PILOT STUDY

Raftlin, presepsin levels and thiol-disulphide homeostasis in acute appendicitis: A pilot study

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Abstract

Objective: To investigate some of the new inflammatory and oxidative stress markers in acute appendicitis.

Methods: This clinical pilot study was conducted at the emergency department of Bezmialem Vakif University, Istanbul, Turkey, between January and July 2015, and comprised patients with definitive diagnosis of acute appendicitis and as many healthy controls. Venous blood was collected to assess white blood cell count, C-reactive protein, raftlin, presepsin, total thiol, native thiol and disulphide levels. Alvarado scores of patients were determined at the time of admission. Surgical excisions were sent for pathological examination. The results of histopathology of appendectomy specimens were categorised as non-perforated or perforated appendicitis.

Results: There were130 subjects with 65(50%) patients and 65(50%) controls. Serum raftlin, presepsin, white blood count, C-reactive protein and disulphide levels were higher, and the total and native thiol levels were significantly lower in patients compared to controls ($p<0.05$). There was no significant difference between the non-perforated and perforated appendicitis patients regarding all the measured parameters ($p>0.05$) except mean Alvarado scores which were higher in perforated than non-perforated appendicitis ($p<0.05$).

Conclusions: Inflammatory and oxidative stress markers were significantly different in acute appendicitis patients compared to healthy controls.

Keywords: Raftlin, Presepsin, White blood cell count, C-reactive protein, Thiol/Disulphide, Appendicitis.

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Introduction

Acute appendicitis is one of the most common surgical emergencies.[1] The importance of early diagnosis is critical to minimise the possibility of injury or rupture, and to avoid morbidity. Generally, the diagnosis of acute appendicitis is primarily based on clinical symptoms, physical examination and laboratory testing.

Today, despite the implementation of various diagnostic tests and imaging techniques for a definitive preoperative diagnosis, there are still some delays seen in the surgical treatment of patients with acute appendicitis. Due to misdiagnosis, a significant number of people are operated on even though they are not actually acute appendicitis patients. The diagnostic error rate ranges from 12% to 23% for men and 24% to 42% for women.[2] Therefore, a biomarker for acute appendicitis that can rapidly and specifically evaluate the severity of the disease is needed. A few laboratory tests are being routinely used as support for the clinical diagnosis of acute appendicitis. Because these tests are used as non-specific markers of inflammation, the probability of a false positive (FP) result rate is expected to be high. The identification of more specific and sensitive markers for appendicitis will guide clinicians in the early and accurate diagnosis and prevention of negative explorations.[3]

White blood cells (WBCs) play a part in the inflammatory response. All WBCs are produced and derived from multipotent cells in the bone marrow known as haematopoietic stem cells. The WBCs have many vital yet subtle roles in promoting and regulating inflammation.[4] Examining the functions of such cells can help us better understand the

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complex relationships between immunity and inflammation as well as allergies and autoimmune diseases.

C-reactive protein (CRP) is an acute phase reactant. It is synthesised by hepatocytes, and stimulated by interleukin-6 (IL-6) and IL-1 genes.[5] The most important role of CRP is to help the body's immune defence mechanisms by reacting with complex systems. Serum CRP level increases in many diseases such as infections, rheumatoid arthritis, rheumatic fever, cancer, and tuberculosis.[6] Serum WBC and CRP levels have been used for many years to determine the degree of inflammation and sepsis. However, it is not specific to any inflammatory disease, and in many of them, the blood WBC and CRP levels are found to be high. Today, although it is used in the diagnosis of acute appendicitis, sensitivity and specificity are low without the support of physical examination and imaging techniques.[3]

Previously, a novel major lipid raft protein, raftlin, was identified in Raji B cells. Raftlin is necessary for the integrity of lipid raft and B cell antigen receptor signal transduction.[7] It is also involved in the nucleocapture complex in induction of toll-like receptor 3 (TLR3) activation and autoimmune responses.[8] The central role of lipid raft in the pathophysiology of vascular inflammatory responses[9,10] led us to hypothesise that raftlin might have been involved in the pathogenesis of acute appendicitis.

The biological role of presepsin has not been exhaustively elucidated. It is a regulatory factor capable of modulating cellular and humoral immune responses by interacting directly with T and B cells. One of the production mechanisms of presepsin is related to the phagocytosis process in response to bacterial infection and cleavage of membrane cluster of differentiation 14 (CD14) with lysosomal enzymes of granulocytes.[11] Although there is a growing body of evidence suggesting that the concentration of blood presepsin was found to be increased in patients with sepsis compared to healthy individuals,[12] the clinical significance of presepsin in the diagnosis of acute appendicitis remains unclear. Appendix vermiform is a lymphoid organ in which T and B cells are very dense. In addition a marked increase is observed especially in B cells during inflammation.[13] Raftlin and presepsin are thought to increase during appendicitis because they originate especially from B cells.

Oxidative stress plays an active role in inflammation. The role of oxidative stress in the etiopathogenesis of acute appendicitis has been demonstrated in many studies.[14,15] Thiol is an organic compound containing a sulfhydryl group that plays a critical role in preventing the formation of any oxidative stress state in cells.[16] Cysteine, which is among the defensive protein mechanisms of the body, plays an important role in the prevention of oxidative damage by the functional thiol group it contains. Thiols can undergo oxidation reaction via oxidants and form disulphide bonds.[17] When the thiol groups of the proteins are oxygenated by oxygen molecules from the environment, they turn into disulphide bonds. The formed disulphide bonds can again be reduced to thiol groups. Thus, dynamic thiol-disulphide homeostasis is maintained.[18] A problem in this homeostasis results in a variety of disorders, as the ratio of thiol to disulphide plays a critical role in antioxidant protection, detoxification, and signal transduction, regulation of enzymatic activity, apoptosis and cellular signalling mechanisms.

The exact diagnosis of appendicitis is made histopathologically. It is very convenient for a clinician to have a marker that can be applied quickly and at a reasonable cost preoperatively in any hospital. Such a marker may fill a very important gap for surgeons in determining the accuracy of the treatment approach, the dose and duration of antibiotic use, and the necessity of drainage during surgery.

The current study was planned to investigate some of the new inflammatory markers such as raftlin and presepsin, andoxidative stress markers, such as thiol and disulphide bonds, in acute appendicitis and to compare these parameters with conventional clinical diagnostic variables WBC and CRP in diagnosing or excluding acute and perforated appendicitis.

Patients and Method

This clinical pilot study was conducted at the emergency department of Bezmialem Vakif University, Istanbul, Turkey, between January and July 2015, and comprised patients with suspicion of acute appendicitis whose Alvarado scores (AS)[19] were determined. Patients with AS $\geq$4 were evaluated with computed tomography (CT) to confirm the diagnosis of appendicitis. Those receiving non-appendicitis diagnosis, deficiencies in the tests, histopathologically confirmed as negative cases of appendectomy and those who did not agree to have surgery were excluded along with subjects who were

alcoholic or smokers, or had obesity, diabetes, hypertension, cardiovascular disease, malignant diseases, and systemic inflammatory diseases. Moreover, subjects who were pregnant, appendicitis-operated, taking vitamins or antioxidant supplements were also excluded. Patients with definitive diagnosis is of acute appendicitis and as many healthy controls were enrolled after obtaining approval was obtained from the institutional ethics committee. Written informed consent was also obtained from the subjects.

Blood was centrifuged for 10 minutes at 1.500 x g, and the serum separated and stored at -80°C until the day of the study. Serum raftlin and presepsin levels were analysed by an enzyme-linked immunosorbent assay (ELISA) reader (Thermo Fisher MA, USA) using commercial kits (Sunred Biological Technology-Shanghai, China). WBC count was measured with an automated laser/impedance cell counter (Abbott Cell Dyn 3700, USA). WBC count greater than 10,000 / mm^3 was considered to be elevated. The concentration of CRP was determined by an immunoturbidimetric method using a CRP kit (Abbott, USA) and an Architect C4000 auto analyser (Abbott). According to the kit's guidelines, concentrations greater than 0.5 mg/dl were considered positive. Total thiol, native thiol, and disulphide levels were measured by a new automated method.[20] This new method is based on the reduction of proteins which convert to a reversible form of disulphides under oxidative conditions to sulfhydryl groups and again to thiol groups of disulphide bonds. Sodium borohydride (NaBH4) is used in the new assay to reduce sulphur bonds to thiol groups. The total and native thiol content of the sample was measured with modified Ellman's reagent. Native thiol levels were subtracted from the total thiol levels and the results were divided into two to determine disulphide bond amount. After dynamic disulphide, native and total thiol levels were determined; disulphide-to-native thiol, disulphide-to-total thiol, and native thiol-to-total thiol ratios were calculated. Final diagnoses were confirmed by histopathological examination. The results of histopathology of appendectomy specimens were categorised as non-perforated or perforated appendicitis.

Statistical analysis was performed using SPSS 20. Kolmogorov-Smirnov test was used to determine the distribution of data. The mean ± standard deviation or median and 25-75th percentile values, interquartile range (IQR) (normally and not normally distributed, respectively) were used to express the continuous variables. All categorical variables were expressed as frequency and percentage. Student's t-test was used to compare variables between the two groups when the variable was normally distributed, and Mann-Whitney U-test when the variable was not normally distributed. Chi-square test was used to compare categorical variables. The relationship between numerical variables was evaluated using Pearson correlation analysis. The differences between the groups were presented at 95% confidence intervals. P<0.05 was considered statistically significant.

Results

There were130 subjects with 65(50%) patients and 65(50%) controls. Of the patients, 30 (46.15%) were female and 35(53.84%) were male with an overall mean age of 31.5±12.7 years (range: 16-63 years). The control group included 31(47.69%) females and 34(52.31%)males with an overall mean age of 31.8±9.2 years (range: 17-60 years) (Table1). Patients with acute appendicitis had significantly lower levels of total thiol and native thiol, and the ratio of native thiol to total thiol compared to the controls (p<0.001). However, WBC, serum levels of raftlin, presepsin, disulphide and CRP, and the ratios of disulphide to total thiol and disulphide to native thiol were higher in patients

Table-1: Comparison between the controls and acute appendicitis patients regarding to measured parameters.

Variables	Acute Appendicitis (n = 65)	Control (n = 65)	p value	t, z values
Age (years)	31.5±12.7	31.8± 9.2	NS	
Gender (n, %)				
Male	35 (53.8)	34 (52.3)	NS	
Raftlin (ng/mL)	15.79 ± 3.13	6.11 ± 3.19	<0.001	t=-17.45
Presepsin(mg/L)	6.49 ± 2.30	1.51 ± 0.44	<0.001	z=-9.80
	6.42 [4.79-7.78]	1.53[1.20-1.72]		
WBC (103/µL)	12.80 ± 3.87	8.20 ± 2.49	<0.001	z=-6.94
	12.48[9.44-15.69]	8.00[6.30-9.35]		
CRP(mg/dL)	3.05 ± 4.60	0.35 ± 0.13	<0.001	z=-4.30
	0.70[0.3-3.5]	0.35[0.27-0.41]		
Total thiol (µmol/L)	340.59 ± 22.58	394.73 ± 30.28	<0.001	t=11.55
Native thiol (µmol/L)	258.53 ± 23.69	332.14 ± 23.34	<0.001	t=17.84
Disulphide (µmol/L)	41.03 ± 15.60	31.29 ± 17.47	<0.001	t=-3.35
Disulphide/total thiol (%)	11.90 ± 4.11	7.72 ± 3.98	<0.001	t=-5.89
Disulphide/native thiol (%)	16.42 ± 7.53	9.66 ± 5.73		
	15.09[10.74-20.16]	8.25[5.70-13.30]	<0.001	z=-5.13
Native thiol/total thiol (%)	76.19 ± 8.22	84.56 ± 7.97	<0.001	t=5.59

t: Data with normal distribution are expressed as mean±SD, Student's t-test, p<0.05. z: Data with not normal distribution are expressed as median and IQR, Mann-Whitney U test, p<0.05. CRP: C-reactive protein, WBC: white blood cell. NS: Non significant

Table-2: Comparison between the non-perforated and perforated appendicitis patients regarding to measured parameters.

Variables	Non-perforated Appendicitis (n = 44)	Perforated Appendicitis (n = 21)	*p* value	t, z values
Age (years)	29.6± 11.9	35.4 ± 13.4	NS	
Gender (n, %)				
Male	26 (59.1)	9 (42.9)	NS	
Raftlin (ng/mL)	15.35 ± 2.76	16.73 ±3.69	0.095	t=-1.69
Presepsin (mg/L)	6.47 ± 2.26	6.54 ± 2.43	0.918	z=-0.35
	6.92[4.61-7.77]	6.21[5.00-7.93]		
WBC(103/µL)	12.24 ± 3.79	13.98 ± 3.86	0.091	z=-1.90
	11.85[9.27-14.99]	13.29[9.97-17.65]		
CRP (mg/dL)	2.64 ± 3.97	3.90 ± 5.72	0.302	z=-1.25
	0.60[0.30-3.10]	0.80[0.40-7.10]		
Total thiol (µmol/L)	339.68 ± 20.49	342.50 ± 26.89	0.641	t=-0.47
Native thiol (µmol/L)	257.60 ± 25.98	260.49 ± 18.41	0.649	t=-0.46
Disulphide (µmol/L)	41.04 ± 14.90	41.01 ± 17.37	0.993	t=0.01
Disulphide/total thiol (%)	11.98 ± 4.13	11.73 ± 4.16	0.819	t=0.23
Disulphide/native thiol (%)	16.57 ± 7.62	16.10 ± 7.49		
	15.05[12.04-21.47]	15.36[8.56-20.16]	0.817	z=-0.03
Native thiol/total thiol (%)	76.03 ± 8.26	76.54 ± 8.32	0.818	t=-0.23
Alvarado score	6.4 ± 1.6	7.8 ± 1.4	<0.001	z=-3.04
	6.0[5.0-7.7]	8.0[7.0-9.0]		

t: Data with normal distribution are expressed as mean±SD, Student's t-test, p<0.05. z: Data with not normal distribution are expressed as median and IQR, Mann-Whitney U test, p<0.05. CRP: C-reactive protein, WBC: white blood cell. NS: Non significant

Table-3: Correlation between the Alvarado scores, and WBC and CRP level in non-perforated and perforated appendicitis patients.

Variables	CRP		WBC	
	r	*p*	*r*	*p*
Alvarado score of non-perforated appendicitis patients	0.348*	0.021	0.707**	0.001
Alvarado score of perforated appendicitis patients	0.549*	0.010	0.663**	0.001

r: Pearson correlation, CRP: C-reactive protein, WBC: white blood cell

compared to the controls(p<0.001).

There was no significant (p>0.05) difference between the non-perforated and perforated appendicitis patients with regard to all the measured parameters except mean Alvarado scores (Table2). The mean Alvarado score of histopathologically confirmed perforated appendicitis patients was significantly higher than those of non-perforated appendicitis patients (p <0.001).

There was a positive correlation (p<0.05) between the Alvarado scores, and WBC and CRP levels in non-perforated and perforated appendicitis patients (Table-3).

Discussion

Appendicitis is the most common cause of emergency abdominal surgeries.[1] Diagnosis is usually made by considering patient history, clinical symptoms and physical examination findings. Clinical symptoms are atypical in about one-third of the cases. It is extremely difficult and time-consuming to diagnose it, especially since it is mixed with other organ damages in women and children. The use of CT and ultrasonography (USG) leads to loss of time and money, although it reduces the incidence of negative appendectomy.[21] In addition, perforated appendicitis, which is the result of prolonged diagnosis period of acute appendicitis, causes prolonged hospitalisation, usage of wide spectrum antibiotics, and financial burden on both patient and social security institutions. Recent studies with high number of cases have shown that the false positivity in acute appendicitis is still 15%.[22] Therefore, inflammatory parameters such as WBC, CRP, bilirubin or fibrinogen elevation and serum procalcitonin (PCT) level,[22,24] and also oxidative stress markers[14,25] have been investigated in acute appendicitis.

The current study found that both WBC and CRP levels were significantly higher in acute appendicitis patients than those in controls. Although there was no difference between the perforated and non-perforated appendicitis patients regarding WBC or CRP levels, there are studies reporting that these two parameters with the association of other laboratory biomarkers and imaging modalities might be used for the diagnosis of perforated appendicitis.[24,26] In addition to studies reporting that a high CRP level and blood leukocyte count are good biomarkers for the diagnosis of perforated and non-perforated appendicitis, there are also studies that report the opposite.[27,28] For a long time, WBC and CRP tests have been used for the diagnosis of appendicitis, but their sensitivities and specificities are low. In literature, the sensitivity and specificity of WBC are reported to vary between 60% and 90% and 44% and 88%, respectively.[29] In addition, the sensitivity and specificity of CRP are reported to vary between 48% and 75% and 57% and 82%, respectively.[21]

Despite the use of so many different biomarkers in the acute appendicitis, there is not yet a highly sensitive and specific marker. To the best of our knowledge, this is the first study that investigated raftlin and presepsin levels in patients with acute appendicitis. Raftlin is a new diagnostic parameter that can be used to diagnose inflammatory

diseases and to characterise the true immune response. Raftlin, which was mostly investigated to determine sepsis and septic shock, was evaluated in appendicitis patients and found significantly higher in patients with appendicitis than healthy controls in the current study. Another new marker of inflammation is the presepsin. Presepsin has three biological features that differ from inflammatory markers such as PCT, CRP and IL-6. Firstly, it can be detected at an earlier stage in the onset of the infection. Secondly, levels are not affected by situations such as severe trauma, burns or invasive surgical intervention. Thirdly, it reflects the clinical course of septic patients.[30] Presepsin has been investigated in various inflammatory diseases. However, there is no study investigating the role of presepsin in appendicitis, which is an inflammatory disease. This is the first study to evaluate the role of presepsin in appendicitis. This study found that the presepsin level was significantly higher in patients with acute appendicitis than in the controls. An earlier study reported a relationship between presepsin and sepsis severity.[31] However, in this study, when the appendicitis patients were divided into two groups as perforated and non-perforated appendicitis according to the histopathological evaluation, there was no significant difference in presepsin levels between the two groups.

It has been reported that the levels of serum oxidative stress markers are affected in patients with acute appendicitis.[14,32] Since acute appendicitis is an inflammatory disease, there is severe reactive oxygen species (ROS) production in acute appendicitis through inflammatory cells, cytokines and chemokines. Excessively increased ROS causes oxidative stress to increase by providing the shift of thiol redox status to thiol oxidation. Similar to other studies, this study also found a high level of serum disulphide and low level of serum thiol in acute appendicitis patients. This indicated that the homeostasis is shifted into the direction of disulphide in acute appendicitis. Serumthiols are free-radical scavengers and serve as antioxidant functions in several ways. Low levels of antioxidant thiols in serum suggest that it may play a role in the pathogenesis of acute appendicitis. Oxidative stress starts the inflammation or continues the inflammation processin the appendix mucosa by stimulating redox sensitive signalling pathways and transcription factors. Previous studies reporting the associations between the degree of appendicitis and variables related to oxidative stress were sometimes described from a diagnostic perspective and at other times from an aetiological perspective.[14,33] In this regard, these serum markers can be used to help diagnose acute appendicitis and determine the extent of the disease.

A study[33] found serum thiol level to be lower in appendicitis patients compared to the healthy controls. Another research[14] reported that both the preoperative and postoperative serum levels of total antioxidant status, total thiol, total oxidant status, catalase, arylesterase and ischemia-modified albumin in patients with acute appendicitis were significantly affected compared to the healthy controls, and there were no differences between the preoperative and postoperative values. A research[15] investigated the thiol-to-disulphide homeostasis in appendicitis patients, and similar to our results found a decrease in total and native thiol levels but an increase in disulphide levels indicating an oxidative stress.

The Alvarado score, consisting of physical examination findings and laboratory tests, is a scoring system used in the diagnosis of acute appendicitis, and used to predict the severity of inflammation. The system has a total of 10 points, 5 is taken as the limit value in some studies[19] while 7 is taken as the limit value in others.[34] Similar to other studies, this study also found that patients with AS >7 had perforated appendicitis and those with AS<7 had non-perforated appendicitis. We also found a positive correlation between the CRP level and WBC, and the AS. This finding indicates that in addition to being useful in the diagnosis of acute appendicitis, AS can also be used to predict the severity of inflammation.

This research is a preliminary study to determine the predictive values of raftlin, presepsin, thioland disulphide parameters in acute appendicitis. However, it has some limitations like its small sample size at a single centre. Second, inclusion of patients only with acute appendicitis pain who were operated on but exclusion of those who had negative appendectomy or had a different disease than appendicitis. Therefore, the sensitivity and specificity of the measured parameters for appendicitis could not be determined by ROC analysis. Third, it did not evaluate other oxidative stress markers such as lipid hydroperoxide, total antioxidant status, total oxidant status, oxidative stress index, paraoxonase and arylesterase.

Conclusion

Serum raftlin and presepsin levels were significantly higher

in patients with acute appendicitis. Thiol-to-disulphide homeostatic balance shifted towards disulphide formation in acute appendicitis patients, indicating an oxidative stress. However, to determine the sensitivity and specificity of raftlin and presepsin in acute appendicitis, a larger group of patients is needed to be studied.

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