Histol Histopathol (2024) 39: 1443-1455

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Prevention of colon enlargement by TNF-α antagonist in a streptozotocin-induced diabetic rat model

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Summary. Summary. Purpose. We investigated the effect of tumor necrosis factor (TNF)-α antagonist on the structure and function of the streptozotocin-nicotinamide (STZ-NA)-induced diabetic rat colon.

Methods. Thirty rats were divided into normal control (NC), diabetic control (DC), and diabetic etanercept (DE) groups. The DE group was injected with etanercept twice a week. Blood glucose, body weight, fecal pellet, colonic transit time, and plasma TNF-α were measured. The colon was dissected out, followed by weight and length measurements. Toluidine blue and Verhoeff's staining, immunohistochemistry for TNF-α, RAGE, iNOS, arginase, and western blot for RAGE were performed on the colonic tissue.

Results. Administration of TNF-α antagonist had no significant effect on the body weight and blood glucose level of the diabetic groups. However, the DE group had a shorter and lighter colon and less coarse and less dense collagen fibers in the submucosal layer than the DC group. Weaker immunoreactivity of TNF-α, RAGE, iNOS, and arginase I was observed in colon tissue sections of the DE groups compared with the DC group. Although the etanercept effect on colonic function was not significantly different, the preventive effect size of etanercept on colon remodeling was considerably large, as shown by calculated-Cohen's d>0.8.

Conclusions. TNF-α signaling in the colonic tissue of diabetic rats has a strong effect on tissue remodeling, leading to colon enlargement. TNF-α antagonists may be beneficial in preventing diabetic-related pathology in the colon in combination with anti-diabetic drugs.

Key words: Diabetic rats, TNF-α antagonist, Colon enlargement, Tissue remodeling

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www.hh.um.es. DOI: 10.14670/HH-18-735

Introduction

Diabetic conditions, which are characterized by chronic hyperglycemia, induce multiple macrovascular and microvascular complications that lead to various symptoms (Zheng et al., 2018). Gastrointestinal symptoms can be found in 75% of diabetic patients and may interfere with their quality of life (Piper and Saad, 2017). These disorders may occur over the entire digestive tract, from the esophagus to the anus of diabetic patients (Du et al., 2018) and diabetic animals (Izbeki et al., 2008; Xu et al., 2013).

The colon is the distal fragment of the intestine that functions in fecal formation and excretion. The colonic wall consists of mucosa that mediates absorption and secretion, followed by two smooth muscle layers responsible for moving the stools. The submucosa, which consists of significant blood and lymphatic vessel branches, separates the mucosa and muscular layer. Altered colon function in diabetic patients results in constipation, diarrhea, or both (Piper and Saad, 2017). In diabetic rat models, colonic enlargement and dysfunction, as well as tissue remodeling, have been reported in several stages of hyperglycemia (Siegman et al., 2016; Esteves-Monteiro et al., 2022).

Tissue inflammation is a crucial feature of diabetic conditions that may induce organ remodeling via several mechanisms, including activation of stromal fibroblasts, which leads to fibrosis (Mack, 2018; Jasso et al., 2022). Some studies suggest that tumor necrosis factor (TNF)-α may induce the pathogenesis of diabetes mellitus (Akash et al., 2018). Therefore, inhibiting TNF-α may be beneficial in reducing the degree of inflammation and subsequently preventing organ damage and dysfunction. The TNF-α antagonist, etanercept, has been shown to reduce colon inflammation in rat models of Crohn's disease and lessen tissue damage (Paiotti et al., 2011). This study aimed to investigate the effects of etanercept on the structure and function of the colon in chronic diabetic rat models induced by streptozotocin (STZ) accompanied with nicotinamide (NA) (Szkudelski, 2012).

Materials and methods

Animals and treatment

Ethical approval no. KE/FK/0701/EC/2022 was obtained from the Medical and Health Research Ethics Committee at the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Thirty male Sprague-Dawley (SD) rats weighing 200-250 g and 8-12 weeks old were used in this study. The rats were divided randomly into three groups: normal control group (NC), diabetic control group (DC), and diabetic etanercept group (DE). The diabetic rat model was established after five days of adaptation and overnight fasting by a single intraperitoneal (i.p.) administration of 65 mg/kg body weight (BW) STZ dissolved in freshly prepared citrate buffer pH 4.5, preceded 15 minutes earlier by an i.p. injection of 100 mg/kg BW NA dissolved in normal saline. One week later, the fasting blood glucose level was determined in blood samples obtained from the retro-orbital vein. Rats with fasting blood glucose levels higher than 200 mg/dL were considered diabetic (Li et al., 2011). Subcutaneous injections of 0.4 mg/kg BW etanercept were administered twice weekly to the DE group. Body weight was measured weekly and blood glucose every two weeks.

Fecal pellet assessment and colonic transit time measurement

Fecal pellet assessment and colonic transit time were performed at week ten, on the first and second days after STZ injection, respectively. The fecal pellet was assessed using a modified method from previous reports (Patel et al., 2017; Van Den Berge et al., 2021). Each rat was placed in a cage without bedding for six hours while being allowed access to food and drink. Dry paper was placed on the floor cage to absorb urine excreted during the test. Fecal pellets were collected every hour, counted, and weighed immediately to measure the wet weight. Subsequently, pellets were dried in an incubator at 60°C overnight and weighed to determine the dry weight. The water content of pellets was calculated by subtracting the wet weight from the dry weight, dividing it by the wet weight, and multiplying it by 100% (Barone et al., 1990).

The colonic transit time was measured by a previously modified technique (Izbeki et al., 2008; Xu et al., 2013). Before colonic transit time measurement, rats were fasted overnight. The rats were briefly anesthetized with ketamine 45 mg/kg BW. Colonic transit time was measured by inserting a single 5 mm colored plastic bead into the distal colon or 3 cm past the anus with a plastic rod. After the rats regained consciousness, they were placed in individual cages without bedding and monitored until bead expulsion. The duration between bead insertion and removal in minutes was recorded as the colonic transit time.

Tissue sampling

The rats were euthanized at the end of week ten after STZ administration. After being anesthetized with ketamine 90 mg/kg BW and xylazine 9 mg/kg BW, blood samples were taken from the retro-orbital vein and used for TNF-α measurements. Transcardial perfusion was performed with ice-cold phosphate-buffered saline (PBS) pH 7.3. Colon segments were removed, measured in length, and cut into 0.8-1.0 cm segments, the fecal matter was cleaned off with cylindrical glass and weighed. Slabs of the colon were sampled in a systematically random manner and fixed in 4% paraformaldehyde in PBS for histological observation. Some slabs were put into liquid nitrogen, and stored at -80°C before being used for western blot analysis.

Plasma levels of TNF-α

Plasma was obtained by centrifuging the blood samples at 1500 rpm, 4°C for 15 minutes. According to the manufacturer's instructions, the plasma was stored at -80°C before being used in an enzyme-linked immunosorbent assay for TNF-α (rat TNF-α ELISA kit, Elabscience, E-EL-R2856). Plasma TNF-α concentrations were calculated from duplicate absorbance results with an ELISA calculator at https://www.arigobio.com/ELISA-calculator.

Histological observation and immunohistochemistry

Fixed colon tissue from one rat was embedded in 2-3 paraffin blocks in an orientation that ensured longitudinal sectioning. The tissue section of the proximal, middle, and distal colonic segments of each paraffin block was obtained with a 4 μm thickness and stained with toluidine blue. Some colon sections were stained with Verhoeff's staining or immunohistochemistry. The immunohistochemistry staining was performed on sections mounted on poly-L-lysine-coated slides. After rehydrating, blocked in normal serum and 3% H_2O_2, the slides were incubated with the primary antibodies (anti-rat TNF-alpha polyclonal antibody, Bioss, bs-2081R, dilution 1: 200; receptor of advanced glycation end-products (RAGE) polyclonal antibody, Invitrogen, #PA1-075, dilution 1:50; anti-inducible nitric synthase (iNOS) antibody, Abcam, ab15323, dilution 1:500; Arginase I antibody, Santa Cruz, sc20150, dilution 1:250) at 4°C overnight. The immunoreactivity was visualized with Mouse and Rabbit-Specific Horseradish Peroxidase/Diaminobenzidine Immunohistochemistry Detection Kit - Micro-polymer (Abcam ab236466) according to the manufacturer's instructions. Positive and negative controls were used to validate the immunohistochemical staining. The sections were observed using an Olympus CX23 microscope connected to a camera and computer monitor equipped with Optilab software (Miconos, Yogyakarta, Indonesia).

Western blot analysis

A western blot detection kit (Elabscience, E-IR-R304) was used to measure the level of RAGE. The colon tissue of three rats from each group was homogenized with a RIPA lysis working solution. The sample was kept on ice and sonicated for 2 min with ten sonication and 10 s intervals to fully lyse the cells and reduce sample viscosity. The colon tissue was centrifuged at 12.000 rpm for 4 min at 4°C, and the supernatant obtained was transferred to a new tube for further analysis. The protein concentration of each sample was determined by the BCA method.

Protein samples were diluted with a 5x SDS loading buffer at the ratio of 4:1 and boiled at 95°C for 5 min. Afterward, samples containing equal amounts of protein (40 µg) were loaded into wells of an SDS-PAGE gel along with pre-stained protein markers for separation and transferred onto a PVDF membrane. The membranes were blocked with Tris buffered-saline with Tween-20 detergent (TBST) containing 5% skim milk powder for 90 minutes with shaking at room temperature and then incubated with the primary antibodies (RAGE polyclonal antibody, Invitrogen, #PA1-075, dilution 1:1000) and β actin (ABclonal, AC026, dilution 1:50000) at 4°C overnight. After washing with Tris-buffered saline (TBS) three times, the membranes were incubated with secondary antibodies at room temperature for one hour with shaking. The membranes were washed with the same buffer, and protein detection was performed using a chemiluminescence reagent according to the manufacturer's instructions. The intensity of the protein band was quantified using Image J software.

Statistical analysis

The microscopic observations were described and analyzed qualitatively. The quantitative data were presented as mean value ± standard error mean (SEM). Analysis of variance (ANOVA) followed by Least Significant Difference (LSD) *post hoc* tests were performed to detect differences among groups only when the data were normally distributed and variances were equal. Otherwise, the Kruskal-Wallis and Mann-Whitney tests were applied. All analyses used GraphPad Prism 9 (Dot Matrix, Inc., USA). Pearson's bivariate correlation was used to measure the strength of the correlation between structural and functional variables. A p-value of <0.05 was considered statistically significant. The value of Cohen's d was calculated when the data of the DC and DE groups were significantly different. Cohen's d ≥ 0.8 was considered a large effect size, 0.5 was medium, and 0.2 was small.

Results

Fasting blood glucose, body weight, and plasma TNF-α of each group

At the beginning of the study, all the rats had the same fasting blood glucose level and BW One week after STZ-NA induction, hyperglycemia was observed in the DC (290.33±22.5 mg/dL) and DE (267.71±16.31 mg/dL) groups, while the NC group had normal blood glucose levels (106.37±6.05 mg/dL; $p<0.05$). After receiving 0.4 mg/kg BW etanercept treatment twice a week for ten weeks, the blood glucose levels of the DE group were not significantly different compared to the DC group (Fig. 1A).

Consistent weight gain was observed in the rats of the NC group throughout the study. In contrast, the BW graphs of both the DC and DE groups tended to be flat (Fig. 1B). Meanwhile, the plasma level of TNF-α was not significantly different between groups (374.08±33.55 pg/mL in the NC group, 437.89±70.85 pg/mL in the DC group, and 337.61±18.63 pg/mL in the DE group, $p>0.05$) (Fig. 1C).

Fecal pellets and colonic transit time

At the end of week 10, the number of pellets was significantly higher in the DC (15.6±1.05) and the DE group (16.7±1.79) compared with the NC group (7.9±1.21; $p<0.05$) (Fig. 2A). The DE group (68.99±1.09 %) had a significantly higher water content in the pellets than the NC group (62.62±1.45; $p<0.05$) (Fig. 2B). The colonic transit time was significantly faster in the DC (105.3±19.12 min) and the DE group (116.6±23.7 min) compared with the NC group (257.8±38.9 min; $p<0.05$) (Fig. 2C). However, no significant differences in these three functional parameters were found between DE and DC groups (Fig. 2).

Etanercept treatment strongly reduced colon enlargement in diabetic rat models

The representative samples of colon length measurement from each group are shown in Fig. 3A. Compared with the length and weight of the colon in the NC group (16.65±0.51 cm; 2007.6±90.9 mg, respectively), both the DC and DE groups had significantly longer (Fig. 3B) and heavier colons (Fig. 3C). The length (20.1±0.45 cm), weight (2908.7±60.8 mg), calculated colon weight/length (145.25±3.98 mg/cm) (Fig. 3D), and colon weight/BW (1.35± 0.051%) (Fig. 3F) in the DE group were significantly lower than the average of the DC group (22.0±0.47 cm; 3576.7±172 mg; 162.2±5.86 mg/cm; 1.61±0.082%, respectively; $p<0.05$). The effect size of the etanercept preventive treatment for colon enlargement parameters was considerably large as the calculated Cohen's d was >0.8 (Table 1). Pearson's correlation study showed no significant correlation between colon size and the water content of pellets, except for the colon length/BW. However, colon size variables correlated positively with the number of pellets and negatively with the colonic transit time. The correlation coefficient was between 0.4-0.6 and considered moderate (Table 2).

No apparent reduction in mucosa and muscular layer thickness upon etanercept treatment in diabetic rats

We noticed the increased thickness of the mucosa and muscular layer in the proximal, middle, and distal colonic segments of the diabetic rats (DC and DE) compared with NC rats (Fig. 4). Hypertrophy of the smooth muscle cells in the circular muscular layer of DC and DE rats was observed (Fig. 4). All three groups had comparable muscle size in their longitudinal muscular layer (data not shown). However, no apparent difference in the thickness of the histological layers or the size of

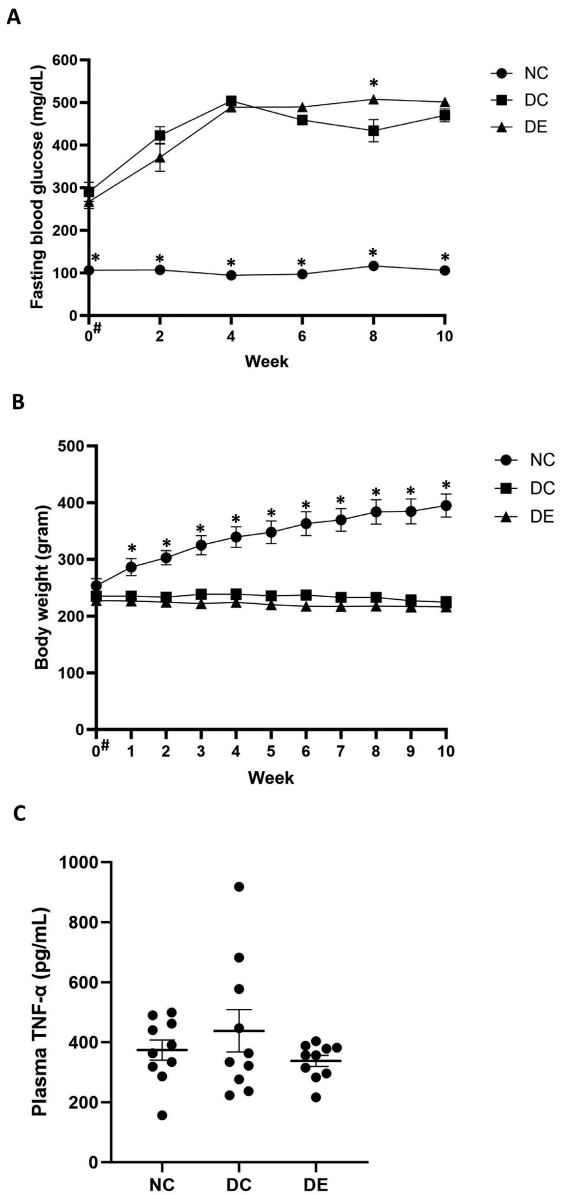

Fig. 1. Effect of etanercept treatment on blood glucose, body weight, and plasma TNF-α. **A.** Serial fasting blood glucose level. Hyperglycemia in the diabetic control (DC) and diabetic etanercept (DE) groups was observed one week after STZ-NA treatment and persisted until the end of the study. **B.** Serial body weight measurement. The DC and DE groups had no significant weight gain throughout the study. **C.** TNF-α concentration in the plasma of each group. With a $p>0.05$, there is no significant difference among groups. Data are presented as mean±SEM (n=10 per group), *$p<0.05$ compared with the DC group; NC: normal control group; #: one week after induction.

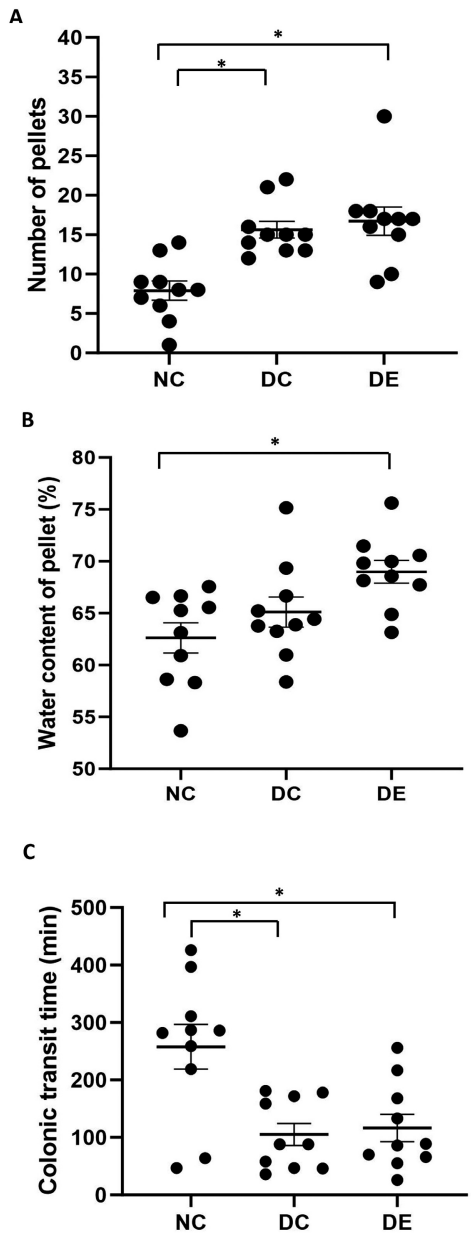

Fig. 2. Colon functional assessments in the rats at week 10. **A.** Number of pellets. The diabetic control (DC) and diabetic etanercept (DE) groups had a higher number of pellets than the normal control (NC) rats. **B.** The water content of pellets. The DE group had a higher water content. **C.** Colonic transit time. The DC and DE groups had faster colonic transit time. There is no significant difference between the DC and DE groups for all three functional parameters. Data are presented as mean±SEM (n=10 per group). *$p<0.05$.

smooth muscle cells was observed between both diabetic groups.

Etanercept reduced the colonic collagen fiber submucosal layer of diabetic rats.

In the sections with Verhoeff's staining (Fig. 5), collagen fibers were primarily observed in the submucosa of the colonic segments. Collagen fibers also formed lamina propria of the mucosa, in between smooth muscle cells of the muscular layer, encircled the myenteric neural plexus, and formed serosa. The submucosa of the DC colon tended to have thicker and coarser collagen bundles (Fig. 5B,E). DE rats had a

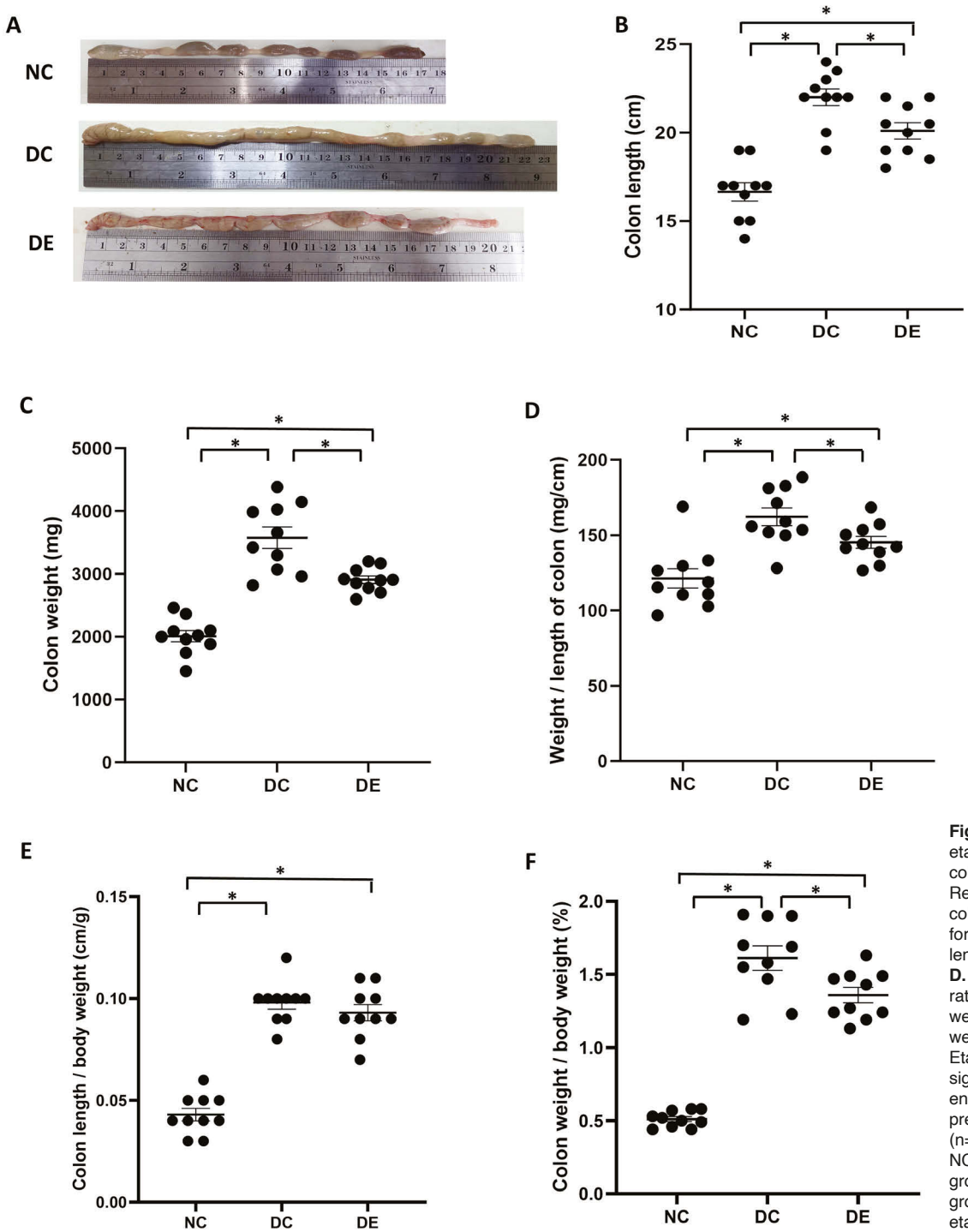

Fig. 3. Effect of etanercept treatment on colon size and weight. **A.** Representative figure of colon length measurement for each group. **B.** Colon length. **C.** Colon weight. **D.** Colon weight/length ratio. **E.** Colon length/body weight ratio. **F.** Colon weight/body weight ratio. Etanercept treatment significantly reduced colon enlargement. Data are presented as mean±SEM (n=10 per group). *p<0.05. NC is the normal control group; DC is the diabetic group; DE is the diabetic etanercept group.

Table 1. The effect size of etanercept treatment on colon size.

	Effect size value (Cohen's d)	95% CI
Colon weight	1.636	0.595-2.644
Colon length	1.284	0.299-2.240
Colon weight / length	1.069	0.113-1.998
Colon weight / body weight	1.162	0.194-2.103

Table 2. Pearson correlation coefficient of colon size and colon function (n=30).

	Number of pellets	Water content of pellet	Colonic Transit time
Colon weight	0.557**	0.181	-0.569**
Colon length	0.562**	0.286	-0.511**
Colon weight / colon length	0.449*	0.42	-0.481**
Colon length / body weight	0.640**	0.441*	-0.636**
Colon weight / body weight	0.637**	0.353	-0.647**

*p<0.05; **p<0.01

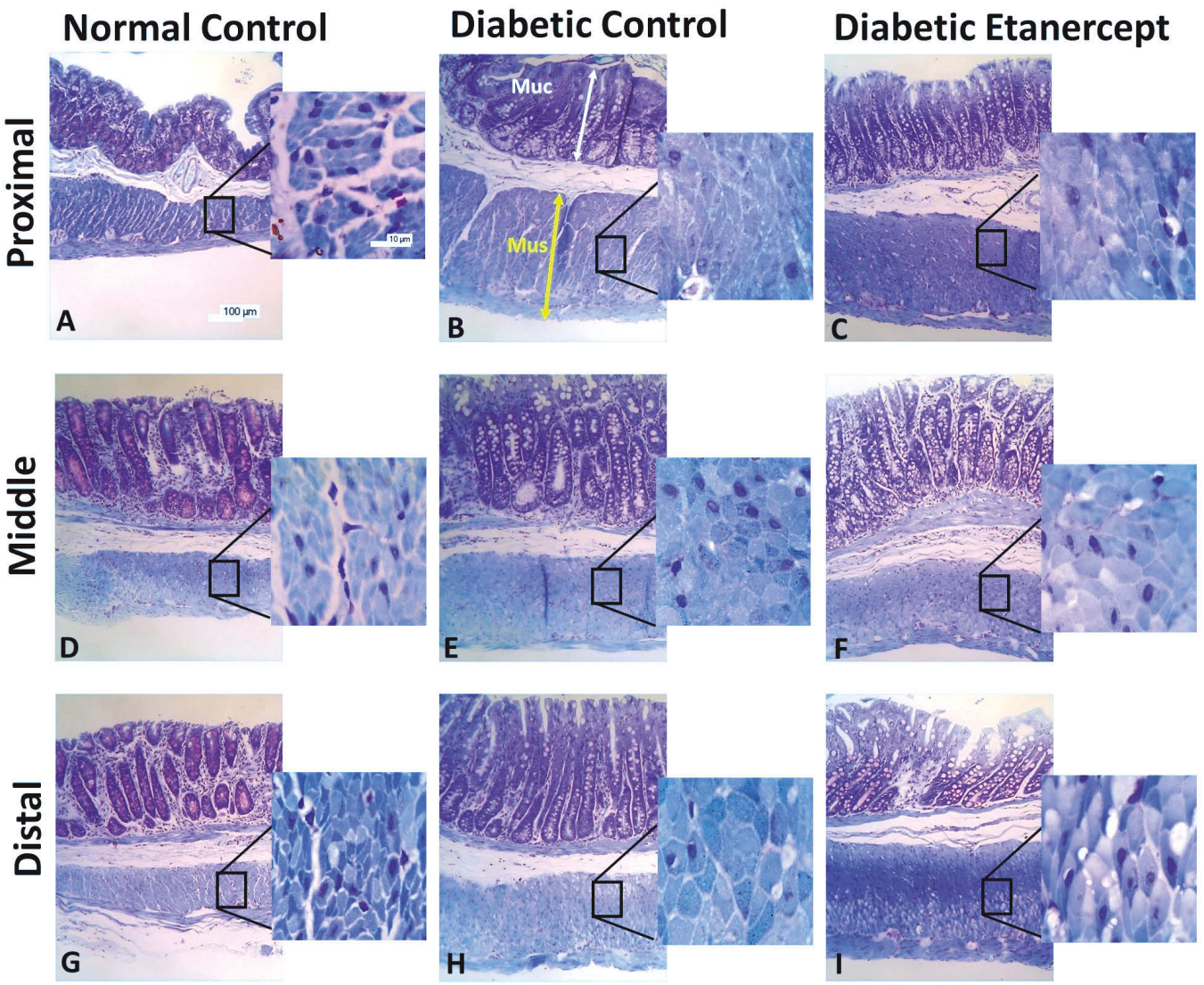

Fig. 4. Toluidine blue staining of the colonic segments in each group. Normal control **(A, D, G)**, diabetic control **(B, E, H)**, and diabetic etanercept **(C, F, I)** rats. Proximal colon **(A-C)**; middle colon **(D-F)**, and distal colon **(G-I)**. Some diabetic rats had slightly thicker colonic mucosa and muscular layer in the proximal, middle, and distal colon. The thickness of the colonic layers in the diabetic etanercept (DE) group was comparable to the diabetic control (DC) group. Hypertrophy of smooth muscle cells of the circular muscular layer was observed in the DC and DE rats. Muc, mucosa; Mus, muscular layer.

similar collagen distribution as NC rats. Black-colored elastic fibers were observed between collagen bundles and tended to be more frequently found in NC and DE rats (Fig. 5).

Etanercept reduced colonic TNF-α and RAGE expression in diabetic rats

Tumor necrosis factor-α immunohistochemistry on paraffin-embedded colon tissue visualized strong TNF-α expression in the mucosa, submucosa, and muscular layer of the colon of the diabetic groups compared with the very weak immunoreactivity found in the colon of the NC group (Fig. 6). Compared with the staining in the DC group (Fig. 6B,E), less robust TNF-α immunopositivity was found in the colon of the DE group (Fig. 6C,F).

Immunohistochemical staining on paraffin-embedded colon tissue visualized RAGE expression in the mucosa, submucosa, and muscular layer (Fig. 7A). The DC group had the most robust RAGE immunoreactivity, and DE had slightly less RAGE immunostaining than the DC group. The NC group showed the weakest immunopositivity (Fig. 7A). This was according to the immunoblotting results, which showed higher RAGE expression in the diabetic groups compared with the NC group. The RAGE bands in the DE group were slightly weaker than in the DC group. However, the semi-quantitative analysis showed no significant difference (Fig. 7B).

Etanercept reduced iNOS and arginase expression in the mucosa and myenteric neural plexus of the colon of diabetic rats

Immunostaining expression of iNOS and arginase I on colon tissue sections is shown in Figs. 8, 9. iNOS and arginase I immunostaining were found in the mucosa and myenteric neural plexus. Most of the positive staining was found in the cytoplasm of epithelial cells of the mucosa and neurons of the myenteric neural plexus. However, the expression of iNOS and arginase I in some other cells of the connective tissue and enteric glia of the myenteric neural plexus were observed. The DC group showed the strongest immunopositivity for both markers (Figs. 8B,E,H, 9B,E,H). Immunostaining of iNOS and arginase I in the DE group was weaker than that of the colon in the DC group but more substantial than in the NC group (Figs. 8C,F,I, 9C,F,I).

Discussion

The colonic dysfunction of diabetic rats shown in this study was decreased colonic transit time

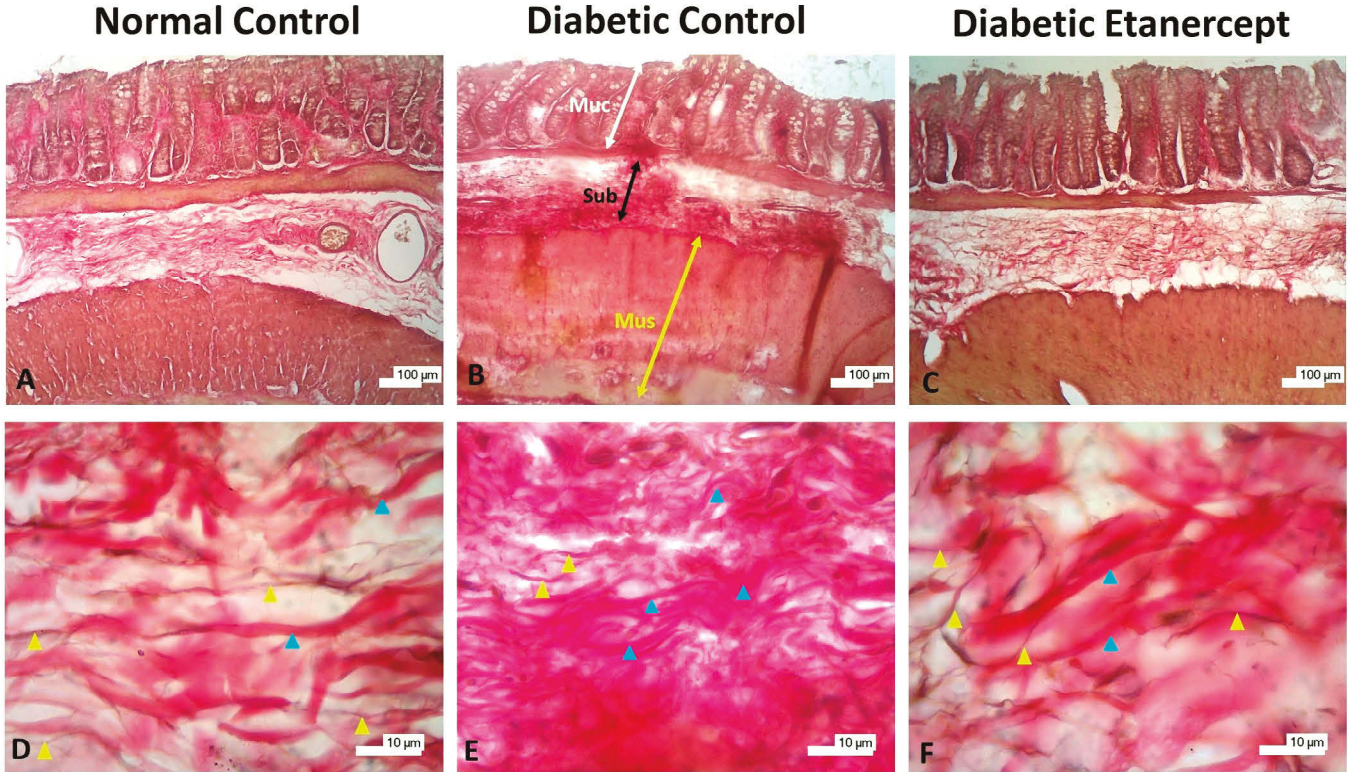

Fig. 5. Distribution of collagen and elastic fibers in samples of distal colon tissue with Verhoeff's staining. **A-C.** Low magnification of the colon segments. Collagen fibers were mainly observed in the submucosa. **D-F.** High magnification of the submucosa. Collagen bundles were stained red (blue arrowheads), and elastic fibers were stained black (yellow arrowheads). Muc, mucosa; Sub, submucosa; Mus, muscular layer.

accompanied by more pellets and water content in the feces. Other studies on STZ-induced diabetic rat models reported a similar effect (Izbeki et al., 2008; Xie et al., 2013), while one study in STZ-induced diabetic mice reported a delayed intestinal transit time (Ha et al., 2021). In diabetic patients, constipation is the main reported symptom, while diarrhea is only found in 22% of cases (Piper and Saad, 2017). Increased TNF-α signaling activated by the NF-κB pathway may trigger increased intestinal epithelial permeability (Al-Sadi et al., 2016), leading to the higher water content observed in this study.

Administration twice weekly of 0.4 mg/kg BW etanercept treatment does not affect the fasting blood glucose level or the BW of diabetic rats. The diabetic rats remained hyperglycemic with almost no weight gain throughout the study. One study reported weight gain as one advantage of using a more frequent administration of etanercept (Ye et al., 2019), while reporting a similar effect on blood glucose levels. The lack of a blood glucose-lowering effect allows the continuation of diabetic autonomic neuropathy and diabetic vasculopathy. Therefore, colonic dysfunction is still observed in the etanercept-treated diabetic rats. Furthermore, blocking TNF-α signaling may interfere with the beneficial effects of TNF-α in inducing mucosa repair (Ruder et al., 2019), negating its good anti-inflammatory and cell death prevention effects.

No difference in the plasma TNF-α level was observed in this study, however, a higher expression of TNF-α in the colonic tissue of diabetic rats revealed colon inflammatory environments. A higher inflammatory environment in the diabetic colon is supported by the more robust expression of the proinflammatory component iNOS, which is involved in the signaling pathway for collagen production. This data supports TNF-α and iNOS involvement in the tissue remodeling induced by hyperglycemia and RAGE (Sha et al., 2018). The prevention of colonic enlargement in diabetic rats by etanercept demonstrated in this study concurs with the reduced colonic RAGE and iNOS expression, which resulted in reduced collagen fiber production. In addition, etanercept may block the cross-talk between RAGE and TGF-β signaling (Serban et al., 2016) in regulating matrix extracellular turnover and cytokine synthesis.

Pro-inflammatory cytokines, including TNF-α, promoted colonic smooth muscle cell growth by stimulating the expression of platelet-derived growth factor receptor β (PDGF-Rβ), an essential mitogen for

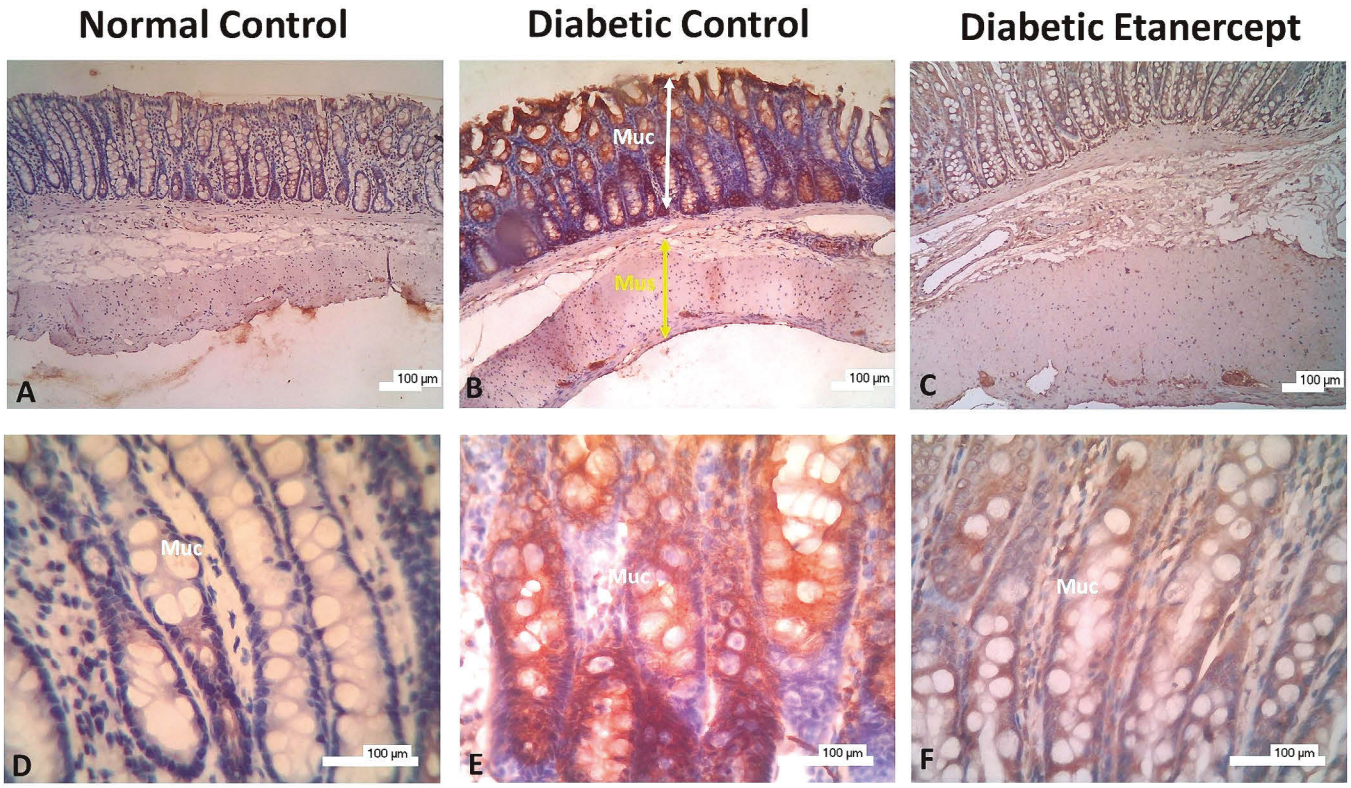

Fig. 6. Immunohistochemical staining for TNF-α expression on a sample of distal colon. **A-C.** Low magnification of TNF-α expression (brown color). TNF-α immunopositivity was primarily detected in the mucosa. Less prominent staining was found in the submucosa and muscular layer. **D-F.** High magnification of the mucosa. The diabetic control (DC) group displayed the most robust expression of TNF-α, and the normal control (NC) group had the weakest immunostaining. The colon of the diabetes etanercept (DE) group had less immunoreactivity than the DC group. Muc, mucosa; Mus, muscular layer.

smooth muscle cells of the rat colon (Nair et al., 2014). A previous study reported that inflammation was positively associated with the degree of hypertrophy of smooth muscle cells (Chen et al., 2017). However, although lower RAGE, TNF-α, and iNOS expression were reported in this study, cellular hypertrophy is not prevented by etanercept administration. In this study, the colonic tissue had stronger immunostaining for arginase

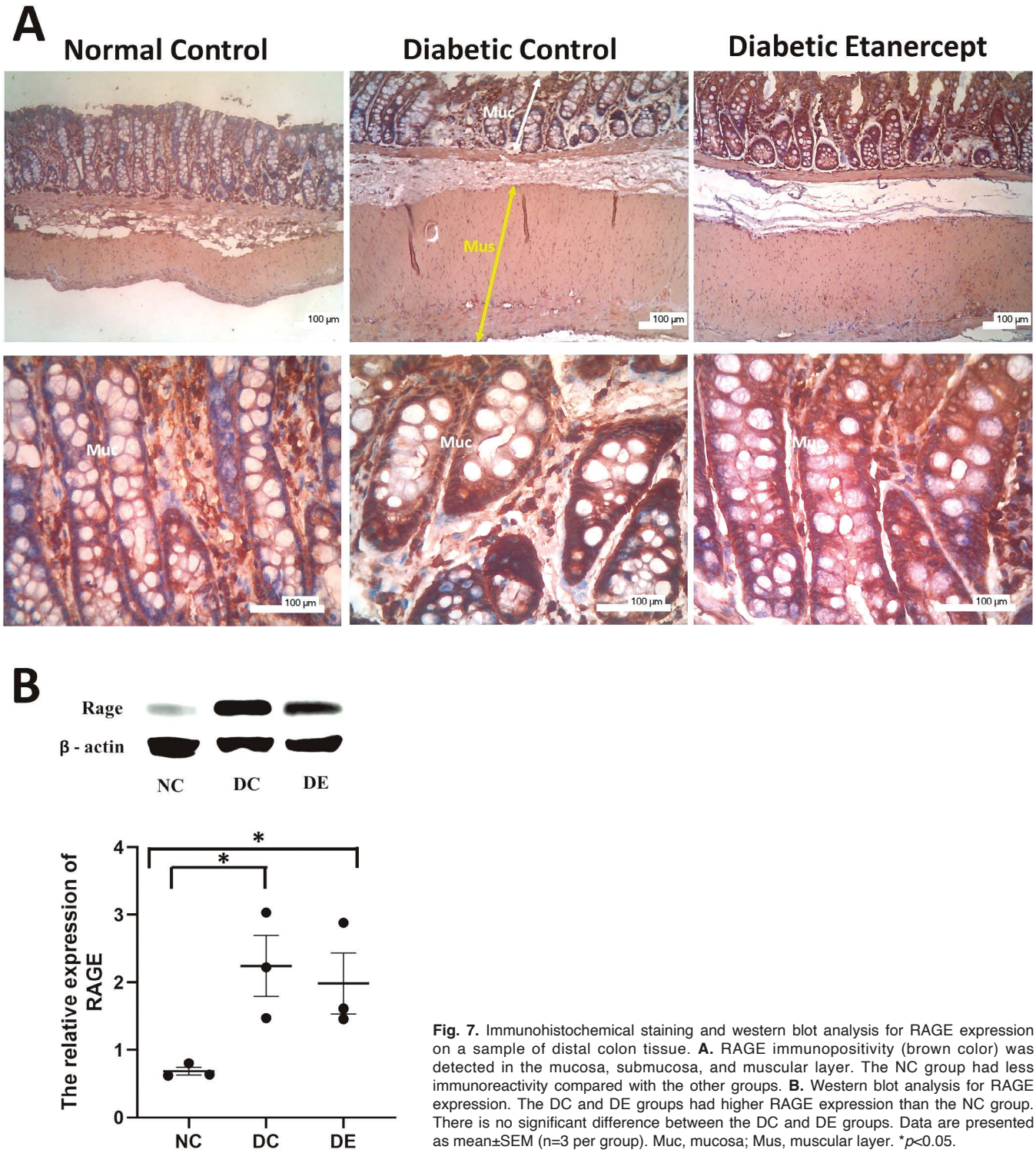

Fig. 7. Immunohistochemical staining and western blot analysis for RAGE expression on a sample of distal colon tissue. **A.** RAGE immunopositivity (brown color) was detected in the mucosa, submucosa, and muscular layer. The NC group had less immunoreactivity compared with the other groups. **B.** Western blot analysis for RAGE expression. The DC and DE groups had higher RAGE expression than the NC group. There is no significant difference between the DC and DE groups. Data are presented as mean±SEM (n=3 per group). Muc, mucosa; Mus, muscular layer. *p<0.05.

I, an enzyme induced by anti-inflammatory cytokines, with a slightly reduced expression upon etanercept treatment in diabetic rats. Its colonic expression is comparable with the expression of iNOS. The arginase I and iNOS expression balance reported in a previous study (Yu et al., 2001) may explain a homeostatic mechanism that prevents worse organ dysfunction in diabetic rats.

The strong expression of iNOS in the neurons of the myenteric neural plexus may be involved in the death of neurons during inflammatory insult (Venkataramana et al., 2015). Higher iNOS expression in the ganglia also supports macrophage involvement in colonic neurodegeneration (Dora et al., 2021), which contributes to colonic dysfunction during inflammation. Neuronal cell death induced by TNF-α signaling (Ruder et al., 2019) may be compensated with a thicker muscular layer and muscle hypertrophy (Kuznik et al., 2020). Inflamed tissue may disrupt the colonic epithelium, interfering with regular epithelial-neuron communication (Najjar et al., 2020). These effects may further disrupt normal colonic motility. Whether TNF-α antagonism by etanercept at this dosage may prevent the neuronal cell death caused by strong TNF-α signaling in the myenteric

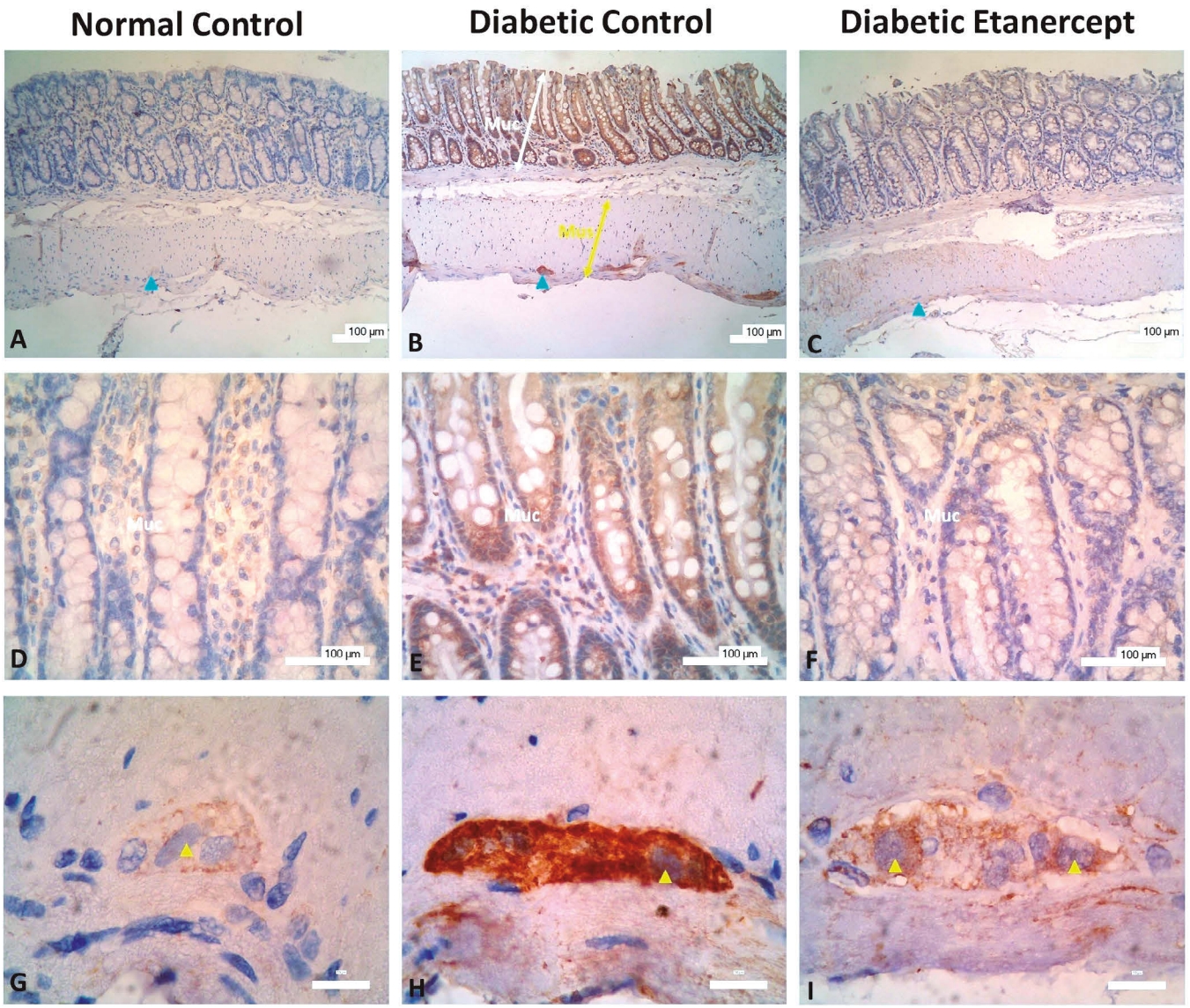

Fig. 8. Immunohistochemical staining for iNOS expression on a sample of distal colon tissue. **A-C.** Low magnification of the colon section showed that iNOS expression (brown color) was strongly expressed in the mucosa and myenteric neural plexus (blue arrowhead) of diabetic rats. **D-F.** High magnification of the mucosa distal colon showed lower iNOS expression in the DE group. **G-I.** High magnification of the myenteric neural plexus showed lower iNOS expression in the DE group. Etanercept reduced iNOS expression in the mucosa colon and myenteric neural plexus of diabetic rats. Yellow arrowhead: neuron; Muc, mucosa; Mus, muscular layer.

neural plexus of the diabetic colon (van Loo and Bertrand, 2023) needs to be elucidated.

Limitations of the study are that some data obtained using a qualitative observation may lead to biased results. Stereological methods, like volume estimation by point counting (Nyengaard and Alwasel, 2014; Wicaksono et al., 2024), may provide unbiased and comparable quantitative data. Whether TNF-α antagonists may prevent dysbiosis (Jones-Hall and Nakatsu, 2016) and cell death that may cause autonomic neuropathy (Marathe et al., 2020) deserves more attention in subsequent studies. The lack of a preventive effect of etanercept in several rat models, while often used as a single treatment (Isic et al., 2008; Grauballe et al., 2015; Kuiken et al., 2017), supports the suggestion to use etanercept in combination therapy. The incomplete effect of etanercept suggests multiple pathways for colon remodeling and functional alteration, which need a more comprehensive study to develop a better treatment.

Conclusions

In conclusion, we showed that although colonic dysfunction in STZ-induced diabetic rats is not entirely

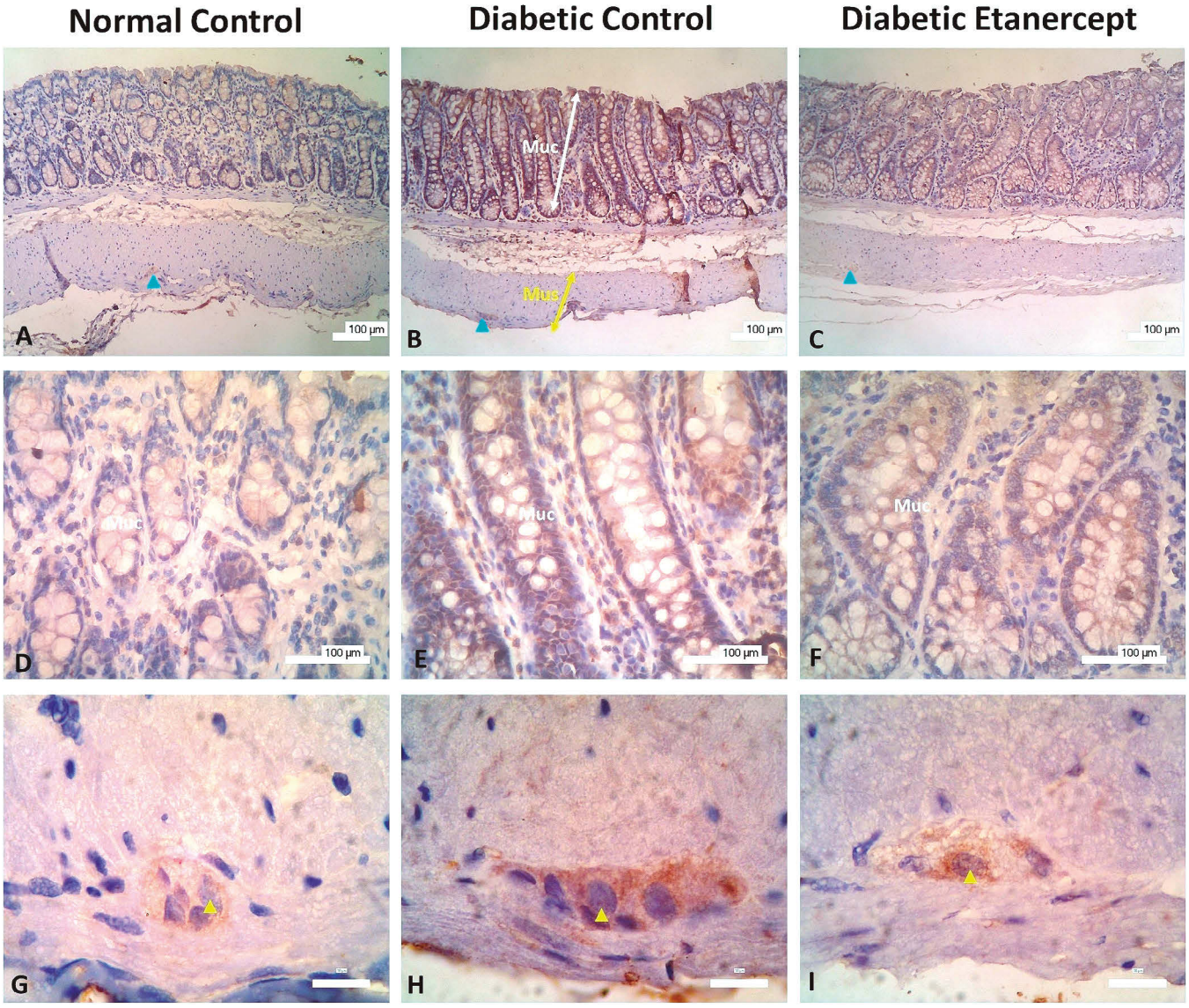

Fig. 9. Immunohistochemical staining for Arginase expression on a sample of distal colon tissue. **A-C.** Low magnification of the colon section. Arginase immunopositivity (brown color) was detected in the mucosa and myenteric neural plexus (blue arrowhead). **D-F.** High magnification of mucosa. **G-I.** High magnification of the myenteric neural plexus. The diabetic control (DC) group displayed the strongest Arginase expression, and the normal control (NC) group had the weakest immunostaining. The colon of the diabetic etanercept (DE) group had less immunoreactivity than the DC group. Yellow arrowhead, neuron; Muc, mucosa; Mus, muscular layer.

prevented by TNF-α antagonists, blocking the inflammatory signal strongly prevents colon remodeling that leads to colon enlargement.

Acknowledgements. We extend our thanks to the Integrated Research and Testing Laboratory (Laboratorium Penelitian dan Pengujian Terpadu) Universitas Gadjah Mada, Yogyakarta, Indonesia, for experimental animal care, Mr. Nur Salisa Siddik Muliyantoro for excellent technical assistance, and to the Office of Research and Publication (ORP) Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, Yogyakarta, Indonesia for English editing.

Funding. This work was supported by the Doctoral Dissertation Research Grant from Universitas Sebelas Maret, Surakarta, Indonesia (Grant Number 254/UN27.22/PT.01.03/2022).

Ethics approval and consent to participate. The study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia with no: KE/FK/0701/EC/2022. All procedures followed the humane treatment of laboratory research animals.

Conflict of interest. The authors declare that they have no conflict of interest.

Author's contributions. TAS participated in study design, data collection, data interpretation, and manuscript writing. DES and YAAS participated in sample and data collection. MM contributed to the study design and data interpretation. RS participated in the study design, sample collection, data collection, and manuscript writing.

References

Akash M.S.H., Rehman K. and Liaqat A. (2018). Tumor necrosis factor-alpha: role in development of insulin resistance and pathogenesis of Type 2 diabetes mellitus. J. Cell. Biochem. 119, 105-110.

Al-Sadi R., Guo S., Ye D., Rawat M. and Ma T.Y. (2016). TNF-α modulation of intestinal tight junction permeability is mediated by NIK/IKK-α Axis activation of the canonical NF-κB Pathway. Am. J. Pathol. 186, 1151-1165.

Barone F.C., Deegan J.F., Price W.J., Fowler P.J., Fondacaro J.D. and Ormsbee H.S 3rd (1990). Cold-restraint stress increases rat fecal pellet output and colonic transit. Am. J. Physiol. 258, G329-337.

Chen W., Lu C., Hirota C., Iacucci M., Ghosh S. and Gui X. (2017). Smooth muscle hyperplasia/hypertrophy is the most prominent histological change in Crohn's fibrostenosing bowel strictures: A semiquantitative analysis by using a novel histological grading scheme. J. Crohn's Colitis. 11, 92-104.

Dora D., Ferenczi S., Stavely R., Toth V.E., Varga Z.V., Kovacs T., Bodi I., Hotta R., Kovacs K.J., Goldstein A.M. and Nagy N. (2021). Evidence of a myenteric plexus barrier and its macrophage-dependent degradation during murine colitis: Implications in enteric neuroinflammation. Cell. Mol. Gastroenterol. Hepatol. 12, 1617-1641.

Du Y.T., Rayner C.K., Jones K.L., Talley N.J. and Horowitz M. (2018). Gastrointestinal symptoms in diabetes: Prevalence, assessment, pathogenesis, and management. Diabetes Care. 41, 627-637.

Esteves-Monteiro M., Menezes-Pinto D., Ferreira-Duarte M., Dias-Pereira P., Morato M. and Duarte-Araújo M. (2022). Histomorphometry changes and decreased reactivity to angiotensin II in the ileum and colon of streptozotocin-induced diabetic rats. Int. J. Mol. Sci. 23, 1-18.

Grauballe M.B., Østergaard J.A., Schou S., Flyvbjerg A. and Holmstrup P. (2015). Effects of TNF-α blocking on experimental periodontitis and type 2 diabetes in obese diabetic Zucker rats. J. Clin. Periodontol. 42, 807-816.

Ha C., Kim H., Cha R., Lee J., Lee S., Ryu J.H., Kim H. and Lee O.J. (2021). Effect of DA-9701 on the gastrointestinal motility in the streptozotocin-induced diabetic mice. J. Clin. Med. 10, 1-12.

Isic A., Täng M.S., Haugen E. and Fu M. (2008). TNFα-antagonist neither improve cardiac remodelling or cardiac function at early stage of heart failure in diabetic rats. Autoimmunity 41, 473-477.

Izbeki F., Wittman T., Rosztoczy A., Linke N., Bodi N., Fekete E. and Bagyanszki M. (2008). Immediate insulin treatment prevents gut motility alterations and loss of nitrergic neurons in the ileum and colon of rats with streptozotocin-induced diabetes. Diabetes Res. Clin. Pract. 80, 192-198.

Jasso G.J., Jaiswal A., Varma M., Laszewski T., Grauel A., Omar A., Silva N., Dranoff G., Porter J.A., Mansfield K., Cremasco V., Regev A., Xavier R.J. and Graham D.B. (2022). Colon stroma mediates an inflammation-driven fibroblastic response controlling matrix remodeling and healing. PLoS Biol. 20, 1-30.

Jones-Hall Y.L. and Nakatsu C.H. (2016). The Intersection of TNF, IBD and the Microbiome. Gut Microbes 7, 58-62.

Kuiken N.S.S., Rings E.H.H.M., Alffenaar J.W.C., Havinga R., Jurdzinski A., Groen A.K. and Tissing W.J.E. (2017). Tumor necrosis factor-alpha inhibitor etanercept does not alter methotrexate-induced gastrointestinal mucositis in rats. J. Pediatr. Gastroenterol. Nutr. 65, e28-e34.

Kuznik E., Dudkowiak R., Adamiec R. and Poniewierka E. (2020). Diabetic autonomic neuropathy of the gastrointestinal tract. Prz. Gastroenterol. 15, 89-93.

Li H.T., Wu X.D., Davey A.K. and Wang J. (2011). Antihyperglycemic effects of baicalin on streptozotocin - Nicotinamide induced diabetic rats. Phytother. Res. 25, 189-194.

Mack M. (2018). Inflammation and fibrosis. Matrix Biol. 68-69:106-121.

Marathe C.S., Jones K.L., Wu T., Rayner C.K. and Horowitz M. (2020). Gastrointestinal autonomic neuropathy in diabetes. Auton. Neurosci. 229, 1-9.

Nair D.G., Miller K.G., Lourenssen S.R. and Blennerhassett M.G. (2014). Inflammatory cytokines promote growth of intestinal smooth muscle cells by induced expression of PDGF-Rβ. J. Cell. Mol. Med. 18, 444-454.

Najjar S.A., Davis B.M. and Albers K.M. (2020). Epithelial-neuronal communication in the colon: implications for visceral pain. Trends. Neurosci. 43, 170-181.

Nyengaard J.R. and Alwasel S.H. (2014). Practical stereology of the stomach and intestine. Ann. Anat. 196, 41-47.

Paiotti A.P.R., Miszputen S.J., Oshima C.T.F., Neto R.A., Ribeiro D.A. and Franco M. (2011). Etanercept attenuates TNBS-induced experimental colitis: Role of TNF-α expression. J. Mol. Histol. 42, 443-450.

Patel B.A., Fidalgo S., Wang C., Parmar L., Mandona K., Panossian A., Flint M.S., Ranson R.N., Saffrey M.J. and Yeoman M.S. (2017). The TNF-α antagonist etanercept reverses age-related decreases in colonic SERT expression and faecal output in mice. Sci. Rep. 7, 1-12.

Piper M.S. and Saad R.J. (2017). Diabetes mellitus and the colon. Curr. Treat. Options. Gastroenterol. 15, 1-16.

Ruder B., Atreya R. and Becker C. (2019). Tumour necrosis factor alpha

in intestinal homeostasis and gut related diseases. Int. J. Mol. Sci. 20, 1-21.

Serban A.I., Stanca L., Geicu O.I., Munteanu M.C. and Dinischiotu A. (2016). RAGE and TGF-β1 cross-talk regulate extracellular matrix turnover and cytokine synthesis in AGEs exposed fibroblast cells. PLoS One 11, e0152376.

Sha H., Tong X. and Zhao J. (2018). Abnormal expressions of AGEs, TGF-β1, BDNF and their receptors in diabetic rat colon-associations with colonic morphometric and biomechanical remodeling. Sci. Rep. 8, 1-14.

Siegman M.J., Eto M. and Butler T.M. (2016). Remodeling of the rat distal colon in diabetes: function and ultrastructure. Am. J. Physiol. Cell. Physiol. 310, C151-C160.

Szkudelski T. (2012). Streptozotocin-nicotinamide-induced diabetes in the rat. Characteristics of the experimental model. Exp. Biol. Med. 237, 481-490.

Van Den Berge N., Ferreira N., Mikkelsen T.W., Alstrup A.K.O, Tamgüney G., Karlsson P., Terkelsen A.J., Nyengaard J.R., Jensen P.H. and Borghammer P. (2021). Ageing promotes pathological alpha-synuclein propagation and autonomic dysfunction in wild-type rats. Brain 144, 1853-1868.

van Loo G. and Bertrand M.J.M. (2023). Death by TNF: a road to inflammation. Nat. Rev. Immunol. 23, 289-303.

Venkataramana S., Lourenssen S., Miller K.G. and Blennerhassett M.G. (2015). Early inflammatory damage to intestinal neurons occurs via inducible nitric oxide synthase. Neurobiol. Dis. 75, 40-52.

Wicaksono S., Sumiwi Y.A., Paramita D. and Susilowati R. (2024). ImageJ-FIJI-Assisted estimation of intestinal layers' volume: Study in Jejunum-Ileum of rats. J. Microsc. Ultrastruct. (in press).

Xie D.P., Li S., Li L., Chang X.W., Xi T.F., Yang X., Jin Z. and Zeng Y. (2013). Beta-arrestin2 is involved in the increase of distal colonic contraction in diabetic rats. Regul. Pept. 185, 29-33.

Xu J., Chen Y., Liu S. and Hou X. (2013). Electroacupuncture regulates apoptosis/proliferation of intramuscular interstitial cells of cajal and restores colonic motility in diabetic constipation rats. Evid. Based Complement. Alternat. Med. 2013, 1-10.

Ye Q., Lin Y.N., Xie M.S., Yao Y.H., Tang S.M., Huang Y., Wang X.H. and Zhu Y.H. (2019). Effects of etanercept on the apoptosis of ganglion cells and expression of Fas, TNF-α, caspase-8 in the retina of diabetic rats. Int. J. Ophthalmol. 12, 1083-1088.

Yu H., Iyer R.K., Kern R.M., Rodriguez W.I., Grody W.W. and Cederbaum S.D. (2001). Expression of Arginase Isozymes in Mouse Brain. J. Neurosci. Res. 66, 406-422.

Zheng Y., Ley S.H. and Hu F.B. (2018). Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat. Rev. Endocrinol. 14, 88-98.

Accepted March 18, 2024