A Study on the Role of Oxidative Stress and Prolidase Enzyme in the Clinical Evaluation of Irritable Bowel Syndrome (IBS)

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ABSTRACT

Introduction: Irritable bowel syndrome (IBS), though a benign disorder is highly prevalent and imposes high cost and substantial morbidity upon general population. Long considered as functional disorder, IBS pathogenesis carries an organic basis at least in a subset of patients. Altered intestinal immune response and low grade intestinal inflammation have been confirmed as pathophysiology of IBS in few studies. Oxidative stress indicates that there is inflammation and, markers of oxidative stress may be developed as diagnostic tool for IBS in future. Study aimed to evaluate oxidative stress in form of total oxidant status (TOS), total anti-oxidant status (TAS), oxidative stress index (OSI) and serum prolidase activity (SPA) as a marker of intestinal inflammation in IBS patients and healthy controls.

Material and methods: In this case –control study done at a teaching medical institute in north India over a period of one year, 120 IBS patients (cases) and 40 healthy volunteers (controls) were evaluated for TOS, TAS, OSI and SPA. Patients with IBS were sub-divided into 3 groups (40 each): diarrhea predominant, constipation predominant and mixed type (IBS-D, C and M respectively). Student t-test, chi-square test and ANOVA tests were used for statistical analysis.

Results: Mean TOS, TOS/TAS (OSI) and prolidase levels were significantly higher in IBS group than control with p value of <0.001,< 0.001, and <0.01 respectively. Level of TOS was highest in IBS-D subgroup followed by IBS-M and Lowest in IBS-C subgroup showing a significant difference between IBS-D and IBS-C, IBS-D and IBS-M and IBS-M and IBS-C with p values <0.001 for each comparison. OSI was highest in IBS-D and lowest in IBS-C with significant differences between the subgroups (P<0.001). Only IBS-M subgroup had significantly higher serum prolidase activity when compared to controls (p<0.001) IBS-D (P=0.013) and IBS-C (P=0.01). TAS level was significantly higher in controls (P<0.001) than cases. There were significant differences between all four subgroups (p<0.001) except between IBS-C and IBS-M subgroups (P=0.294).

Conclusion: This study observed that there is increased oxidative stress and decreased antioxidant capacity in patient with IBS. To support our results further prospective and randomized controlled trials are necessary.

Keywords: Total Anti-Oxidant Status, Total Oxidant Status, Irritable Bowel Syndrome

INTRODUCTION

Irritable bowel syndrome (IBS) is highly prevalent bowel disorder that carries substantial morbidity and enormous costs. Global prevalence of IBS is 8-23% of the adult population.[1] The pathophysiology of IBS is not well understood and may involve impaired brain-gut function, altered intestinal motility and visceral hypersensitivity.[2] Although IBS is considered as a functional disease, recent studies show a role for alterations in the intestinal immune function and low grade inflammation in its pathogenesis.[3-5] Earliest evidence for an inflammatory component in IBS was identified in 1960 where IBS patients had a higher number of mast cells in their intestinal wall compared to healthy subjects.[6] Further studies have described a number of histopathological abnormalities in biopsies from the intestinal mucosa of patients with IBS, including increased numbers of activated immune-competent cells, like: intraepithelial lymphocytes, lamina propria CD3+ cells, CD25+ cells, neutrophils and mast cells compared with healthy controls.[7,8]

About 6-17% of IBS patients have their onset of symptoms following an acute episode of gastrointestinal infection; referred to as "post-infectious IBS" (PI-IBS)[9,10] supporting a role of low grade inflammation. Studies have found ongoing alterations in enteroendocrine cells, higher number of T-cell lymphocytes[11] and increased expression of interleukin 1β in mucosal biopsies of patients with PI-IBS.[12] Effect of this local inflammation can also expand systemically which has been demonstrated in peripheral blood. Examples include genetic studies showing reduced levels of IL-10 expression, findings of increased ratio of pro- to anti-inflammatory cytokines (i.e., IL-10 to IL-12)[13], increased production of pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNF-α) from peripheral blood mononuclear cells (PBMCs)[14] and increased numbers of activated T cells in the peripheral blood of patients with IBS compared to controls.

A number of markers of inflammation has been studied in patients with IBS including hsCRP (high sensitive c-reactive protein)[15], tumor necrosis factor alpha (TNF-alpha), IL-17, IL-10, malondialdehyde (MDA), total anti-oxidant capacity[16], total oxidant status, total anti-oxidant status,

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serum prolidase activity[17,18] and many others.

Oxidative stress

Disturbance in the balance between reactive oxygen species (free radicals) produced and antioxidant defenses is defined as oxidative stress.[19] Generation of oxidants and antioxidants are under regulation. Oxidants are produced by different metabolic pathways such as respiration and in response to inflammation. Excess of these oxidants, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are harmful to host system. In response, host system triggers its protective system alarming the presence of excess oxidants inducing the production of different antioxidants such as Superoxide dismutase, catalase, glutathione peroxidase and so forth to nullify the effects of these excess oxidants.[20]

The persistence of oxidative stress may be responsible for the pathogenesis of different chronic diseases including irritable bowel syndrome. Chronic inflammation is characterized by infiltration of lymphocytes and macrophages, proliferation of hyper permeable and heterogeneous small blood vessels, necrosis and fibrosis.[21] These activated lymphocytes and macrophages actively participate in releasing inflammatory cytokines and other mediators which intensify the immune reactivity. The prolonged inflammatory reaction in chronic inflammation leads to tissue destruction and repair.[22] Collagen is a structural component of the connective tissues. It also acts as ligand for integrin family of cell surface receptors. It interacts between extracellular matrix proteins and cells, e.g. collagen regulates intracellular ion transport, kinase activation, cytoskeleton reorganization, lipid metabolism, gene expression, cell cycle regulation and metastasis. Any change in the structure, quantity, and collagen distribution can affect the cellular function and metabolism. Human prolidase acts on the C-terminal imidodipeptide of Gly-L-Pro residue. The sources of this imidodipeptides are collagen (extracellular form of collagen), intracellular degradation of procollagen (intracellular form of collagen) and other proline containing protein as well as dietary proteins. Due to their unique characteristics, in the way of collagen gene expression, prolidase enzyme activity may be considered as rate limiting factor in the collagen biosynthesis regulation. Prolidase or proline dipeptidase degrades tri- or di-peptides at the C-terminal position (Xaa-Pro) of proline or hydroxyproline residues and result in generation of proline or hydroxyproline.[23] Thus, these are also called as Xaa-Pro peptidases. Most of prolidases show metal dependent activity and require two divalent cations such as Mn^{2+}, Zn^{2+} or Co^{2+} at the active site of enzyme for maximal activity.[23]

Objectives of this study were to examine the role of oxidative stress in irritable bowel syndrome in terms of-Total oxidant status (TAS), Total anti-oxidant status (TOS), Activity of prolidase enzyme (SPA) in patients with irritable bowel syndrome as compared to healthy controls and to observe the correlation of Total oxidant status, Total anti-oxidant status and Activity of prolidase enzyme within the subgroups of irritable bowel syndrome (IBS-C, IBS-D and IBS-M).

MATERIAL AND METHODS

It was a cross-sectional study conducted at a tertiary care hospital, associated with a medical institution in north India. Study included 120 patients with irritable bowel syndrome as defined by Rome III criteria[1] (40 patients with IBS-D; 40 with IBS-C and 40 patients with IBS-M) and 40 healthy controls which included staff and medical students. All IBS patients must have had symptoms for 6 months or more and a normal colonoscopy examination at any time since onset of symptoms. Healthy controls were assessed by a gastroenterologist for any bowel symptom and any other organic disease based on history and examination. Subjects fulfilling Rome III criteria for IBS who were older than 18 years and younger than 50 years were defined as cases. Asymptomatic subjects older than 18 years and younger than 50 years were defined as healthy controls. Healthy controls didn't undergo colonoscopic examination. Patients with alarm symptoms [Table 1], abnormal stools examination, chronic diseases like diabetes mellitus, hypertension, cardiac disease or renal disease, abnormal complete blood count, abnormal liver function tests, abnormal renal function tests, abnormal thyroid function and abnormal celiac serology were excluded from study.

Experimental details
Measurement of Total Antioxidant and Oxidant Status

Total antioxidant status (TAS) was measured by an automated method, developed by Erel O et al.[24] In this assay, Fe2+ o-dianisidine complex reacts with a standardized solution of hydrogen peroxide by a Fenton-type reaction, producing OH. These potent ROS oxidize the reduced colorless o-dianisidine molecules to yellow-brown colored dianisidyl radicals at low pH. The oxidation reactions progress among dianisidyl radicals and further oxidation reactions develop. The color formation is increased with further oxidation reactions. Antioxidants present in sample suppress the oxidation reactions and color formation, which can be measured spectrophotometrically. The results are expressed as μmol Trolox equiv. /L.

Total oxidative status (TOS) of serum samples was measured by an automated measurement method. Ferrous ion–o-dianisidine complex oxidized by serum oxidants to ferric ion. Glycerol molecules enhance the oxidation reactions which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, measured spectrophotometrically, reflects total amount of oxidants present in serum sample expressed as μmol H_2O_2 equiv. /L. The ratio of the total oxidants to the total antioxidants gives the OSI, the degree of oxidative stress.[25,26]

Measurement of Prolidase Activity

Proline level produced by prolidase was determined by a spectrophotometric method. The supernatant was diluted two fold with serum. A pre-incubation solution (50 mmol/L TrisHCl buffer pH 7.0 containing 1 mmol/L glutathione and 50 mmol/L MnCl2) 75 μL and diluted supernatant 25 μL were pre-incubated at 37°C for 30 min. The reaction

mixture, which contained 144 mmol/L gly– pro, pH 7.8 (100 μL), was incubated with 100 μL of pre- incubated sample at 37°C for 5 min. One mL glacial acetic acid was added to stop incubation reaction. After adding 300 μLTrisHCl buffer, pH 7.8 and 1 mL ninhydrin solution (3 g/dL ninhydrin was melted in 0.5 mol/L orthophosphoric acid), the mixture was incubated at 90°C for 20 min and then cooled with ice. At a 515 nm wavelength absorbance was measured to determine proline level.[27,28]

Instrument- UV-VIS spectroscopy was used to determine the concentration of the absorber in a solution.

STATISTICAL ANALYSIS

The data was analyzed by statistical software SPSS version 16.0.The distribution of patients for continuous variables was represented by Mean ± St Dev and for categorical data frequency with their relative percentages were given. For comparison of quantitative group means student –t test was used and for Qualitative/categorical data Chi-square test was used. For comparison between multiple groups ANOVA test was used. A p- value <0.05 was considered as statistically significant.

RESULTS

Mean age of controls was 25.95±5.09 years and IBS patients was 29.698.95 years (P<0.05) [Table 2]. Control group included 1st year medical students primarily explaining the significant age difference between cases and controls. There was male predominance in both control and IBS group with overall male to female ratio of 1.31:1 without any significant difference between two groups. There also was male predominance in IBS subgroups [Table 3].

Mean duration of symptoms in IBS group was 25.52±29.36 months, without any significant difference between IBS subgroups IBS-D, IBS-C and IBS-M. Mean hemoglobin level in controls and IBS group was similar (13.50±1.13 and 13.22±0.95 g/dL respectively).

Mean TOS, TOS/TAS (OSI) and prolidase levels were significantly higher in IBS group (11.54±3.09 μmol H_2O_2 Eq/L; 12.06±5.81 and 210.07±82.94 mmol $Min^{-1}L^{-1}$ respectively) than control group (7.00±1.44 μmol H_2O_2 Eq/L; 4.28±1.44 and 165.03±77.22 mmol $Min^{-1}L^{-1}$ respectively) with p value of <0.001,< 0.001, and <0.01 respectively [Table 4].

Level of TOS was highest among IBS-D subgroup (15.28±1.23 μmol H_2O_2 Eq/L) followed by IBS-M (10.671.36 μmol H_2O_2 Eq/L) and Lowest in IBS-C subgroup (8.68±1.51 μmol H_2O_2 Eq/L) showing a significant difference between IBS-D and IBS-C, IBS-D and IBS-M and IBS-M and IBS-C with p values <0.001 for each comparison upon post hoc analysis of subgroups [Table 5].

OSI in controls, IBS-D, IBS-C and IBS-M subgroups were 4.28, 19.31, 7.45 and 9.43 respectively, being highest in IBS-D and lowest in IBS-C with significant differences

History	Physical Examination
Blood in the stool	Abdominal mass
Chronic diarrhea	Arthritis (active)
Family history of colon cancer, IBD, or celiac disease	Dermatitis herpetiformis or Pyodermagangrenosum
Fever	Overt blood or mass on rectal examination
Onset after age 50 years	Signs of anemia
Night-time symptoms (awakening the patient from sleep)	Signs of intestinal malabsorption
Progressive dysphagia	Signs of intestinal obstruction
Recurrent vomiting	Signs of thyroid dysfunction
Short history of symptoms	
Travel history to locations endemic for parasitic diseases	
Weight loss	

Table-1: Alarm features considered to potentially relevant in diagnosing organic disease as opposed to IBS

Groups	Mean ± SD	ANOVA
Controls (n=40)	25.95±5.09	F=3.292
IBS-D (n=40)	28.08±8.61*	
IBS-C (n=40)	31.48±9.41*	
IBS-M (n=40)	29.52±8.70*	
ANOVA; * P<0.05		

Table-2: Age distribution in IBS subgroups

Sex	Controls (n=40)	IBS-D (n=40)	IBS-C (n=40)	IBS-M (n=40)
Female	17 (42.5%)	16 (40.0%)	19 (47.5%)	17 (42.5%)
Male	23 (57.5%)	24 (60.0%)	21 (52.5%)	23 (57.5%)
Total	40 (100.0%)	40 (100.0%)	40 (100.0%)	40 (100.0%)

Table-3: Sex distribution in subgroups

Parameters	Controls (n=40)	IBS (n=120)	t-value
TOS (μmol H_2O_2 Eq/L)	7.00±1.44	11.54±3.09*	t=-8.973
TAS (mmol Trolox Eq/L)	1.72±0.34	1.05±0.24*	t=13.506
TOS/TAS (OSI)	4.28±1.44	12.06±5.81*	t=8.351
Prolidase (mmol $Min^{-1}L^{-1}$)	165.03±77.22	210.07±82.94*	t=3.023
Student t-test; *P values < less than 0.001, 0.001, 0.001 and 0.01 for TOS, TAS, OSI and Prolidase respectively.			

Table-4: Measures of oxidative stress in healthy controls and IBS patients

Groups	TOS Mean ± SD (μmol H_2O_2 Eq/L)	ANOVA
Controls (n=40)	7.00±1.44	F=264.255
IBS-D (n=40)	15.28±1.23*	
IBS-C (n=40)	8.68±1.51*	
IBS-M (n=40)	10.67±1.36*	
ANOVA; *P values < 0.001 for control vs all IBS subgroups and between subgroups.		

Table-5: TOS level in subgroups

Groups	Mean ± SD	ANOVA
Controls (n=40)	4.28±1.44	F=298.241
IBS-D (n=40)	19.31±3.60*	
IBS-C (n=40)	7.45±2.01*	
IBS-M (n=40)	9.43±1.84*	
ANOVA; *P values < 0.001 for control versus all IBS subgroups and between subgroups.		

Table-6: TOS/TAS (OSI) in subgroups

Groups	Mean ± SD	ANOVA
Controls (n=40)	165.0371.22	F=6.354
IBS-D (n=40)	197.031±70.37	
IBS-C (n=40)	191.57±68.67	
IBS-M (n=40)	241.62±98.97*	
ANOVA; *P values < .001, 0.013 and 0.01 for IBS-M versus controls, IBS-D and IBS-C respectively. Other subgroup comparisons were not significant.		

Table-7: Serum prolidase activity in subgroups

Groups	TAS Mean ± SD (mmol Trolox Eq/L)	ANOVA p-value
Controls (n=40)	1.72±0.34	F=111.658 p<0.001 (HS)
IBS-D (n=40)	0.81±0.14*	
IBS-C (n=40)	1.20±0.20*	
IBS-M (n=40)	1.15±0.14*	
ANOVA; *P values <0.001 for controls versus all IBS subgroups, IBS-D versus IBS-C and IBS-D versus IBS-M; Difference between IBS-C versus IBS-M was not significant.		

Table-8: TAS level in subgroups

between the subgroups (P<0.001) [Table 6].

Mean levels of serum prolidase were 197.03, 191.57 and 241.64 mmol Min^{-1}L^{-1} in IBS-D, IBS-C and IBS-M subgroups respectively as compared to control group where serum prolidase activity was 165.03 mmol Min^{-1}L^{-1}. Only IBS-M subgroup had significantly higher serum prolidase activity when compared to controls (p<0.001) IBS-D (P=0.013) and IBS-C (P=0.01). Although prolidase activity was higher in IBS-D and IBS-C subgroups than control group, it was not significant [Table 7].

TAS level in controls and cases (IBS) were 1.72±0.34 mmol Trolox Eq/L and 1.05±0.24 mmol Trolox Eq/L, a significantly higher value in controls (P<0.001). Subgroup analysis showed TAS values 0.81, 1.20 and 1.15 mmol Trolox Eq/L in IBS-D, IBS-C and IBS-M subgroups respectively. There was

significant difference between all four subgroups (p<0.001) except between IBS-C and IBS-M subgroups (P=0.294) [Table 8].

DISCUSSION

Present study demonstrated that mean TOS, TOS/TAS (OSI) and serum prolidase activity were significantly higher in IBS group than control group with p value of <0.001,< 0.001, and <0.01 respectively suggesting a role of oxidative stress in IBS patients. This is in accordance to a study where 50 patients diagnosed with IBS using the Rome III criteria and a control group of 50 healthy subjects were included. In that study TOS, TAS and OSI values were assessed and it was found that TOS and OSI values were significantly higher, and the TAS value was significantly lower in IBS patients (p < 0.001 for all) as compared to controls.[17]

Similarly our study demonstrated that TAS is significantly lower and OSI is significantly higher in IBS group as compared to controls. TAS level in controls and cases (IBS) were 1.72 and 1.05 mmol Trolox Eq/L observing a significantly higher value in controls (P<0.001).

In addition serum prolidase, an enzyme involved in collagen metabolism and a marker of oxidative stress and inflammation is also significantly elevated in IBS patients than controls. It has been reported that plasma prolidase activity might be useful in evaluating bowel disorders and fibrotic processes in chronic liver disease patients.[27,29] However, there probably is no study in literature evaluating serum prolidase activity as a marker of oxidative stress in patients with IBS.

Highest TOS seen in IBS-D patients followed by IBS-M and lowest in IBS-C subgroup suggesting that patients with diarrheal symptoms have greater load of oxidative stress and hence inflammation as compared to patients with predominant constipation [Table 5]. OSI in IBS-D, IBS-C and IBS-M subgroups were 19.31, 7.45 and 9.43 respectively, being highest in IBS-D and lowest in IBS-C with significant differences between the subgroups (P<0.001) [Table 6].

Subgroup analysis showed TAS values 0.81, 1.20 and 1.15 in IBS-D, IBS-C and IBS-M subgroups respectively. There was significant difference between all four subgroups (p<0.001) except between IBS-C and IBS-M subgroups (P=0.294). This finding is in parallel to TOS and OSI values explaining that patients with diarrhea predominant symptoms have higher oxidative stress as compared to healthy controls and constipation predominant symptoms [Table 8]. Similarly, total anti-oxidant capacity was found to be significantly lower in patients with IBS patients compared to healthy controls in one recent study.[16]

Findings of higher TOS and OSI and lower TAS in IBS patients in this study are in accordance to study by Karakas et al.[17] Additional finding from our study is patients with diarrhea predominant IBS have higher TOS/OSI and lower TAS as compared to patients who have purely constipation or have mixed symptoms of diarrhea and constipation. Another finding of this study that SPA though was significantly higher in IBS patients than controls, only patients with mixed symptoms (IBS-M) have significant elevation in SPA

when compared to IBS-D, IBS-C and controls on subgroup analysis.

On the basis of these findings it can be inferred that there is an oxidative injury in the IBS which may be involved in its pathogenesis or may be the effect of inflammation caused by some other etiology. Traditionally, IBS has been considered as a condition arising from brain-gut dysregulation and classified as one of the functional gastrointestinal disorders; hence its symptoms are not explained by structural or biochemical abnormalities.[30] Studies have shown that increased oxidative stress is involved in various inflammatory, degenerative and endocrine diseases which affect tissues integrity and metabolic rate of body. There were several studies demonstrating higher concentrations of oxidative stress products and antioxidants in the chronic inflammatory diseases of gastrointestinal tract such as crohn's disease, ulcerative colitis and chronic hepatitis. However there is limited number of studies which evaluate oxidative stress products in IBS. Mete R. et al.[31] evaluated the plasma concentrations of malondialdehyde (MDA) and nitric oxide (NO) and the plasma activities of oxidant and antioxidant enzymes; Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSK-Px), xanthine oxidase (XO), adenosine deaminase (ADA) activities in patients with IBS. Plasma XO and ADA activities, MDA and NO concentrations were significantly higher in IBS patients than in controls. The SOD, CAT, and GSH-Px activities in the serum of patients with IBS were significantly lower than that of controls. Oran et al.[32] demonstrated the presence of oxidative stress and increased inflammation in patients with IBS by showing the drop in paraoxonase and arylesterase activities accompanied with an increase in conjugated diene levels.

CONCLUSION

In conclusion this study observed that there is increased oxidative stress and decreased antioxidant capacity in patient with IBS; therefore, we should consider that antioxidants might be beneficial in the supportive treatment of IBS. To support our results further prospective and randomized controlled trials are necessary.

Limitations: Major limitations of this study include small number of subjects and inclusion of medical students as healthy controls which may not truly represent healthy general population.

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