Effect of tofacitinib on the phenotype and activity of Caco-2 cells in a model of inflammatory bowel disease

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Abstract. Reports have indicated that autoimmune bowel disorders affect an increasing number of people on every continent; therefore, it is important to better understand inflammatory bowel disease (IBD) and to explore new treatment options for patients suffering from it. Research has indicated the important role of enterocytes in IBD. Understanding the role of the intestinal epithelium in the pathogenesis of IBD may contribute to a better understanding of the inflammatory processes and aid in the identification of potential therapeutic treatments. The present study aimed to evaluate the effects of tofacitinib on Caco-2 cells cultured in an inflammatory environment induced using cytokines naturally found in patients with ulcerative colitis. Tofacitinib is an orally administered inhibitor of Janus kinases (JAKs) which, by modifying the JAK/STAT signaling pathway, reduces the effect of inflammatory cytokines in the gut. Caco-2 cells were used to model the intestinal epithelium and the culture conditions included the proinflammatory cytokine TNFα and tofacitinib. At the end of the culture period, enzymes involved in oxidative stress (superoxide dismutase 1, catalase, nicotinamide adenine dinucleotide phosphate), a marker of apoptosis (Bcl-2) and a key player in intracellular inflammatory signaling (nuclear factor κB) were assessed by quantitative PCR and western blotting. The *in vitro* phenotype of Caco-2 cells exposed to an inflammatory environment was observed to be similar to that observed in ulcerative colitis. Notably, tofacitinib was able to improve TNFα-induced changes in an *in vitro* model of ulcerative colitis, and a reduction in the activity of enzymes associated with oxidative stress was observed. In addition, tofacitinib-induced upregulation of Bcl-2 and claudin-1 may contribute to the beneficial effects of tofacitinib on the intestinal epithelium. Tofacitinib appears to have a protective effect on Caco-2 cells. Notably, in the present study, exposure to TNFα stimulated oxidative stress and apoptotic effects, and disrupted intercellular connectivity. The addition of tofacitinib decreased the activity of the examined parameters of oxidative and apoptotic stress, while increasing the activity of the parameter examined to evaluate the degree of intercellular connections. In conclusion, the inhibitory effects of tofacitinib on oxidative stress, as well as its anti-apoptotic and regenerative effects, provide important information regarding the positive effect of tofacitinib on Caco-2 cells, and therefore constitute potential information about the beneficial effect of the evaluated drug in UC.

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Introduction

The Caco-2 cell line (colon adenocarcinoma cell line) cultured under certain conditions may resemble enterocytes present in a normal intestine and previously have been used as a model system in research (1). The intestinal epithelium has the ability to function as a barrier between the external and internal environment. This ability is essential for human health, and disorders in this functioning due to increased intestinal permeability are associated with diseases such as: inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) or celiac disease (1). There are reports that tofacitinib may improve the functioning of the cellular barrier in the form of the intestinal epithelium in patients with enteritis (2). Most of the *in vitro* studies that have been conducted to date have used the Caco-2 and HT-29 cell lines to follow these reports. Therefore, an experiment was conducted using the Caco-2 cell line to verify the effect of tofacitinib on intestinal epithelial cells after inducing inflammation in them. The cell line used is not a reflection of the healthy intestinal epithelium found in the human body, as it is a cancer cell line, but the model is similar to that found in the human body.

The TNFα used in the experiment was aimed at inducing an inflammatory phenotype in the tested cell line, the markers of which were: SOD, NADPH, CAT as an assessment of oxidative stress, NF-κB and Bcl-2 as an assessment of the percentage of cells in apoptosis and CLD-1 representing assessment of the state of intercellular connections. TNFα is also mentioned

in the literature as being involved in causing inflammation associated with UC (3,4).

Ulcerative colitis (UC) belongs to the group of inflammatory bowel diseases (IBD). It is a disease with a complex and so far, not fully understood etiology. Histopathological features of inflammation include hyperemia of the mucosa accompanied by edema, inflammatory infiltration covering only the mucosa, as well as the presence of desquamated epithelial cells, or the presence of granulocytes on the surface of the mucosa, but also in between epithelial cells (2). More and more research results indicate the important role of enterocytes in IBD. The intestinal epithelium is the physical, protective barrier of the intestinal microflora and actively contributes to the protection of the immune system of the intestinal mucosa. This barrier is mainly formed by a single layer of specialized intestinal epithelial cells that are crucial in maintaining intestinal homeostasis. Therefore, damage to the epithelium may increase intestinal permeability, lead to disturbances in the interactions between intestinal epithelial cells and immune cells, and thus disturb the homeostasis of the intestinal immune system (3). Functional defects occurring on the intestinal epithelial barrier in the course of UC, which result in the lack of expression of P-glycoprotein, may be one of the causes of the development of inflammation. Some of the drugs commonly used in the treatment of IBD are substrates of P-glycoprotein and seem to have a potential influence in regards to the treatment of patients with UC (4). The above issues seem to be crucial for the course of IBD. It remains an open question whether new drugs used in inflammatory bowel diseases affect the phenotype and function of the intestinal epithelium.

A plethora of novel drugs affecting intracellular signaling pathways have recently been approved for therapy or are under development (5). Moving the site of blockage of the inflammatory signal inside the cell has numerous advantages. It allows for even more precise modification of the effects of the inflammatory stimulus, and moreover, these compounds usually have a low-molecular weight (i.e. they are relatively simple chemical entities), which may significantly reduce the costs of therapy in the future. An example of a drug with such properties is tofacitinib (TBF), which has been used, among others, in the treatment of ulcerative colitis. It is an orally administered inhibitor of Janus kinases (JAK) which, by modifying the JAK/STAT signaling pathway, reduces the effect of inflammatory cytokines in the gut. However, its effect on intestinal epithelial cells is not fully understood (6).

In our work, we tested the effect of TBF on Caco-2 cells by evaluating its effect on oxidative stress observed by gene analysis-superoxide dismutase (SOD-1), catalase (CAT), nicotinamide adenine dinucleotide phosphate oxidase (NADPHox), cell apoptosis and inflammatory signaling assessed by nuclear factor κB (NF-κB) and a family of regulatory proteins involved in apoptosis (Bcl-2) and the condition of intercellular junctions, which included the expression of claudin 1 (CLD-1) were observed. These parameters were assessed in the cell after inducing inflammation with TNF alpha and after using TBF.

It seems that with the existing state of knowledge, the search for information on the effect of TBF on the intestinal epithelium and new biological ways of UC therapy may contribute to a reduction in the risk of exacerbation of the disease.

Materials and methods

Cell culture. Human colon adenocarcinoma cells-Caco2 (colon adenocarcinoma) from the European Collection of Authenticated Cell Cultures (ECACC) cell line collection, purchased from Sigma-Aldrich (Poznań, Poland), were used to conduct the experiment. Cells were cultured in Eagle's Minimum Essential Medium (EMEM, Lonza, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA), 1% MEM Non-essential Amino Acid Solution 100x (Sigma-Aldrich, Poznań, Poland) and 1% antibiotics/antifungal compounds (Antibiotic-Antimycotic Solution 100x) (Sigma-Aldrich, Poznań, Poland). The culture was carried out at 37˚C and in a CO_2-enriched atmosphere (5.3%) in a 24-well culture plate (Nunc™, Sigma-Aldrich, Poznań, Poland) in duplicate. The cells were cultured for 24-48 h in a medium free of inflammatory substances. The medium was replaced with fresh and TNFα 10 ng/ml (Sigma-Aldrich, Poznań, Poland) was added to induce inflammation in the cells, the next step was to add TBF (Sigma-Aldrich, Poznań, Poland) at a concentration of 100 nM.

Confirmation of the presence of Caco-2 cells in the culture. The cell phenotype, normal culture and lack of contamination with other cell populations were confirmed by immunofluorescence staining showing the presence of retinol binding protein 2 RBP-2 in Caco-2 cells, which is commonly used for their detection (4). The cultured cells were flooded with cold methanol and incubated in the freezer for 10 min, then double permeabilization was performed with 0.1% Triton x100 (Sigma-Aldrich, Poznań, Poland). The next step was to block the fixed preparation in 3% BSA (Sigma-Aldrich, Poznań, Poland) and treat it with the primary antibody 1:500 (Anti-CRABP2, Sigma-Aldrich, Poznań, Poland) overnight at 4 degrees C, after which the secondary fluorescent antibody 1:300 (Alexa Flour™ 546 goat anti mouse IgG (H+L), Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) was added and rinsed with PBS, observations were made using a Delta Optical IB-100 inverted microscope (Delta Optical, Mińsk Mazowiecki, Poland) with a fluorescent attachment. We compared the obtained images with images of Normal Human Astrocyte (NHA) primary cells from the ECACC cell line collection, purchased from Lonza (Lonza, USA), to show differences in the detection of a specific marker for Caco-2 cells. NHA cells were cultured in astrocyte basal medium (Lonza, USA) supplemented with 10% FBS (Gibco, USA), supplements for a complete growth medium developed especially for NHA (AGM SingleQuots™; Lonza, USA) and 1% antibiotics/antifungal compounds (Antibiotic-Antimycotic Solution 100X; Sigma-Aldrich, Poznań, Poland). The culture was carried out at 37˚C in a CO_2-enriched atmosphere (5.3%) in a 24-well culture plate (Nunc™; Sigma-Aldrich, Poznań, Poland) in duplicate. The cell phenotype, normal culture and lack of contamination with other cell populations were confirmed by immunofluorescence staining showing the presence of RBP-2 in Caco-2 cells, which is commonly used for their detection. We performed the same experiment to confirm the phenotype of Caco-2 cells, using the correct culture as aforementioned. We subjected the NHA cell culture to immunofluorescence staining to show the absence of the RBP-2 in NHA cells; this staining is characteristic only of Caco-2 cells.

Cell viability assessment-Trypan Blue. Cell viability was assessed using a Bio-rad® TC20 (Bio-Rad Poland, Warszawa, Poland) automated cell counter. Samples from various stages of the experiments were taken from the cell culture. 10 μl of trypan blue 0,4% was added to 10 μl of the cell suspension after trypsinization, waited 5 min and 10 μl of the sample was collected and placed on a special cell viability plate. The next step was to insert the plate into the device and measure the number of live and dead cells in the sample (7).

Assessment of proliferation-MTT method. Cell proliferation was examined by the MTT method (8). It is a colorimetric test that evaluates the metabolic activity of cells. For this purpose, the enzyme NADPH-dependent cellular oxidoreductase is used, which in active mammalian cells reduces MTT to a colored formazan product. After dissolution, the formazan absorbance was measured with a Bio-rad® (Bio-Rad Poland, Warszawa, Poland) microplate absorbance reader. Cells were grown in a 96-well plate and exposed to reagents for 24 h. The absorbance was then read at 750 nm.

Cell lysis with RIPA buffer and protein isolation. After completion of the cell culture, supernatant was collected and afterwards, using 150 μl of RIPA Buffer (Sigma-Aldrich, Poznań, Poland) supplemented with protease inhibitors [Thermo Scientific Halt™ Protease Inhibitor Cocktail (100X) (Thermo Fisher Scientific, Massachusetts, USA)] adherent cells lysis was obtained (9). The samples were subjected to vortexing, sonication, and finally centrifugation at 4˚C at 12,000 x g for 5 min. The protein concentration was determined using the BCA method, i.e. the quantitative determination of protein with bicinchoninic acid. This method uses the binding of the protein to the Brilliant Blue G dye. The XMark microplate Spectrophotometer (Bio-Rad Poland, Warszawa, Poland) detector was used for the measurement, which uses the maximum absorbance of the dye at 562 nm. The absorbance value is proportional to the protein concentration of the standard samples (bovine serum albumin-BSA, Sigma-Aldrich, Poznań, Poland), which allows the drawing of a standard curve.

Western blot technique. The western blot technique is used to detect and identify proteins. The first step is protein electrophoresis in a 10% polyacrylamide gel. Electrophoresis is the separation of proteins (20 μl protein and 5 μl Sample Buffer, Laemmli 2X Concentrate, Sigma-Aldrich, Poznań, Poland) on a polyacrylamide gel carried out at a constant voltage of 175-180 V.

The next step of this technique is the transfer of proteins onto the membrane. The gel and membrane are placed in the transfer buffer and transferred overnight.

After transfer we block the membrane in 10X casein solution (Vector Laboratories Inc., Newark, USA). Blocking is a step to prevent non-specific binding of the antibodies. On the following step of procedure binding of unlabeled antibodies (primary antibody in concentration 1:1,000: Anti-ACTB, Anti-SOD-1, Anti-CAT, Anti-NADPH, Invitrogen, Thermo Fisher Scientific, Massachusetts, USA; Anti-CLD1, Millipore Corp. Merck, Sigma-Aldrich, Poznań Poland; Anti-NF-κB, Sigma-Aldrich, Poznań, Poland; Anti-Bcl-2, Cell Signaling Technology, Lab-JOT, Warszawa, Poland) to the antigens present on the membrane takes place. Next a secondary antibody in concentration 1:2,000 (Anti-Rabbit IgG in goat, Sigma-Aldrich, Poznań, Poland) is added to mark the unlabeled primary antibody used (10).

The last step is protein detection using the horseradish peroxidase reaction (SuperSignal™ Pierce™ Thermo Fisher Scientific, Massachusetts, USA). Visualization is done by chemiluminescence using a LI-COR C-DiGit Chemiluminescence Western Blot Scanner (Polygen, Wrocław, Poland). The results were developed using the ImageJ program (11). Beta actin (ACTB) was chosen as the reference gene for analysis.

Cell lysis with TRI Reagent (TRIzol) and RNA isolation. Cells from the culture plate intended for RNA analysis are purified from the supernatant, and then 1 ml of TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Massachusetts, USA) is added to the well. This reagent is used to isolate total RNA from a sample.

The next step was to add 200 μl of chloroform to the samples and incubate them at room temperature for 2-3 min. The material was then centrifuged and two layers were obtained. The upper layer, called the DNA-containing aqueous phase, was transferred to a new tube, 500 μl of isopropanol was added and incubated for 10 min at room temperature. The next step was to centrifuge the samples to obtain a visible precipitate at the bottom of the tubes, which was dissolved in 1 ml of cold 75% ethanol by vortexing followed by centrifugation. The resulting supernatant was removed and the resulting samples were dried at room temperature for 5-10 min. 50 μl of highly purified analytical water Molecular Biology Grande Water Eppendorf® (Thermo Fisher Scientific, Massachusetts, USA) was added to the prepared sediment and dissolved in a Dry Block Thermostat Bio-TDB (Biosan, Biogenet, Józefów, Poland) at 55˚C for 10 min. BioPhotometer Model #6131 (Eppendorf® Marshall Scientific, Cambridge, United Kingdom) spectrophotometer was used to measure the concentration of the obtained RNA at the absorbance of 260-280 nm.

Reverse transcription-quantitative PCT (RT-qPCR). The qPCR technique is used to quantitatively analyze the mRNA expression of the studied genes. The analysis was carried out using the Roche LightCycler 480 (Roche Polska, Warszawa) apparatus. The first step was to perform a reverse transcription reaction using the Applied Biosystem protocol (Thermo Fisher Scientific, Massachusetts, USA). This process consists in transcribing the RNA template into cDNA while creating appropriate reaction conditions. The reaction is performed using an MJ Mini Personal Thermal Cycler (Bio-Rad® Poland, Warszawa Poland).

The second step was to perform qPCR analysis with the use of cDNA obtained in the process of reverse transcription. The method using the dye SYBR Green I (Roche Poland, Warszawa, Poland) was used. This mixture is placed in tubes suitable for the Roche LightCycler 480 (Roche Poland, Warszawa, Poland) instrument. he sequence of the primers for the tested genes was obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/GenBank Accession NM_000207): SOD1: forward-GAAGGTGTGGGGAAGCATTA; reverse-CCA CCGTGTTTTCTGGATAGA. CAT: forward-TCAGGC

AGAAACTTTTCCATT; reverse-TGGGTCGAAGGCT ATCTGTT. NADPHox: forward-GAAGAAGATGTGGGA ACGGG; reverse-GTATGTCTTTGCCTCCCACC. CLD1: forward-CCTATGACCCCAGTCAATGC; reverse-TCC CAGAAGGCAGAGAGAAG. NF-κB: forward-TTGCTG GTCCCACATAGTTG; reverse-ATGTATGTGAAGGCC CATCC. Bcl-2: forward-CGGAGGCTGGGATGCCTTTG; reverse-TTTGGGGCAGGCATGTTGAC.

This stage of the research was carried out with the following profile: 95˚C/5 min, 45 repetitions (95˚C/30 sec, 63˚C/50 sec, 58˚C/30 sec, 72˚C/40 sec) the profile was as follows: 95˚C/5 min, 35 repetitions (95˚C/30 sec, 63˚C/50 sec, 58˚C/30 sec, 72˚C/40 sec) with single signal effect at the end each stage of primer annealing (12).

ACTB was used as the reference gene (forward: TCATGA AGTGTGACGTGGACC, revers: CAGGAGGAGCAATGAT CTTGATCT). The ΔΔCq method was used to determine the mRNA expression of the studied genes (13).

Statistical analysis. All experiments were repeated three times. All data were analyzed using Statistica TIBCO, version 13. The normality of variable distribution was confirmed by the Shapiro-Wilks test and visual analysis of data distribution. The one-way ANOVA and post-hoc Tukey test were used to analyze the data. Data are represented by means and SEM. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Retinol binding protein 2-RBP-2. At the first stage of the experiments, the correctness of the culture was confirmed by showing the specific marker of Caco-2 cells, which is RBP-2. In Fig. 1 (Fig. 1A-D), protein expression in Caco-2 cells is shown and compared to the low expression of RBP-2 in astrocytes-NHA, cells that do not produce retinol binding protein.

Evaluation of cell viability-trypan blue. The use of TNFα and TBF resulted in changes compared with in the control group, but we didn't observe differences between used reagents (Fig. 2B).

Evaluation of cell proliferation-MTT method. Analyzing the results of the experiment, we found that the proliferation was higher after the addition of TBF relative to TNFα, although still lower than the control [TNFα with TBF (TNFαt) vs. TNFα 36.28%; P=0.001] (Fig. 2A).

Expression of proteins involved in the maintenance of redox balance (SOD1, CAT, NADPH) SOD1. In the RT-qPCR study, after added TBF to the culture, an increase in SOD expression was observed in cells exposed to TNFα (TNFαt vs. TNFα 123.92%; P=0.0456), as well as compared to the control sample (Con.) (TNFαt vs. Con. 24.5%; P=0.0001) (Fig. 3A). This is confirmed by the result of the western blot analysis, where increased in the expression of the tested gene in relation to TNFα (TNFαt vs. TNFα 62.56%; P=0.0001) and control (TNFαt vs. Con. 111.44%; P=0.0001) were noted (Fig. 3A).

CAT. The results of the RT-qPCR analysis shown that medium containing TNFα with TBF decreased the expression relative to medium containing only TNF (TNFαt vs. TNFα 69.41%; P=0.0322). This decrease is also observed compared to controls (TNFαt vs. Con. 45.5%; P=0.0001) (Fig. 3B). The results are consistent with western blot analysis (TNFαt vs. TNFα 45.05%; P=0.0001; TNFαt vs. Con. 27.47%; P=0.0001) (Fig. 3B).

NADPH. RT-qPCR analysis in the case of TNFα together with TBF showed that the expression of the tested gene in relation to TNFα was reduced (TNFαt vs. TNFα 71.80%; P=0.0015), which was also observed in relation to the control culture (TNFαt vs. Con. 57.41%; P=0.0033) (Fig. 3C). Western blot results show a decrease in NADPH expression for TNFα with TBF relative to TNFα (TNFαt vs. TNFα 46.77%; P=0.0001), but an increase relative to controls (TNFαt vs. Con. 2.76%; P=0.0001) (Fig. 3C).

Evaluation of cell phenotype-CLD1 expression. TNFα increased CLD-1 expression. The addition of TBF to the medium containing TNF by RT-qPCR (TNFαt vs. TBF 47.44%; P=0.0001) (Fig. 3D). This was confirmed by western blot for TNFα (TNFαt vs. TNFα 67.48%; P=0.0005) (Fig. 3D).

NF-κB expression. TBF reduces gene expression in the TNF experiment (TNFαt vs. TNFα 36.97%; P=0.0001) when analyzed by RT-qPCR (Fig. 3E). Western blot analysis did not reach statistical significance for TNF with TBF (Fig. 3E).

Apoptosis assessment-Bcl-2 expression. For this analysis, a statistically significant result was obtained only when TBF was added to culture medium containing TNFα, where a slight increase in Bcl-2 expression was observed (TNFαt vs. TNFα 110.57%; P=0.0068) (Fig. 3F). Statistical significance was not obtained in the control case by western blot analysis and confirmation of this result by RT-qPCR analysis (Fig. 3F).

Discussion

In our study, we assessed the effect of TBF on Caco-2 cells after prior induction of inflammation by the pro-inflammatory cytokine-TNFα, based on the analysis of parameters such as anti-inflammatory, anti-apoptotic activity and markers of oxidative stress. It was observed that the NADPHox (nicotinamide adenine dinucleotide phosphate oxidase) expression was higher after exposure to TNFα than after the use of TBF, which reduced the expression of the studied gene. In addition, it was observed that in the case of TNFα, there was an increase in the expression of CLD-1, which may indicate an improvement in the tightness of the cell barrier, and thus a protective effect on the epithelium. Similar to observations in another study that showed a positive effect of TBF on the cell barrier of IBD patients (3). In the first case, the authors induced inflammation using IFNγ, which was responsible for increasing permeability of the cell barrier and that effect was reduced after the use of TBF (3). In the second publication, the authors showed that TBF corrected defects in cell barrier permeability both *in vitro* and *in vivo* (14). Therefore, it can be assumed that TBF has a beneficial effect on the regeneration of the intestinal epithelium in people suffering from UC, and this contributes to the remission of intestinal inflammation associated with this disease.

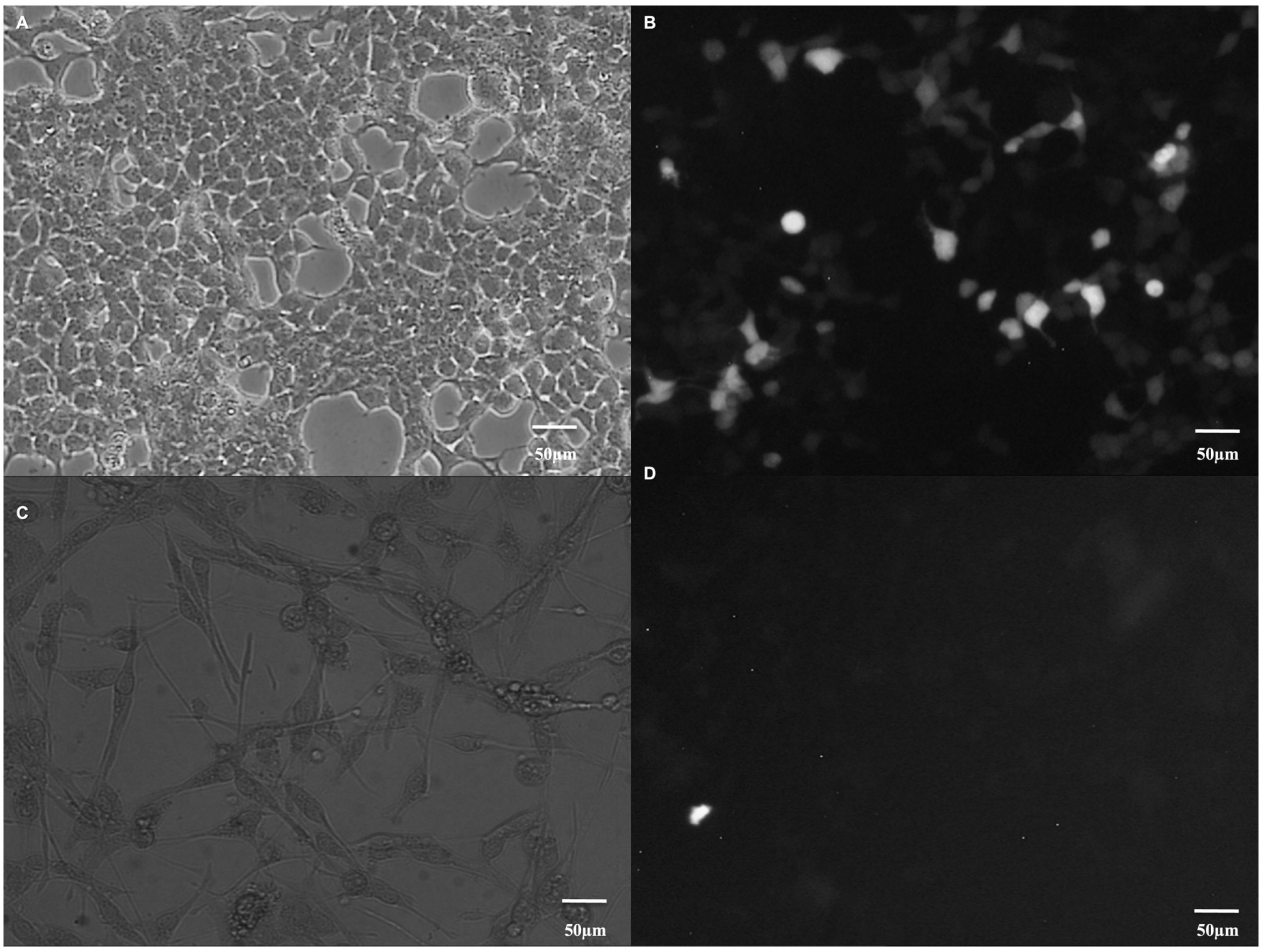

Figure 1. *In vitro* microscopic image of cells (original magnification x40). Confirmation of the culture using the specific Caco-2 cell marker-retinol binding protein. (A) Caco-2 cell culture, (B) Caco-2 cell culture with visible staining of retinol binding protein 2, (C) NHA cell culture, (D) NHA cell culture with staining of retinol binding protein 2.

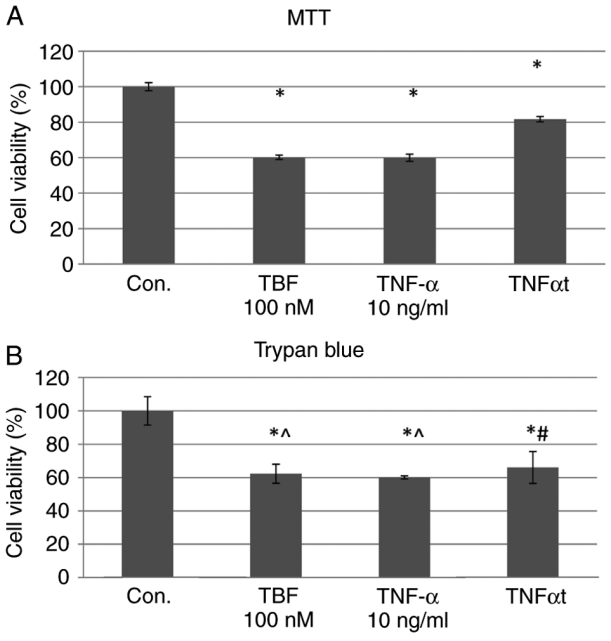

Figure 2. (A) Results of cell viability assessment using the trypan blue method and (B) results of proliferation assessment using the MTT method. Data are expressed as the mean ± SEM. *P<0.05 vs. Con; #P<0.05 vs. TBF; ^P<0.05 vs. TNFαt. Con-control; TBF, tofacitinib; TNFα, tumor necrosis factor α; TNFαt, TNFα with TBF.

In our experimental model, we observed an increase in NADPHox and CAT expression and a decrease in SOD expression in the case of TNFα. TBF reduced the increase in NADPHox and CAT expression. In order for the oxygen explosion to occur, NADPHox must be activated, which is associated with the appearance of ROS, hence it can be assumed that ROS appeared in the experiment, which secondarily triggered the cascade of cell protection against oxidative stress. A decrease in SOD expression and an increase in NADPH expression may indicate a direct SOD stimulation or an indirect one involving p22 NADPHox oxidase. The increase in CAT expression is caused by the action of reactive oxygen species derived from ROS activation, which under the influence of SOD form a hydrogen peroxide compound affecting CAT activation, and this increases its expression in tests, which is a response to oxidative stress caused by this situation. Similar to the observations of Yang and Xie, it can be observed that TBF has an effect on the mitigation of oxidative stress (15), which also confirms our observations.

The increase in NF-κB expression with TNFα, followed by a decrease in expression with the addition of TBF, illustrates the involvement of NF-κB in cell protection processes. In addition, SOD expression increases upon addition of TBF as observed by Zuo *et al* in studies on the treatment of squamous cell carcinoma of the esophagus, where it was observed that

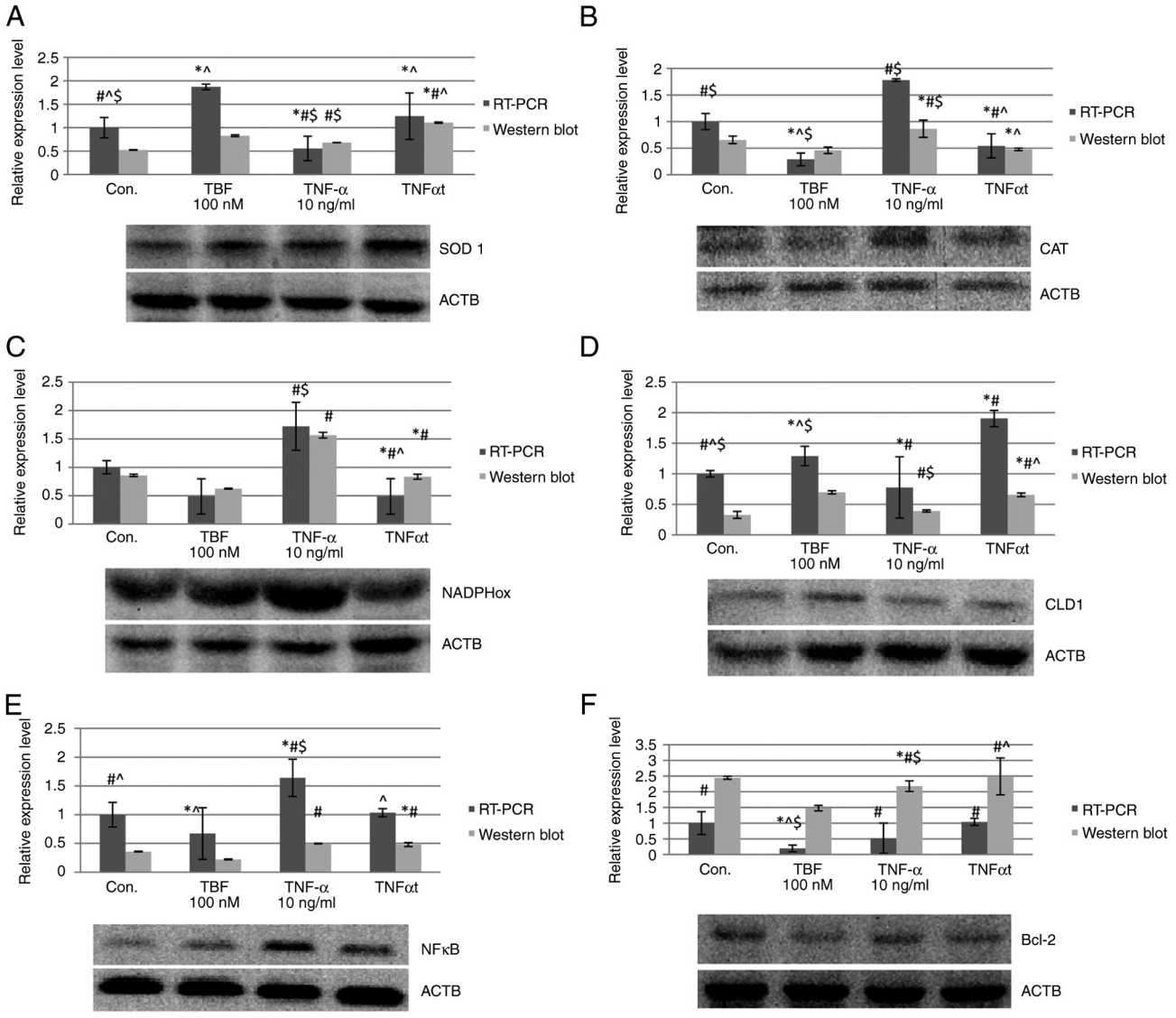

Figure 3. Expression results of the assessed genes by RT-qPCR and western blotting. (A) SOD-1, (B) CAT, (C) NADPHox, (D) CLD1, (E) NF-κB (nuclear factor κB) and (F) Bcl-2 (family of regulatory proteins involved in apoptosis) (F). Data expressed as the mean ± SEM. *P<0.05 vs. Con.; #P<0.05 vs. TBF; ^P<0.05 vs. TNFα; $P<0.05 vs. TNFαt. ACTB, β-actin; Con-control; TBF, tofacitinib; TNFα, tumor necrosis factor α; TNFαt, TNFα with TBF; SOD-1, superoxide dismutase 1; CAT, catalase; NADPHox, nicotinamide adenine dinucleotide phosphate oxidase; CLD1, claudin 1; NF-κB, nuclear factor κB.

higher expression of SOD-2 is associated with the expression of TNFα (16). It has also been shown that TNFα can regulate SOD-2 and increase its level through the NF-κB pathway, contributing to cell proliferation (16). ROS stimulate cells to produce cytokines during inflammation, which leads to their damage and consequently, to the aggravation of inflammation through TNFα, which induces the production of intracellular mitochondrial ROS (17). Similar to the observations of Zhao *et al*, the present study observed an increase in NADPHOx expression in the case of TNFα, which may indicate the appearance of ROS in Caco-2 cells (17).

Studies have shown that administration of TBF increases the expression of Bcl-2. This may indicate a protective effect of TBF on intestinal epithelial cells by inhibiting apoptotic processes induced by TNF-induced inflammation. This was also demonstrated in a study by Yang and Xie, who observed similar results in their study where they checked cell viability with Bcl-2 after inflammation and when fed into a TBF culture. It has been shown that TBF can increase cell viability and at the same time inhibit apoptosis (15). In another study, TBF was also observed to inhibit the expression of anti-apoptotic BCL-A1 and BCL-XL and induce apoptosis in human dendritic plasmacytoids (PDC). Stimulation of Toll-like 7 receptors increased the level of anti-apoptotic Bcl-2 family members and initiated the induction of PDC apoptosis by TBF (18).

The increase in CLD-1 expression after the addition of TBF may suggest a positive effect of TBF on the intestinal epithelium, stimulating it to regenerate by increasing intercellular connections after TNFα stimulation. The activation of cells by an inflammatory factor followed by inhibition by TBF in the case of CLD-1 appears to be related to the JAK-STAT pathway, which is involved in cell repair processes. Similarly to the observations obtained by Sayoc-Becerra *et al*, the use of IFNγ as an inflammatory factor and then TBF confirmed its positive effect on intestinal epithelial cells (19). As observed in another study on the epithelial-mesenchymal transition (EMT)

in cancer progression and malignancies, including colorectal cancer, TNF also increased the expression of claudin-1, accompanied by an increase in proliferation and wound healing rate (20). Zhang *et al* reported that tight junction dysfunction plays an important role in some chronic inflammatory diseases. TNFα acts as a disruptor of the tight junctions of the intestinal epithelium in inflammatory conditions. It was observed that the expression of claudin-2 was induced by TNFα (21) which is confirmed by our observations.

The increase in NF-κB TNFα expression may result from the stimulation of the immune system through contact of the intestinal microbiome with the pathogen. As observed by Mahalanobis *et al*, the immune system maintains a balance with the gut microbiome and recognizes molecular patterns associated with pathogens that activate transcription factors, including NF-κB and related inflammatory pathways (22). Immune cells of the intestinal mucosa are also involved in the inflammatory process, producing pro-inflammatory factors such as TNFα and IFNγ. This activates pro-apoptotic signaling involving proteins such as caspase-1 (23).

The increase in CLD-1 expression relative to the control in the case of TNFα with TBF may be related to the phenomenon of autophagy. Foerster *et al* in their work described the phenomenon of macroautophagy/autophagy, the role of which in maintaining the intestinal epithelium seems to be an interesting process (24). Several autophagy-related genes have been linked to gut diseases. It is a catabolic cellular process responsible for the destruction of cellular pathogens and the processing of organelles and protein aggregates involving lysosomes. It has been observed that the autophagy process is involved in intestinal repair, but also supports the function of the intestinal barrier by regulating tight junctions and protecting against cell death, and is responsible for maintaining proper homeostasis (24). The phenomenon of autophagy may explain the increase in CLD-1 expression for TNFα from TBF observed in my study. Autophagy is of great importance for intestinal stem cells (IECs), taking part in their metabolic processes and affecting their proliferative and regenerative abilities, and is also of great importance for reducing oxidative stress caused by ROS (24). Therefore, we can assume that the phenomenon of autophagy occurred in our experiment, which seems to be confirmed by the obtained results.

In conclusion, studies using TBF as a molecule to mitigate inflammation caused in Caco-2 cells contribute to a positive effect on cells and cellular connections. In our studies, we showed that TBF positively affected the mitigation of oxidative stress associated with inflammation, had an anti-apoptotic effect by reducing Bcl-2 expression, and also affected the reconstruction of cellular connections lost as a result of the irritating effect of burning cytokines. Many of our studies have been cited in publications supporting the hypotheses.

Due to of the use of only the Caco-2 cell line as a model of the intestinal epithelium in the study, this led to limitations in the results. These are cancerous cells and incompletely represent healthy, non-cancerous intestinal epithelium. In addition, *in vitro* conditions do not fully reflect the complexity of pathways between cells occurring in living organisms. The reaction of tumor cells to inflammatory factors after the use of classical inducers and inhibitors of inflammation may differ from the reaction of normal enterocyte cells.

Due to the described limitations, we plan to conduct similar *in vivo* studies using laboratory animals in the future to confirm the obtained results.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ES conceived the study, performed the data analysis and wrote the manuscript. LB designed the study, conducted the data analysis and reviewed the manuscript. GM made substantial contributions to study conception and design, or acquisition of data, reviewed the experiments and laboratory studies, and reviewed the manuscript. BO was responsible for data interpretation, and critically revised the manuscript for important intellectual contents. LB and GM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' information

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