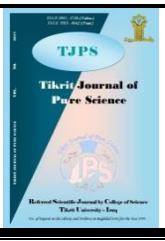
Tikrit Journal of Pure Science (2023) 28 (5): 1-11
Doi: https://doi.org/10.25130/tjps.v28i5.1308

TJPS

IASJ
المجلات الاكاديمية العراقية

Tikrit Journal of Pure Science
ISSN: 1813 – 1662 (Print) --- E-ISSN: 2415 – 1726 (Online)

Journal Homepage: http://tjps.tu.edu.iq/index.php/j



Isolation and identification of some predominant bacteria and assessment of TNF-α level in serum of patients with gingivitis

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Keywords: periodontal disease, gingivitis, TNF-α, bacteria, antibiotic sensitivity.

ARTICLEINFO.

Article history:

-Received:	**13 June 2023**
-Received in revised form:	**14 Aug. 2023**
-Accepted:	**15 Aug. 2023**
-Final Proofreading:	**24 Oct. 2023**
-Available online:	**25 Oct. 2023**

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ABSTRACT

This study was conducted to diagnose the aerobic bacterial species causing gingivitis, reveal the antibiotic susceptibility pattern and assess the level of tumor necrosis factor-alpha (TNF-α) in serum of patients with gingivitis. In total, 110 samples (including patient group and control group) were collected during the period from November 2021 until March 2022. Of which, 80 samples were collected by using oral swabs from patients attending the College of Dentistry at Tikrit University and outpatient clinics of both gender with different age groups. While the remaining 30 samples were collected from healthy individuals representing the control group. Identification of bacterial isolates was performed depending on micro and macroscopic cultural characteristics and biochemical tests. In addition to assessing the biochemical characteristics of the isolates, a VITKE2 compact system was used to ensure the identification of species level. The TNF-α concentrations in the serum were determined using an enzyme-linked immunosorbent assay known as a sandwich ELISA. Out of 80 samples, 60 (75%) samples showed positive bacterial growth cultures, while 20 (25%) samples showed no bacterial growth. The most common isolated bacteria species was *Streptococcus mutans* (18%), followed by *Streptococcus mitis* (13%), *Staphylococcus aureus* (12%) *Streptococcus salivarius, Streptococcus pyogenes* (8%) *Staphylococcus epidermidis, Enterococcus feacalis, Escherichia coli, Klebsilla pneumoniae* (6%), *Rothia dentocariosa, Pseudomonas aeruginosa* (4%), and *Streptococcus pneumonia, Kocuria kristinae, pneumonia, Kocuria kristinae, Granulicatella adiacens* (3%). The sensitivity of the bacterial isolates under study was tested to 11 antibiotics. Different species of bacteria showed various sensitivity patterns to several kinds of antibiotics. The study recorded a high significant difference (P=0.0007) between the patients (56.54 + 9.32 pg/ml) and the control group (31.88 + 7.44 pg/ml) concerning the level of TNF-α. In conclusion, the predominant bacteria identified from gingivitis patients were *S. mutans* and *S. mitis*. In addition, the levels of TNF-α in gingivitis patients were significantly higher than in the control group.

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عزل وتشخيص بعض البكتيريا المسببة لالتهاب اللثة وتقييم عامل نخر الورم الفا في مصل المرضى

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الملخص

أجريت هذه الدراسة لتشخيص الأنواع البكتيرية الهوائية المسببة لالتهاب اللثة ولأجراء اختبار الحساسية للمضادات الحيوية، وتقييم مستوى TNF-alpha في مصل مرضى التهاب اللثة. تم جمع العينات خلال فترة تشرين الثاني ٢٠٢١ إلى آذار ٢٠٢٢. بالمجموع ١١٠ عينة من (المرضى والاصحاء)، تم اخذ ٨٠ عينة من مسحة من الفم والدم من المرضى المراجعين الى كلية طب الاسنان جامعة تكريت والعيادات الخارجية من كلا الجنسين بمختلف الفئات العمرية. تم تشخيص العزلات بالاعتماد على الفحوصات المجهرية والبكتيرلوجية والاختبارات الكيموحيوية، وتم استخدام جهاز vitek 2 للتأكيد، تم قياس مستوى TNF-alphaفي الدم باستخدام تقنية الاليزا (sandwich ELISA). وقد أظهرت النتائج من بين ٨٠ عينة ٦٠(٧٥٪) نموا بكتيريا موجبا و٢٠(٢٥٪) لم تعط نمواً بكتيرياً، وكانت أعلى نسبة لبكتيريا *Streptococcus mutans* بنسبة (١٧٪) تليها *Streptococcus mitis* (12%) و*Staphylococcus aureus* بنسبة (١١٪) *Streptococcus salivarius, Streptococcus pyogenes* بنسبة (٧٪) وكانت كل من *Enterococcus feacalis, Escherichia coli, Klebsilla pneumonia Staphylococcus epidermidis* بنسبة (٦٪).

اما *Rothia dentocariosa, Streptococcus pneumonia, Kocuria kristinae, Granulicatella adiacens* و *Pseudomonas aeruginosa* بواقع (٤٪)، اختبرت حساسية العزلات البكتيرية قيد الدراسة ل ١١ مضاد حيوي وكانت العزلات حساسة لأنواع عديدة من المضادات الحيوية. سجلت الدراسة فرقا معنويا عاليا P=0.0007بين المرضى (152.8 + 19.5 pg/ml) والاصحاء (82.8 + 12.3 pg/ml) فيما يتعلق بـ TNF-alph.

الكلمات المفتاحية: امراض اللثة، التهاب اللثة، عامل نخر الورم الفا، البكتيريا، حساسية المضادات الحيوية.

Introduction

Gingivitis and periodontitis are two conditions listed under the umbrella term periodontal disease. Periodontal disease refers to a range of conditions that affect the supporting tissues of the teeth [1]. Typically, one of the first indications of gingivitis is bleeding gums, which is a common symptom of the disorder [2]. In the absence of treatment, gingivitis can progress to periodontitis, which is characterized by the loss of periodontal attachment and alveolar bone and ultimately results in tooth loss. Antibiotics can be used to treat gingivitis [3].

Dentists refer to the inflammation of the gums as gingivitis. It occurs as a result of inadequate tooth cleaning, which leads to the deposition of bacterial plaque on the surface of the teeth. Therefore, effective tooth brushing is vital for achieving enough food debris clearance, as it helps to avoid the formation of plaque in the future. Gingivitis is considered local when less than 30% of the gingival tissue bleeds during periodontal probing; however, it is termed generalized when the percentage is larger than 30%. Gingivitis is separated from periodontitis by the absence of x-ray evidence of periodontal tissue degeneration or tooth attachment loss [4].

Depending on the conditions, the relationship between immune surveillance and the oral microbe-induced host immunological response might take several forms. When local stimulation and the host's immune response are in a healthy balance, immunological monitoring and an optimal immune response will prevail [5]. The bacteria that cause the disease emit chemicals that stimulate the innate immune system, resulting in the release of proinflammatory cytokines that contribute to the course of the disease. Cytokines and chemokines released during a continuous immunological response have the ability to damage periodontal ligaments, gingiva, and alveolar bone [6]. Cytokines are crucial peptide mediators whose primary function is to facilitate cell-to-cell communication and signaling. The control of cell proliferation and differentiation, immunological and inflammatory responses, and immune responses are among the many activities of cytokines. The size of cytokines ranges from less than 5 kilodaltons to greater than 20 kilodaltons. These cytokines have the ability to bind to

specific receptors on specific cells, resulting in diverse genetic and chemical regulation. Certain cells produce cytokines, which then influence the activity of numerous other cells [7]. TNF-alpha, also known as tumor necrosis factor alpha, is a pro-inflammatory cytokine that is released by macrophages. TNF-alpha is principally responsible for periodontitis-related bone resorption [8]. TNF-a is one of the key periodontal pathogens-induced early inflammatory cytokines in destructive periodontal disease. Microorganisms that cause periodontal disease are referred to as periodontal pathogens. It is well known that elevated TNF-a levels represent a risk factor for devastating periodontal disease [9].

Materials and Methods

Sample Collection and Bacterial Isolation and Identification

Using cotton swabs, 110 samples (including patient group and control group) were collected from gingivitis patients identified at the College of Dentistry at Tikrit University and outpatient clinics during the period from November 2021 to March 2022. The samples were transferred directly to the microbiology laboratory. Specimens were cultured on Blood agar, MacConkey agar, Nutrient agar and Mannitol salt agar for the growth of bacteria species. They were incubated at 37°C for 18-24 hours. The bacteria were diagnosed through employing phenotypic and microscopic examinations, as well as biochemical tests that included (oxidase, Coagulase, Catalase, IMVIC) [10,11]. The diagnosis was confirmed by using VITKE2 system.

Blood Samples

A sample of three to five milliliters of venous blood was taken from each individual in the patient group and the control group. A tourniquet was applied directly to the skin around the arm, and the skin over the vein was sterilized with 70% of ethyl alcohol. Then, centrifugation was applied at 3000 g for 2 minutes. After that, the serum was collected in a sterile extension tube in three replicates and kept frozen at −20 °C until they were assayed.

Bacterial Susceptibility Antibiotics

On Muller Hinton Agar (MHA), antibiotic susceptibility testing of various bacterial species was conducted using the Kirby-Bauer disc diffusion method (Bauer et al., 1966). Utilizing various marketed antibiotic discs, the sensitivity pattern of various isolated species was determined. On the basis of CLSI recommendations, the zone of inhibition of each antibiotic disc against various bacterial species was interpreted [12].

Statistical Analysis

The data was statistically examined using the T-test with P-values of (0.01) and (0.05). Then, it was compared using the Duncan's Multiple Range statistical tool in Microsoft Office Excel 2010 [13].

Results and Discussion

In this study, a total of 110 samples were collected from the patient group and control group. The patient group included eighty samples collected by using oral swab. Out of these 80 samples, 60 (75%) samples showed positive bacterial growth cultures, while 20 (25%) samples showed no bacterial growth. In addition, the remaining 30 samples were collected from healthy individuals representing the control group, showing no bacterial growth cultures. This result is in agreement with [14,15], where they found that 25% and 18% did not show growth. In this study, the rate of infection was higher in males as compared to that in females, as shown in table (1). This result is consistent with [16], which showed that 50% of males suffered from periodontal disease compared with females (10%). This may indicate that females did not reach a threshold of inflammation that might have otherwise been associated with severe periodontal infections. In the same context, the study of [17] shows that periodontal disease was more among males (68%) as compared to females (32%). The gender differences reported might be attributable to the treatment bias, practice differences, or socioeconomic determinants.

The rates of dental care utilization were lower among men than women, due to ignorance of oral hygiene and negligence of or wrong tooth brushing [16,18].

Table 1. Gender distribution among patients with periodontal disease

Gender	No. (%) of patients
Males	46 (76.6%)
Females	14 (23.4%)
Total	60 (100%)

The patients' ages ranged between 15-75 years. The highest rate of infections (53%) was among patients aging 15-25, followed by 17% for the age group 6-10 and 33% for the age group 26-35. The lowest percentage of all infections (13%) was for the age group >36 years. As for ages groups, the most affected age group was 15-25 years old. These results are almost similar to those of [19], but differ from [20] which showed that the high percentage of periodontitis was at age 33-57 years old. The prevalence and severity of periodontal disease tends to increase with patient age. Degenerative changes in periodontal tissues are assumed to be the cause of this condition. For certain extent, poverty, lower income and lower education may be associated with higher levels of periodontal disease among adults [21].

This study revealed that the most common isolated species causing bacterial infections was *S. mutans* (18%), followed by *S. mitis* (13 %), *S. aureus* (12%), *S. salivarius and S. pyogenes* (8%), *Staph. epidermidis, E. faecalis, E. coli and K. pneumonia* (6%), *R. dentocariosa and P. aeruginosa* (4%). While the least common isolated bacterial species represented by (3%) recorded for *S. pneumonia, K. kristinae and G. adiacens*, as shown in table (2).

Table 2. Number and percentage of bacterial species isolated from gingivitis

No.	Bacteria	Gram stain	Number	Percentage
1	*Streptococcus mutans*	Gram positive	11	18 %
2	*Streptococcus mitis*		8	13 %
3	*Staphylococcus aureus*		7	12 %
4	*Streptococcus salivarius*		5	8 %
5	*Streptococcus pyogenes*		5	8 %
6	*Staphylococcus epidermidis*		4	6 %
7	*Enterococcus faecalis*		4	6 %
8	*Escherichia coli*	Gram negative	4	6 %
9	*Klebsilla pneumoniae*		4	6 %
10	*Pseudomonas aeruginosa*		3	4 %
11	*Rothia dentocariosa*	Gram positive	3	4 %
12	*Streptococcus pneumoniae*		2	3 %
13	*Kocuria kristinae*		2	3 %
14	*Granulicatella adiacens*		2	3 %
	Total		**64**	**100 %**

The current study detected a number of bacterial species. The majority of bacterial isolates (82%) were Gram-positive bacteria, whereas Gram-negative bacteria were found in only a few cases (18%). In accordance with the current findings, [15] find that 89% of the bacterial isolates were Gram positive and 11% were Gram negative. Moreover, [14] and [22,23] demonstrate that Gram-positive bacteria were the most prevalent pathogens detected in patient cultures (88%). This result contradicts that of [24], as only 41% of the bacterial isolates were Gram-positive bacteria. Viridans streptococci were the

most common bacterial isolates from gingivitis. These results are in agreement with [15], but disagree with [24] which demonstrated that *S. aureus* was the most common bacterium. Viridans streptococci are present in the dental plaque surrounding the teeth. It is generally accepted that the primary etiology for gingivitis and periodontitis is the dental plaque bacteria, bacterial products, and the resulting inflammatory cascade [25]. Oral streptococci species, such as *S. mutans* and *Streptococcus mitis*, play an important role in the formation of supragingival plaque and dental caries [26]. *E. faecalis* was detected in 6% of the samples, *R. dentocariosa* in 4%, *K. kristinae* and *G. adiacens* in 3%. [14] and [27] reveal the same results. Similarly, the current study indicated that 41.7% of individuals with chronic periodontitis had *E. faecalis* in their subgingival plaque samples. In addition, it was shown that *E. faecalis* was completely absent in people with healthy gums. *S. pneumonia* was isolated in 3% of the cases, followed by *S. aureus* in 11% of the cases and *S. epidermis* in 6% of the cases. These results are in agreement with [28]. The results of antibiotic susceptibility test for bacteria isolated from CSOM are shown in table (5).

Table 3. Antibiotic susceptibility test of isolated bacteria

Bacteria	Sensitivity of isolates to antibiotics										
	AM	AX	ATM	C	CN	CIP	CD	E	TE	MET	CFM
	S%	S%	S%	S%	S%	S%	S%	S%	S%	S%	S%
S.mutans	63.36	90.91	27	81.82	27	90.91	63.64	36.36	63.64	36.36	27.27
S.mitis	37.5	100	37.5	0	37.5	75	87.5	100	100	62.5	87.5
S.salivarius	80	100	80	60	20	60	80	60	40	40	100
S.pyogen	100	80	100	80	60	40	100	40	80	60	80
S.pneumoniae	50	50	0	0	0	100	50	50	0	50	0
S. aureus	85.71	71.43	71.43	28.57	0	85.71	85.71	28.57	42.86	71.43	28.57
S.epidermidis	100	100	50	25	0	75	75	100	0	75	75
E. Faecalis	0	50	50	25	25	25	0	0	50	75	0
Rothia. dentocariosa	100	33.33	66.67	100	66.67	100	66.67	100	100	33	100
K.kristinae	100	50	100	50	0	100	100	50	80	50	100
G. Adicens	100	100	80	100	50	0	80	50	100	0	0
E.coli	100	50	25	50	25	75	0	25	0	25	25
Klebsiella. Pneumonia	100	25	25	50	0	75	50	25	75	25	50
Pseudo. Aeruginosa	0	66.67	33.33	0	33.33	0	33.33	33.33	0	66.67	66.67

AM: ampicillin, AX: amoxicillin, ATM: Azithromycin, C: chloramphenicol, CN: gentamicin CIP: ciprofloxacin, CD: Clindamycin, E: Erythromycin, TE: tetracycline, MET: Metronidazole, CFM: Cefixime.

The results of antibiotic suscetibility test for bacteria isolated from gingivitis are shown in table (5). It is found that with *S.mutans* bacterium, amoxicillin, ciprofloxacin, and chloramphenicol were the most effective antibiotics (90.91, 81.82%, respectively). These results are in agreement with [15] which showed that *S.mutans* isolates were sensetive to chloramphenicol, ciprofloxacin, amoxicillin and ampicillin. In the current study, *S.mitis* isolates were 100% sensitive to tetracycline, erythromycin and amoxicillin. These results are close to [29], but disagree with [30] which found that *S.mitis* was highly resistant to tetracycline (78.12%), followed by 65.62 to ciprofloxacin and 28.12% to gentamycin.

The majority of *S. salivarius* isolates were 100% sensitive to amoxicillin and cefixem, 80% to ampicillin, azithromycin, and clindamycin. These results are close to [15], showing that these isolates were 62.50% resistant to gentamycin and 50% to tetracycline. *Streptococcus pnumonia* showed a high sensitivity of 100% to ciprofloxacin, 50% to amoxicillin, ampicillin, clindamycin, erythromycin and metronidazole, while it had no resistance to azithromycin, chloramphenicol, gentamicin, tetracyclin, and cefixem. These results are in agreement with [28] and [31] which revealed that *S.pneumonia* was highly resistant to ampicillin, amoxicillin, cefixem, and azithromycin.

Staphylococcus aureus showed 85,71% sensitivity to ampicillin, clindamycin and ciprofloxacin, 71,43% to amoxicillin, metronidazole and azithromycin. While it showed no resistance to gentamycin 0%. These results are in agreement with [15] which revealed that *Staph. aureus* was highly sensitive to ampicillin, clindamycin, ciprofloxacin, amoxicillin and azithromycin.

Staphylococcus epidermidis showed a high sensitivity 100% to ampicillin, amoxicillin, erythromycin, 75% to clindamycin, ciprofloxacin, metronidazole and cefixem, 50% for azithromycin. While it showed low resistance to chloramphenicol 25%, and 0% for gentamycin. These results are almost in a close agreement to [28] which revealed that *Staph.epidermides* was highly sensitive 100% to ampicillin, amoxicillin and gentamycin.

Enterococcus faecalis showed high susceptibility 75% to metronidazole and 50% to tetracyclin, amoxicillin and azithromycin.

Granulicatella adiacens isolates were susceptible 100% to ampicillin, amoxicillin, chloramphenicol and tetracycline. This result is near to that of [31].

K. pneumoniae showed 75% sensitivity to tetracyclin and ciprofloxacin, 50% to clindamycin, chloramphenicol, and cefixime, while showing high resistance 100% to ampicillin. These results are in agreement with those of [32].

P. aeruginosa showed 66,67% sensetivity to amoxicillin, metronidazole, and cefixim, while showing no resistance 0% to ampicillin, chloramphenicol, ciprofloxacin, and tetracyclin, 33,33% for each of gentamycin, azithromycin, clindamycin,

and erythromycin. In this regard, [33] and [15] find that all *Pseudomonas aeruginosa* isolates were resistant to all antibiotics.

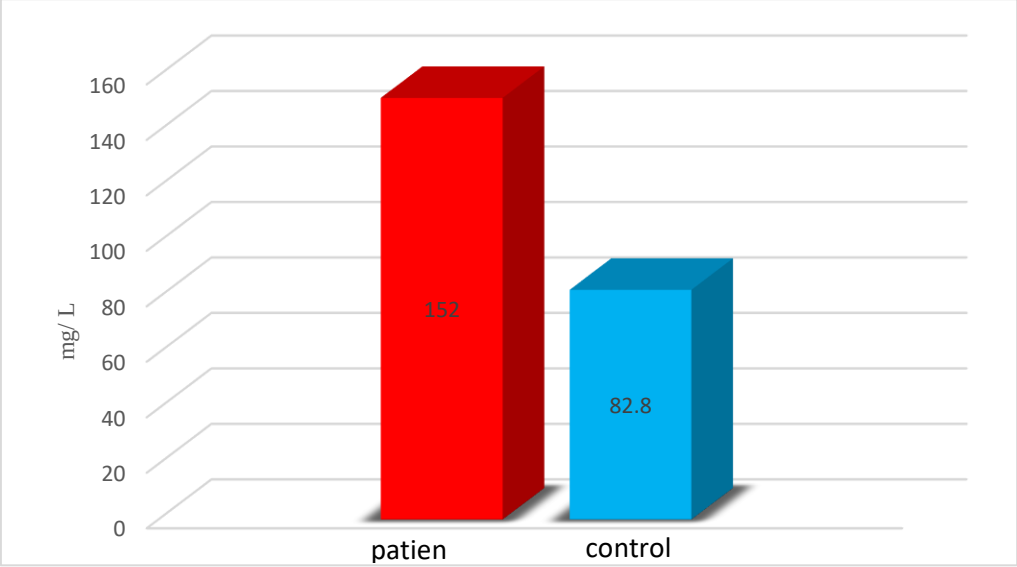
The study reported a highly significant increase in the levels of TNF-α in patients with gingivitis (152.8 ± 19.5 mg/L) compared to the control group (82.8 ± 12.3 mg/L) at (P<0.05), as shown in table (4) and figure (1).

Table 4. TNF-α level in patients with gingivitis and control group

Group	Patients	Control
No.	60	30
TNF-α (pg/mL)	152.8±19.5	82.8±12.3
P. value: 0.0007	**T test: 20.74**	

The results of this study correspond with [34]. These observations suggest a positive association between periodontal disease and increased levels of TNF-α in serum. In this context, [35] reveal that there were increased levels of TNF-α in gingival tissues of periodontitis patients. They suggest that TNF-α is related with the inflammatory condition of the periodontium.

The results of the current study are in accordance with a study conducted by [36], estimating the salivary TNF-α in chronic and aggressive periodontitis and control participants. They conclude that the salivary TNF-α levels were significantly higher in chronic periodontitis than in healthy controls; however, there was no significant correlation with the clinical parameters.



P. value: 0.0007 **t. test: 20.74**

Figure 1. TNF- α Level in patient and control group

The results of descriptive statistics for immunological parameter according to gender are shown in table (5), indicating a highly significant level of TNF-α (P=0.00004) among (males and females) between the groups of control and patients.

In serum of male, the mean of TNF-α concentration was (155.12±20.42) and (81.18 ± 10.97) for the patients and control groups, respectively. While in female, the mean value of TNF-α concentration was (145.18 ± 14.22) and (88.01 ± 15.83) for the patients and control groups, respectively.

Table 5. Descriptive statistics for immunological parameter according to gender

Group	Sex	TNF-α
Patient	Male	155.12±20.42
	Female	145.18±14.22
Control	Male	81.18±10.97
	Female	88.01±15.83
F test		111.5
P value		0.00004

Females exhibited greater humoral and cell mediated immune responses to antigenic stimulation, vaccination, and infection than do males, due to their innate and adaptive immune responses than males. This can result in faster clearance of pathogens, but also contributes to increased susceptibility to inflammatory and autoimmune diseases in females compared with males. Estrogens may increase the immunological activity of vitamin D, thus enhancing the outcomes of infections [37].

The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) is produced by macrophages and is associated with periodontitis-induced bone loss [38]. This is present in both periodontitis and healthy saliva, gingival crevicular fluid (GCF), and serum [39]. The higher concentration observed in patients with periodontitis is directly linked to tissue damage and immune response [40,41].

TNF-α is a proinflammatory cytokine known for its substantial role in periodontitis mediated bone loss. Increased concentration observed in periodontitis is correlated closely with the tissue destruction and immunologic response. These can, in turn, induce an elevated expression of matrix metalloproteinase (MMP) in periodontal tissues. This impairs the conventional host response in bacterial clearance and neutralizing the infection. The granulocyte function is impaired, these cells react to a bacterial challenge by releasing the serine proteases elastase and matrix metalloproteinase to which they are associated with degradation of connective tissue [42].

Conclusions

In this study, *Streptococcus* spp. and *Staphylococcus aureus* were the predominant bacteria, colonizing gingivitis patients. All of the isolated bacteria in this study were resistant to the vast majority of broad-spectrum antibiotics. Metronidazole, azithromycin, erythromycin, tetracycline, ciprofloxacin, and clindamycin were the most effective periodontal drugs. In

gingivitis, the TNF- levels of patients were significantly higher than those of the control group.

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