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Expression Levels of Interleukin-1 Beta (IL-1β), Inducible Nitric Oxide Synthase (iNOS), and Tumor Necrosis Factor-Alpha (TNF-α) in Patients with Irritable Bowel Syndrome at Baghdad Hospital, Iraq

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ABSTRACT

The etiology of irritable bowel syndrome (IBS) is unknown. However, it has been suggested that immune activation and low-grade mucosal inflammation may be involved in the pathogenesis of IBS. This study was undertaken to estimate the blood levels of interleukin-1 beta (IL-1β), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-alpha (TNF-a) in Iraqi IBS patients and healthy controls.

Blood samples were drawn from 20 IBS patients and 20 healthy volunteers and were studied using the real-time PCR technique in the present study. Results of the analyzed data showed a significant increase in TNF-a levels ($p < 0.05$) between IBS patients and controls ($p > 0.05$). However, there was no significant difference in the mRNA levels of iNOS and IL-1β between groups.

This study suggests that patients with IBS show altered mRNA levels of TNF-a. However, further studies are required to clarify the roles of this cytokine in the pathogenesis of IBS.

Keywords: *Irritable Bowel Syndrome, Interleukin-1beta, iNOS, Iraq, TNF-a, Cytokines, Blood Cells*

1. INTRODUCTION

Irritable bowel syndrome (IBS) is a complex functional disorder of the bowel that affects about twelve percent of the world's population (1). The prevalence and symptoms of IBS can vary due to factors such as geographic region and the diagnostic criteria used (2). IBS can be categorized into four types: IBS with constipation-predominant (IBS-C), IBS with diarrhea-predominant (IBS-D), IBS with mixed bowel habits (IBS-M), and unsubtyped IBS, where stool consistency does not meet the criteria for any of the other subtypes. The disease is most prevalent among young individuals and women (3). It significantly impacts the well-being of those affected and leads to considerable healthcare costs (4). In the United States, the annual direct costs associated with IBS exceed $1 billion (3).

Since there is no structural, organic or biochemical abnormalities in IBS, it is diagnosed clinically (5). The precise underlying mechanisms associated with IBS remain largely unclear, but it seems to be multifactorial (6, 7).

Immune activation and low-grade mucosal inflammation have been reported in patients with IBS (5). An imbalance in the ratio of anti-inflammatory and pro-inflammatory cytokines could contribute to the persistence of low-grade inflammation (8). Cytokines are a type of signaling molecules that regulate immunological responses (5). Some of the main cytokines include monokines, lymphokines, interleukins, tumor necrosis factor (TNF), and interferons, which are secreted by various cells, particularly white blood cells (9). Cytokine gene mutations could make individuals more vulnerable to infectious gastroenteritis, which could consequently lead to the development of primary IBS or cause post-infectious IBS (PI-IBS) (10). Although cytokine imbalances are observed in patients with IBS, there is no clear or consistent pattern of cytokine levels in the blood (8). Therefore, further studies should be conducted in this area.

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Additionally, nitric oxide (NO) may assist in the development of IBS pathogenesis (11). One of NO's key roles is regulating immune responses. It is synthesized by nitric oxide synthase (NOS) enzymes. NO production notably increases during inflammation, primarily due to the activation of inducible nitric oxide synthase (iNOS) (12, 13). Factors like bacteria and cytokines have been reported to cause iNOS expression (12). Research by Koçak et al. revealed elevated NO levels in the colonic tissues of IBS patients (14). Despite this, there is still much that needs to be known about the particular role of iNOS in IBS, hence necessitating further studies.

This study aims to investigate the levels of IL-1β, TNF-α, and iNOS in blood samples from Iraqi patients with IBS compared to healthy controls. Given the evidence suggesting that immune activation and inflammation play a role in IBS, our goal is to clarify the potential roles of these markers in the pathogenesis of IBS and to explore their utility in diagnosing the condition and treatment.

2. METHODS

Study population and sample collection

A total of Twenty patients with IBS and Twenty healthy individuals (serving as the control group) were recruited from Baghdad Hospital in Iraq, following the Rome IV criteria. The presence of other chronic inflammatory diseases, a history of abdominal surgery, celiac disease, malignancies, or use of immunosuppressive drugs were considered exclusion criteria.

Venous blood samples (five milliliters) were drawn after 8 hours of fasting into ethylenediaminetetraacetic acid (EDTA) tubes. All samples were collected on the same day and immediately transferred to the laboratory. The samples were frozen at -80°C for further processing.

The study received approval from the Ethics Committee of the Urmia University, Faculty of Veterinary Medicine, and consent was gained from all participants.

Cytokine analysis

Total RNA was isolated from the Blood using the RNA Isolation Kit (DNAZist, Iran; Cat. No.: S-1021-1), following the manufacturer's guideline. Isolated RNA molecules were used as templates for the synthesis of complementary DNA (cDNA). This process was performed using the cDNA Synthesis Kit (DNAZist, Iran; Cat. No. S-1074-50). The amplification program consisted of the following steps: 25°C in 10 minutes, 55°C in 30 minutes, and 80°C in 5 minutes. The synthesized cDNA was then stored at −20°C for future use.

Then, the synthesized cDNA from the target genes was quantified using a Real-Time PCR System (ABI step one, America). The reaction mixture's final volume was 20 µL, comprising 10 µL of master mix (Denazist, Iran), 0.5 µL each of primer, 0.5 µL of synthesized cDNA, and 8.5 µL of water (nuclease-free). Specific primers for each gene, as shown in Table 1, were obtained from SinaClone, Iran. β-Actin served as endogenous control. The PCR program consisted of four steps: an initial denaturation step at 95 ℃ for 10 min, 40 cycles that included denaturation at 95°C for 15 seconds, annealing at 60°C for 60 seconds, and followed by a melting step at 60 to 95°C. The gene expression data were presented regarding $2^{-\Delta\Delta CT}$ for both control and patient groups.

Table 1. Primer sequences for the expression of target genes (FP = forward primer; RP = reverse primer; IL-1β = interleukin-1 beta; iNOS = inducible nitric oxide synthase; TNF = tumor necrosis factor)

Gene name	Sequences	Reference
iNOS	Forward-TTCAGTATCACAACCTCAGCAAG	(15)
	Reverse-TGGACCTGCAAGTTAAAATCCC	
IL-1β	Forward-CCACAGACCTTCCAGGAGAATG	(16)
	Reverse-GTGCAGTTCAGTGATCGTACAGG	
β-Actin	Forward-CACCATTGGCAATGAGCGGTT	
	Reverse-AGGTCTTTGCGGATGTCCACGT	
TNF_alpha	Forward-CTTCTGCCTGCTGCACTTTG	(17)
	Reverse-GTCACTCGGGGTTCGAGAAG	

Statistical analysis

The expression levels of IL-1β, TNF-α, and iNOS are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 10 (GraphPad Software, USA). The Kolmogorov-Smirnov test was used to consider data normality, and group comparisons were performed using independent t-test. A probability value (p-value) below 0.05 was considered statistically significant.

3. RESULTS

Blood samples were collected from twenty healthy individuals and twenty IBS patients to analyze the levels of TNF-α, IL-1β, and iNOS. The results, shown in Figure 1, indicate that TNF-α levels were higher in IBS patients in comparison to healthy individuals, while the differences in iNOS and IL-1β levels were not statistically significant.

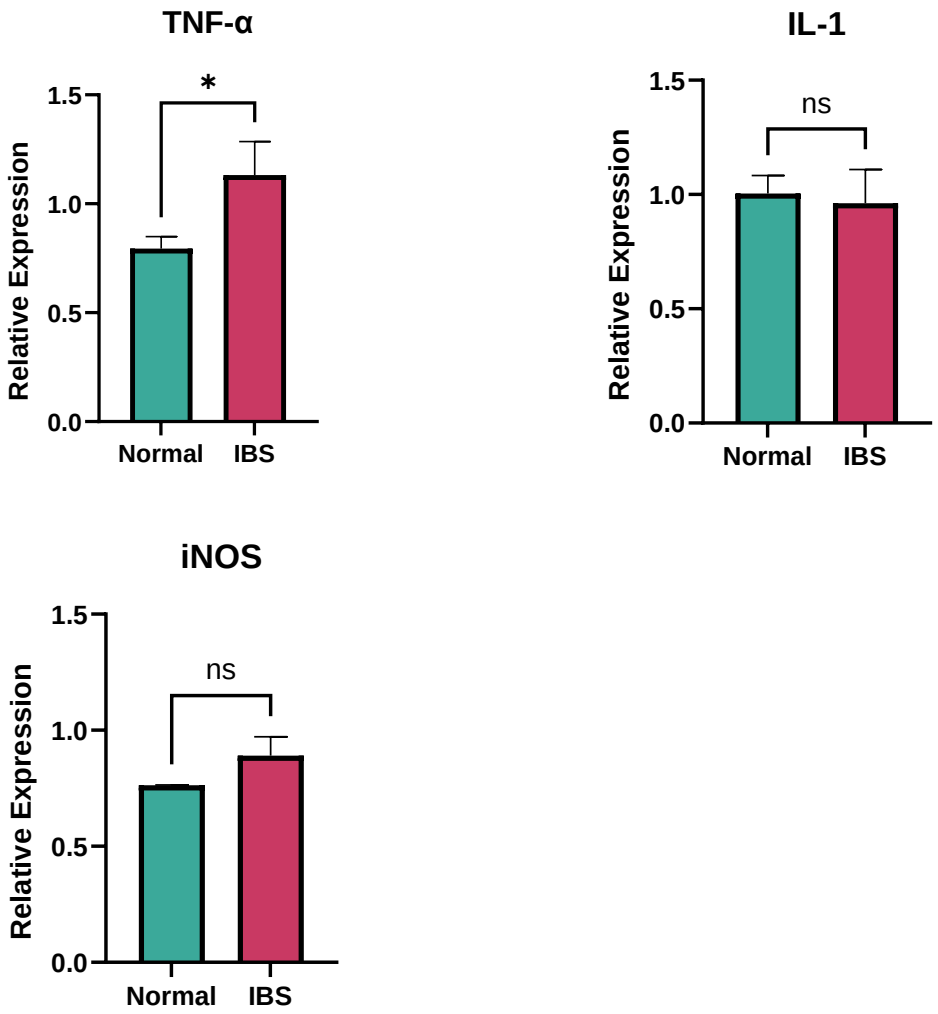

Figure 1. Levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and inducible nitric oxide synthase (iNOS) in patients with irritable bowel syndrome (IBS) compared to healthy individuals. Statistical significance: ns (not significant, $p > 0.05$), * $p < 0.05$.

4. DISCUSSION

The exact cause of IBS remains unclear. Understanding the underlying pathogenesis of IBS is essential, particularly as newer medications are starting to focus on the known pathophysiological processes of the disorder. Factors such as increased visceral sensitivity, altered gastrointestinal motility, brain-gut interactions, post-infectious changes, shifts in fecal microbiota, food intolerances, bacterial overgrowth, intestinal inflammation, and carbohydrate malabsorption have all been linked to the pathogenesis of IBS (18). Evidence suggests that immune activation, both systemic and intestinal, may play a role in IBS.

Early studies show that targeting the immune system could be promising (19). This study compared TNF-α, IL-1β, and iNOS levels between Iraqi patients with IBS and healthy controls. Results showed a significant increase in TNF-α levels ($p < 0.05$) in IBS patients, while IL-1β and iNOS levels were not significantly different.

Consistent with our results, numerous studies have reported a significant increase in TNF-α expression in IBS (20-23). Furthermore, research has examined TNF-α levels in subgroups of IBS. For instance, Rana et al. demonstrated elevated TNF-α in individuals with IBS-D compared to controls (24). Similarly, Scully et al. found that while overall TNF-α levels did not change significantly between IBS patients and controls, they were elevated in IBS patients with extra-intestinal comorbidities (25). TNF-α is a key regulator of inflammatory responses and plays a role in the pathogenesis of autoimmune and inflammatory diseases. Given TNF-α's role in the autoimmune diseases, TNF-alpha inhibitors have been effectively used in the treatment of conditions like rheumatoid arthritis and Crohn's disease (26). Consistent with prior research demonstrating elevated TNF-α in IBS, our findings support its potential clinical significance. Moreover, Barkhordari et al. showed that TNF-α and IL-6 cytokine gene polymorphisms could change individual vulnerability to IBS, and they might play a role in the pathophysiology of IBS (27). However, further comprehensive studies are warranted to elucidate its role in IBS pathogenesis and explore its utility as a diagnostic biomarker or therapeutic option.

IL-1β, as a crucial mediator of the inflammatory reaction, is vital for resistance to pathogens and host defense. However, it can also worsen damage through acute tissue injuries and chronic diseases (28). There is limited and inconsistent data regarding the effects of IL-1β on individuals with IBS, underscoring the need for further research in this field. The cytokine IL-1β was also examined in this study; however, it did not show any significant changes. Similar to our study, Mitselou et al. also did not observe a significant change in IL-1β levels. They also found that the concentrations of IL-1β were consistent across different subgroups of IBS, with no significant variations in the cecum, terminal ileum, or rectum (29). Likewise, Scully et al. reported findings that were similar to our study. However, they also highlighted that IBS patients with extra-intestinal co-morbidities exhibited higher IL-1β levels. (30). Unlike our study and the previously mentioned ones, Bennett et al. reported elevated serum IL-1β levels in IBS patients compared to healthy individuals (21).

There are very few studies investigating the relationship between iNOS and IBS. An et al. observed increased iNOS levels in colonic tissue samples obtained from individuals with IBS-D (15). However, in our study, iNOS levels in blood samples from IBS patients did not significantly change. These variations may be attributed to An et al.'s evaluation of iNOS changes within specific IBS subgroups (IBS-D) or the disparity in sample types: they used colonic mucosal samples, whereas we used blood samples.

5. CONCLUSION

The understanding of pathogenic mechanisms underlying the development of IBS is essential. It helps the development of novel biological therapies and diagnoses. In the present study, a dysregulated TNF-α expression was observed in Iraqi IBS patients when compared to healthy control. On the other hand, there were no statistically significant changes in IL-1β and iNOS mRNA levels. The findings are consistent with previous research indicating that dysregulated cytokine secretion could contribute to IBS.

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