrol.* 2021;16(7):1131–1139.
11. Hong WP, Lee YJ. The association of dialysis adequacy, body mass index, and mortality among hemodialysis patients. *BMC Nephrol.* 2019 Oct 22;20(1):382. doi: 10.1186/s12882-019-1570-0
12. Ammirati AL. Chronic kidney disease. *Revista da Associação Médica Brasileira.* 2020;66:s03–9.
13. Jahnen-Dechent W, Ketteler M. Magnesium basics. *Clinical kidney journal.* 2012;5(Suppl_1):i3–14.
14. Volpe SL. Magnesium in disease prevention and overall health. *Adv Nutr.* 2013;4(3):378S–83S.

15. Ray E, Mohan K, Ahmad S, Wolf MT. Physiology of a Forgotten Electrolyte-Magnesium Disorders. *Advances in Kidney Disease and Health.* 2023;30(2):148–63.
16. Chubanov V, Mittermeier L, Gudermann T. Role of kinase-coupled TRP channels in mineral homeostasis. *Pharmacology & therapeutics.* 2018;184:159–76.
17. De Baaij JH, Hoenderop JG, Bindels RJ. Regulation of magnesium balance: lessons learned from human genetic disease. *Clinical kidney journal.* 2012;5(Suppl_1):i15–24.
18. De Baaij JH, Hoenderop JG, Bindels RJ. Magnesium in man: implications for health and disease. *Physiol Rev.* 2015;95(1):1–46.
19. Tinawi M. Disorders of Magnesium Metabolism: Hypomagnesemia and Hypermagnesemia. *Archives of Clinical and Biomedical Research.* 2020;4(2020):205–220.
20. Gröber U, Schmidt J, Kisters K. Magnesium in Prevention and Therapy. *Nutrients*. 2015;7(9):8199–226.
21. Gragossian A, Bashir K, Bhutta BS, Rotem Friede. *Hypomagnesemia*. Updated 2022 Nov 4. In: StatPearls (Internet).
22. Barbagallo M, Veronese N, Dominguez LJ. Magnesium in Aging, Health, and Diseases. *Nutrients.* 2021;13(2):463.
23. Van Laecke S. Hypomagnesemia and hypermagnesemia. *Acta Clinica Belgica.* 2019;74(1):41–7.
24. Larsson SC, Orsini N, Wolk A. Dietary magnesium intake and risk of stroke: a meta-analysis of prospective studies. *The American journal of clinical nutrition*. 2012;95(2):362–6.
25. Martin KJ, Gonzalez EA, Slatopolsky E. Clinical consequences and management of hypomagnesemia. *Journal of the American Society of Nephrology*. 2009;20(11):2291–5.
26. Ferrè S, Li X, Adams-Huet B, et al. Low serum magnesium is associated with faster decline in kidney function: the Dallas Heart Study experience. *Journal of Investigative Medicine*. 2019;67(6):987–94.
27. Cunningham J, Rodríguez M, Messa P. Magnesium in chronic kidney disease Stages 3 and 4 and in dialysis patients. *Clinical kidney journal.* 2012;5(Suppl_1):i39–51.
28. De Francisco AL, Varas J, Ramos R, et al. Proton pump inhibitor usage and the risk of mortality in hemodialysis patients. *Kidney international reports.* 2018;3(2):374–84.
29. Pérez-García R, Jaldo MT, Puerta M, et al. Hypomagnesemia in hemodialysis is associated with increased mortality risk: Its relationship with dialysis fluid. *Nefrología (English Edition).* 2020;40(5):552–62.
30. Liamis G, Hoorn EJ, Florentin M, Milionis H. An overview of diagnosis and management of drug-induced hypomagnesemia. *Pharmacology Research & Perspectives.* 2021;9(4):e00829.
31. Dey R, Rajappa M, Parameswaran S, Revathy G. Hypomagnesemia and atherogenic dyslipidemia in chronic kidney disease: surrogate markers for increased cardiovascular risk. *Clinical and experimental nephrology.* 2015;19:1054–61.
32. Pethő ÁG, Tapolyai M, Browne M, Fülöp T. Hypomagnesemia as a Risk Factor and Accelerator for Vascular Aging in Diabetes Mellitus and Chronic Kidney Disease. *Metabolites.* 2023;13(2):306.
33. Sakaguchi Y. The emerging role of magnesium in CKD. *Clin Exp Nephrol.* 2022;26(5):379–384.
34. Pham PC, Pham PM, Pham PT. Patients with diabetes mellitus type 2 and hypomagnesemia may have enhanced glomerular filtration via hypocalcemia. *Clinical nephrology.* 2012;78(6):442–8.
35. Ter Braake AD, Shanahan CM, de Baaij JHF. Magnesium Counteracts Vascular Calcification: Passive Interference or Active Modulation? *Arterioscler Thromb Vasc Biol.* 2017;37(8):1431–1445.
36. Fang L, Tang B, Hou D, et al. Effect of parathyroid hormone on serum magnesium levels: the neglected relationship in hemodialysis patients with secondary hyperparathyroidism. *Renal Failure.* 2016;38(1):50–6.
37. Ohya M, Negi S, Sakaguchi T, et al. Significance of serum magnesium as an independent correlative factor on the parathyroid hormone level in uremic patients. *The Journal of Clinical Endocrinology & Metabolism.* 2014;99(10):3873–8.
38. Jandaghi E, Yarmohammadi M, Ghorbani R, et al. Comparison of Sevelamer and Calcium Carbonate in Prevention of Hypomagnesemia in Hemodialysis Patients. *Int J Prev Med.* 2021;12:104. doi: 10.4103/ijpvm.IJPVM_464_19
39. Chrysant SG, Chrysant GS. Association of hypomagnesemia with cardiovascular diseases and hypertension. *International Journal of Cardiology Hypertension.* 2019;1:100005.
40. Zhang Y, Deng D, Zhang R, et al. Relationship between Proton Pump Inhibitors and Adverse Effects in Hemodialysis Patients: A Systematic Review and Meta-Analysis. *Kidney Blood Press Res*. 2022;47(9):545–555.
41. Gommers LM, Hoenderop JG, de Baaij JH. Mechanisms of proton pump inhibitor-induced hypomagnesemia. *Acta Physiologica.* 2022;235(4):e13846.
42. Gröber U. Magnesium and drugs. *International journal of molecular sciences.* 2019;20(9):2094.
43. Azem R, Daou R, Bassil E, et al. Serum magnesium, mortality and disease progression in chronic kidney disease. *BMC Nephrol.* 2020;21(1):49. doi: 10.1186/s12882-020-1713-3

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Investigation of Various Immunological Parameters in Post-Menopause Osteoporotic Women in Basrah

Conflict of interest: nothing to declare.
Authors' contribution: Bassam Dawood – conceptualization, data curation, investigation, methodology, project administration, resources, software, validation, visualization, writing – original draft and writing – review & editing; Naael Ali – conceptualization, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing – original draft and writing – review & editing; Shereen Al-Ali – conceptualization, data curation, investigation, project administration, resources, software, supervision, validation, visualization, writing – original draft and writing – review & editing.
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Abstract

Introduction. Osteoporosis is a progressive skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with increased bone fragility and fracture susceptibility.
Purpose. To investigate the role of various immunological parameters including soluble receptor activator of nuclear factor kappa B ligand (sRANKL), sclerostin, macrophage colony stimulating factor (M-CSF), cathepsin K, and IL-17 in postmenopausal osteoporotic women.
Materials and methods. A total of 128 women were included in the current study and they were categorized as postmenopausal osteoporotic women (PO) (n=43), postmenopausal non-osteoporotic women (PNO) (n=45), and non-osteoporotic young women (YA) (n=40) according to their DXA score. A venous blood sample was taken from each woman to estimate the serum levels of sRANKL, sclerostin, M-CSF, cathepsin K, and IL-17 by ELISA, the data were statistically analyzed using suitable tests.
Results. The current study showed that sRANKL was significantly increased in PO compared to PNO and YA, with no significant difference between PNO and YA groups. Sclerostin level is significantly elevated in PO compared to PNO with no differences between PNO and YA groups. M-CSF level is significantly elevated in PO compared to PNO and YA groups, with no statistical differences between PNO and YA. Finally, no significant differences among study groups regarding the cathepsin K and IL-17 levels were found.
Conclusion. A crucial rule of soluble RANKL, sclerostin, and M-CSF in inducing postmenopausal osteoporosis is proved and their estimation in blood may be beneficial in detecting these cases. Blood level may not give a real estimate of cathepsin K in osteoporotic cases. The observed osteoporotic effect of IL-17 at elevated levels cannot proven.
Keywords: osteoporosis, post menopause, sRANKL, M-CSF, sclerostin, cathepsin K, IL-17

INTRODUCTION

Osteoporosis is a common disease [1]. World Health Organization (WHO) defined osteoporosis as a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with increased bone fragility and susceptibility to fracture [2]. Osteoporosis has been called "the silent epidemic of the 21st century" because of its public health impact. It is a severe, chronic, progressive and clinically silent disease that leads to serious sequelae of morbidity, mortality and increased social and economic burdens at an individual and population level [3–5]. A local study conducted in Basrah – Iraq in 2018 on 172 women complaining of bone pain and investigated by DXA scan showed (54.08%) of post-menopausal women were in the osteoporotic range, while in premenopausal women (7.48%) had osteoporosis [6].

Osteoporosis is classified into primary and secondary osteoporosis [7]. Primary osteoporosis is an age-related condition affects both sexes, induced by hormonal and nutritional factors such as vitamin D, however, connection between vitamin D and BMD is not yet proven [8], low levels of some electrolytes are observed in postmenopausal osteoporosis, especially calcium, magnesium and potassium [9]. Bone health is affected by decreased osteocalcin hormone, in obese men with fatty liver [10]. Secondary osteoporosis results from various medical conditions and medications [12, 13].

Bone homeostasis requires balanced activity of osteoclast and osteoblast cells, therefore factors that disturb this balance result in greater bone resorption than bone formation with a consequent decrease in bone strength [14]. However, it is stated that loss of bone mass is more often associated with dysregulated osteoclast production and function rather than impaired osteoblast activity [15]. Thus, applying successful osteoporosis preventive measures requires a full understanding of the mechanisms regulating the proliferation, differentiation, and activation of osteoclast cells [16]. Osteoclasts can resorb bone and play a central role in bone homeostasis and pathological bone loss such as osteoporosis [17]. Osteoclasts are large, multinucleated cells originating from hematopoietic stem cells, formed by the fusion of myeloid progenitor cells [18, 19].

M-CSF binds to its specific receptor, colony stimulating factor 1 (c-Fms) receptor, present on the surface of osteoclast precursor cells leading to the activation of various intracellular signals essential for the growth and survival of osteoclast precursor cells [20, 21]. Furthermore, M-CSF/c-Fms binding upregulates the expression of receptor activator of nuclear factor kappa-B (RANK) on osteoclast precursors. The subsequent binding of RANKL with RANK triggers the terminal differentiation into mature osteoclast [22, 23]. RANKL is indispensable for osteoclast differentiation [24, 25]. RANKL is present in 2 forms: soluble in serum and membrane-bound on the surface of RANKL secreting cells [26]. Soluble RANKL (sRANKL) and membrane RANKL (mRANKL) often have comparable roles in osteoclast development. On the other hand, excessive bone resorption brought on by overexpressed sRANKL might result in osteoporosis [27]. Recently, researchers identified a binding location via molecular dynamic simulations that permits a tiny chemical to specifically disrupt sRANKL-RANK connection while leaving the mRANKL-RANK relationship intact [28]. Through the RANK receptor, RANKL and M-CSF can stimulate osteoclast growth and activation [29]. Macrophages are distinguished from monocytes by M-CSF and are crucial in the activation and development of osteoclasts [30]. During fracture-associated bone regeneration, macrophages have an impact on bone production. On the other hand, proinflammatory cytokines secreted by activated macrophages can

cause osteoclastogenesis, which leads to bone loss and can also trigger bone resorption. In patients with osteoporosis, targeting activated macrophages at the right time may help prevent or delay the deterioration of bone loss [31].

The SOST gene encodes sclerostin, a protein found in human bone tissue. Sclerostin participates in the anti-anabolic processes of bone formation. The transcription of SOST gene is regulated by many components in the gene. Winkler et al.'s investigation yielded the first detection of sclerostin in adult human osteocytes. Research has also demonstrated that hypertrophic chondrocytes contain this protein. Sclerostin is a potent inhibitor of osteoblastogenesis [32, 33].

Activated osteoclasts are the main source of cathepsin K (CatK), which breaks down collagen and other matrix proteins during bone resorption [34]. The crucial pro-osteoclastogenic transcriptional factor NFATc1 (nuclear factor of activated T cells cytoplasmic protein 1) is stimulated to commence the transcription of CatK when the RANKL-RANK signaling pathway is active in osteoclast precursors [35].

IL-17 as a proinflammatory cytokine is elevated in inflammatory diseases like Rheumatoid arthritis [36]. IL-17 enhances osteoclast differentiation by stimulating osteocytes and osteoblasts, via upregulation of RANK production, to produce higher levels of RANKL [37], upregulates TNF-α and IL-1 production by macrophages [38], also promotes osteoclast formation through activating JNK signaling [39]. Additionally, IL-17 induces expression of M-CSF and RANKL on human mesenchymal stem cells to support osteoclastogenesis [40]. On osteoblasts; IL-17 has a positive effect on the early differentiation of primary osteoblasts and an inhibitory effect on osteoblast calcification [41].

■ PURPOSE OF THE STUDY

To investigate the role of various immunological parameters including soluble receptor activator of nuclear factor kappa B ligand (sRANKL), sclerostin, macrophage colony stimulating factor (M-CSF), cathepsin K, and IL-17 in postmenopausal osteoporotic women.

■ MATERIALS AND METHODS

A case control study was conducted from December 2022 to August 2023 on 128 women who attended Rheumatology and Rehabilitation Clinic at Al Basrah Teaching Hospital: 88 of them were postmenopausal women with/without osteoporosis and 40 young female controls, after obtaining their signed permission on a consent form, all were examined by bone densitometry for the lumbar spines (L1-L4) to estimate Bone Mineral Density (BMD) and Trabecular Bone Score (TBS), expressed as T-score value for postmenopausal women and Z-score value for the young control females. According to WHO criteria, women with a T-score ≤–2.5 represent postmenopausal osteoporotic group (PO) (n=43), women with T-score >–2.5 represent postmenopausal non-osteoporotic group considered as age-match control (PNO) (n=45), and young females with Z-score >–2 as baseline control group (YA) (n=40). A peripheral whole blood sample was taken from each participant and the serum level of sRANKL, sclerostin, M-CSF, cathepsin K, and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA) using various kits including; Human Cathepsin K ELISA Kit (FY-EH6483), Human Interleukin 17 ELISA kit (FY-EH6678), Human M-CSF ELISA kit (FY-EH6380), Human Sclerostin ELISA Kit (FY-EH6388),

and Human sRANKL ELISA Kit (FY-EH6391). The resulting data were statistically analyzed using normality test at the beginning then the Mann – Whitney U test.

■ RESULTS

The results of the current study regarding the age of the participants showed that the median age of the postmenopausal osteoporotic group (PO) was 65 (50–80) years and the median age of the postmenopausal non osteoporotic (PNO) was 60 (42–78) years while the control group represented by the young females (YA) was 27 (16–31) years.

The results of the current study regarding the sRANKL showed that the level of sRANKL was significantly increased in PO group in compare to YA and PNO groups, while there was no statistical difference between PNO and YA: 108.2 (12.5–919.82), 62.9 (8.86–175.49) and 33.4 (135–158.18) pg/ml respectively. The p values between PO vs YA, PO vs PNO and PNO vs YA were p=0.019, p=0.023 & p=0.39, respectively (fig. 1A).

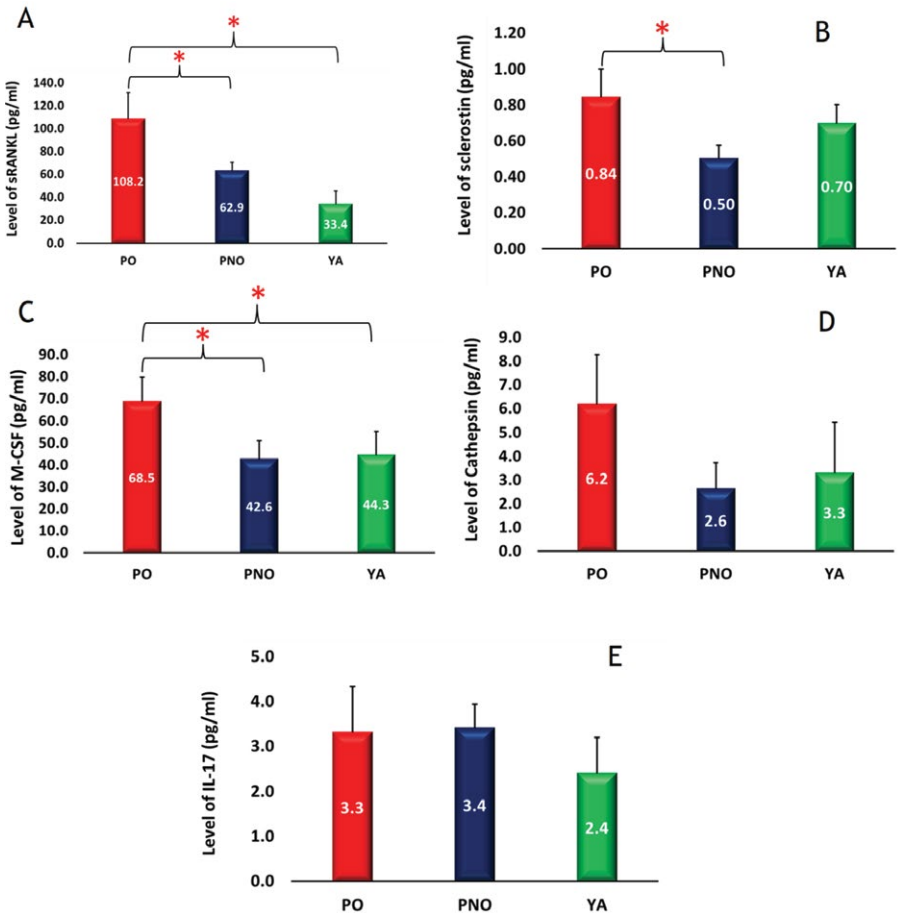

Fig. 1. A – The serum level of sRANKL in study groups; B – The serum level of sclerostin in study groups; C – The serum level of M-CSF in study groups; D – The serum level of Cathepsin K in study groups; E – The serum level of IL-17 in study groups

The result regarding sclerostin level showed that the median level in PO, PNO and YA groups were 0.84 (0.5–5.21), 0.50 (0.5–2.35) and 0.70 (0.5–1.76) pg/ml, respectively. The results showed a significant elevation in PO compared to PNO (p value = 0.033), with a non-significant elevation in PO compared to YA (p value = 0.45). While, there was non-significant downregulation in PNO compared to YA groups (p=0.41) as shown in (fig. 1B).

The result regarding M-CSF level showed that the median level in PO, PNO and YA groups were 68.5 (8.75–348.94), 42.6 (3.75–182.72) and 44.3 (2.5–161.24) pg/ml, respectively. The results showed a significant elevation in PO in compare to PNO and YA with a p value = 0.035 and p=0.036 respectively. While there were no statistical differences between PNO and YA with p=0.61 (fig. 1C).

The result regarding Cathepsin K level showed that the median level in PO, PNO and YA groups were 6.2 (0.67–57.2), 2.6 (1.24–22.09) and 3.3 (1.03–24.86) pg/ml, respectively. The results showed non-significant elevation in PO compared to PNO and YA with a p value = 0.48, 0.74 respectively, while there was no difference between PNO and YA with a p value = 0.62. As shown in fig. 1D.

The result regarding IL-17 level showed that the median level in PO, PNO and YA groups were 3.3 (0.8–33), 3.4 (1–13.4) and 2.4 (1.2–9.8) pg/ml, respectively. The results showed a non-significant difference among PO, PNO and YA with a p value = 0.94, 0.68 and 0.55 in PO vs PNO, PO vs YA and PNO vs YA, respectively (fig. 1E).

■ DISCUSSION

RANKL is a cytokine that has an absolutely essential role in osteoporosis; since this secreted ligand is the sole master cytokine that specifically induces osteoclastogenesis and to date, there is no RANKL independent osteoclastogenesis [24]. Increased RANKL will lead to elevation in the osteoclast population, with a consequent increase in bone absorption. RANKL have three isoforms; isoforms 1 and 3 are trans-membrane, while isoform 2 is soluble form [26].

The sRANKL is either directly secreted from helper T cells or detached from its position on the cell membrane by metalloproteinases. It is reported that sRANKL also enhances osteoclastogenesis [42]. The present study found that the level of serum sRANKL was significantly increased in PO group in compare to PNO and YA group, while there was a statistically non-significant difference in sRANKL level in PNO in compare to YA. This finding agrees with the reported finding that osteoporotic postmenopausal women have higher sRANKL levels than non-osteoporotic postmenopausal women [26]. Consequently, indicates a high osteoclastogenesis rate in PO group.

In the current study, the level of M-CSF showed a significant elevation in PO group in compare to PNO and YA groups, while there is no significant difference in M-CSF level between PNO and YA groups. This result agrees with the reported findings of high M-CSF levels found in post-menopausal osteoporotic women [44], the complementary role of M-CSF and RANKL as essential stimulating factors for osteoclastogenesis [43], and elevated M-CSF levels cause elevation of sRANKL levels via upregulating RANK expression[45]. This finding supports the immunological role in the pathogenesis of postmenopausal osteoporosis through osteoclasts, RANKL and M-CSF [43].

Sclerostin is one of the most vital products of the osteocyte cells, this secreted ligand binds to Frizzled receptor and Low density lipoprotein receptor-related protein

5 on osteoblast precursor cells leading to blocking Wingless/Integrase-1 (Wnt) proteins signalling pathway thereby preventing the activation and nuclear translocation of the transcription factor β-catenin which induces transcription of genes involved in osteoblast differentiation [46]. The current study found that sclerostin level is significantly elevated in PO group compared to PNO, while there were no significant differences between PNO and YA. This finding agrees with the reported effect of high sclerostin levels on decreasing bone mass [47], through suppressing bone formation[11], augmenting RANKL production and suppressing osteoprotegrin which is a decoy receptor for RANKL [55].

Cathepsin K is a cysteine protease, highly expressed by osteoclasts, secreted into the resorption lacune, a space formed between ruffled membrane of the osteoclast cell and the adhering bone surface. Cathepsin-K degraded type I collagen [48], elastin, gelatin, osteopontin and osteonectin which are bone matrix proteins [30, 49]. The expression of cathepsin K in osteoclast cells is largely mediated by RANKL induced activation of NFATc1 [50].

The current study found an elevated level of Cathepsin-K in PO group in compare to PNO and YA groups, although not reach statistical significance, this finding may still be important since clinical data about serum cathepsin K levels are limited; because the concentrations of this enzyme in the circulation are very low and accurate estimation of this enzyme remains challenging [51]. In addition, the limited number of included women in the current study may participate in not reaching the significance limits. Accordingly, this finding may agree with the reported effect of cathepsin K as an osteoporotic enhancing factor [52]. Importantly, the current study found that the level of Cathepsin K was lower in PNO group compared to YA group, this result agrees with the reported findings that serum Cathepsin K level decreases with age in both healthy women and men, contrasting the effect of ageing in rapid bone loss [53].

IL-17 induces RANKL production by osteoblasts and osteocytes via upregulating RANK expression on osteoclast precursors, thereby enhancing osteoclastogenesis and bone resorption [37]. In the current study, the level of interleukin-17 showed non-significant differences among PO, PNO, and YA groups. This finding may agree with the reported ambiguous effect of IL-17 on osteoclastogenesis; because various studies reported contradictory results [54]. Importantly, studies demonstrate that high concentrations of IL-17 prevent the differentiation of osteoclast precursors into osteoclasts, also high concentrations of IL-17 prevent matrix protein hydrolysis during bone resorption by downregulating the cathepsin K and MMP-9 expression in osteoclasts so the function of IL-17 changes with increasing concentrations [56]. On the other hand, IL-17 is known to direct mesenchymal stem cell differentiation from adipogenesis to osteogenesis and enhance differentiation of pre-osteoblast to osteoblast [54].

■ CONCLUSION

A crucial rule of soluble RANKL, sclerostin, and M-CSF in inducing postmenopausal osteoporosis is proved and their estimation in blood may be beneficial in detecting these cases. Blood level may not give a real estimate of cathepsin K in osteoporotic cases. The observed osteoporotic effect of IL-17 at elevated levels cannot proven.

REFERENCES

1. Salari N, Ghasemi H, Mohammadi L, et al. The global prevalence of osteoporosis in the world: a comprehensive systematic review and meta-analysis. *J Orthop Surg Res.* 2021 Oct 17;16(1):609. doi: 10.1186/s13018-021-02772-0
2. Curtis EM, Harvey NC, Cooper C. *The burden of osteoporosis*. In: *Harvey NC, Cooper C (eds) Osteoporosis: a life course epidemiology approach to skeletal health.* CRC Press, BocaRaton, 2018;1–20. Available at: https://www.taylorfrancis.com/books/edit/10.1201/9781351234627/osteoporosis-nicholas-harvey-cyrus-cooper
3. González LA, Vásquez GM, Molina JF. Epidemiología de la osteoporosis. *J. Rev. Colomb. Reumatol,* 2009;16:61–75.
4. Wade S.W., Satram-Hoang S., Nadkar A., et al. Impact of Medication Adherence on Health Care Utilization and Productivity: Self-Reported Data From a Cohort of Postmenopausal Women on Osteoporosis Therapy. *Clinical Therapeutics.* 2011;33(12):2006–2015. Available at: https://doi.org/10.1016/j
5. Weycker D, Li X, Barron R, et al. Hospitalizations for osteoporosis-related fractures: Economic costs and clinical outcomes. *Bone Rep.* 2016;5:186–191. doi: 10.1016/j.bonr.2016.07.005
6. Mustafa A Almajeed, Thamer A Hamdan. Dual energy x-ray absorptiometry for diagnosis of bone mineral density in pre and post-menopausal women. *J. Phys.,* 2019. Conf. Ser. 1294.062024
7. Aibar-Almazán A., Voltes-Martínez A., Castellote-Caballero Y. et al. Current Status of the Diagnosis and Management of Osteoporosis. *Int. J. Mol. Sci.* 2022;23:9465. Available at: https://doi.org/10.3390/ijms23169465
8. Kamal Breesam Lafta, Muntadher Abdulkareem Abdullah, Fatih A. Al Khaqani, Akeel M. Jaber. The Prevalence of Osteoporosis in Chronic Liver Disease Patients Seen in Basrah Gastroenterology and Hepatology Hospital. *The Egyptian Journal of Hospital Medicine,* 2023;91:4707–4711.
9. Hussein Mustafa, AL-Maliki Sami. Changes in electrolyte ions level in healthy and osteoporosis women in Basra province / Iraq. *Corresponding Author. World Journal of Pharmaceutical Research,* 2019;7:23–33. doi: 10.20959/ wjpr201817-13333
10. Al-Akabi D, Kata FS. Effect of Nonalcoholic Fatty Liver Diseases on Some of Bone Biomarkers in Men. *Open Access Maced J Med Sci.* 2021;9(A):924–927. Available at: https://doi.org/10.3889/oamjms.2021.6892
11. Nusse R, Clevers H. Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. *Cell*. 2017;169:985–999. Available at: https://doi.org/10.1016/j.cell.2017.05.016
12. Keen MU, Reddivari AKR. *Osteoporosis in Females*. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. 2023.
13. Sözen T, Özı¸sık L, Ba¸saran N. An overview and management of osteoporosis. *Eur. J. Rheumatol.* 2017;4:46–56.
14. Kenkre JS, Bassett JHD. The bone remodelling cycle. *Ann. Clin. Biochem.* 2018;55:308–327. doi: 10.1177/0004563218759371
15. Feng X, Teitelbaum SL. Osteoclasts: new insights. *Bone Res.* 2013;1:11–26. doi: 10.4248/BR201301003
16. Park-Min KH. Mechanisms involved in normal and pathological osteoclastogenesis. *Cell. Mol. Life Sci.* 2018;75:2519–2528. doi: 10.1007/s00018-018-2817-9
17. Tsukasaki M, Takayanagi H. Osteoclast biology in the single-cell era. *Inflammation and Regeneration.* 2022;42. Available at: https://doi.org/10.1186/s41232-022-00213-x
18. Ikeda K., Takeshita S. The role of osteoclast differentiation and function in skeletal homeostasis. *J. Biochem.* 2016;159:1–8.
19. Abdelmagid SM, Sondag GR, Moussa FM, et al. Mutation in osteoactivin promotes receptor activator of NFkB ligand (RANKL)-mediated osteoclast differentiation and survival but inhibits osteoclast function. *J Biol Chem.* 2015;290:20128–46. doi: 10.1074/jbc.M114.624270
20. Weilbaecher KN, Motyckova G, Huber WE, et al. Linkage of M-CSF signaling to Mitf, TFE3, and the osteoclast defect in Mitfmi/mi mice. *Mol. Cell.* 2001;8:749–758. doi: 10.1016/S1097-2765(01)00360-4
21. McGill GG, Horstmann M, Widlund HR, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell.* 2002;109:707–718. doi: 10.1016/S0092-8674(02)00762-6
22. Ansalone C. et al. TNF is a homoeostatic regulator of distinct epigenetically primed human osteoclast precursors. *Annals of the Rheumatic Diseases*. 2021;80(6):748–757.
23. Okamoto K, Nakashima T, Shinohara M, et al. Osteoimmunology: The conceptual framework unifying the immune and skeletal systems. *Physiol Rev.* 2017;97(4):1295–1349. Available at: https://doi.org/10.1152/physrev.00036.2
24. Takayanagi H. RANKL as the master regulator of osteoclast differentiation. *J Bone Miner Metab.* 2021;39:13–18. Available at: https://doi.org/10.1007/s00774-020-01191-1
25. Palagano E, Menale C, Sobacchi C, Villa A. Genetics of osteopetrosis. *Curr. Osteoporos. Rep.* 2018;16:13–25. doi: 10.1007/s11914-018-0415-2
26. Yunaini Luluk, Dwi Anita Suryandari, Lindawati Soetanto Kusdhany, et al. The Role of Receptor Activator of Nuclear Factor Kappa B (RANK) and Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) in Osteoporosis Risk: Gene Polymorphism and Soluble RANKL Level in Indonesia Post-Menopausal Women. *Journal of Biological Sciences.* 2018;18:358–364.
27. Lacey DL, Timms E, Tan HL, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 1998;93(2):165–76. doi: 10.1016/s0092-8674(00)81569-x
28. Huang D., Zhao C., Li R. et al. Identification of a binding site on soluble RANKL that can be targeted to inhibit soluble RANK-RANKL interactions and treat osteoporosis. *Nat Commun.* 2022;13:5338. Available at: https://doi.org/10.1038/s41467-022-33006-4
29. Chen X, Wang Z, Duan N, et al. Osteoblast-osteoclast interactions. *Connective tissue research.* 2018;59(2):99–107.
30. Drake MT, Clarke BL, Oursler MJ, Khosla S. Cathepsin K Inhibitors for Osteoporosis: Biology, Potential Clinical Utility, and Lessons Learned. *Endocrine reviews*. 2017;38(4):325–350. Available at: https://doi.org/10.1210/er.2015-1114
31. Yang DH, Yang MY. The Role of Macrophage in the Pathogenesis of Osteoporosis. *International journal of molecular sciences.* 2019;20(9):2093. Available at: https://doi.org/10.3390/ijms20092093
32. Winkler DG, Sutherland MK, Geoghegan JC, et al. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *The EMBO journal.* 2003;22(23):6267–6276. Available at: https://doi.org/10.1093/emboj/cdg599
33. Suen PK, Qin L. Sclerostin, an emerging therapeutic target for treating osteoporosis and osteoporotic fracture: A general review. *Journal of orthopaedic translation.* 2015;4:1–13. Available at: https://doi.org/10.1016/j.jot.2015.08.004
34. Costa AG, Cusano NE, Silva BC, et al. Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis. Nature reviews. *Rheumatology.* 2011;7(8):447–456. Available at: https://doi.org/10.1038/nrrheum.2011.77
35. Dai R, Wu Z, Chu HY, et al. Cathepsin K: The Action in and Beyond Bone. *Frontiers in cell and developmental biology.* 2020;8:433. Available at: https://doi.org/10.3389/fcell.2020.00433
36. Alaa A. Saleh, Shereen Al-Ali. The correlation between vitamin D3 and IL-17 in rheumatoid arthritis patients. *Eurasian Research Bulletin.* 2022;7:114–119. Available at: https://geniusjournals.org/index.php/erb/article/view/1139

37. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*. 2015;74:5–17. doi: 10.1016/j.cyto.2014.09.011
38. Hwang SY, Kim JY, Kim KW, et al. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF kappa B- and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther.* 2004;6(2):R120–8. doi: 10.1186/ar1038
39. Ke D, Fu X, Xue Y, et al. IL-17A regulates the autophagic activity of osteoclast precursors through RANKL-JNK1 signaling during osteoclastogenesis in vitro. *Biochem Biophys Res Commun.* 2018;497(3):890–6. doi: 10.1016/j.bbrc.2018.02.164
40. Huang H, Kim HJ, Chang EJ, et al. IL-17 stimulates the proliferation and differentiation of human mesenchymal stem cells: implications for bone remodeling. *Cell Death Differ.* 2009;16(10):1332–43. doi: 10.1038/cdd.2009.74
41. Wang Z, Tan J, Lei L, et al. The positive effects of secreting cytokines IL-17 and IFN-g on the early-stage differentiation and negative effects on the calcification of primary osteoblasts in vitro. *Int Immunopharmacol.* 2018;2–18;57:1–10. doi: 10.1016/j.intimp.2018.02.002
42. Rubin J, Greenfield E. *Osteoclast: Origin and Differentiation*. In: Bronner F, Farach-Carson MC and Rubin J (Eds.). Bone Respiration, Springer, Verlag London, 2005; 1–16.
43. Nakashima T, Hayashi M, Takayanagi H. New insights into osteoclastogenic signaling mechanisms. *Trends Endocrinol Metab.* 2012;23(11):582–90. doi: 10.1016/j.tem.2012.05.005
44. Hajer Mahmood, Eiman AA Abass. Macrophage Colony Stimulating Factor as Predictive Marker of Osteoporosis in T2DM Patients. *PJMHS.* 2022;6(06). Available at: https://doi.org/10.53350/pjmhs22166539
45. Mun SH, Park PS. The M-CSF receptor in osteoclasts and beyond. *Experimental & Molecular Medicine*. 2020;52(8):1239–1254. doi.org/10.1038/s12276-020-0484-z
46. Dar HY, Azam Z, Anupam R, et al. Osteoimmunology, The Nexus between bone and immune system. *Front Biosci.* 2018;23:464–92. doi: 10.2741/4600
47. Poole KES, van Bezooijen RL, Loveridge N, et al. Clerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J.* 2005;19:1842–1844. doi: 10.1096/fj.05-4221fje
48. Ross PE. In Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism (ed. Rosen CJ). 2008;16–22 (American Society for Bone and Mineral Research, Washington, D. C., 2008).
49. Liang W, Wang F, Chen Q, et al. Targeting cathepsin K diminishes prostate cancer establishment and growth in murine bone. *J. Cancer Res. Clin. Oncol.* 2019;145:1999–2012.
50. Troen BR. The regulation of cathepsin K gene expression. *Ann N Y Acad Sci.* 2006;1068:165–172.
51. Garnero P, Szulc P. *Assessment of Bone Turnover in Men Using Biochemical Markers. Osteoporosis in Men* (2nd edition). 2009;25–40.
52. Mukherjee K, Chattopadhyay N. Pharmacological inhibition of cathepsin K. A promising novel approach for postmenopausal osteoporosis therapy. *Biochem. Pharmacol.* 2016;117:10–19. doi: 10.1016/j.bcp.2016.04.010
53. Kerschan-Schindl K, Hawa G, Kudlacek S, et al. Serum levels of cathepsin K decrease with age in both women and men. *Exp. Gerontol.* 2005;40:532–535.
54. Youngkyun LEE. The role of interleukin-17 in bone metabolism and inflammatory skeletal diseases. *BMB reports.* 2013;46(10):479.
55. Nakashima T, Hayashi M, Fukunaga T, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nature Medicine.* 2011;17:1231–1234. Available at: https://doi.org/10.1038/nm.2452
56. Kitami S, Tanaka H, Kawato T, et al. IL-17A suppresses the expression of bone resorption-related proteinases and osteoclast differentiation via IL-17RA or IL-17RC receptors in RAW264.7 cells. *Biochimie*. 2010;92(4):398–404. doi: 10.1016/j.biochi.2009.12.011

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Effect of Hyaluronic Acid Filler on Skin Layers Thickness in Laboratory Rats

Conflict of interest: nothing to declare.
Authors' contribution: Afrah Ali Abdulameer – conceptualization, data curation, investigation, methodology, project administration, resources, software, visualization, writing – original draft and writing – review & editing; Satar Abood Faris – conceptualization, data curation, project administration, supervision, validation, visualization, writing – original draft and writing – review & editing.
Ethics statement: this study was conducted on laboratory rats with the approval of the Ethics Board of the Department of Life Sciences / College of Education for Pure Sciences, University of Thi Qar, according to the established protocol (No. 7 dated 10/4/2023).
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Abstract

Introduction. The majority of dermal fillers used for aesthetic reasons are hyaluronic acid fillers. The ease of application of fillers derived from this substance for both cosmetic and medicinal purposes, as well as its influence on the thickness of skin layers and the density of collagen, are the reasons behind this attribution.
Purpose. To determine the effect of hyaluronic acid filler on the thickness of different skin layers experimentally.
Materials and methods. 100 rats were divided into two groups, 50 males and 50 females, and each group was divided into 5 subgroups, each with a control group, for time periods of 13, 10, 9, 4, and 2 weeks, respectively. We treated these animals with dermal filler type Neuramis Volume Lidocaine in a dose of 0.1 ml in the subcutaneous layer.
Results. The findings demonstrated a statistically significant ($p<0.05$) augmentation in the thickness of the epidermal layer over the 2–4 week intervals, as well as a similarly significant rise during the 9–10 week intervals. Additionally, there was a pronounced increase during the 13 week period. A statistically significant ($p<0.05$) augmentation in the thickness of the dermis layer was detected, particularly during the 13 week period. Additionally, there was a steady increase in thickness at the 2, 4, 9, and 10 week periods, respectively. The statistical analysis revealed a noteworthy rise ($p<0.05$) in the thickness of the subcutaneous layer throughout the course of 13 weeks. Additionally, there was a comparable increase observed during the 2–4 week intervals, and a progressive increase during the 9–10 week period. The histology study corroborated the statistical findings, demonstrating a progressive rise over time in the rats subjected to dermal fillers.
Conclusion. Due to a variety of complications and potential long- or short-term functional and physiological changes, dermal fillers gradually increase the thickness of the various subcutaneous layers over time. Histological examination of the skin samples showed a gradual thickening of layers during the time periods, this change includes the epidermis, dermis, and subcutaneous layer.
Keywords: dermal filler, hyaluronic acid, skin thickness, laboratory rat, subcutaneous layer

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INTRODUCTION

Hyaluronic acid (HA)-based dermal fillers dominated the US market in 2015, representing over 92% of all fillers. Cross-linking often involves the chemical modification of the lengthy chains of hyaluronic acid (HA) included in these fillers [1]. Hyaluronic acid (HA) dermal fillers are considered the gold standard for dermal fillers due to their excellent performance and safety profile [2]. At this time, they are the most popular fillers. Dermal fillers made of HA are a type of glycosaminoglycan polymer. It is formed of two disaccharide molecules, D-glucuronic acid and N-acetyl-glucosamine, and is joined together by β-1,4 and β-1,3 glycosidic connections, respectively. Clear and viscous, HA is a thick liquid composed of unbranched single-chain polymers having molecular weights ranging from 105 to 107 kDa. The capacity of HA to bind and hold water molecules makes it a vital part of organs and connective tissues, ensuring that tissues remain hydrated [3]. Its absence of adverse reactions makes dermal fillers the most suitable choice for replenishing additional volume in soft tissues and skin, as many medical or cosmetic clinical indications have approved dermal fillers containing hyaluronic acid [4].

Hyaluronic acid (HA) has shown high efficacy in skin tightening, high elasticity, and improvement in cosmetic results with reduced wrinkle scars. Good results and efficacy in cosmetics have proven this, justifying the use of dermal fillers as a basic factor in cosmetic products [5]. The most common side effect of filler injection is pain at the injection site. Currently, doctors combine HA fillers with lidocaine to alleviate patient discomfort during injection [6]. Side effects may develop for several days up to one year after injection and include redness, pruritus, and swelling [7]. Histological analysis reveals various degrees of inflammation, including a moderate to severe inflammatory reaction involving lymphocytes, macrophages, and plasma cells surrounding the injected material, as well as a mild to chronic inflammatory infiltration [8]. The inflammation spreads to the subcutaneous layer, and fibrosis can be seen in the lower dermis [9]. Lumping is one of the alterations linked to HA dermal fillers that have been studied in clinical settings [10].

The continuation of early side effects of dermal fillers for several days or more may be due to a hypersensitivity reaction [11]. Nodules manifest as edematous, sclerosing, or cystic structures and manifest as clear and defined lesions shortly after injection [12]. One of the most important late symptoms is permanent telangiectasia with erythema at the injection site [13]. Filler injections in patients with telangiectasia can worsen the condition in terms of size and appearance [14]. Granulomas appear after a late period in all soft-tissue fillers, regardless of their type, and may persist for several months to years after filler injection [13]. Therefore, there is still a need to develop an effective filler associated with fewer side effects. With the development and emergence of new and different types and compositions of HA dermal fillers, it became necessary to prove their biodegradability using hyaluronidase as a safety measure against unexpected events. Researchers consider the in vivo animal studies system to be more reliable and efficient for evaluating HA dermal fillers, as it allows for monitoring of the degradation process in a setting that closely mimics the clinical environment [15]. Also, in the event of incorrect injection or at a high or inappropriate dose, it can be removed or repaired by hyaluronidase [16, 17].

Injectable fillers have a range of properties that make them ideal, including efficacy, safety, inexpensiveness, painlessness, resistance to infection, biocompatibility, volume retention upon application, ability to stabilize and stabilize in the surrounding tissue, and preferably minimal reaction to the foreign substance injected. The ideal injectable

material should be non-allergenic, require no testing, and be stable when stored at room temperature [11]. Several factors that reflect the local tissue environment and treatment modalities can be accounted for in an in vivo animal model. These factors include the placement of the hyaluronidase injection in relation to the dermal filler injection site, the swelling of the filler and integration of the tissue, the diffusion of the enzyme from the injection site, the inactivation and clearance of the enzyme, cellular interactions, and other inflammatory responses. When these factors are taken into account, the breakdown potential of HA dermal fillers by hyaluronidase can be more precisely evaluated [18, 19].

■ PURPOSE OF THE STUDY

To determine the effect of hyaluronic acid filler on the thickness of different skin layers experimentally.

■ MATERIALS AND METHODS

This study was conducted on male and female laboratory rats, Rattus norvegicus type albino rats aged between (3–4) months and their average weight between 200 to 250 gm, where their clinical health was confirmed before the experiment was conducted. As for the HA filler that was purchased, it belongs to the NEU Neuramis Volume Lidocaine (NVL) series from (Medytox Inc., Seoul, South Korea). One option is a monophasic dermal filler made by Medytox. It has a concentration of 20 mg/mL of hyaluronic acid and 0.3% lidocaine. The filler is unique because it was developed using double cross-linking of molecules, which made it very cohesive [10, 20]. The animals were then divided into 50 males, 50 females, then into 5 subgroups for each group (5 treated rats: 5 control rats). The treated groups were injected with the dermal filler at different time intervals of 2, 4, 9, 10, 13 weeks, as the injection was done with a dose of (0.1 mL) in the subcutaneous layer, which forms a prominent opus [15, 21] on the central dorsal skin of hairless mice [22]. Dissecting the animals after the trial was over allowed researchers to remove the filler. To observe the tissue changes caused by the dermal filler, histological slices were prepared and stained with hematoxylin and eosin [23]. These slices were then examined under a light microscope to determine the histological measurements of the skin tissue using an Ocular micrometer OM attached to the light microscope. This study [24] examined the skin thickness of all groups.

Statistical analysis

Statistical analysis was performed on all data using SPSS v.27 and Microsoft Excel 2019 for graphics. The data is presented as the mean plus or minus the standard deviation. The statistical analyses included both one- and two-way ANOVA tests, as well as Duncan's post hoc test for multiple group comparisons. At $p<0.05$, differences were found to be significant [25].

■ RESULTS

Effect of dermal fillers on skin thickness

The results demonstrated that the average thickness of the epidermal layer increased over time in both the male and female treatment groups. The male and female group that received 13 weeks of treatment had significantly thicker epidermis layers (1.57±0.08) and (1.54±0.09) compared to the control group (0.21±0.06) and the other groups (0.24±0.09). In addition, when comparing the groups that were treated for 9, 10 weeks, the researchers

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discovered no significant change in the thickness of the epidermis layer (1±0.19, 1.08±0.12) in males and 0.94±0.15, 1.06±0.11) in females (Table 1). When comparing the male and female groups treated for 13 weeks to the control group and other groups, the study found a clear and significant increase in dermal thickness (13.52±0.58) and 1.54±0.09, respectively, as compared to the control group and other groups (Table 2). The dermal layer thickness progressively increased ($p<0.05$) in the male and female groups treated for 2, 4, 9, 10, and 13 weeks, respectively. Table 3 shows that the treated groups' subcutaneous layer thickness increased significantly over time. The male rats (6.66±0.24) and female rats (6.64±0.23) treated for 13 weeks showed a substantial increase ($p<0.05$) compared to the control group (2.55±0.37) and other groups. There was no significant difference ($p<0.05$) between the male (3.68±0.24, 3.85±0.37) and female (3.66±0.23, 3.76±0.3) groups treated for 2, 4 weeks in Table 3.

Table 1
The effect of dermal fillers on the average thickness of the epidermis layer

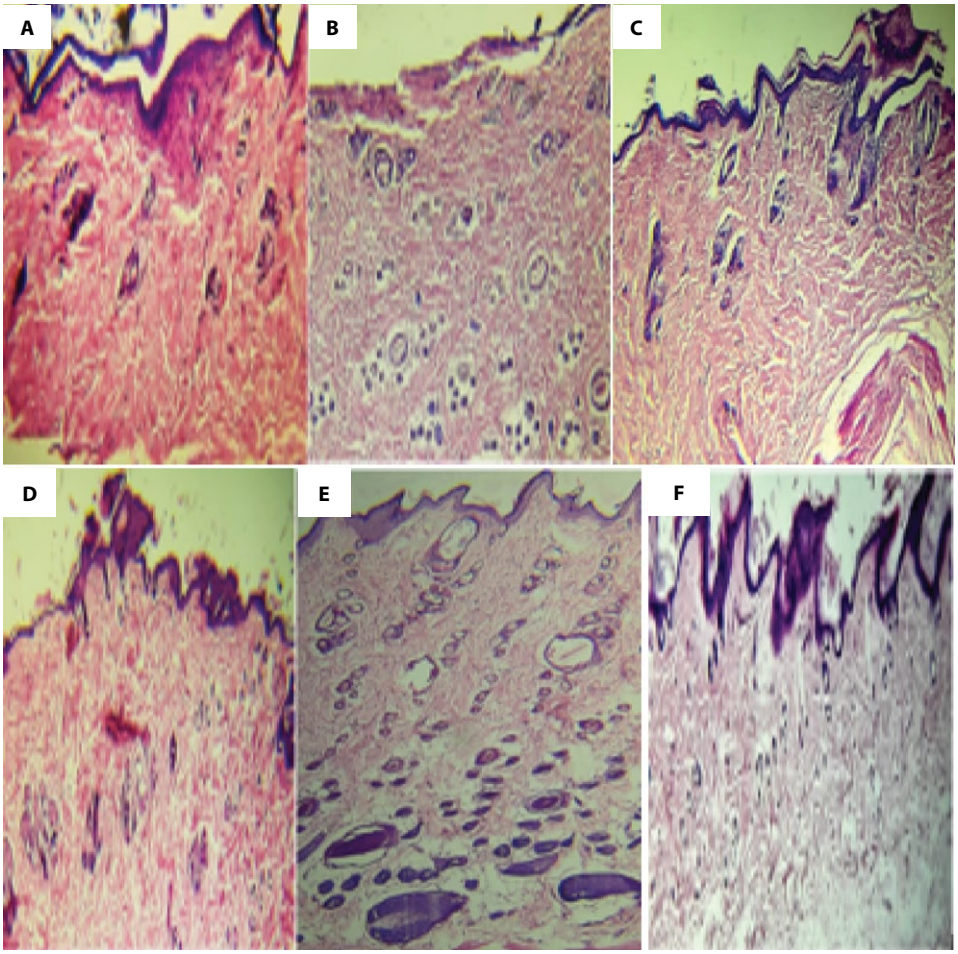
Treatment groups	Mean±SD Epidermal thickness (µm)					
	2W	4W	9W	10W	13W	Total
Male control	0.17±0.09	0.21±0.06	0.2±0.06	0.19±0.07	0.21±0.06	0.2±0.06
Male treatment	0.69±0.15	0.86±0.06	1±0.19	1.08±0.12	1.57±0.08	1.04±0.32
Female control	0.2±0.1	0.22±0.09	0.23±0.08	0.22±0.09	0.24±0.09	0.22±0.08
Female treatment	0.66±0.15	0.82±0.08	0.94±0.15	1.06±0.11	1.54±0.09	1±0.32
Total	0.43±0.28	0.53±0.33	0.59±0.41	0.64±0.45	0.89±0.69	
p-value	0.0001*					

Table 2
The effect of dermal fillers on the average thickness of the dermis layer

Treatment groups	Mean±SD Dermal thickness (µm)					
	2W	4W	9W	10W	13W	Total
Male control	5.46±0.44	5.49±0.42	5.57±0.31	5.53±0.35	5.53±0.36	5.52±0.35
Male treatment	6.42±0.39	7.86±0.29	8.73±0.38	10.53±0.76	13.52±0.58	9.41±2.54
Female control	5.44±0.44	5.45±0.45	5.49±0.4	5.48±0.41	5.50±0.39	5.47±0.38
Female treatment	6.38±0.4	7.72±0.45	8.70±0.4	10.52±0.77	13.5±0.58	9.36±2.57
Total	5.93±0.62	6.63±1.25	7.13±1.67	8.02±2.63	9.51±4.12	
p-value	0.0001*					

Table 3
The effect of dermal fillers on the average thickness of the subcutaneous layer

Treatment groups	Mean±SD Subcutaneous thickness (µm)					
	2W	4W	9W	10W	13W	Total
Male control	2.52±0.37	2.57±0.45	2.58±0.42	2.59±0.37	2.55±0.37	2.56±0.36
Male treatment	3.68±0.24	3.85±0.37	4.74±0.25	5.76±0.27	6.66±0.24	4.94±1.19
Female control	2.5±0.37	2.54±0.41	2.56±0.37	2.57±0.38	2.52±0.37	2.54±0.35
Female treatment	3.66±0.23	3.76±0.3	4.7±0.22	5.74±0.27	6.64±0.23	4.90±1.2
Total	3.09±0.66	3.18±0.73	3.64±1.14	4.16±1.66	4.59±2.13	
p-value	0.0001*					



Histological analysis of skin layers in hairless laboratory rats treated with hyaluronic acid dermal filler after staining with H&E stains (10×) for different time periods (A/Control/B/2W/C/4W/D/9W/E/10W/F/13W) respectively, where (W) represents one week

Histological examination

Histological examination of the skin samples showed a significant and gradual thickening of the skin layers during the time periods of 13, 10, 9, 4, and 2 weeks, respectively, compared to the control group. This change includes the skin layers: the epidermis, dermis, and subcutaneous layer, as shown in Figure.

■ DISCUSSION

The findings of the present study revealed that administering dermal filler Hyaluronic acid (HA) to male and female laboratory animals for varying durations resulted in a significant increase in the thickness of the epidermis layer. Specifically, the thickness was notably higher after 9, 10, and 13 weeks compared to the periods of 2 and 4 weeks.

These results were observed when comparing the treated groups to the control groups over the same time periods. Regarding the dermis, it had a progressive increase in thickness during the dermal filler treatments. The subcutaneous layer exhibited greater thickness at 9, 10, and 13 weeks in comparison to the groups treated for 2 and 4 weeks, as well as the control groups within the same time intervals. There was no discernible disparity between males and females in terms of treatment outcomes across all time periods. The histological analysis of the skin layers, following staining with hematoxylin and eosin, corroborates these statistical findings. The reason for this is that HA has a strong affinity for water molecules and can retain water up to 10,000 times its own weight [26, 27]. On the other hand, dermal filler revitalizes skin cells and enhances the proportion of collagen. These findings are consistent with the results published by Kim [28] regarding the utilization of dermal fillers in a group of female patients aged 38 years. The mean dermal thickness exhibited a significant rise during a duration of 1.4 years of injections. Hyaluronic acid (HA) is crucial for skin hydration and tissue regeneration due to its exceptional ability to bind water and regulate tissue volume [29].

In accordance with Kwon et al. [22], a study was conducted with 24 female hairless mice (SKH1-Hrhr). These mice were injected with 100 µl of several dermal fillers, including polydioxanone (PDO) filler, poly-l lactic acid (PLLA), and polycaprolactone (PCL) fillers. The durability of the fillers was assessed at various time intervals, including 0, 3 days, and 1, 4, 8, and 12 weeks after injection. The filler injection groups exhibited moderate increases in epidermal thickness and the deposition of dermal collagen fibers, which were slower compared to the control group. The study aligns with Kwon et al. [22], where 24 female hairless mice (SKH1-Hrhr) were administered 100 µl of various dermal fillers, including polydioxanone (PDO) filler, poly-l lactic acid (PLLA), and polycaprolactone (PCL) fillers. The durability of the fillers was assessed at certain time intervals (0, 3 days, 1, 4, 8, and 12 weeks) after injection. The filler injection groups exhibited incremental increases in epidermal thickness and deposition of dermal collagen fibers, which were slower compared to the control group.

It agreed with Aziz et al. [30] who explained that the thin skin of female laboratory rats injected with hyaluronic acid (HA) at a dose of 0.1 ml showed a significant increase ($p<0.05$) in the total subcutaneous tissue (SC) thickness, a significant increase in collagen and a significant increase in endothelial cells in the animals of the group for the time periods of 90, 60, and 7 days compared to the control group, with a significant difference of $P<0.05$, where the highest peak of the total thickness was detected for the rats in the group treated for 7 days in the thickness of the subcutaneous tissue. The detection of basophilic areas of fluid accumulation with a small number of collagen fibers accounts for this significant increase. The animals in the group that was treated for 60 days had a significant increase in thickness, with a significant difference ($P<0.05$) compared to the control group. This was because collagen fibers formed between the fluid buildup. The group treated for 90 days showed a decrease in subcutaneous tissue thickness. Some authors have demonstrated that hyaluronic acid-based filler can extend the adjacent dermal fibroblasts by absorbing interstitial fluid in the first few weeks after injection, as evidenced by the disappearance of fluid spaces compared to the 7, 60-day group [31]. This supports the histological results of our current study, while there were insignificant changes in the average thickness of the epidermal and dermal layers in female laboratory rats of the 30-, 7-, 60-, and 90-day groups compared to the control group ($P>0.05$).

Aziz et al. [30] found that rats that were injected for 30 days had major increases in the epidermal and dermal layers, which was different from rats that were not treated ($P<0.05$).

After 30 days of injection, the skin, dermis, and subcutaneous tissues of laboratory-aged rats given HA looked almost identical to the control group's adult rats, according to research by Aziz et al. [30]. Consistent with our findings, the statistical analysis showed that the total thickness of the dermis, epidermis, and subcutaneous tissues in these animals was significantly higher than in untreated aged rats. A person's skin is composed of four layers: the basal, spinosum, granulosum, and corneum layers [32], the final layer remains unchanged in thickness because the epidermis's cellular structure determines the layer's thickness, which rises somewhat but not significantly. Collagen fibers are found scattered throughout the dermis and the skin becomes noticeably flatter as a result. Young skin has big bundles of collagen, but as we age, it becomes more apparent that there is a network of fine, small fibers surrounding the fibroblasts. The total thickness of the epidermis decreases by 15 mm as we age, but the position of the fibrous layer remains constant. There were notable alterations to the structure of collagen fibers and bundles; furthermore, there were no statistically significant differences in skin measurements between male and female volunteers, according to Aziz et al. [30].

Polycaprolactone fillers (PCL) can improve skin texture and quality by significantly and long-term increasing the thickness of the dermis and stimulating new collagen formation. The mean skin thickness increased significantly ($P<0.001$) in female samples after 1 year of intradermal filler injection. The injectable PCL filler effectively treats skin atrophy, wrinkles, and pores by stimulating the production of new collagen in the dermis, hence enhancing skin texture. Following a period of 4 years, there was a notable and statistically significant rise in the average thickness of the skin ($P<0.001$). Ultrasound imaging verified that the thickness of the skin progressively and modestly augmented in all patients following the injection of PCL filler into the dermis, aligning with the findings of our investigation. Polycaprolactone (PCL) fillers have the ability to remain in the dermis for a duration exceeding 4 years. Subdermal filler injections can augment volume and address wrinkles, but they do not have a direct impact on the dermis. The injection elevates the dermis, but it seldom augments its thickness. In this instance, the dermis underwent a change. The investigation revealed a substantial augmentation in thickness not just between the epidermis and the appendage, but also between the appendage and the fat. Hence, the real disparity in skin thickness may exceed that. There is a common belief that injecting fillers into the layer of fat might increase the thickness of the skin. Nevertheless, the dermis lacks the ability to generate collagen completely, therefore subcutaneous injections are incapable of directly modifying the thickness of the skin. Subcutaneously injected PCL fillers can improve skin texture and quality by increasing skin thickness, promoting the production of new collagen, and delivering a potent treatment for skin wrinkles. Kim (28) has shown that intradermal injection leads to a much longer duration of residence compared to subdermal injection. Microscopic analysis of hyaluronic acid fillers showed predominantly vacant regions, occasionally interspersed with traces of amorphous material [33]. The histological images from our current study support this conclusion.

Ten women were given hyaluronic acid filler (Derma Hyal®) injections into each nasolabial fold at 1, 3-, 6-, 9-, and 12-month intervals, in accordance with the findings of Salles et al. [34]. In the control group, the average thickness of the nasolabial folds was 0.38 ± 0.14 mm. After 1 month, it increased to 0.69 ± 0.19 mm. After 3 months, it stood at

0.65±0.17 mm. After 6 months, it dropped to 0.61±0.22 mm. After 9 months, it rose to 0.57±0.23 mm. Finally, after 12 months, it reached 0.55±0.14 mm. As a result, the statistical analysis proved that the soft sections' thickness increased the most after 12.6 months, and then reduced gradually thereafter.

A study by Yeom et al. [35] found that injecting 400 µL of the fillers Sodium hyaluronate and sodium salt of hyaluronic acid (HA) as Restylane and HAHMDA into the dorsal skin of hairless mice at 6 weeks old caused big changes in the epidermis, dermis, and subcutaneous tissues in aging mice. The skin got wrinkled and then became the same as the control group's skin. The study observed a significant renewal of the dermis layer compared to the control group, with a clear increase in dermis thickness. The study's statistical results showed that 12 weeks after treatment with hyaluronic acid, the dermis was thicker and collagen density was higher in the HA-HMDA group than in the Restylane filler group (* $P<0.05$) or the aging group (** $P<0.01$), but not significantly different from the control group. Collagen is what makes skin strong and flexible [36]. There was a significant increase in collagen bundles after Restylane filler injection. This is consistent with our study, which found that the dermis layer thickness was very large in the treatment group for a period of 13 weeks. Kim et al. [37] showed that ADM/HA filler can maintain the appropriate volume over a period of 7 weeks without any complications. The ADM/HA filler group maintained 80% and 83% of the filler's volume and weight, respectively. Therefore, small quantities of ADM/HA filler can serve as an alternative to autologous fat grafts.

According to Su et al. [38], the wounds that were treated with HA scaffolds showed improved healing and achieved greater healing rates after 5 days. This occurred because the dermal and epidermal layers were surgically removed and had not yet regrown, and the wounds treated with HA scaffolds also had a thicker hypodermis and epidermis. In addition, skin appendages were observed on the 20th day in the wounds that were treated with scaffolds containing hyaluronic acid. Previous research discovered that mice with wounds that were completely covered with cotton gauze had a wound healing rate of 40% and did not develop any epidermis after a period of two weeks [39]. On the other hand, the wounds that were treated with decellularized scaffolds containing hyaluronic acid also showed a wound healing rate of 70.94% and the creation of a clean epidermis after 15 days. Within the pig population, a previous investigation found that 87.41% of wounds that were treated with scaffolds containing HA successfully healed after a period of 21 days. In contrast, 49.9% of wounds in the dorsal midline region, which were full thickness in nature, did not exhibit the formation of skin tags [40].

The filler material may resist degradation and remain isolated in macrophages, which in turn secrete many inflammatory substances and cytokines that will attract other monocytes and macrophages. Macrophages, epithelioid histiocytes, may grow or merge to form the giant multinucleated cells of the foreign body, leading to the formation of a granuloma [13]. In their study, Lemperle et al. [41] discovered the formation of a granuloma after dermal filler injection [42]. After injection, macrophages may rapidly absorb biodegradable fillers, which then undergo slow biodegradation through phagocytosis into small, irregularly shaped particles. This explains the disappearance of the fillers after a certain period. After 3 months of injection, histological examination reveals that the filler particles remain spherical, surrounded by giant cells, macrophages, and lymphocytes. After 6 months, most particles exhibit a porous, cracked, and occasionally distorted outer surface, encircled by giant cells and macrophages. These symptoms may be present at least 18 months after injection [43].

Regardless of the injection site, one should exercise caution when using large amounts of dermal fillers, as the current study's volume may have exceeded 0.1 ml. A high dose is thought to carry numerous risks. Because dermal filler is a gel and not a liquid, it requires a lot of pressure on the syringe plunger, necessitating clinical measures by the physician to determine the appropriate volume of filler for each injection site and each disease condition [44].

■ CONCLUSION

Due to a variety of complications and potential long- or short-term functional and physiological changes, dermal fillers gradually increase the thickness of the various subcutaneous layers over time. Histological examination of the skin samples showed a gradual thickening of layers during the time periods, this change includes the epidermis, dermis, and subcutaneous layer.

■ REFERENCES

1. Ballin AC, Brandt FS, Cazzaniga A. Dermal fillers: an update. *American journal of clinical dermatology.* 2015;16:271–83.
2. Betemps JB, Marchetti F, Lim T, et al. Projection capacity assessment of hyaluronic acid fillers. *Plast Aesthet Res.* 2018;5(6):1-8.
3. Mero A, Campisi M. Hyaluronic acid bioconjugates for the delivery of bioactive molecules. *Polymers.* 2014;6(2):346–69.
4. Choi MS, Kwak S, Kim J, et al. Comparative analyses of inflammatory response and tissue integration of 14 hyaluronic acid-based fillers in mini pigs. *Clinical, Cosmetic and Investigational Dermatology.* 2021; 765–78.
5. Bukhari SN, Roswandi NL, Waqas M, et al. Hyaluronic acid, a promising skin rejuvenating biomedicine: A review of recent updates and pre-clinical and clinical investigations on cosmetic and nutricosmetic effects. *International journal of biological macromolecules.* 2018;120:1682–95.
6. Smith L, Cockerham K. Hyaluronic acid dermal fillers: can adjunctive lidocaine improve patient satisfaction without decreasing efficacy or duration? *Patient preference and adherence.* 2011;133–9.
7. Jones DH, Fitzgerald R, Cox SE, et al. Preventing and treating adverse events of injectable fillers: evidence-based recommendations from the American Society for Dermatologic Surgery Multidisciplinary Task Force. *Dermatologic Surgery.* 2021;47(2):214–26.
8. Micheels P. Human anti-hyaluronic acid antibodies: is it possible? *Dermatologic surgery.* 2001;27(2):185–91.
9. Jesús Fernández-Aceñero M, Zamora E, Borbujo J. Granulomatous foreign body reaction against hyaluronic acid: report of a case after lip augmentation. *Dermatologic surgery.* 2003;29(12):1225–6.
10. Flynn TC, Sarazin D, Bezzola A, et al. Comparative histology of intradermal implantation of mono and biphasic hyaluronic acid fillers. *Dermatologic surgery.* 2011;37(5):637–43.
11. Kim JH, Ahn DK, Jeong HS, Suh IS. Treatment algorithm of complications after filler injection: based on wound healing process. *Journal of Korean medical science.* 2014;29(Suppl 3):S176–82.
12. Alam M, Gladstone H, Kramer EM, et al. ASDS guidelines of care: injectable fillers. *Dermatologic Surgery.* 2008;34:S115–48.
13. Funt D, Pavicic T. Dermal fillers in aesthetics: an overview of adverse events and treatment approaches. *Plastic and Aesthetic Nursing.* 2015;35(1):13–32.
14. Lemperle G, Duffy DM. Treatment options for dermal filler complications. *Aesthetic Surgery Journal.* 2006;26(3):356–64.
15. Kwak SS, Yoon KH, Kwon JH, et al. Comparative analysis of hyaluronidase-mediated degradation among seven hyaluronic acid fillers in hairless mice. *Clinical, Cosmetic and Investigational Dermatology.* 2021;8:241–8.
16. Kim JE, Sykes JM. Hyaluronic acid fillers: history and overview. *Facial Plastic Surgery.* 2011;27(06):523–8.
17. Vartanian AJ, Frankel AS, Rubin MG. Injected hyaluronidase reduces restylane-mediated cutaneous augmentation. *Archives of Facial Plastic Surgery.* 2005;7(4):231–7.
18. Juhász ML, Levin MK, Marmur ES. The kinetics of reversible hyaluronic acid filler injection treated with hyaluronidase. *Dermatologic Surgery.* 2017;43(6):841–7.
19. Brandt FS, Cazzaniga A. Hyaluronic acid gel fillers in the management of facial aging. *Clinical interventions in aging.* 2008;3(1):153–9.
20. Joo HJ, Woo YJ, Kim JE, et al. A randomized clinical trial to evaluate the efficacy and safety of lidocaine-containing monophasic hyaluronic acid filler for nasolabial folds. *Plastic and Reconstructive Surgery.* 2016;137(3):799–808.
21. Hwang SH, Kim DH, Nam BM, et al. Efficacy and safety of lyophilized articular cartilage matrix as an injectable facial filler. *Aesthetic Plastic Surgery.* 2021;45:1266–72.
22. Kwon TR, Han SW, Yeo IK, et al. Biostimulatory effects of polydioxanone, poly-d, l lactic acid, and polycaprolactone fillers in mouse model. *Journal of cosmetic dermatology.* 2019;18(4):1002–8.
23. Bancroft JD, Gamble M. *Histological techniques.* In: Suvarna KS, Layton C, Bancroft JD (editors). Theory and practice of histological techniques (7th edtition). Elsevier health sciences, NY. 2008;203–210.
24. Galigher AE, Kozloff EN. *Microscpic Analysis.* In: Galigher AE, Kozloff EN (editors). Essentials of practical microtechnique (2nd edition). Lea & Febiger, UK. 1971;41–44.
25. Sullivan LM. *Essentials of biostatistics for public health* (4th edition). Jones & Bartlett Learning, US. 2022;184–195.
26. Romagnoli M, Belmontesi M. Hyaluronic acid – based fillers: theory and practice. *Clinics in dermatology.* 2008;26(2):123–59.
27. Heldin P, Basu K, Olofsson B, et al. Deregulation of hyaluronan synthesis, degradation and binding promotes breast cancer. *The journal of biochemistry.* 2013;154(5):395–408.

28. Kim JS. Changes in dermal thickness in biopsy study of histologic findings after a single injection of polycaprolactone-based filler into the dermis. *Aesthetic surgery journal.* 2019;39(12):NP484–94.
29. Gold MH. Use of hyaluronic acid fillers for the treatment of the aging face. *Clinical interventions in aging.* 2007;2(3):369–76.
30. Aziz M, Gamal El Din E, Ahmed SK, Ahmed Mekway M. Histological study on the effect of hyaluronic acid injection (dermal filler) on the skin of the adult and aged albino rat. *Egyptian Journal of Histology.* 2019;42(2):245–61.
31. Tezel A, Fredrickson GH. The science of hyaluronic acid dermal fillers. *Journal of Cosmetic and Laser Therapy.* 2008;10(1):35–42.
32. Faris SA. Histochemical and ultrastructural observations of the skin in one humped camel (Camelus Dromedarius). *Basrah Journal of Veterinary Research.* 2019;18(1).
33. Rosa SC, Macedo JL, Magalhães AV. An experimental study of tissue reaction to hyaluronic acid (Restylane) and polymethylmethacrylate (Metacrill) in the mouse. *The American Journal of Dermatopathology.* 2012;34(7):716–22.
34. Salles A, Remigio A, Zacchi V, Ferreira O. Clinical and dermal thickness assessment 1 year after hyaluronic acid filler treatment. *Revista Brasileira de Cirurgia Plástica.* 2001;26(1):66–9.
35. Yeom J, Bhang SH, Kim BS, et al. Effect of cross-linking reagents for hyaluronic acid hydrogel dermal fillers on tissue augmentation and regeneration. *Bioconjugate chemistry.* 2010;21(2):240–7.
36. Tsukahara K, Nakagawa H, Moriwaki S, et al. Inhibition of ultraviolet-B-induced wrinkle formation by an elastase-inhibiting herbal extract: implication for the mechanism underlying elastase-associated wrinkles. *International journal of dermatology*. 2006;45(4):460–8.
37. Kim JH, Kim SE, Kim YJ, et al. Comparison of volume retention and biocompatibility of acellular dermal matrix/hyaluronic acid filler to autologous fat grafts in a mouse model. *Aesthetic Plastic Surgery.* 2020;44:986–92.
38. Su Z, Ma H, Wu Z, et al. Enhancement of skin wound healing with decellularized scaffolds loaded with hyaluronic acid and epidermal growth factor. *Materials Science and Engineering: C.* 2014;44:440–8.
39. Huang S, Zhang Y, Tang L, et al. Functional bilayered skin substitute constructed by tissue-engineered extracellular matrix and microsphere-incorporated gelatin hydrogel for wound repair. *Tissue Engineering Part A.* 2009;15(9):2617–24.
40. Intaraprasita S, Faikruab A, Sittichokechaiwutc A, Viyocha J. Efficacy evaluation of the fibroblast-seeded collagen/chitosan scaffold on application in skin tissue engineering. *ScienceAsia.* 2012;38(3):268–77.
41. Lemperle G, Rullan PP, Gauthier-Hazan N. Avoiding and Treating Dermal Filler Complications Table 1. Classification of US Food and Drug Administration – Approved Injectables. *Plastic and reconstructive surgery.* 2006;118(3S):92S–107S.
42. Carruthers A, Carruthers J. Non-animal-based hyaluronic acid fillers: Scientific and technical considerations. *Plastic and reconstructive surgery.* 2007;120(6S):33S–40S.
43. Zimmermann US, Clerici TJ. The histological aspects of fillers complications. *In Seminars in cutaneous medicine and surgery.* 2004;23(4):241–250.
44. Maruyama S. A histopathologic diagnosis of vascular occlusion after injection of hyaluronic acid filler: findings of intravascular foreign body and skin necrosis. *Aesthetic Surgery Journal.* 2017;37(9):NP102–8.

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Biological Activity of Cinnamomum Zeylanicum J Prel Extract on Growth of C. Albicans, C. Tropicalis, C. Glabrata and C. Krusei Isolated from Patients with Dermatitis

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Abstract

Purpose. To identify the activity of Cinnamomum zeylanicum J Prel ethanol extracts with different concentrations and compare their activity with antifungal therapeutics as Amphotericin B, Fluconazole and Miconazole.
Materials and methods. All edges of skin rash of dermatitis lesions were sampled (fungal growth is most likely to be active in these lesion edges). Scrapings of the skin done gently by removed the surface of the skin and placed into a laboratory specimen container. The fungi used in this research were C. albicans, C. krusei, C. glabrata and C. tropicalis were isolated from patients with dermatitis. The isolated fungi were identification based in both morphological properties and biochemical characterization by Epi-Candida.
Results. The ethanolic extract with concentration 100% exhibited the high activity against all Candida spp in both 12 and 24 hours of experiment. Also, noted the antifungal activity increased with increasing concentration, the study also showed a significant difference in activity between 12 and 24 hours, the ethanolic extract with 100% concentration exhibited same activity obtained by MCL against C. albicans, against C. tropicalis the ethanolic extract with 100% exhibited activity more than FLC and less than PB and MCL, while against C. krusei and C. glabrata the ethanolic extract with 100% exhibited activity more than all antifungal therapeutics.
Conclusion. The ethanolic extract has a high activity against all Candida spp. in concentration 100%, and their activity was reduced with decreasing concentration.
Keywords: Cinnamomum zeylanicum J Prel, Ethanol Extracts, Candida spp., Amphotericin B, Fluconazole, Miconazole

■ INTRODUCTION

One element that is essential to the preparation of food is spice. Around the world, more than a hundred plant species are used to make spices and condiments [1]. These fragrant, dried plant components are typically obtained from seeds, fruits, leaves, roots,

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and bark, etc. Since ancient times, they have typically been used to improve the flavor and quality of food [2]. Moreover, spices have qualities such as antioxidant, antibacterial, anti-inflammatory, antidiabetic, and anticancer due to their abundance of biologically active chemicals, etc. One of the first recognized spices utilized in culinary art is cinnamon [3]. Although many species in this genus are sold as cinnamon, Cinnamomum zeylanicum J. Presl, a member of the Lauraceae family, is thought to be real cinnamon. Many biologically active chemicals found in cinnamon, a spice tree, can be used to combat a range of microorganisms. Cinnamon's raw bark extract has frequently been said to have antifungal properties. These activities were attributed mostly to the chemicals cinnamaldehyde and eugenol [4]. These compounds are essential to the plant's defense against insects and vertebrate herbivores, as well as its defense against UV radiation and cold stress. Flavonoids, tannins, glycosides, saponins, alkaloids, and essential oils with biological actions including bacteriostatic, fungistatic, and anti-inflammatory make cinnamon bark a major source of these compounds [5]. Because cinnamon species have substantial antimicrobial properties, lotions, soaps, and toothpastes utilize their volatile oils as antibacterial and antifungal ingredients [4].

In the clinical environment, Candida spp. constitute the most common source of invasive fungal infection. In a recent multistate point prevalence study of hospitalized patients, Candida spp. was identified as the microbial species responsible for the majority of bloodstream infections 22% [6]. These infections frequently have disastrous effects because invasive candidiasis has the greatest mortality rate of all nosocomial diseases, with up to 47% attributed mortality (Pappas et al., 2016). Medical devices such indwelling vascular catheters have been associated in epidemiological studies to the emergence of invasive candidiasis and Candida bloodstream infection (candidemia) [7]. Candida adheres to the surface of artificial objects and proliferates as adherent microbial communities [8]. Within the biofilms the fungal cells exhibit resistance to almost all available drug therapies and weaken host defenses. The most common species of Candida that has been used as a model organism for research on fungus biofilms is Candida albicans [9]. C. albicans commonly goes through filamentation as biofilms form, resulting in extended pseudo-hyphae and hyphae as well as preserving cells in the yeast shape [10]. Several C. albicans strains and clinical settings appear to exhibit varying degrees of this morphologic transformation. The significance of non-albicans species, such as C. tropicalis, C. parapsilosis, and C. glabrata, in addition to C. albicans, has come to be understood more and more recently [11]. Like this, the recently discovered species C. auris easily creates biofilms on synthetic objects, which most likely explains why this species prefers to infect individuals who have indwelling medical equipment with disease [12]. Many of the non-albicans Candida spp ack the ability to filament and instead produce yeast-only biofilms [13]. Yet, all Candida species must create a matrix, an extracellular sticking polymeric component that is essential to biofilm formation [14]. In addition to its functions in adhesion and cohesiveness, this substance also contributes significantly to Candida biofilms' tolerance of antifungals and resistance to host defenses [15]. Many of the non-albicans Candida spp lack the ability to filament and instead produce yeast-only biofilms. However, all Candida species produce a matrix, an extracellular polymeric substance that serves as the biofilm's defining characteristic [16].

■ PURPOSE OF THE STUDY

To identify the activity of Cinnamomum zeylanicum J Prel ethanol extracts with different concentrations and compare their activity with antifungal therapeutics as Amphotericin B, Fluconazole and Miconazole.

■ MATERIALS AND METHODS

Plant bark collection

The barks of cinnamon barks were bought from local market in Thi-Qar. The plant was identified as cinnamon as Cinnamomum zeylanicum L (Breyn). The l Cinnamon barks were ground into powder and kept in plastic bags until used.

The ethanolic extract preparation

The ethanolic crude extracts of barks of cinnamon was prepared to test against Candida spp. The method of Mahmoud [17], was used to process the ethanolic extracts. Stock solutions and various concentrations (dilutions) of ethanolic extract, twenty grams of dried powder were added to 200 ml of ethanol to create the extract. Soxhlet continuous extraction was then used to extract the mixture, which was then filtered using Whatmann No. 13 filter paper. The filtrate was then concentrated under reduced pressure on a rotary evaporator at 50 °C and dried at 25 °C, and the extract was then collected in sterilized glass tubes until use.

The four concentrations were prepared by adding 1 gm from ethanol extract and 10 ml of DMSO:

1. 100% concentration was prepared by adding 100 µl of stoke solution only.
2. The 75% concentration was prepared by adding 5 µl stoke solution and 25 µl DMSO.
3. The 50% concentration was prepared by adding 50 µl stoke solution and 50 µl DMSO.
4. The 25% concentration was prepared by adding 25 µl stoke solution and 75 µl DMSO.

Isolation and identification of fungi

The fungi used in this research were C. albicans, C. krusei, C. glabrata and C. tropicalis were isolated from patients with dermatitis. The isolated fungi were identification based in both morphological properties and biochemical characterization by Epi-Candida.

Samples collection

All edges of skin rash of dermatitis lesions were sampled (fungal growth is most likely to be active in these lesion edges). Scrapings of the skin done gently by removed the surface of the skin and placed into a laboratory specimen container.

Antifungal therapeutics

The isolated Candida spp, were tested against antifungal drugs such as Amphotericin B (PB), Fluconazole (FLC), and Miconazole (MCL).

■ RESULTS

Phytochemical analysis of ethanolic extracts of cinnamon barks

Phytochemical analysis of Cinnamomum zeylanicum bark extracts established the presence of numerous bioactive compounds, alkaloids, essential oil, flavonoids, saponins,

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and terpenoids were detected in ethanolic extract of cinnamon, whereas the steroids and tannins were non-detected in ethanolic extract of cinnamon as show in table 1.

Table 1
Bioactive compounds in ethanolic extract of cinnamon barks

Constituents	Cinnamomum zeylanicum result
Alkaloids	+
Essential oil	+
Flavonoids	+
Saponins	+
Steroids	–
Tannins	–
Terpenoids	+

Activity of CZ J Prel ethanol extracts on Candida spp. after 12 hours in different concentration compared with anti-fungal therapeutics

The present study recorded the high activity against C. albicans in both ethanolic extract with concentration 100% and MCL, followed activity showed in ethanolic extract 75% and FLC, in third grad activity was noted in PB, while the low activity in ethanolic extract 50%, and 25% extract was has not antifungal activity. In C. tropicalis the study noted the high activity was showed in MCL, followed in PB, followed in ethanolic extract 100%, followed in FLC, while the other ethanolic extract exhibited antifungal activity with increase concentration. In C. krusei the study showed the high activity in ethanolic extract 100%, followed in FCL and ethanolic extract 75%, following in PB and ethanolic extract 50%. In C. glabrata the study noted the high activity ethanolic extract 100%, following in FLC, following in PB and MCL, at p. value <0.01 as in table 2.

Table 2
Activity of CZ ethanol extracts on Candida spp. after 12 hours in different concentration compared with anti-fungal therapeutics

Concentration	Cases No.	C. albicans	C. tropicalis	C. krusei	C. glabrata
		Inhibitions zone Mean±SD			
25%	3 cases in each group	0.00 ± 0.00^{d}	5.93 ± 0.51^{g}	14.7 ± 0.75^{e}	7.70 ± 0.62^{f}
50%		8.10 ± 0.65^{c}	13.6 ± 0.70^{f}	17.7 ± 0.86^{cd}	14.1 ± 0.75^{e}
75%		15.7 ± 0.94^{ab}	15.9 ± 0.30^{e}	22.6 ± 1.15^{b}	18.5 ± 0.50^{d}
100%		16.5 ± 0.81^{a}	20.9 ± 0.96^{c}	24.2 ± 0.94^{a}	25.6 ± 0.71^{a}
PB		14.4 ± 0.40^{b}	23.2 ± 1.55^{b}	18.3 ± 0.41^{c}	20.5 ± 0.86^{c}
FLC		15.3 ± 0.47^{b}	19.8 ± 0.76^{d}	22.8 ± 1.04^{b}	24.0 ± 0.50^{b}
MCL		16.4 ± 0.51^{a}	24.8 ± 0.80^{a}	16.8 ± 1.17^{d}	19.4 ± 0.55^{cd}
p. value		$<0.001^{**}$	$<0.001^{**}$	$<0.001^{**}$	$<0.001^{**}$
LSD		1.07	1.54	1.64	1.14

Notes: a non-significant difference is shown by a similar small litter, whereas a significant difference is indicated by a distinct small letter; PB – Amphotericin B, FLC – Fluconazole, MCL – Miconazole.

Table 3
Activity of CZ ethanol extracts on Candida spp after 24 hours in different concentration compared with anti-fungal therapeutics

Concentration	Cases No.	C. albicans	C. tropicalis	C. krusei	C. glabrata
		Inhibitions zone Mean±SD			
25%	3 cases in each group	3.66±0.28^{d}	6.40±0.52^{f}	15.5±0.40^{e}	10.7±0.70^{f}
50%		10.3±0.75^{c}	14.4±0.60^{e}	18.4±0.55^{d}	14.6±0.65^{e}
75%		19.7±0.73^{b}	22.3±1.52^{d}	29.1±1.20^{b}	24.8±0.52^{d}
100%		20.1±0.76^{ab}	25.9±0.90^{c}	31.4±0.50^{a}	31.9±0.17^{a}
PB		18.5±0.86^{b}	28.7±1.57^{b}	25.2±0.70^{c}	26.8±1.02^{c}
FLC		21.1±1.04^{a}	25.2±0.75^{c}	30.1±1.08^{ab}	30.4±0.68^{b}
MCL		20.1±0.32^{ab}	30.6±0.80^{a}	25.7±0.87^{c}	25.9±0.25^{c}
p. value		<0.001**	<0.001**	<0.001**	<0.001**
LSD		1.27	2.10	1.41	1.11

Notes: a non-significant difference is shown by a similar small litter, whereas a significant difference is indicated by a distinct small letter; PB – Amphotericin B, FLC – Fluconazole, MCL – Miconazole.

Table 4
Compartment between ethanol extracts and anti-fungal therapeutics on Candida spp. after 12 and 24 hours

Concentration		C. albicans	C. tropicalis	C. krusei	C. glabrata
		Inhibitions zone Mean±SD			
25%	12 hours	0.00±0.00	5.93±0.51	14.7±0.75	7.70±0.62
	24 hours	3.66±0.28	6.40±0.52	15.5±0.40	10.7±0.70
p. value		0.002**	0.334	0.200	0.005**
50%	12 hours	8.10±0.65	13.6±0.70	17.7±0.86	14.1±0.75
	24 hours	10.3±0.75	14.4±0.60	18.4±0.55	14.6±0.65
p. value		0.019*	0.213	0.312	0.435
75%	12 hours	15.7±0.94	15.9±0.30	22.6±1.15	18.5±0.50
	24 hours	19.7±0.73	22.3±1.52	29.1±1.20	24.8±0.52
p. value		0.005**	0.016*	0.003**	0.001**
100%	12 hours	16.5±0.81	20.9±0.96	24.2±0.94	25.6±0.71
	24 hours	20.1±0.76	25.9±0.90	31.4±0.50	31.9±0.17
p. value		0.005**	0.003**	0.001**	0.003**
PB	12 hours	14.4±0.40	23.2±1.55	18.3±0.41	20.5±0.86
	24 hours	18.5±0.86	28.7±1.57	25.2±0.70	26.8±1.02
p. value		0.006**	0.013**	< 0.001**	0.001**
FLC	12 hours	15.3±0.47	19.8±0.76	22.8±1.04	24.0±0.50
	24 hours	21.1±1.04	25.2±0.75	30.1±1.08	30.4±0.68
p. value		0.004**	0.001**	0.001**	< 0.001**
MCL	12 hours	16.4±0.51	24.8±0.80	16.8±1.17	19.4±0.55
	24 hours	20.1±0.32	30.6±0.1.80	25.7±0.87	25.9±0.25
p. value		0.001*	0.018*	0.001**	0.001**

Notes: a non-significant difference is shown by a similar small litter, whereas a significant difference is indicated by a distinct small letter; PB – Amphotericin B, FLC – Fluconazole, MCL – Miconazole.

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Activity of CZ J Prel ethanol extracts on candida spp. after 24 hours in different concentration compared with anti-fungal therapeutics

The present study recorded the high activity against C. albicans in FCL, MCL and ethanolic extract 100%, followed activity showed in PB, while the low activity in ethanolic extract with 50%, and 25% concentration. In C. tropicalis the study noted the high activity was showed in MCL, followed in PB, followed in both ethanolic extract 100% and FCL, while the other ethanolic extract exhibited antifungal activity with increase concentration. In C. krusei the study showed the high activity in both ethanolic extract 100% and FCL, followed in ethanolic extract 75%, following in PB and MCL. In C. glabrata the study noted the high activity in ethanolic extract 100%, following in FLC, following in PB and MCL, at p. value <0.01 as in table 3.

A Compartment between activity of CZ J Prel ethanol extracts on candida spp after 12 and 24 hours in different concentration compared with anti-fungal therapeutics

The results noted the antifungal activity of ethanolic extract with 25% concentration increased significantly against C. albicans and C. glabrata only, while against C. tropicalis and C. krusei increased non-significantly. In ethanolic extract with concentration 50%, the study noted the antifungal activity increased significantly against C. albicans only, while against other Candida spp increased non-significantly. In ethanolic extract with both concentrations 75%, and 100%, and other antifungal therapeutics, the antifungal activity against all Candida spp increased significantly with increasing time at p. value <0.01 and <0.05, as show in table 4.

■ DISCUSSION

Testing for antifungal susceptibility continues to be of great interest. Testing for susceptibility can be utilized in epidemiology and drug development. There are numerous papers demonstrating the effectiveness of CZ essential oils as antibacterial agents Sharafati Chaleshtori [18]. According to reports, the oil extracted from, the bark and leaves has fungicidal effect against fungi that cause diseases in both people and plants, such as banana crown rot disease. Cinnamaldehyde and eugenol were identified to be the primary components in CZ bark and leaf oils with antifungal action, respectively. The presence of other chemicals with fungicidal properties in bark and leaves has also been documented [19].

The results obtained by the current study showed the activity of barks of Cinnamomum zeylanicum with 100% concentration have a highest antifungal activity against all isolated fungi, the study also noted the activity increased with increasing both time and concentration. This suggests that the existence of these bioactive chemicals may be the cause of the inhibitory effect of cinnamon extract extracts against the four fungi that were the subject of the study. The antifungal activity that has been reported may be due to the presence of flavonoids, alkaloids, tannins, saponins, terpenes, steroids, and essential oil in cinnamon extracts Mahmoud [17]. Yet, the different components of plant extracts and the essential oils may work together in a synergistic manner to produce the overall antibacterial activity. According to reports, cinnamon's bark exhibits antifungal properties due to the presence of the essential oil components cinnamaldehyde, eugenol, and cinnamic acid El Atki et al. [20].

Both antifungal and antibacterial activities are possessed by these bioactive components, eugenol has been shown by several researchers to have antifungal effects against Candida SPP (Marchese et al. [21]. One of the main bioactive components in cinnamon's bark is cinnamaldehyde, which according to some studies, cinnamaldehyde responsible for 80% of bacteria and fungi killing (Gilani et al. [22]. In addition to flavonoids, alkaloids, tannins, and saponins, some researchers have identified cinnamaldehyde, eugenol, and cinnamic acid as antifungal compounds (Djarot et al. [23]. 1,8-cineole, a distinctive chemical of the many components of Cinnamomum zeylanicum essential oil, several researchers have discovered that this ingredient is substantially responsible for a variety of its antimicrobial and pesticidal effects [24]. Essential oils abound in cinnamon inhibition of a variety of microorganisms, including fungus and bacteria, has been attributed by several researchers to the presence of essential oil and its various compounds in cinnamon extract [25].

The composition of essential oils, which are made up of numerous different volatile molecules, frequently changes between plant species. The antibacterial actions appear to be the consequence of numerous components working together synergistically. This indicates that the separate chemicals are less potent on their own Gann et al. [26].

The antifungal activity of cinnamon extracts observed in this study against Candida spp may be due to the presence of different phytochemical components in the extracts. In this research, cinnamon extracts exerted higher antifungal activity after 24 hours compared to the same extract after 12 hours of the extracts and this may be related to the content of bioactive compounds in cinnamon that are more effective against fungi over time. The methanolic fraction of cinnamon bark demonstrated the strongest antifungal activity against four fungi isolates when compared to the same fraction from other plants, followed by the hexane and aqueous fractions, according to results obtained by Pappas et al. [27].

In a different experiment against the A. alternata fungus, it was discovered that the methanolic extract of cinnamon and eucalyptus and other medicinal plants had powerful antifungal effects in preventing the formation of the mycelia, whereas the aqueous extracts had either weaker or no effects [28].

■ CONCLUSION

The ethanolic extract has a high activity against all Candida Spp in concentration 100%, and their activity was reduced with decreasing concentration.

■ REFERENCES

1. Singh N, Rao AS, Nandal A, et al. Phytochemical and pharmacological review of Cinnamomum verum J. Presl-a versatile spice used in food and nutrition. *Food Chemistry.* 2021;338:127773.
2. Diniz do Nascimento L, Barbosa de Moraes AA, Santana da Costa K, et al. Bioactive natural compounds and antioxidant activity of essential oils from spice plants: New findings and potential applications. *Biomolecules*. 2020;10(7):988.
3. Łuczaj Ł, Dumanowski J, Marszałek C, Parasecoli F. Turmeric and Cumin Instead of Stock Cubes: An Internet Survey of Spices and Culinary Herbs Used in Poland Compared with Historical Cookbooks and Herbals. *Plants*. 2023;12(3):591.
4. Hashemian M, Poustchi H, Murphy G, et al. Turmeric, pepper, cinnamon, and saffron consumption and mortality. *Journal of the American Heart Association.* 2019;8(18):e012240.
5. Salem MZ, Mervat EH, Ali HM, et al. Plants-derived bioactives: Novel utilization as antimicrobial, antioxidant and phytoreducing agents for the biosynthesis of metallic nanoparticles. *Microbial Pathogenesis.* 2021;158:105107.
6. Magill SS, Edwards JR, Bamberg W, et al. Multistate point-prevalence survey of health care–associated infections. *New England Journal of Medicine.* 2014;370(13):1198–208.

7. Nett JE, Andes DR. Contributions of the biofilm matrix to Candida pathogenesis. *Journal of Fungi.* 2020;6(1):21.
8. Mourer T, El Ghalid M, Pehau-Arnaudet G, et al. The Pga59 cell wall protein is an amyloid forming protein involved in adhesion and biofilm establishment in the pathogenic yeast Candida albicans. *npj Biofilms and Microbiomes.* 2023;9(1):6.
9. Falanga A, Maione A, La Pietra A, et al. Competitiveness during dual-species biofilm formation of Fusarium oxysporum and Candida albicans and a novel treatment strategy. *Pharmaceutics.* 2022;14(6):1167.
10. Maile SJ. *An investigation into alternative materials to silicone rubber for reducing Candida albicans biofilm formation in in-dwelling urinary catheters.* PhD Thesis. University of Central Lancashire.
11. Du H, Bing J, Hu T, et al. Candida auris: Epidemiology, biology, antifungal resistance, and virulence. *PLoS pathogens.* 2020;16(10):e1008921.
12. Govender NP, Avenant T, Brink A, et al. Federation of Infectious Diseases Societies of Southern Africa guideline: Recommendations for the detection, management and prevention of healthcare-associated Candida auris colonisation and disease in South Africa. *S Afr J Infect Dis.* 2019;34(1):163. doi: 10.4102/sajid.v34i1.163
13. Archambault LS. *Intravital Imaging in a Zebrafish Model Elucidates Interactions Between Mucosal Immunity and Pathogenic Fungi.* Theses of The University of Maine, 2019. Electronic Theses and Dissertations. 3066. Available at: https://digitalcommons.library.umaine.edu/etd/3066
14. Fulaz S, Vitale S, Quinn L, Casey E. Nanoparticle – biofilm interactions: the role of the EPS matrix. *Trends in microbiology.* 2019;27(11):915–26.
15. Costa-de-Oliveira S, Rodrigues AG. Candida albicans antifungal resistance and tolerance in bloodstream infections: The triad yeast-host-antifungal. *Microorganisms.* 2020;8(2):154.
16. Cheeseman S, Shaw ZL, Vongsvivut J, et al. Analysis of pathogenic bacterial and yeast biofilms using the combination of synchrotron ATR-FTIR microspectroscopy and chemometric approaches. *Molecules.* 2021;26(13):3890.
17. N Mahmoud S. Antifungal Activity of Cinnamomum zeylanicum and Eucalyptus microtheca Crude Extracts Against Food Spoilage Fungi. *Euphrates Journal of Agriculture Science*. 2012;4(3):218–31.
18. Sharafati Chaleshtori F, Taghizadeh M, Rafieian-kopaei M, Sharafati-chaleshtori R. Effect of chitosan incorporated with cumin and eucalyptus essential oils as antimicrobial agents on fresh chicken meat. *Journal of food processing and preservation.* 2016;40(3):396–404.
19. Mishra AK, Mishra A, Kehri HK, et al. Inhibitory activity of Indian spice plant Cinnamomum zeylanicum extracts against Alternaria solani and Curvularia lunata, the pathogenic dematiaceous moulds. *Annals of clinical microbiology and antimicrobials.* 2009;8:1–7.
20. El Atki Y, Aouam I, El Kamari F, et al. Antibacterial activity of cinnamon essential oils and their synergistic potential with antibiotics. *Journal of advanced pharmaceutical technology & research.* 2019;10(2):63–7.
21. Marchese A, Barbieri R, Coppo E, et al. Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. Critical reviews in microbiology. 2017;43(6):668-89.Gilani S, Najafpour G. Evaluation of the extraction process parameters on bioactive compounds of cinnamon bark: a comparative study. *Process Biochemistry.* 2022;114:93–101.
22. Djarot P, Utami NF, Putra AM, et al. Bioactivities and Chemical Compositions of Cinnamomum burmannii Bark Extracts (Lauraceae). *Sustainability*. 2023;15(2):1696.
23. Merghni A, Noumi E, Hadded O, et al. Assessment of the antibiofilm and antiquorum sensing activities of Eucalyptus globulus essential oil and its main component 1, 8-cineole against methicillin-resistant Staphylococcus aureus strains. *Microbial pathogenesis.* 2018;118:74-80.
24. Boire NA, Riedel S, Parrish NM. Essential oils and future antibiotics: new weapons against emerging 'superbugs'. *J Anc Dis Prev Rem.* 2013;1(2):105.
25. Gann LD. *Antimicrobial activity of essential oils and their components against lactic acid bacteria* (Master's Thesis, University of Tennessee), 2013. Available at: https://trace.tennessee.edu/utk_gradthes/2604
26. Pappas PG, Kauffman CA, Andes D, et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America.* 2009;48(5):503.
27. Da Cruz Cabral L, Pinto VF, Patriarca A. Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. *International journal of food microbiology.* 2013;166(1):1–4.

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In Vitro Evaluation of Photodynamic Disinfection and Conventional Root Canal Irrigation Protocol

Conflict of interest: nothing to declare.
Authors' contribution: Mohammed Al-Jaberi – conceptualization, data curation, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing – original draft and writing – review & editing; Salman Sahab Atshan – conceptualization, data curation, investigation, methodology, project administration, resources, validation, visualization, writing – original draft and writing – review & editing; Sonia Zouiten – conceptualization, data curation, investigation, methodology, project administration, validation, visualization, writing – original draft and writing – review & editing.
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Abstract

Introduction. The root canal treatment can lead to great success, but the infection within insufficiently treated root canal led to post-treatment disease.
Purpose. To evaluate photodynamic disinfection (PDD) efficacy in preventing the growth of microorganisms with conventional root canal irrigations regime in vitro.
Materials and methods. Sixty teeth were extracted and gathered with a single root and the crowns of all teeth were trimmed to have 14 mm root teeth. The autoclave was used for sterilizing all teeth, which were fitted in glass tube perpendicular, at a temperature of 121 °C for a period of 15 minutes. Afterwards, a sterile broth containing Enterococcus faecalis (E. faecalis) was added to the teeth. After that, the tubes were kept in an aerobic environment at 37 °C for a period of thirty days, every two days fresh broth was added. To evaluate the effectiveness of various disinfectants teeth were randomly divided into 6 groups according to disinfections protocol, each group contained 10 post-infected teeth. After completing the treatment, using a size #35 Hedstrom file, dentin chip samples were obtained from within the canals, and the samples were cultured on Mueller – Hinton agar and then bacterial colonies were counted in each group types. The experiments were repeated in triplicate and duplicate. The data consisted of the number of colonies forming units (CFUs) both before and after the treatments.
Results. The current study showed a significant difference between pre- and post-treatment results in each group. Irrespective of the photosensitizer used in photodynamic disinfection (toluidine blue O or riboflavin), PDD was significantly better than control group ($P<0.05$). There was no significant difference between PDD with toluidine blue O or riboflavin ($P>0.05$). Among the other groups, there was not a significant difference in colony count (PDD, NaOCl; $P>0.05$).

Conclusion. This study presents evidence that the employment of toluidine blue O and riboflavin in photodynamic disinfection to the regular chemo-mechanical irrigation procedure yielded the most favorable outcomes. These substances effectively reduced the quantity of (E. faecalis) in infected root canals. Therefore, they may serve as a valuable addition to root canal disinfection procedures.
Keywords: photodynamic disinfection, enterococcus faecalis, root canal, LED, biofilms

■ INTRODUCTION

The main goal of endodontic therapy is to eradicate or substantially reduce the amount of bacteria in the root canal [1]. Conventional endodontic treatments are frequently unable to entirely eliminate microorganisms due to the intricate anatomy of root canals. Evidence indicates that residual bacteria can be found in over half of the prepared root canal surfaces just prior to obturation [2].

The main cause of pulpal and periapical illnesses is persistent pathogenic microorganisms within the root canal system [3, 4].

E. faecalis strains primarily contribute to the development of pulpal and periradicular disorders [5]. While there is less information available on the various genotypes of this bacterium species, it possesses multiple types of surface and secretory virulence factors [4]. Biofilm creation is a crucial element in the pathogenicity of E. faecalis [6], biofilm-related endodontic infections pose a special challenge for E. faecalis because biofilm modifications enhance the pathogen's pathogenicity and resistance to antimicrobial agents and immune system components [7]. It is difficult and complex to completely eradicate the bacteria causing persistent root canal infections using traditional treatment methods [8].

Culturing investigations reveal that the frequently isolated microorganisms in persistent periapical lesions (mostly endodontic failures) is (E. faecalis), a facultative anaerobic gram-positive cocci [9], 18% of primary endodontic infections and 67% of secondary endodontic infections have been linked to E. faecalis [10].

Furthermore, lasers can be employed to conduct final root canal disinfection in addition to irrigant. In endodontic research, antimicrobial photodynamic disinfection (PDD) has been investigated more frequently over the past decade [11, 12].

As opposed to photo thermal, PDD has minimal to no heating and is primarily an indirect effect. A photo-activated material (the dye) is needed for PDD. After being applied, the microorganisms absorb the pigment. Next, the bacteria are subjected to light of a particular wavelength. Intracellular generation of reactive oxygen species (ROS) takes place as light activates the absorbed dye. These extremely reactive chemicals interfere with regular metabolic processes [12, 13].

PDD is a process that utilizes non-toxic photosensitizer (PS) chemicals which are activated by a certain wavelength of light [14]. Upon irradiation, the photosensitizer undergoes a reaction with molecular oxygen, resulting in the generation of high ROS. These ROS are responsible for causing damage and ultimately leading to the killing of microorganisms [12]. The photosensitizer attaches to the bacteria cell membranes, which then triggers the production of singlet oxygen upon exposure to radiation. In the end, the bacteria die because the singlet oxygen breaks down their cell wall [13].

PS is a crucial element of PDD that becomes active when exposed to light of a specific wavelength. Toluidine blue O (TBO) is a photosensitizer with a blue tint. It is amphiphilic. TBO has a positive charge and a low molecular weight. It absorbs red light most strongly at wavelengths between 620 and 660 nanometers. Root canal infections consist of a combination of both Gram-negative and Gram-positive bacteria. However, the amphiphilic nature of TBO makes it a suitable option for disinfecting root canals and has antibacterial impact on both types of bacteria. Furthermore, research has demonstrated that the firm attachment of TBO to E. faecalis makes it a more potent agent than methylene blue photosensitizer for root canals disinfection [15, 16].

Riboflavin (RFV) is a photosensitizer with a yellow tint that shows great potential as an alternative photosensitizing substance. A micronutrient and an intrinsic photosensitizer, it produces ROS upon exposure to blue light [17]. This material has excellent biocompatibility and may be easily activated using available LED lamps. That is specifically designed for the purpose of curing composite materials in dental offices. Due to its favorable and widely recognized toxicological and pharmacokinetic characteristics [18].

This study utilized a light-emitting diode (LED) as a source of light instead of a laser, which is worth emphasizing. Many research investigations examining the antibacterial properties of PDD utilize lasers that are more expensive than LED lamps and are subject to strict power output restrictions to prevent harm to surrounding tissues.

Currently, there is a shortage of studies examining the effectiveness of toluidine blue O and riboflavin, both used with LED light, in reducing E. faecalis biofilm on extracted teeth.

■ PURPOSE OF THE STUDY

To evaluate photodynamic disinfection (PDD) efficacy in preventing the growth of microorganisms with conventional root canal irrigations regime in vitro.

■ MATERIALS AND METHODS

Tooth selection and preparation

This experimental in vitro study was performed on 60 extracted human teeth. The teeth were maxillary and mandibular canines, maxillary incisors, and mandibular premolars. These teeth were extracted due to periodontal issues or as part of an orthodontic treatment plan. The teeth exhibited a single canal and a single root, with straight and intact roots of nearly similar length. There was no evidence of apical resorption or having endodontic disinfection. Consequently, teeth that had pulpal calcification, decay in the crown or root, fractures in the root, multiple roots, bent roots, or had undergone previous endodontic disinfection were not included. Prior to the experiment, the teeth had been disinfected and preserved in a saline solution.

Root canal preparation

All the teeth are cleaned with dental scaler and periodontal curette for removal of residual tissue. Using a dental laboratory engine and a sectioning disc under running water, the crowns of the teeth are removed. This process results in a 14 mm-long root. By using gates Glidden drills widen and taper the root canal orifice. By visually inspecting a size 10 manual K-file and placing it 1 mm from the apical foramen to assess the working length. Root canals were shaped with Rotary files (plex-v, Orodeka file, LTD China) from size 15 to 40 and irrigated with 2 mL of 3% sodium hypochlorite (NaOCl) after the use

of each instrument. One skilled researcher handled all aspects of root canal preparation. 2 ml of 5% sodium thiosulfate were used to neutralize the remaining intracanal NaOCl [19]. Each canal was irrigated with 2 mL of 17% Ethylenediaminetetraacetic acid (EDTA) after that being cleaned with distal water. Next the teeth were autoclaved at 121 °C for 15 min at 26 psi.

We used glass tube vial (K3 EDTA Tube 7 ml 13×75mm) after cleaning it in distal water to fixed the roots by using silicon impression material. Teeth were inserted with the root embedded in the silicon material in the glass tube in which the canal orifice is free of silicon in upright position where the cervical portion facing upwards and left 10 mm free space in the top of the tube to the broth material so that the broth could cover the tooth's root and then autoclaved again at 121 °C for 15 min at 26 psi, then aseptically stored until use.

Biofilm formation and bacterial inoculation

Following sterilization, 100 µL of E. faecalis at a concentration of 0.5 McFarland was introduced into the root canals to create biofilm. After that, the specimens were incubated for 30 days at 37 ºC in an aerobic environment. To maintain bacterial viability, fresh broth was added every two days.

Distribution of experimental groups

Group 1: teeth treated only with distal water it represents the control group.

Group 2: teeth treated with riboflavin + 480 nm LED.

Group 3: teeth treated with toluidine blue O +630 nm LED.

Group 4: teeth subjected to treatment of 3% NaOCl and 17% EDTA,

Group 5: teeth subjected to treatment of 3% NaOCl and 17% EDTA and riboflavin + 480 nm LED.

Group 6: teeth subjected to treatment of 3% NaOCl and 17% EDTA and toluidine blue O + 630 nm LED.

Procedure of disinfection

Sodium hypochlorite (NaOCl). Using a 30-gauge irrigation needle to disinfect specimens with 10 ml of 3% NaOCl at a flow rate of 3–3.5 ml per minute. Using rotating file agitation (extending up to the working length) with 35 No. file (plex-v, Orodeka file, LTD China), After that, irrigate with 2 ml 17% EDTA (Ultradent, South Jordan, USA), which was left in the canal for one minute.

Antimicrobial photodynamic disinfection with toluidine blue O. For the red-light source in groups 3 and 6, a light-emitting diode (LED) device (Fotosan 630, Korea, MDD, CMS Dental Denmark) was employed. This system has a wavelength spectrum of 630 nm and an output power of 2,000_4,000 mW/cm^2. TBO (0.1 mg/mL) was utilized as a PS agent. The root canal was filled with a TBO solution using a 30-gauge irrigation needle. The endodontic plastic tip was attached to the LED device and placed into the root canal without exerting any pressure, and light activation was performed for a duration of 30 seconds.

Antimicrobial photodynamic disinfection with riboflavin. A 0.1% riboflavin solution containing the photoactive material was injected into the root canals, thereafter, employing a blue light activation LED for 30 seconds using light cure device (O-Star, Guilin Woodpecker medical instrument Co.) at 1000–2000 mW/cm^2, 385–515 nm.

NaOCl and PDD. The specimens from Group 5 and Group 6 were initially disinfected using NaOCl and EDTA, and then underwent photodynamic disinfection.

SEM. Scanning electron microscope (SEM) was used to confirm the presence of the E. faecalis biofilm. From the available samples, five teeth were randomly selected. The root surfaces were prepared with longitudinal grooves using a high-speed diamond bur, while ensuring that the inner part of the root canal was not affected. After that, a stainless-steel chisel was used to split the roots into two sections via these grooves. After being fixed for 24 hours in 2% formalin, the samples were incubated in a graded sequence of ethanol concentrations (50, 70%, 90%, and 100%, twice) for 20 minutes. Afterward, they were allowed to dry overnight at room temperature in the open. Subsequently, the specimens were then sputtered coated with gold alloy and examined by using scanning electron microscope (ThermoFisher Scientific, Oregon, USA) at 1 kV.

Microbial sampling and quantification of colony-forming units

A preliminary sampling. Prior to disinfection of the teeth, a 100 µl sample of the microbial suspension was obtained and transferred to a sterile tube containing 1 milliliter of distilled water. The mixture was then vigorously mixed for 20 seconds and subsequently serially diluted 10 times. Subsequently, 100 µl of each solution was evenly distributed on a Mueller–Hinton agar (Merck Co., Germany) plate and subjected to incubation at 37 °C for a duration of 24 hours. The bacterial colonies were quantified based on the colony-forming units per milliliter (CFU/mL).

Final sampling. To inactivate the remaining NaOCl present in the canal, the teeth in the NaOCl groups (group 4, 5, 6) were irrigated for one minute using 2 ml of 5% sodium thiosulphate. The root canal was cleaned with 5 ml of sterile saline and left for 30 seconds in order to standardize all the groups. After that, the root canals were filed aggressively with an H-file size 35 [20]. This process made it possible for the biofilm to come down and for the bacteria that remained inside but were inaccessible to paper points to be extracted. Subsequently, paper points were inserted into the root canals up to the working length (WL) for a duration of 1 minute to collect samples for microbiological analysis. After that, the paper point was put into microtubes with 500 µl of the nutrient broth solution and vortexed for 20 seconds to thoroughly mix it. Subsequently, the sample was serially diluted. Subsequently, 100 µl of each solution was evenly distributed onto a Mueller – Hinton agar plate and subjected to incubation at 37 °C for a duration of 24 h. The quantity of bacterial colonies was assessed based on the CFU/ml measurement as showed in Figures (A–G).

Statistical analysis

The data was analyzed using SPSS software version 21. Descriptive statistics and statistical measures, including the t-test and one-way analysis of variance (ANOVA), were employed to examine the data. Nonparametric tests were utilized when the normality hypothesis was not proven. The tests were conducted using a significant threshold of 0.05.

■ RESULTS

Based on the findings of our investigation, all the groups that received treatment showed a noticeable decrease in the number of E. faecalis biofilms in the root canal compared to the control groups (specifically, the group that used water as a control; $p<0.05$). The group that had the lowest average value of colony-forming units per milliliter (CFUs/mL) was the

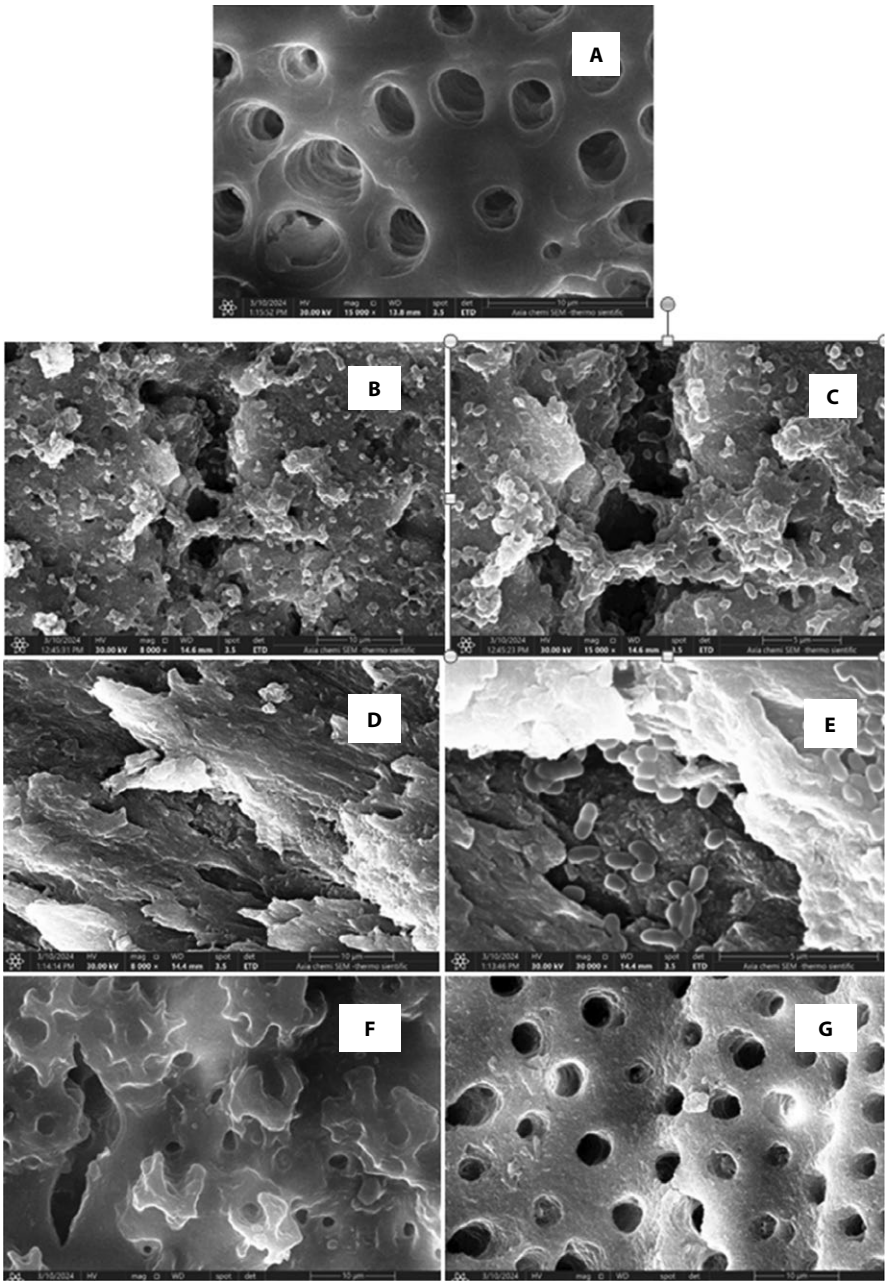

Figure: A – Clean dentinal tubule in sterile tooth specimen (15 000×); B – Biofilm colonization of E. Faecalis in the dentinal tubule in the root canal lumen before disinfection treatment (8000×); C – Biofilm colonization of E. Faecalis before disinfection treatment (15 000×); D – Biofilm colonization of E. Faecalis in the dentinal tubule in the fracture surface (8000×); E – Biofilm colonization of E. Faecalis the fracture surface (30 000×); F – Root canal lumen after disinfection with PDD (8000×); G – Root canal lumen after disinfection with NaOCl and PDD (8000×)

one that used sodium hypochlorite (NaOCl) and toluidine blue O (TBO) as PDD. This was followed by the group that used NaOCl and riboflavin PDD. The distal water group had the greatest mean value. Each group exhibited a noteworthy decrease in the quantity of biofilm generated by E. faecalis both before and after the treatment. Furthermore, there was no statistically significant difference (P>0.05) between the photodynamic treatment alone and the combination of NaOCl and photodynamic disinfection. On biofilm forms of E. faecalis strains, the effects of TBO PDD and NaOCl solutions, as well as riboflavin PDD and NaOCl solutions, were comparable to those of conventional irrigation solutions with NaOCl. The CFU/mL mean values (standard deviation) for the groups acquired at the two evaluation times are displayed in Table.

CFUs of bacteria before and after disinfection and significant differences in comparison with control group

Group	No.	Before treatment		After treatment			
		Mean	SD	Mean	SD	% of the reduction in comparison with the control	P value
Distal water (control group)	10	65×10^{11}	15×10^{12}	19×10^{5}	29×10^{5}	–	–
Riboflavin	10	14×10^{11}	20×10^{11}	15×10^{4}	26×10^{4}	99.99	0.001
TBO	10	32×10^{11}	50×10^{11}	18×10^{3}	14×10^{3}	99.99	0.001
NaOCl	10	41×10^{11}	71×10^{11}	67×10^{1}	63×10^{1}	99.99	0.001
NaOCl + Riboflavin	10	23×10^{11}	42×10^{11}	49×10^{1}	51×10^{1}	99.99	0.001
NaOCl + TBO	10	74×10^{11}	79×10^{11}	44×10^{1}	53×10^{1}	99.99	0.001

■ DISCUSSION

E. faecalis is the most commonly found species in endodontic infections and is a significant factor in the development of persistent periradicular lesions following root canal treatment [21]. The use of disinfecting chemicals to disinfect root canals is the primary and essential step in removing germs from the root canal system, dentinal tubules, and the periapical region [22]. The most common approach for reducing bacteria in infected root canals is through mechanical instrumentation. However, completely eliminating germs from the root canal is a challenging endeavor. Multiple studies have indicated that chemomechanical methods are not effective in completely eradicating germs from diseased root canals [23].

Paper points or dentin chips collected from the canal walls can be used to collect samples from within the root canal. Both of these methods are viable options for root canal sampling. While paper point sampling has been commonly utilized in several research because it's simple to use, it alone gathers specimens of intracanal fluid-containing planktonic microorganisms [24]. We collected dentin chips in our study because they allow us to sample biofilm-like formations holding to the canal wall, including microorganisms that have entered deep into the dentinal tubules by using Hedstrom file [2, 24].

An essential aim of modern clinical microbiology is to develop innovative approaches that can efficiently reduce the prevalence of biofilm infections in the wall of the affected root canal.

PDD is a form of antimicrobial treatment that has been the subject of various research [25] and the effectiveness of which has been proven in endodontics.

One of its advantages is the targeted elimination of bacteria without harming normal tissue or hurting neighboring tissues [26].

Based on the current study, groups 2 and 3, who underwent PDD alone, demonstrated a significant decrease in microbial content compared to the control group. Nevertheless, the total eradication of E. faecalis from root canal samples was not achieved. The results agree with previous research, which similarly observed significant reductions in microbial content with the use of PDD. However, this reduction was not enough to completely eliminate E. faecalis [27, 28].

Riboflavin (RFV) has several benefits as a photosensitizer for the purpose of PDD opportunities on a theoretical level. It is an extremely biocompatible chemical that is safe to employ intraorally [29]. Due to its pale-yellow hue, it does not cause as much discoloration on the tooth's hard tissues compared to TBO. This is particularly beneficial in areas of the teeth that are aesthetically significant. Additionally, it can be stimulated using readily available blue light generating LED light cure device. As a result, there would be little effort required to implement PDD with RFV and blue light in dental procedures. Prior research has demonstrated that blue light producing LED lamps used for composite curing can stimulate RFV, leading to the generation of ROS [17]. Nevertheless, our investigation unequivocally demonstrates that the antibacterial efficacy of PDD with RFV and blue light was inferior to that of PDD utilizing TBO and red light. The limited efficacy of PDD RFV can be attributed to a reduced generation of ROS from RFV in comparison to TBO [17].

As comprehensive root canal disinfectant, PDD cannot be implemented in the absence of chemical irrigation. According to these findings, PDD is believed to possess an extra antibacterial impact during root canal irrigation, particularly against resistant pathogens.

NaOCl is recommended as the main irrigation solution in endodontics because of its broad-spectrum antibacterial effectiveness and its capacity to dissolve organic compounds [30].

Research investigations have demonstrated that PDD has shown greater efficacy compared to NaOCl [31] or as effective as NaOCl [32], others, however, experienced different outcomes and felt it was less successful [28], these studies were using laser or another photosensitizer. The documented variances can be attributed to variations in methodology, NaOCl concentrations, and the diversity of PDD processes.

In this study, groups 5 and 6 (NaOCl + EDTA + PDD) demonstrated the most effective outcomes in terms of lowering the presence of E. faecalis within the root canal area being better than NaOCl and EDTA with no PDD. The discovery has validated the hypothesis of the current study, which posited that photodynamic disinfection would enhance the decontamination of the root canal system, and this in harmony with a study of Vaid et al. the study examined the collective impact of methylene blue dye and diode laser as PDD and a 2.5% (NaOCl) solution on E. faecalis, in comparison to the effects of saline and NaOCl irrigation alone, they found that PDD and NaOCl solution was the most efficient method for root canals disinfection that contained mature E. faecalis biofilms [33]. Also, Garcez et al [34] have a study on the impact of diode laser PDD in endodontic retreatments while in vivo. The researchers discovered that the use of PDD in addition to traditional endodontic treatment results in a notable decrease in bacterial presence following the use of NaOCl, hydrogen peroxide, and EDTA for irrigation. Furthermore, PDD proves to be effective against bacteria that are resistant to several drugs.

Although PDD is believed to possess an extra antibacterial impact during root canal irrigation, particularly against resistant pathogens. Additional research is required, particularly focusing on roots that have multiple canals, to evaluate the elimination of bacteria from challenging anatomical regions. Additionally, the effectiveness of PDD should be tested on other bacterial species, and additional evaluation should be conducted through clinical trials.

■ CONCLUSION

Within the constraints of the study, the combination of root canal irrigation using NaOCl and EDTA, along with photodynamic disinfection using LED light, demonstrated the most effective antimicrobial outcomes.

■ REFERENCES

1. Samiei M, Pakdel SMV, Rikhtegaran S, et al. Scanning electron microscopy comparison of the cleaning efficacy of a root canal system by Nd: YAG laser and rotary instruments. *Microscopy and Microanalysis*. 2014;20(4):1240–5.
2. Tennert C, Feldmann K, Haamann E, et al. Effect of photodynamic therapy (PDT) on Enterococcus faecalis biofilm in experimental primary and secondary endodontic infections. *BMC Oral Health.* 2014;14(1):1–8.
3. De Oliveira BP, Aguiar CM, Câmara AC. Photodynamic therapy in combating the causative microorganisms from endodontic infections. *Eur J Dent.* 2014;8(03):424–30.
4. Pourhajibagher M, Bahador A. Is antimicrobial agents can considered as effective weapons against endodontic infections by Enterococcus faecalis. *Der Pharma Chemica*. 2015;7:196–200.
5. Dioguardi M, Di Gioia G, Illuzzi G, et al. Inspection of the microbiota in endodontic lesions. *Dent J (Basel).* 2019;7(2):47.
6. Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: a review. *J Int Soc Prev Community Dent.* 2015;5(1):1.
7. Neelakantan P, Romero M, Vera J, et al. Biofilms in endodontics – current status and future directions. *Int J Mol Sci.* 2017;18(8):1748.
8. Pourhajibagher M. Adjunctive antimicrobial photodynamic therapy to conventional chemo-mechanical debridement of infected root canal systems: A systematic review and meta-analysis. *Photodiagnosis Photodyn Ther.* 2019;26:19–26.
9. Ørstavik D. Root canal disinfection: a review of concepts and recent developments. *Australian Endodontic Journal.* 2003;29(2):70–4.
10. Afkhami F, Akbari S, Chiniforush N. Entrococcus faecalis elimination in root canals using silver nanoparticles, photodynamic therapy, diode laser, or laser-activated nanoparticles: an in vitro study. *J Endod.* 2017;43(2):279–82.
11. Chrepa V, Kotsakis GA, Pagonis TC, Hargreaves KM. The effect of photodynamic therapy in root canal disinfection: a systematic review. *J Endod.* 2014;40(7):891–8.
12. Demidova TN, Hamblin MR. Photodynamic therapy targeted to pathogens. *Int J Immunopathol Pharmacol.* 2004;17(3):245–54.
13. Komine C, Tsujimoto Y. A small amount of singlet oxygen generated via excited methylene blue by photodynamic therapy induces the sterilization of Enterococcus faecalis. *J Endod.* 2013;39(3):411–4.
14. Benov L. Photodynamic therapy: current status and future directions. *Medical principles and practice.* 2014;24(Suppl. 1):14–28.
15. Singh S, Nagpal R, Manuja N, Tyagi SP. Photodynamic therapy: An adjunct to conventional root canal disinfection strategies. *Australian endodontic journal.* 2015;41(2):54–71.
16. Plotino G, Grande NM, Mercade M. Photodynamic therapy in endodontics. *International Endodontic Journal. Blackwell Publishing Ltd.* 2019;52:760–74.
17. Bouillaguet S, Wataha JC, Zapata O, et al. Production of reactive oxygen species from photosensitizers activated with visible light sources available in dental offices. *Photomed Laser Surg.* 2010;28(4):519–25.
18. Corbin F. Pathogen inactivation of blood components: current status and introduction of an approach using riboflavin as a photosensitizer. *Int J Hematol.* 2002;76(Suppl 2):253–7.
19. Peters OA, Bardsley S, Fong J, et al. Disinfection of root canals with photon-initiated photoacoustic streaming. *J Endod.* 2011 Jul;37(7):1008–12.
20. Bago I, Plečko V, Gabrić Pandurić D, et al. Antimicrobial efficacy of a high-power diode laser, photo-activated disinfection, conventional and sonic activated irrigation during root canal treatment. *Int Endod J.* 2013;46(4):339–47.
21. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *J Endod.* 2006;32(2):93–8.
22. Plotino G, Cortese T, Grande NM, et al. New technologies to improve root canal disinfection. *Brazilian Dental Journal. Associacao Brasileira de Divulgacao Cientifica.* 2016;27:3–8.
23. Borzini L, Condò R, De Dominicis P, et al. Root canal irrigation: Chemical agents and plant extracts against Enterococcus faecalis. *Open Dent J.* 2016;10:692.
24. Rios A, He J, Glickman GN, et al. Evaluation of photodynamic therapy using a light-emitting diode lamp against Enterococcus faecalis in extracted human teeth. *J Endod.* 2011;37(6):856–9.
25. Hecker S, Hiller K, Galler KM, et al. Establishment of an optimized ex vivo system for artificial root canal infection evaluated by use of sodium hypochlorite and the photodynamic therapy. *Int Endod J.* 2013;46(5):449–57.
26. Bhatti M, MacRobert A, Meghji S, et al. Effect of dosimetric and physiological factors on the lethal photosensitization of Porphyromonas gingivalis in vitro. *Photochem Photobiol.* 1997;65(6):1026–31.

27. Souza LC, Brito PRR, de Oliveira JCM, et al. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis. *J Endod.* 2010;36(2):292–6.
28. Rios A, He J, Glickman GN, et al. Evaluation of photodynamic therapy using a light-emitting diode lamp against Enterococcus faecalis in extracted human teeth. *J Endod.* 2011;37(6):856–9.
29. Nielsen HK, Garcia J, Væth M, Schlafer S. Comparison of riboflavin and toluidine blue O as photosensitizers for photoactivated disinfection on endodontic and periodontal pathogens in vitro. *PLoS One.* 2015;10(10):e0140720.
30. Zehnder M. Root canal irrigants. *J Endod.* 2006;32(5):389–98.
31. Garcez AS, Núñez SC, Lage-Marques JL, et al. Efficiency of NaOCl and laser-assisted photosensitization on the reduction of Enterococcus faecalis in vitro. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology.* 2006;102(4):e93–8.
32. Yildirim C, Karaarslan ES, Ozsevik S, et al. Antimicrobial efficiency of photodynamic therapy with different irradiation durations. *Eur J Dent.* 2013;7(04):469–73.
33. Vaid D, Shah N, Kothari D, Bilgi P. Additive effect of photoactivated disinfection on the antibacterial activity of QMix 2in1 against 6-week Enterococcus faecalis biofilms: An in vitro study. *J Conserv Dent.* 2017;20(1):41.
34. Garcez AS, Nuñez SC, Hamblim MR, et al. Photodynamic therapy associated with conventional endodontic treatment in patients with antibiotic-resistant microflora: a preliminary report. *J Endod.* 2010;36(9):1463–6.

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Correlation between Melatonin Hormone and Superoxide Dismutase in Patients with Obstructive Sleep Apnea

Conflict of interest: nothing to declare.
Authors' contribution: Mahdi Maid Yousif – conceptualization, data curation, investigation, methodology, project administration, resources, software, visualization, writing – original draft and writing – review & editing; Husam Kredy – conceptualization, data curation, investigation, methodology, supervision, validation, visualization, writing – original draft and writing – review & editing; Majeed Mohan ALhamami – conceptualization, data curation, investigation, project administration, resources, supervision, validation, visualization, writing – original draft and writing – review & editing.
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Abstract

Introduction. Obstructive sleep apnea syndrome (OSAS) is the obstruction of the upper airway occurs during sleep, with interruption (apnea) or reduction (hypopnea) of airflow, followed by brief awakening leading to restoration of airway permeability in the upper airway.
Purpose. To detect the relationship between Melatonin (MT) and Superoxide dismutase (SOD) in patients with obstructive sleep apnea (OSA).
Materials and methods. The present study has been conducted on all numbers of healthy people and patients. Group A: 84 patients OSA syndrome (mild, moderate and sever) with high blood pressure (47 male, 37 female) and age range (40–65). Group B: control group, consist of 64 healthy people (33 male, 31 female) and age range (40–65).
Results. The results showed that there was a significant increase in concentrations of serum Melatonin and significant decrease in concentration of serum SOD in patient groups comparison with control group (P≤0.05). Results indicated that there was a weak positive correlation between Melatonin with SOD in patients with Obstructive sleep apnea.
Conclusion. OSA has a significant effect on melatonin secretion, but the exact mechanisms remain to be elucidated. In our study, in accordance with other studies, we found a significant increase in melatonin levels in OSA patients when compared to the control group. The decrease in concentration of serum SOD in patients OSA indicate systemic oxidative stress in patients with OSA. Therefore, we recommend measurement of serum Melatonin and serum SOD in the OSA patients as potential risk predictors for cardiovascular diseases and use for diagnosis of this disease.
Keywords: obstructive sleep apnea, melatonin, superoxide dismutase, upper airway, hypopnea

INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is when partial or complete obstruction of the upper airway occurs during sleep, with interruption (apnea) or reduction (hypopnea) of airflow, followed by brief awakening leading to restoration of airway permeability in the upper airway. These apnea/hypopnea cycles repeat several times per hour, resulting in sleep that is fragmented and almost unrecoverable. Within the upper respiratory tract, the pharynx, particularly the oropharynx and hypopharynx, is the area most susceptible to obstructive processes leading to OSAS [1]. OSAS negatively affects the health and behavior of millions of adolescents worldwide. It is an independent risk factor for many diseases, including hypertension, heart failure, heart disease, cardiovascular events, and arrhythmias. Unfortunately, it is a common chronic disease that seriously affects patients' lives [2]. The relationship between OSA and cardiovascular comorbidities stands out as a point of interest. In OSA patients, the periodic collapse of the upper airway during sleep leads to chronic intermittent hypoxia, which is believed to foster cardiovascular disease by way of triggering oxidative stress and sympathetic activation plus systemic and vascular inflammation [3–5]. Research at present highlights two main areas: the connection between inflammatory biomarkers and the severity of OSA and predicting cardiovascular events among patients with OSA [6].

The pineal gland in vertebrates is responsible for the production and release of melatonin, a small indoleamine that enters the bloodstream. This release of melatonin is most pronounced during nighttime and plays a crucial role in transmitting information about circadian rhythms to the body. In humans, melatonin exhibits various mechanisms of action, including its ability to bind to melatonin receptors coupled with G proteins, thereby regulating numerous aspects of circadian rhythms, sleep patterns, and even cancer suppression. Furthermore, melatonin also possesses receptor-independent functions, such as its capacity to neutralize free radicals and safeguard vital molecules against oxidative stress caused by conditions like ischemia, drug toxicity, or exposure to ionizing radiation [7].

Melatonin exhibits various biological effects by effectively neutralizing free radicals and enhancing the function of antioxidant enzymes [8]. Melatonin receptors are present in numerous tissues throughout the body, including pancreatic islet cells, which highlights the diverse impact of melatonin on physiological processes like energy metabolism and regulation of body weight [9].

The metalloprotein known as SOD plays a crucial role in transforming superoxide anions ($O2^{\bullet-}$) into hydrogen peroxide (H2O2). Within the realm of antioxidants, SOD reigns supreme as the most powerful enzyme in the human body. Its primary function is to neutralize superoxide anions, preventing them from interacting with NO and halting the creation of peroxynitrite (ONOO–) [10], SOD one of the antioxidant enzymes, it is catalyzes the reaction between two superoxide radicals to yield one molecule each of oxygen and hydrogen peroxide [23].

Within the cell, there are three distinct isoforms of SOD (EC 1.15.1.1) that play a crucial role in the generation of reactive oxygen species (ROS). These isoforms, namely cytosolic SOD1, mitochondrial SOD2, and extracellular SOD3, are localized in specific areas. To exert their activity, these isoforms rely on different metal cofactors and dimerization. The activity of SOD1 dimers and SOD3 tetramers is reliant on the binding of copper (Cu) and

zinc (Zn), leading to their classification as Cu-ZnSOD. On the other hand, SOD2 tetramers, also known as MnSOD, utilize manganese (Mn) as a cofactor [11]. The significance of SOD2 lies in its role as the initial defense mechanism within the mitochondrial matrix against $O2^{\bullet-}$, a byproduct generated during oxidative phosphorylation [10]. Furthermore, it has been documented that SOD1 exhibits a peroxidase activity that is reliant on CO2, with this dependence attributed to the occurrence of two consecutive reactions involving H2O2. These reactions result in the formation of a potent oxidizing agent at the copper site of the enzyme, which subsequently facilitates the oxidation of CO2 to carbonate radicals, initiating a series of subsequent oxidation reactions [12]. There have been reports on the correlation between SOD and cardiovascular diseases. One such example is the observation that when SOD2 expression is inhibited, it leads to heightened oxidative stress in mitochondria and hypertrophy in cardiomyocytes [13].

■ PURPOSE OF THE STUDY

To detect the relationship between Melatonin and Superoxide dismutase in patients with obstructive sleep apnea.

■ MATERIALS AND METHODS

The research was carried out at AL-Nasiriyah General Hospital in Thi-Qar, especially, the Respiratory Diseases Unit and the clinical biochemistry laboratory in AL-Haboubi Teaching Hospital, as well as specialist clinics during the timeframe from 1/12/2022 to 1/11/2023. The study comprised a total of (148) individuals, with (64) being control subjects and (84) being patients.

The present study has been conducted on all numbers of healthy people and patients. Group A: 84 patients OSA syndrome (mild, moderate and sever) with high blood pressure (47 male, 37 female) and age range (40–65). Group B: control group, consist of 64 healthy people (33 male, 31 female) and age range (40–65).

Blood samples were obtained post the PSG polysomnography recorded in the Respiratory Diseases Unit at Nasiriyah General Hospital. Approximately (5 mL) of blood samples from both OSA patients and control groups were collected and left to clot in plain disposable tubes at room temperature. The samples were then centrifuged at 3000 rotations per minute (rpm) for 10 minutes; thereafter, the serum samples were separated and preserved at (-20^{o}C) for future evaluation of biochemical parameters unless needed on an urgent basis.

Serum Melatonin levels were measured by a commercially available ELISA kit (Human Melatonin (MT) ELISA Kit, Shanghai YL Biotech Co., Ltd). The assay ranges from 5 to 1000 ng/L. Serum SOD levels and were measured by a commercially available ELISA kit (Human Super Oxidase Dismutase (SOD) ELISA Kit, Shanghai YL Biotech Co., Ltd). The assay ranges from 3 to 900 U/L.

Statistical analysis

Statistical analysis was performed using the statistical package for the social sciences version – 23, and findings were reported in the (mean ± standard deviation) format. All groups that were analyzed used the T-test to compare various parameters. P-values ($P \leq 0.05$) were taken into consideration.

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RESULTS

Characteristic data for all studied groups shown in Table 1.

Table 1
Demographic between groups

Groups	No.	Age year, mean±SD	BMI (Kg/m²), mean±SD	Male/female, No.	Smoker, no/yes	
					Male/Female	
Control	64	47.09±5.82	28.60±4.6	33/31	18/15	31/0
Patients	84	52.89±6.50	35.71±4.88	47/37	27/20	37/0

Melatonin level in OSAS patients and control group

The results of the present study showed that there was significant increase in concentrations of serum Melatonin (MT) in patient groups comparison with control group (P≤0.05) described in Table 2.

Table 2
Serum melatonin concentrations of groups

Group	No.	MT concentration (ng/L), mean±SD	t	P-value
Control	64	7.71±3.02	11.60	0.00
Patients	84	36.39±22.38		

Note: * P-value less than 0.05 consider significant.

SOD level in OSAS patients and control group

Table 3 ashow a decrease in concentration of serum SOD in patient groups in comparison with control group (P≤0.05).

Table 3
Serum SOD concentrations of groups

Group	No.	SOD concentration (U/L), mean±SD	t	P-value
Control	64	24.74±7.12	11.8	0.00
Patients	84	12.51±4.82		

Note: legend as in the table 2.

Correlation between melatonin and SOD

Results indicated that there was a weak positive correlation between Myeloperoxidase with SOD in patients with OSA (Table 4).

Table 4
Correlation between Myeloperoxidase with SOD in patients with OSA

		SOD
Melatonin	Correlation (R)	0.118
	P-value	0.284

■ DISCUSSION

The release of melatonin follows a consistent circadian pattern and is widely recognized as a reliable indicator of circadian rhythms [14]. Sleep disorders, seasonal affective disorder, night shift work, and time zone changes have all been shown to have a significant impact on circadian rhythms, as supported by substantial evidence [15]. The circadian phase can be either advanced or delayed due to the sleep disturbances caused by intermittent hypoxia and frequent awakenings experienced by individuals with OSA. Consequently, the biological profile of hypothalamic hormones and neuropeptides may be modified [16].

In the realm of antioxidant enzymes that eradicate reactive oxygen species (ROS), SOD plays a vital role, much like catalase and peroxidase. However, individuals with OSA exhibit diminished levels of SOD activity compared to individuals in good health [17]. Patients with mild to moderate OSA exhibit a notable decrease in SOD activity [18].

Within the realm of endogenous antioxidant defense, there exists a comprehensive system comprising enzymatic and non-enzymatic antioxidants. Of particular significance is the presence of SOD, an essential enzyme with potent antioxidant properties. Numerous research endeavors have been undertaken to explore the potential of SOD levels as a reliable biomarker for assessing oxidative stress in individuals diagnosed with OSA [19]. In comparison to the control group, these patients have exhibited a more significant reduction in SOD levels, as noted by certain researchers [20, 21].

Oxidative stress is characterized by the heightened generation of oxygen free radicals and/or diminished capacity to eliminate them from tissues. This results in the buildup of different reactive oxygen species (ROS) within cells, including superoxide anions, nitric oxide, hydrogen peroxide, peroxynitrite, and hydroxyl free radicals, ultimately leading to oxidative harm [22]. OSA-related diseases may be attributed, in part, to oxidative stress. The organism's defense mechanisms against free radicals, known as the antioxidant system, play a crucial role in protecting against damage. SOD, the key enzyme of this system, is not only the most important metabolic enzyme for free radicals but also serves as a stable and easily measurable marker of oxidative stress in various clinical scenarios [23]. By scavenging lipid peroxides, SOD repairs cell damage caused by superoxide anion free radicals. It effectively mitigates peroxidative damage to tissue cells and shields arterial endothelial cells from harm caused by oxygen free radicals. In the regulation of oxidative stress, SOD plays a vital role. However, OSA patients exhibit diminished antioxidant levels and reduced activity of antioxidant enzymes, along with increased production of ROS and lower circulating SOD levels [20].

■ CONCLUSION

OSA has significant effect on melatonin secretion, but the exact mechanisms remain to be elucidated. In our study, in accordance with other studies, we found significant increase in melatonin levels in OSA patients when compared to the control group. The decrease in concentration of serum SOD in patients OSA indicate systemic oxidative stress in patients with OSA. Therefore, we recommend measurement of serum Melatonin and serum SOD in the OSA patients as potential risk predictors for cardiovascular diseases and use for diagnosis of this disease.

REFERENCES

1. Lynn RC. Obstructive sleep apnea syndrome (OSAS) in children. Diagnostic challenges. *Sleep*. 1996;19:274–7.
2. Laube I, Seeger R, Russi EW, Bloch KE. Accidents related to sleepiness: review of medical causes and prevention with special reference to Switzerland. *Schweiz Med Wochenschr.* 1998;128:1487–99.
3. Arnaud C, Dematteis M, Pepin JL, et al. Obstructive sleep apnea, immuno-inflammation, and atherosclerosis. *Semin Immunopathol.* 2009;31(1):113–25.
4. Lévy P, Pépin JL, Arnaud C, et al. Obstructive sleep apnea and atherosclerosis. *Prog Cardiovasc Dis.* 2009;51(5):400–10.
5. McNicholas WT. Obstructive sleep apnea and inflammation. *Prog Cardiovasc Dis.* 2009;51(5):392–9.
6. Kent BD, Ryan S, McNicholas WT. Obstructive sleep apnea and inflammation: relationship to cardiovascular co-morbidity. *Respir Physiol Neurobiol.* 2011;178(3):475–81.
7. Reiter RJ, Tan DX, Galano A. Melatonin: exceeding expectations. *Physiology (Bethesda).* 2014;29(5):325–33.
8. Daiekhmin S. Relationship between melatonin hormone and lipid profile of patients with myocardial infarction in Thi-Qar Governorate. *University of Thi-Qar Journal of Science.* 2021;8(2):72–75.
9. Al-Fartosi KG, Jabbar EA, Jabbar AA. Assessment of melatonin level and genetic aspect of type 2 diabetes mellitus patients in Thi-Qar province, Iraq. *University of Thi-Qar Journal of Science,* 2019;7(1):1–6.
10. Fukai T, Ushio-Fukai M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid Redox Signal.* 2011;15(6):1583–606.
11. Sharifi-Rad M, Anil Kumar NV, Zucca P, et al. Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. *Front Physiol.* 2020;11:694.
12. Liochev SI, Fridovich I. Mechanism of the peroxidase activity of Cu, Zn superoxide dismutase. *Free Radical Biology and Medicine*, 2010;48(12):1565–1569.
13. Dubois-Deruy E, Cuvelliez M, Fiedler J, et al. MicroRNAs regulating superoxide dismutase 2 are new circulating biomarkers of heart failure. *Sci Rep.* 2017;7(1):14747. doi: 10.1038/s41598-017-15011-6
14. Arendt J. Melatonin and human rhythms. *Chronobiol Int.* 2006;23(1–2):21–37.
15. Sack RL, Auckley D, Auger RR, et al. Circadian rhythm sleep disorders: Part I, Basic principles, shift work and jet lag disorders: an American academy of sleep medicine review. *Sleep.* 2007;30(11):1460–83.
16. Papaioannou I, Twigg GL, Kemp M, et al. Melatonin concentration as a marker of the circadian phase in patients with obstructive sleep apnoea. *Sleep Med.* 2012;13(2):167–71.
17. Lira AB, de Sousa Rodrigues CF. Evaluation of oxidative stress markers in obstructive sleep apnea syndrome and additional antioxidant therapy: a review article. *Sleep Breath.* 2016;20(4):1155–1160.
18. Wysocka E, Cofta S, Cymerys M, et al. The impact of the sleep apnea syndrome on oxidant-antioxidant balance in the blood of overweight and obese patients. *J Physiol Pharmacol.* 2008;59 Suppl 6:761–9.
19. Tian Z, Sun H, Kang J, et al. Association between the circulating superoxide dismutase and obstructive sleep apnea: a meta-analysis. *Eur Arch Otorhinolaryngol.* 2022;279(4):1663–1673.
20. Murri M, García-Delgado R, Alcázar-Ramírez J, et al. Continuous positive airway pressure therapy reduces oxidative stress markers and blood pressure in sleep apnea-hypopnea syndrome patients. *Biol Trace Elem Res.* 2011;143(3):1289–301.
21. Yang XH, Liu X, Shang J, et al. Correlation between the serum level of advanced oxidation protein products and the cognitive function in patients with obstructive sleep apnea hypopnea syndrome. *Zhonghua Jie He He Hu Xi Za Zhi.* 2013;36(4):274–9.
22. Eleutherio ECA, Silva Magalhães RS, de Araújo Brasil A, et al. SOD1, more than just an antioxidant. *Arch Biochem Biophys.* 2021;697:108701.
23. AL-Slem BAM. Effect of deficiency thyroid hormone on superoxide dismutase activity enzyme and their related metals in hypothyroid patients in Basrah Governorate/Iraq. *University of Thi-Qar Journal of Science.* 2013;3(4):112–116.