

Evaluation of antioxidant and cell death status in people with acute appendicitis: a preliminary study

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Abstract:

Objective: The present study was conducted to investigate the levels of antioxidant glutathione (GSH), lactate dehydrogenase (LDH) and lipid peroxidation (LPx) in plasma of patients with acute appendicitis.

Material and Methods: Blood samples were obtained (n = 34) from people requiring appendectomy (n = 30) and people with normal appendix but requiring gastrointestinal surgery for some other GI diseases and willing for complementary appendectomy (n = 4). The levels of GSH, LPx and LDH were evaluated and statistically compared. Additionally, statistical analysis to observe for correlation between these parameters with histological grading and Alvarado scores were also performed.

Results: The levels of LPx and LDH were higher and GSH was lower in plasma. Negative correlation for GSH and positive correlation for LPx was observed. With respect to LDH positive for plasma was observed with both histopathological and clinical grading. Multiple Regression analysis for the biochemical endpoint in plasma levels showed these parameters to contribute 90.3 and 39.3 respectively towards the histopathological and clinical grading.

Conclusions: The results from this study indicate that GSH, LPx and LDH may be useful surrogate markers for evaluating the progression, stage and complexity of acute appendicitis.

Key words: appendicitis, glutathione, lipid peroxidation, lactate dehydrogenase, Alvarado score

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Introduction:

Appendicitis, an acute inflammation of the appendix is a very common gastrointestinal disease needing immediate surgical intervention.¹ Appendicitis has a lifetime risk of approximately 8.6% in males and 6.7% in females respectively, and when neglected progress from a simple inflammatory condition to abscess, ileus, peritonitis, and may also result in the death of the patient.² Reports indicate that early diagnosis and treatment has minimal risk of mortality, while when neglected and undiagnosed increases the risk of death, especially when perforation has ensued.¹ The diagnosis of acute appendicitis is predominantly based on clinical findings and the Alvarado scoring system developed by Alvarado in 1986 for the diagnosis of acute ap-

pendicitis in emergency situation is globally the most reliable and followed scale. The Alvarado scoring system is easy to apply because it relies purely on clinical history, examination and a few simple laboratory tests (Table 1).³

The cause of acute appendicitis is unknown but is best suggested to be a multifactorial luminal obstruction and, aspects like dietary and familial factors have all been suggested to play cardinal role in the underlying pathogenesis.^{4,5} Reports also suggest that generation of free radicals and triggering of inflammatory reactions are implicated in the etiopathogenesis of many clinical conditions and this also extends to acute appendicitis.^{4,5} Previous studies have shown that patients with acute appendicitis had low levels of

antioxidants and a concomitant increase in the levels of oxidative stress and lipid peroxidation in the blood, thereby substantiating the hypothesis that free radical generation and the resulting stress does indeed have a role to play in the underlying pathogenesis.^{4,5,6,7} In this study we assessed the levels of the cellular antioxidant glutathione (GSH), levels of lipid peroxide (LPx) and lactate dehydrogenase (LDH) the important markers of oxidative stress and cell death, respectively in the plasma and excised tissue of patients with acute appendicitis. Additionally, we also correlated these biochemical parameters with both histopathological and clinical grading (Alvarado score), to assess for a possible correlate if any.

Materials and Methods:

The present study was a collaborative endeavour of the Department of Surgery and Department of Biochemistry of the Father Muller Medical College, Mangalore, Karnataka, India. The study was performed after the approval of the institutional Ethical Committee. The inclusion criteria included patients with typical features of acute appendicitis (in accordance to the Alvarado score) and admitted for appendectomy and people with normal appendix but requiring gastrointestinal surgery for some other GI diseases and willing for complementary appendectomy. Additionally, for the baseline cohort for the blood assays, healthy normal volunteers who were non diabetic and not on any medication for common illness (malaria, bacterial infection, viral fever) and those who were on vitamins or anti-inflammatory drugs in the past one month were excluded. In these healthy volunteers only blood samples were collected. A written consent for the study was taken from all adult volunteers and patients, while it was taken from one of the parent of children and adolescents below the age of 18.

The clinical scoring was carried out according to the Alvarado scoring system in the patients suspected to have acute appendicitis.³ For correlation purpose, we used the mean score derived from adding the individual scores and dividing it by 10. The blood samples were collected preop-

eratively from the appendicitis patients together with routine haematological and biochemical tests, while the appendix was collected after the surgical process. The harvested tissue was fixed in 10% buffered formalin for histopathological studies by the standard H&E staining. A pathologist unaware of the clinical diagnosis examined the specimens and categorized them as either acute focal, acute suppurative, acute gangrenous and perforated appendix.⁸

The blood samples were collected taking aseptic precautions from both patients and volunteers, and centrifuged at 5,000 g in cold. The plasma was separated and stored in prelabelled tubes in deep freezer (-70°C). The samples were analysed by investigators who were not aware of the clinical condition. On the day of the analysis, the samples were thawed and used for various assays. All the biochemical assays were done in UV-visible spectrophotometer of Shimadzu. The levels of GSH, LPx and LDH were estimated in plasma.

Estimation of LPx:

MDA, the sensitive and convenient marker of lipid peroxidation, was assayed as thiobarbituric acid-reactive substances (TBARS), by the method of Ohkawa et al., (1979).⁹ MDA reacts with thiobarbituric acid at 100°C in acidic medium to form pink coloured complex. The colour intensity of MDA-TBA complex is measured at 535 nm. To 0.75 mL of plasma homogenate, 3 mL of MDA reagent (75 mg thiobarbituric acid + 15 gm trichloroacetic acid, in 2.08 mL of 0.2N HCl, made up to 100 mL with distilled water) was added, mixed and kept in boiling water bath for 20 minutes. Then cooled under tap water, centrifuged at 3000 rpm for 10 minutes, and absorbance was measured at 535 nm against reagent blank. The level of MDA was calculated using the molar extinction coefficient of MDA-thiobarbituric acid complex. Plasma MDA level expressed in nanomoles/L. MDA standard (1, 1, 3, 3-Tetramethoxypropane) procured from Sigma-Aldrich was used for standardising the assay and the standard curve was plotted using various concentration.

Estimation of GSH:

The GSH levels were estimated by the method described by the Method of Beutler et al.¹⁰ GSH reduces 5, 5'-dithio, bis-nitrobenzoic acid (DTNB) to yellow colour 5-thionitrobenzoic acid (TNB). Absorbance measured at 412 nm is directly proportional to the concentration of GSH. Plasma homogenate was deproteinized with 2% metaphosphoric acid, and an aliquot of the supernatant was treated with DTNB and 0.3 M Disodium phosphate solution. The yellow colour obtained was measured at 412 nm against the reagent blank. GSH standards ranging in concentration from 25 to 100 mg/dl were run simultaneously, and the GSH level was calculated from the standard curve. The plasma GSH was expressed in terms of micromoles/L.

Estimation of LDH:

The assay for LDH was performed by the kinetic spectrophotometric method described by Demetriou et al.¹¹ The reagent kit obtained from Roche diagnostics was used. The assay was based on LDH-catalyzed reduction of pyruvate with NADH to form NAD⁺. The rate of oxidation of NADH to NAD⁺ was measured as a decrease in absorbance at 340 nm. Plasma LDH was expressed in terms of Units/L. Internal and External quality control programme of Biorad was used to ensure accuracy and precision of plasma LDH values.

Statistical analysis:

The values were expressed as mean with standard deviation. Significance of the difference of the values between the groups was evaluated by Analysis of Variance (ANOVA), Bonferroni multiple comparison and correlation between the levels in blood and tissue was analyzed by Karl Pearson's Correlation Analysis.

Results:

The study population consisted of 19 females and 14 males with appendicitis, whose age ranged from 12 to 48 years (mean age of 24.93±8.5). The appendicitis cases were classified as per the histopathological features and the details are mentioned in table 2. The values of LDH, GSH and LPx in appendicitis subjects are

presented in table 3 and figure 1. The ANOVA test showed significant differences between the different grades of appendicitis with respect to plasma levels of LPx, GSH and LDH (P values 0.001). (Figure 1, Table 3).

With respect to correlation between the levels of GSH, LPx and LDH of plasma with the histopathological grading, the results showed that a negative association of $r = -0.846$ for GSH in plasma; a positive association of $r = 0.93$ for the LPx; and positive correlation of $r = 0.939$ for plasma levels of LDH was observed (Figure 2). Correlation of GSH, LPx and LDH of plasma with the Alvarado grading showed a negative association of $r = -0.566$ for GSH in plasma; a positive association of $r = 0.608$ for the LPx; and positive correlation of $r = 0.593$ for plasma levels of LDH (Figure 3).

Multiple regression analysis for GSH, LPx and LDH of blood with histopathological grading, the results showed GSH to have a negative ($\beta = -0.198$, $p = 0.009$), while LPx ($\beta = 0.335$, $p \geq 0.029$) and LDH ($\beta = 0.45$, $p \geq 0.006$) showed a positive correlation (Table 5). With respect to the analysis of the biochemical endpoints of the plasma with clinical grading the correlation was again insignificant ($R^2 = 0.39$) and contributed only 39.3% (Table 3).

Table 1: Alvarado grading (Alvarado, 1986)

Symptoms Score	
Migratory right iliac fossa pain	1
Nausea /Vomiting	1
Anorexia	1
Signs	
Tenderness in right iliac fossa	2
Rebound tenderness in right iliac fossa	1
Elevated temperature	1
Laboratory Findings	
Lecucocytosis	2
Shift to the left of neutrophils	1
Total	10

Table 2: Histo-pathological grading

Grade 1: Acute focal appendicitis	Grossly the serosa appears dull, granular and red membranous. Microscopically scanty neutrophilic exudate is found throughout the mucosa, sub mucosa, and muscularis propria. Subserosal vessels are congested.
Grade 2: Acute suppurative appendicitis	Prominent neutrophilic exudates generate a fibro purulent reaction over the serosa. As the inflammatory process worsens, there is abscess formation within the wall, along with ulcerations and foci of suppurative necrosis in the mucosa.

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Grade 3: Acute gangrenous appendicitis	Large areas of hemorrhagic green ulceration of the mucosa and green black gangrenous necrosis through the wall, extending to the serosa.
Grade 4: Perforated appendix	Ulcerated mucosa with transmural inflammatory infiltrate, perforated site shows area of hemorrhage with pus within lumen comes out through perforated site.

Table 3: Results of the regression analysis for the correlation of GSH, LPx and LDH levels in the plasma with the histopathological and clinical grading (Alvarado grading)

Plasma	Parameters	Unstandardized coefficient	Standardized coefficient (β)	t	Significance
		B	Std. error		
Histo-pathological	GSH	-0.005	0.002	-0.198	0.009
	LPx	0.0001	0.0001	0.335	0.029
	LDH	0.03	0.001	0.451	0.006
	Constant	0.90	0.463	-	0.193
Clinical Alvarado grading	GSH	0.0001	0.004	-0.04	0.905
	LPx	0.0001	0.0001	0.356	0.256
	LDH	0.001	0.001	0.261	0.411
	Constant	-0.267	0.950	-	0.282

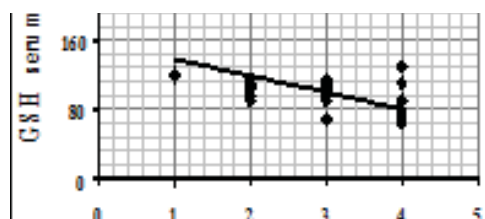


Fig.3 graphs shows correlation between various biochemical parameters

Discussion:

Oxidative stress, which refers to a condition of imbalance in oxidant-antioxidant balance due to increased generation of free radicals and impaired functioning of antioxidants, is implicated in the etiopathogenesis of many clinical conditions.^{2,13} In the current study it was observed that the levels of the cell's principal antioxidant the GSH was significantly decreased and that of the LPx, a marker of oxidative marker was significantly increased in plasma and correlated well with the clinical and histopathological grades of severity.^{12,13} These observations are in agreement to earlier reports where reports do indicate that antioxidant capacity and total thiols were decreased in the blood of people with acute appendicitis and also that an increase in indicators of stress like nitric oxide, TBARS and C-reactive protein were increased in the blood of patients with acute appendicitis.^{4,5,6}

GSH is very important nucleophilic molecule and scavenges the reactive species of both oxygen and nitrogen (ROS/RNS).¹³ It is also a co-substrate for the antioxidant enzyme GSHPx and for the GST mediated phase II detoxification reactions.¹³ Additionally, depletion of GSH initiates lipid peroxidation which then progressively leads to severe cellular damage and cell death.¹²

The estimation of LDH is a routine procedure to assess the cell health and turn over. LDH is a ubiquitous enzyme and its levels are increased in the plasma in various inflammatory diseases, disease of the heart, liver, skeletal muscle, blood, kidneys, and lungs.¹⁴ In our study it was

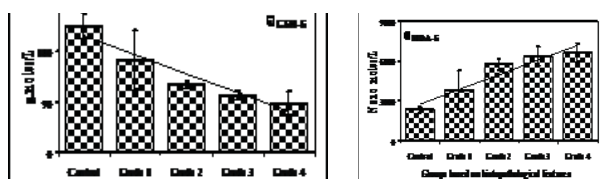


Fig. 1: Levels of various biochemical parameters in the serum

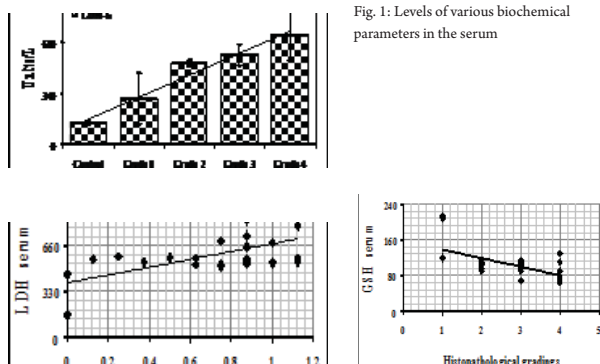


Fig. 2: Graphs shows correlation between various biochemical parameters with histopathological grading

also observed that the levels of plasma LDH were elevated. These divergent observations suggest that the enhanced cell damage and increased membrane permeability caused leakage of cellular enzyme to the plasma.

In our study, we found that the antioxidant enzymes like LDH, LPx levels increased with advanced stages of appendix while GSH decreased which is in agreement with the studies conducted by Satomi et al 1996 and Koltuksuz U et al 2000 and in contrary to the results obtained by Ozdogan et al (2006) where plasma total anti-oxidant activity was used instead of enzymes and found negative co-relation with the progression of disease.^{7,15,16} These findings suggest that anti-oxidant enzyme level increases as the disease progresses may have a role in anti-oxidant defense capacity of an organism and anti-oxidant enzymes can be an indicator for disease progression and sepsis as observed by Vega and coworkers (2002).¹⁷

The results indicated that a correlation was seen between the three biochemical parameters and the clinical and histopathological observations. These observations suggest that estimating the levels of LDH, LPx and GSH in plasma could possibly be used as additional biomarkers to the clinical examination. The observation of changes in the levels of GSH with that of LPx and LDH indicate that the status of GSH in the cell is of importance and that its reduction increases lipid peroxidation and release of LDH. The limitation of the study was the small sample size. Future studies with larger sample size involving assessment of biochemical parameters with correlation to histopathological and clinical examination need to be done to establish oxidative stress indicators as markers of appendicitis, and to establish cut-off values of these parameters which are clinically critical.

Conflict of Interest: None

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