Rom J Morphol Embryol 2023, 64(1):57–63 ISSN (print) 1220–0522, ISSN (online) 2066–8279 doi: 10.47162/RJME.64.1.07

ORIGINAL PAPER

RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/

Investigation of distribution of GSK-3*β* signal pathway by age groups in cases of ulcerative colitis

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Abstract

Ulcerative colitis (UC), one of the inflammatory bowel diseases, has been reported to increase in recent years. Although the exact cause is unknown, disruptions in the molecular pathways are thought to trigger UC. We aimed to examine the distributions of glycogen synthase kinase-3beta (GSK-3*β*), nuclear factor-kappa B (NF-*κ*B) and wingless/int-1 (Wnt-1) in different age groups diagnosed with UC. Patients diagnosed with UC were divided into four groups according to their ages: Group 1, aged 18–30 (*n*=20); Group 2, aged 31–45 (*n*=20); Group 3, aged 46–60 (*n*=20); Group 4, aged 61–75 (*n*=20). Tissue sections were histochemically stained to examine the parameters of epithelial cell height, length of crypt, thickness of *muscularis mucosa* and extent of submucosal fibrosis. The immunohistochemistry assay was performed using cell survival and for GSK-3*β*, NF-*κ*B and Wnt-1 cell growth markers. Immunoreactivities were evaluated using *H*-score and analyzed using the one-way analysis of variance (ANOVA) test for statistics. It was detected a decrease in the histopathological parameters whereas the immunoreactivities of GSK-3*β*, NF-*κ*B and Wnt-1 were increased with increasing age. The levels of GSK-3*β* immunoreactivity were similar in both epithelium and submucosa in all groups. NF-*κ*B immunoreactivity was higher in submucosa of Groups 1, 2 and 3, while Wnt-1 was enhanced in Groups 1 and 3. The results of histopathology showed that the integrity of the epithelial tissue in the colon deteriorated with increasing age. The expressions of GSK-3*β*, NF-*κ*B and Wnt-1 were detected in all age groups. We thought that there was a synergistic activation between these three markers. Nevertheless, studies are needed to investigate this molecular pathway.

Keywords: inflammatory bowel diseases, GSK-3*β*, NF-*κ*B, ulcerative colitis, Wnt-1.

Introduction

Ulcerative colitis (UC), one of the inflammatory bowel diseases (IBDs), has an increasing incidence recently [1, 2]. It is characterized by the loss of function of the epithelial barrier and inflammation in the colon mucosa, and the inflammation causes mucosal ulcers. Although the etiology of UC is not known clearly, it is an autoimmune disease [3, 4]. Studies have shown that down-regulation or up-regulation of some signal molecules play a role in the occurrence of UC. Some of these signal molecules are proinflammatory cytokines, such as interleukins (IL-1*β*, IL-6, IL-12p40), tumor necrosis factor-alpha (TNF-*α*), interferon-gamma (IFN-*γ*), nuclear factor-kappa B (NF-*κ*B), wingless/int-1 (Wnt-1), Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), glycogen synthase kinase-3beta (GSK-3*β*) [2, 4, 5-9].

During UC, the epithelial cells try to repair the damaged tissue area by proliferation and differentiation [10]. But, if there is a major malfunction of signal molecules in the inflammation zone, the damage repair mechanism becomes ineffective. At this point, up-regulation or down-regulation of signal molecules gains prominence. Wnt signaling pathway is crucial to cell proliferation and survival. It has been determined that it is involved in the regeneration of many organs (heart, bone, etc.), especially skin wound healing [11]. The Wnt molecule works in conjunction with the *β*-catenin. The Wnt/*β*-catenin signaling pathway is associated with many pathways. One of them is the NF-*κ*B signaling pathway. It has been reported in both clinical and experimental animal (such as murine) studies that NF-*κ*B is a factor in the formation of inflammatory response in IBD, and also in UC by regulating the inflammatory cytokines [2, 6, 12, 13]. The Wnt/*β*-catenin signaling has both an inhibitory and an activator effect on NF-*κ*B. These effects occur according to the cell type and pathophysiology of the diseases [14]. However, the Wnt/*β*-catenin and NF-*κ*B signaling are also associated with the GSK-3 signaling. In the up-regulation of Wnt, GSK-3 binds to *β*-catenin, and its signaling is initiated. The relationship between NF-*κ*B and GSK-3 is in two-way. Namely, the activation of GSK-3 can affect NF-*κ*B both negatively and positively [13]. GSK-3, whose primary function is in the glycogen metabolism, works with many signaling pathways in cell survival, apoptosis, autophagy, cancer, and other diseases. GSK-3 has two isoforms, *α* and *β*. It is reported that both isoforms have different roles, and GSK-3*β* is associated with UC [8, 14, 15].

Aim

In our study, we aimed to reveal the relationship between these three markers, GSK-3*β*, NF-*κ*B and Wnt-1, in patients with UC of different age groups.

Patients, Materials and Methods

Patients

The study was approved by the Niğde Ömer Halisdemir University Non-Interventional Clinical Research Ethics Committee (Ethical Protocol No. 2019/39, date: 04/10/2019). In our study, paraffin-embedded colonoscopic tissue biopsies were taken from patients diagnosed with UC according to Mayo Clinic score (4–9) from Niğde Ömer Halisdemir University Training and Research Hospital [16]. Architectural and inflammatory changes, such as crypt distortion, ulceration and neutrophilic infiltration with crypt destruction were sought for the diagnosis of UC using Geboes Index [17]. Patients were divided into four different age groups: Group 1, aged 18–30 (n=20); Group 2, aged 31–45 (n=20); Group 3, aged 46–60 (n=20); Group 4, aged 61–75 (n=20).

Histopathology

Four μm thick sections were taken from the paraffin blocks with a microtome and each adhesive slide was arranged in 3–4 pieces. Sections were deparaffinized at 56°C in an oven; after dewaxing and dehydration stages, they were stained with Hematoxylin–Eosin (HE) for the histopathological (HP) examination. HP parameters were used: epithelial cell height, length of crypt and thickness of *muscularis mucosa* (μm) [3, 18].

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut at 4 μm thickness and transferred to positively charged slides. Leica Horseradish peroxidase (HRP) conjugated compact polymer system 3,3'-Diaminobenzidine (DAB) detection kit was used (Leica DS9800, Newcastle, UK) on Leica Bond™ Max automated immunohistochemistry *in situ* hybridization system. The sections were heated and dewaxed after the Ethylenediaminetetraacetic acid (EDTA) heat-induced antigen retrieval for 20 minutes and hydrogen peroxide 10 minutes. Then, the primary antibodies (rabbit polyclonal, Abcam, Waltham, MA, USA), anti-GSK-3β antibody (Ab227208), anti-NF-κB antibody (Ab231481), and anti-Wnt-1 antibody (Ab189001) were applied to the tissue sections for 30 minutes. The tissues were stained with DAB and Mayer's Hematoxylin to make visible the immunoreactivity. The slides were mounted using Entellan™, and tissue images were taken by a light microscope (IX71, Olympus, Japan). The staining was evaluated as no (0), weak (+), moderate (++) and strong (+++), respectively, and stained cells were counted for each staining degree in five high-power fields chosen randomly. The *H*-score formula was used: *Pi* (intensity of staining + 1). *Pi* explains the percentage of stained cells for each intensity. Two observers blinded to age groups evaluated the staining scores independently. The immunohistochemical (IHC) assay was performed thrice [19].

Statistical analysis

The results of histopathology and IHC stainings were analyzed by one-way analysis of variance (ANOVA) by Tukey–Kramer multiple comparisons test. The differences were given as mean ± standard deviation (SD) among groups, and the p-value as ≤0.05 was considered statistically significant [19].

Results

Patient sample collection

Samples were selected from patients diagnosed with UC according to Mayo Clinic score (4–9), and histological evaluation of UC was made using the Geboes Index [16, 17]. In each group, there were patients who were diagnosed with the disease for the first time and followed-up. The relevant information was given in Table 1.

Table 1 – ***Number and gender of patients diagnosed with UC for the first time and followed-up***

Group	Gender	Clinical diagnosis	
		Initial	*Follow-up*
Group 1: aged 18–30, n=20	*F*	4 (20%)	4 (20%)
	M	8 (40%)	4 (20%)
Group 2: aged 31–45, n=20	*F*	5 (25%)	5 (25%)
	M	5 (25%)	5 (25%)
Group 3: aged 46–60, n=20	*F*	4 (20%)	5 (25%)
	M	4 (20%)	7 (35%)
Group 4: aged 61–75, n=20	*F*	5 (25%)	2 (10%)
	M	4 (20%)	9 (45%)

F: Female; M: Male; *n*: No. of patients; UC: Ulcerative colitis; %: Percentage in the group.

When patient information is checked, some patients do not have information after the initial diagnosis. Or the patient applied to the specified health unit for control purposes only and informed that the first diagnosis of the disease was made in another institution. Therefore, in our study, gender differences were ignored, and the HP and IHC examination was carried out considering the age of the patients [Group 1, aged 18–30 (n=20); Group 2, aged 31–45 (n=20); Group 3, aged 46–60 (n=20); Group 4, aged 61–75 (n=20)].

Histopathology

The samples taken from patients diagnosed with UC were stained with HE, and they were examined in terms of epithelial cell height, length of crypt, and thickness of *muscularis mucosa* (μm). The method of measurements was illustrated in Figure 1 (A and B). Firstly, the general histological structure was evaluated, and it was stated that the integrity of epithelium was impaired in all groups (Figure 2, A–H). Together, because of the UC, there were fibrotic areas in the submucosa, so the layer of *muscularis mucosa* displayed a fragmented appearance (Figure 2, A–H). The epithelial cell height was the highest in Group 1 in comparison with the other groups. There was no significant difference between Group 1 and Group 2 (p>0.05), while the differences between Group 1 and Group 3, and also Group 1 and Group 4 were statistically significant (***p<0.001). It was determined that the length of crypt decreased with increasing age (Table 2). There was an obvious difference between the Group 1 and the other groups (***p<0.001). Also, the difference between the Group 2 and Group 3 was significant (**p<0.01). In the Group 4, this parameter was diminished compared to the Group 3 (***p<0.001). When the samples from all groups were evaluated in terms of the thickness of *muscularis mucosa*, this parameter was the highest in the Group 2, however the values of Group 1 and Group 2 were close to each other (p>0.05). It was seen that the lowest group was Group 4.

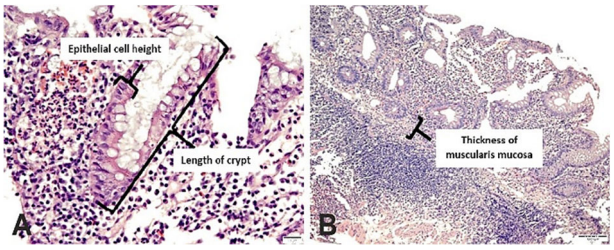

Figure 1 – ***Measuring of epithelial cell height, length of crypt (A), and thickness of muscularis mucosa (B) in the histological sections. Hematoxylin–Eosin (HE) staining. Scale bars: (A) 20 µm; (B) 100 µm.***

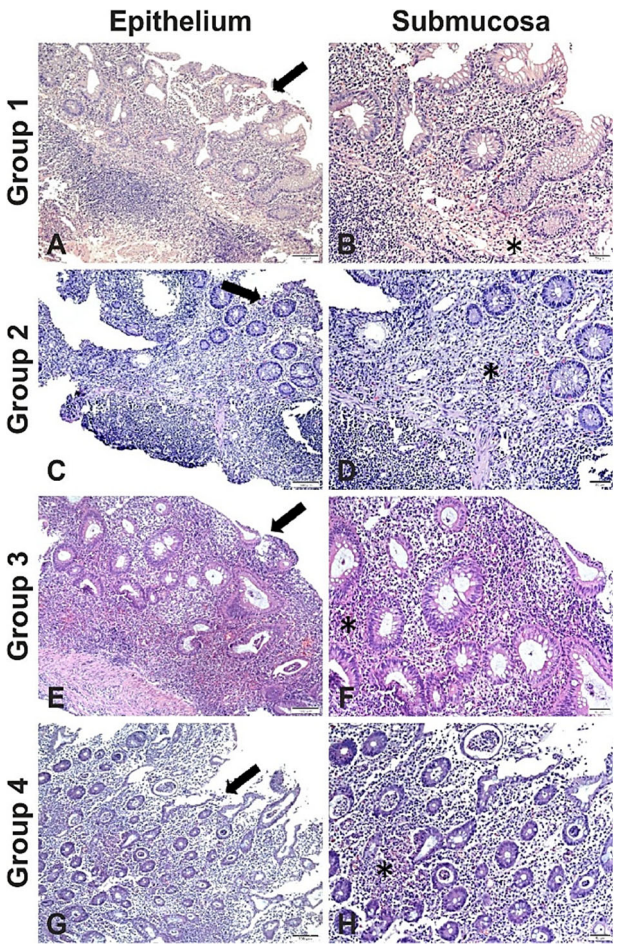

Figure 2 – ***Histological images of ulcerative colitis (UC) samples from all groups. Arrows: Damaged epithelium. Asterisks: Fibrotic area. HE staining. Scale bars: (A, C, E and G) 100 µm; (B, D, F and H) 50 µm.***

Table 2 – ***Comparison of epithelial cell height, length of crypt and thickness of muscularis mucosa in the four groups (mean ± SD, µm)***

Parameters / Groups	Epithelial cell height	Length of crypt	Thickness of *muscularis mucosa*
Group 1	22.035± 7.558	234.183± 65.702	26.983± 7.202
Group 2	17.418± 5.255	174.820± 29.375	29.822± 10.465
Group 3	13.026± 2.530	154.896± 19.322	19.186± 3.589
Group 4	14.678± 3.738	137.345± 15.041	17.306± 4.614

SD: Standard deviation.

Immunohistochemistry

All samples from the patients with UC were immunohistochemically stained to determine the distributions of GSK-3β, NF-κB and Wnt-1, and the staining results were given by *H*-score. The immunoreactivity intensities of these markers were evaluated in both epithelium and submucosa and were given in Figure 3 (A–C). The number of patients whose expression levels of these markers were determined was shown as % in Table 3.

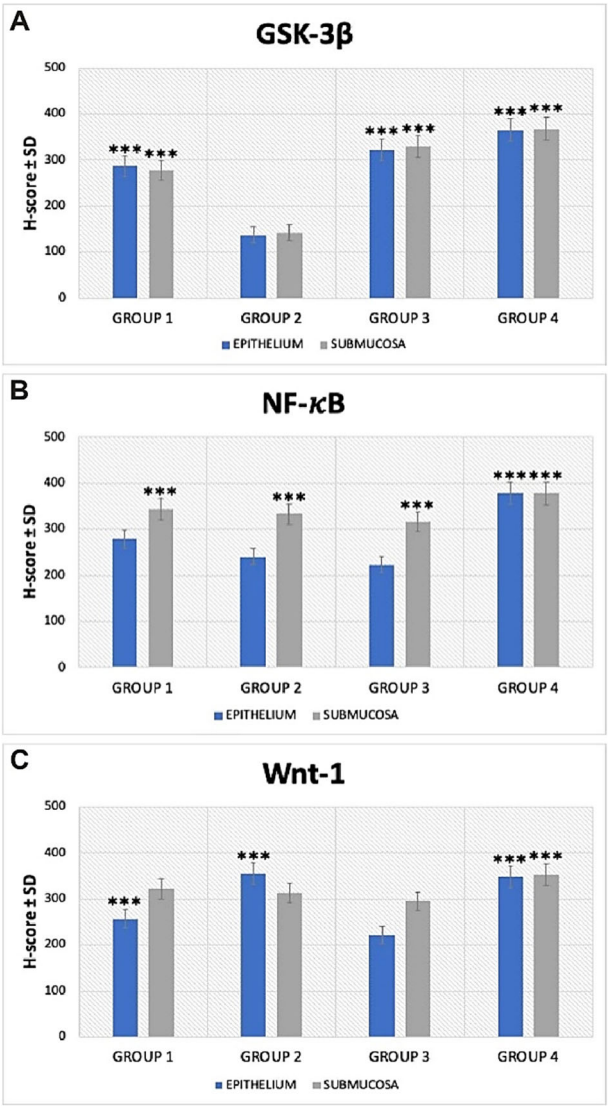

Figure 3 – ***H-score analysis of GSK-3β (A), NF-κB (B) and Wnt-1 (C) immunohistochemistry in the epithelium and submucosa from all groups. GSK-3β: Glycogen synthase kinase-3beta; NF-κB: Nuclear factor-kappa B; SD: Standard deviation; Wnt-1: Wingless/Int-1.***

The level of GSK-3β immunostaining ascended with increasing age, but in Group 2 there was a prominent decrease interestingly. The immunoreactivity of GSK-3β was the lowest in Group 2, in both epithelium (136.7±17.4) and submucosa (142.4±18.1). GSK-3β was enhanced in Group 3 and Group 4 in comparison with Group 1 and Group 2 (***p<0.001). The highest levels were seen in the epithelium (364.8±24.3) and submucosa (367.1±24.7) of Group 4 (Figure 3A; Figure 4, A–H). When evaluated in terms of the number of patients with positive staining, the number of patients with weak staining in Group 2 was the

highest in both epithelium and submucosa. There was no patient with moderate or strong staining. The percentage of patients with moderate or strong staining was the highest in Group 4 (Table 3).

Table 3 – ***Distribution percentages of GSK-3β, NF-κB and Wnt-1 expression levels in patients***

Immunomarker / Group / Sample	No staining (0)	Weak (+)	Moderate (++)	Strong (+++)
GSK-3β				
Group 1: aged 18–30, n=20				
▪ Epithelium	3 (15%)	10 (50%)	5 (25%)	2 (10%)
▪ Submucosa	5 (25%)	11 (55%)	3 (15%)	1 (5%)
Group 2: aged 31–45, n=20				
▪ Epithelium	8 (40%)	12 (60%)	0	0
▪ Submucosa	6 (30%)	14 (70%)	0	0
Group 3: aged 46–60, n=20				
▪ Epithelium	4 (20%)	8 (40%)	7 (35%)	1 (5%)
▪ Submucosa	2 (10%)	8 (40%)	7 (35%)	3 (15%)
Group 4: aged 61–75, n=20				
▪ Epithelium	2 (10%)	6 (30%)	8 (40%)	4 (20%)
▪ Submucosa	2 (10%)	8 (40%)	7 (35%)	3 (15%)
NF-κB				
Group 1: aged 18–30, n=20				
▪ Epithelium	4 (20%)	9 (45%)	4 (20%)	3 (15%)
▪ Submucosa	3 (15%)	7 (35%)	7 (35%)	3 (15%)
Group 2: aged 31–45, n=20				
▪ Epithelium	6 (30%)	9 (45%)	3 (15%)	2 (10%)
▪ Submucosa	3 (15%)	7 (35%)	6 (30%)	4 (20%)
Group 3: aged 46–60, n=20				
▪ Epithelium	7 (35%)	9 (45%)	3 (15%)	1 (5%)
▪ Submucosa	2 (10%)	9 (45%)	6 (30%)	3 (15%)
Group 4: aged 61–75, n=20				
▪ Epithelium	1 (5%)	5 (25%)	9 (45%)	5 (25%)
▪ Submucosa	1 (5%)	7 (35%)	8 (40%)	4 (20%)
Wnt-1				
Group 1: aged 18–30, n=20				
▪ Epithelium	4 (20%)	11 (55%)	4 (20%)	1 (5%)
▪ Submucosa	2 (10%)	8 (40%)	6 (30%)	4 (20%)
Group 2: aged 31–45, n=20				
▪ Epithelium	2 (10%)	7 (35%)	7 (35%)	4 (20%)
▪ Submucosa	3 (15%)	9 (45%)	6 (30%)	2 (10%)
Group 3: aged 46–60, n=20				
▪ Epithelium	4 (20%)	12 (60%)	3 (15%)	1 (5%)
▪ Submucosa	2 (10%)	10 (50%)	6 (30%)	2 (10%)
Group 4: aged 61–75, n=20				
▪ Epithelium	2 (10%)	8 (40%)	7 (35%)	3 (15%)
▪ Submucosa	3 (15%)	7 (35%)	7 (35%)	3 (15%)

GSK-3β: Glycogen synthase kinase-3beta; NF-κB: Nuclear factor-kappa B; *n*: No. of patients; Wnt-1: Wingless/Int-1; %: Percentage in the group.

When the samples from all patients were examined in terms of the staining intensity of NF-κB, the presence of NF-κB immunoexpression was observed in all of them. The levels of NF-κB immunoreactivity were close to each other in Group 1 (343.4±23.4), Group 2 (332.5±22.3) and Group 3 (315.4±21.1) for submucosa (Figure 3B). The highest levels were assigned in epithelium (378.3±24.6) and submucosa (377.7±24.4) of Group 4 (***p<0.001). The expressions of NF-κB in epithelium were diminished compared to submucosa in Group 1, Group 2, and Group 3 (***p<0.001) (Figure 5, A–H). As seen in the *H*-score results, the percentage of patients with moderate or strong staining was higher in Group 4 compared to other groups (Table 3).

When Wnt-1 immunoreactivity was examined, there was not a difference in the intensity of staining correlated with the rising age (Figure 3C; Figure 6, A–H). The levels of Wnt-1 staining in the epithelium of Group 2 (355.2±23.8) and Group 4 (347.6±23.1) were close to each other (p>0.05). The lowest levels were determined in Group 3 (221.5±18.7 for epithelium and 295.4±20.0 for submucosa), and there was a prominent difference between Group 3 and Group 4 (***p<0.001). Therewithal, submucosae of Group 1, Group 2 and Group 3 showed the same level of staining (p>0.05). The percentage of patients with moderate or strong staining in Group 3 was less than in other groups. So, *H*-score values of epithelium and submucosa in Group 3 were decreased. In Group 2 and Group 4, the percentage of patients with moderate or strong staining in the epithelium were higher than the other groups (Table 3).

In the epithelium of Group 1, the immunoreactivities of GSK-3β, NF-κB and Wnt-1 were close to each other, and there was not a significant difference between these markers (p>0.05). In submucosa, the level of NF-κB was higher than the levels of GSK-3β (p>0.05) and Wnt-1 (***p<0.001). The expression of GSK-3β was the lowest in both epithelium and submucosa of Group 2 compared to NF-κB and Wnt-1 (***p<0.001). However, the Wnt-1 and NF-κB expressions in the epithelium were higher than submucosa (**p<0.01 and ***p<0.001, respectively). In Group 3, the level of GSK-3β was increased in comparison with NF-κB and Wnt-1 (***p<0.001). Finally, the expressions of GSK-3β, NF-κB and Wnt-1 were similar, and they were enhanced compared to other groups. And as seen in Table 3, the distribution of these markers in patients reflected the *H*-score results.

Discussions

In the current study, we compared the expressions of GSK-3β, NF-κB and Wnt-1 between four groups [Group 1 (aged 18–30), Group 2 (aged 31–45), Group 3 (aged 46–60), Group 4 (aged 61–75)] by immunohistochemistry. And also, we used the HP parameters, epithelial cell height, length of crypt and thickness of *muscularis mucosa* to detect the differences between the groups. After immunohistochemistry, there was a moderate expression of GSK-3β, NF-κB and Wnt-1, it was found to be a link between these markers and age in patients with UC.

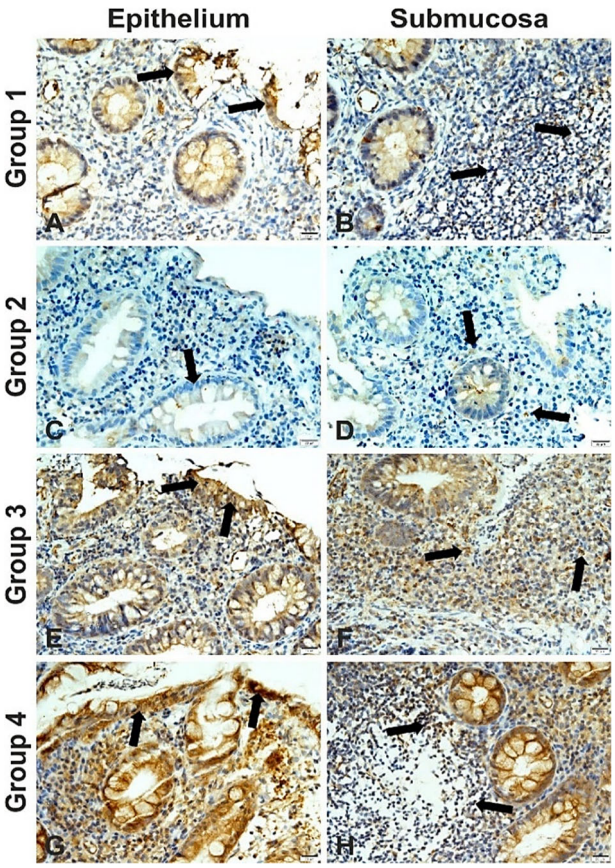

Figure 4 – ***Immunohistochemical (IHC) distribution of GSK-3β in the epithelium and submucosa from all groups. Arrows: Immunopositive cells. Scale bar: (A–H) 20 μm.***

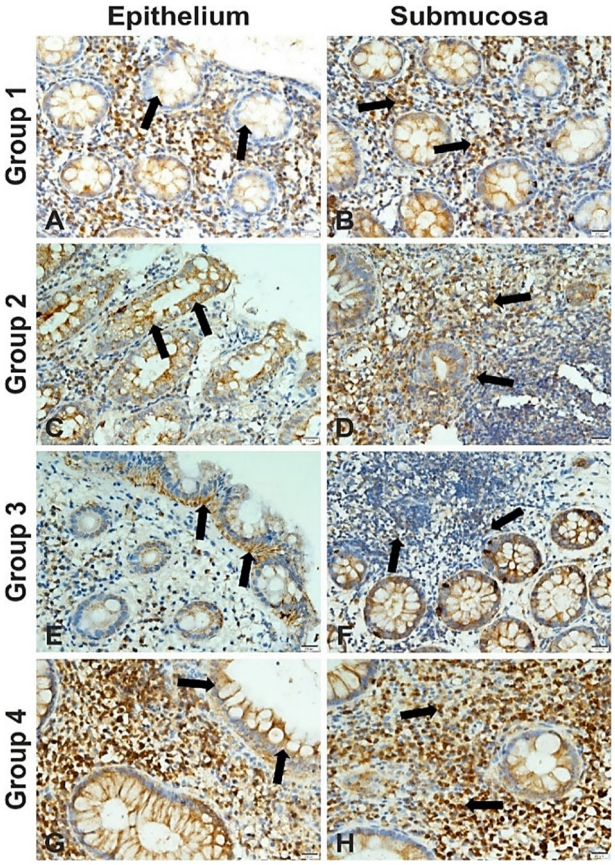

Figure 5 – ***IHC distribution of NF-κB in the epithelium and submucosa from all groups. Arrows: Immunopositive cells. Scale bar: (A–H) 20 μm.***

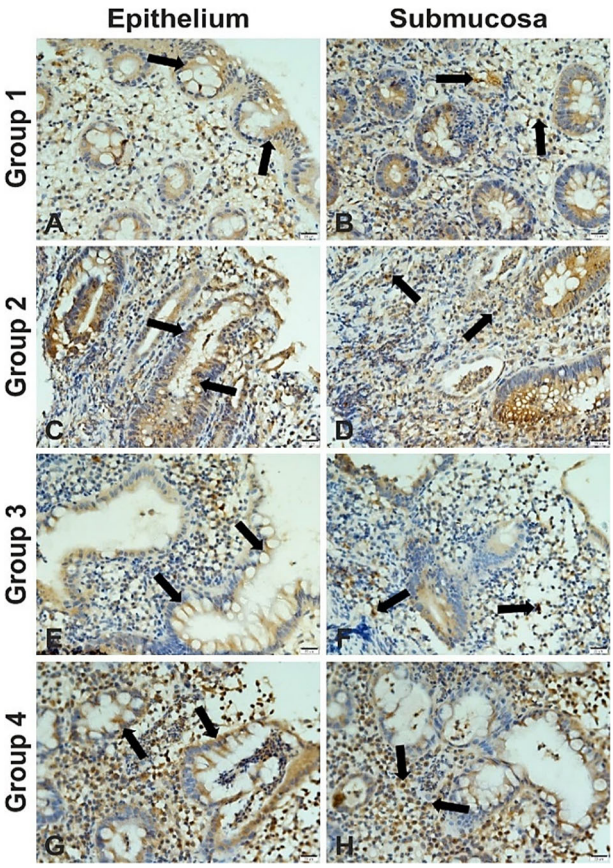

Figure 6 – ***IHC distribution of Wnt-1 in the epithelium and submucosa from all groups. Arrows: Immunopositive cells. Scale bar: (A–H) 20 μm.***

UC is a chronic disease resulting from ulceration and inflammation; therefore, it is observed that tissue integrity in the colon is impaired. In a clinical study, colon samples taken from patients with chronic UC and newly diagnosed were histopathologically examined, and tissue damage was graded as 1 (normal mucosa), 2 (dilated crypts), 3 (increased intercrypt distance), and 4 (absence of crypts). The dilated and branching crypts and a considerable distance between crypts were seen in samples belonging to both patient groups, and no HP significant difference was found [20]. In that study, the patients with chronic and newly diagnosed UC were compared, whereas we aimed to evaluate the histopathology caused by UC among age groups in our study. The parameters of the epithelial cell height, length of crypt and thickness of *muscularis mucosa* were found to decrease with age, and hence the tissue damage increased.

In UC, ulceration and inflammation were triggered in the colon by down- and up-regulation of some signal molecules [21]. Studies explaining the role of the drug in the mechanism of UC are limited, and most of them are experimental. In an experimental UC model [induced with Dextran Sodium Sulfate (DSS) salt] in mice, it was observed that the high level of GSK-3β was detected in colon with UC. Concurrently, TNF-α and inducible nitric oxide synthase (iNOS) levels were enhanced in UC model. It has been defined that the expressions of these markers regressed with carbon monoxide inhalation [8]. It has been determined that the GSK-3β marker plays a role in the epithelial–mesenchymal transition (EMT) mechanism. After induction of UC in mice with 2.5% (*w/v*) DSS, although the expression of GSK-3β increased, no significant difference

was found when compared with the non-UC group. With the application of a peroxisome proliferator-activated receptor gamma (PPARγ) modulator, GED-0507-34-Levo (GED), the level of GSK-3β significantly enhanced, and interestingly, the formation of fibrosis was inhibited in this group. GED is thought to trigger other signaling pathways because of GSK-3β activation and inhibition of fibrosis [22]. In our study, we stated the expression of GSK-3β in colon samples of patients with UC who did not receive treatment. In these patients, the presence of NF-κB and Wnt-1, at the same time, suggested that GSK-3β plays a role in the formation of UC by working synergistically with these markers.

Another experimental study showed that mice with UC exhibited high level of NF-κB, and also mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) [2]. Similarly, in male rats with UC model, the messenger ribonucleic acid (mRNA) level of NF-κB was determined as high *via* enzyme-linked immunosorbent assay (ELISA) [6]. In various studies, it has been reported that the NF-κB signaling pathway plays a role in the inflammation by regulating the immune response. In the presence of NF-κB, the regeneration of tissue wound is triggered in the IBDs. However, the up-regulation of Wnt signaling causes the inhibition of NF-κB [10]. There is not considerable information to reveal the relationship between these two markers in patients with UC. So, we noted that these two markers, NF-κB and Wnt, were expressed in the samples of patients with UC.

Like NF-κB, Wnt is involved in cell renewal and tissue repair, or occurrence of diseases. Wnt signaling mediated by canonical (Wnt-1, Wnt-2b, Wnt-3a, and Wnt-10a) and noncanonical pathways (Wnt-5a) [23, 24]. The Wnts canonical pathways are expressed from macrophages, mucosal dendritic cells, or T-lymphocytes during inflammation to trigger epithelial regeneration [24]. On the other hand, the accumulation of Wnt-synthesizing stromal cells may cause recurrence of inflammation and, in advanced stages, colorectal cancer. Also, Koch (2017) reviewed that during high inflammation of UC, canonical Wnts were highly expressed, especially Wnt-1, Wnt-2b, Wnt-3a, whereas noncanonical Wnt-5a was synthesized during regeneration stage [25]. A previous study supported this data, the expression of Wnt-3a increased in rats 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA)-induced colitis, while the level of Wnt-5a was diminished compared to control and mesenchymal stem cell treated groups (**p<0.01) [24]. Soubh *et al.* showed the increase of Wnt signaling in rats with UC model *via* Wnt/GSK-3β/β-catenin pathway [5]. In our study, we detected the existence of Wnt-1, a canonical Wnt, in the non-treated patients with UC, and it was enhanced with age.

Conclusions

We demonstrated the expression of GSK-3β, NF-κB and Wnt-1 in colon samples of patients with UC, and the synthesis of all three markers suggests that they are activated by a synergistic signaling pathway in these patients. Although each of these markers has a two-way effect on both tissue homeostasis and diseases, it is striking that there is an activation towards the formation of UC.

Conflict of interests

The authors declare no conflict of interests.

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Received: February 1, 2023

Accepted: April 24, 2023