Original Article

Extracellular DNA is Increased in Dextran Sulphate Sodium-Induced Colitis in Mice

(inflammatory bowel disease / extracellular DNA / DNAse / dextran sulphate sodium / inflammation)

M. MARONEK[1], B. GROMOVA[1], R. LIPTAK[1], D. KLIMOVA[2], B. CECHOVA[1], R. GARDLIK[1]

[1]Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, Bratislava, Slovak Republic
[2]Cancer Research Institute of the Slovak Academy of Sciences, Bratislava, Slovak Republic

Abstract. Ulcerative colitis and Crohn's disease constitute the two main forms of inflammatory bowel disease. Prevalence of these diseases increases. In the present day, inadequate and inefficient therapy causes complications and frequent relapse. Extracellular DNA (ecDNA) is the DNA that is outside of cells and may be responsible for activation of the inflammatory response. To determine whether colitis is associated with higher concentration of ecDNA we used male mice of the C57BL/6 strain. Colitis was induced by 2% dextran sulphate sodium (DSS). After 7 days, mice exhibited considerable weight loss compared to the control group. Also, there was a higher stool consistency score and the colon was significantly shorter in comparison to the control group. Higher concentration of ecDNA was found in the DSS group. Interestingly, deoxyribonuclease activity was lower in the colon of the DSS group compared with the negative control. These findings may point to ecDNA as a potential pathogenetic factor and marker of inflammation.

Introduction

Inflammatory bowel disease (IBD) is a group of chronic inflammatory diseases characterized by long-lasting recurrent inflammation of the intestinal epithelium. The two main forms of IBD are ulcerative colitis and Crohn's disease. The number of patients suffering from IBD is increasing (Molodecky et al., 2012). The present day therapy focuses on symptoms rather than causes of IBD, and therefore results in ineffective and costly treatment (Click et al., 2016). Despite extensive research in this field, the exact pathological mechanisms leading to the development or recurrence of IBD still remain to be elucidated.

Extracellular DNA (ecDNA) is defined as any DNA molecule that is present outside the cell. This DNA can be of nuclear, mitochondrial, or even bacterial origin. EcDNA is present in the plasma, urine or saliva (O'Driscoll, 2007). It can be found even under physiological conditions (Zhong et al., 2000; Lui et al., 2003). EcDNA can have immunogenic potential because it has been proved to bind toll-like receptor 9 and thereby induce the cascade leading to the inflammatory response (Krieg, 2002; Sharma and Fitzgerald, 2011). It has therefore been suggested that ecDNA could be viewed as a potential marker of inflammation. Elevated levels of ecDNA were detected in a pig model of late-onset sepsis (Nguyen et al., 2017). However, lower concentration of ecDNA has been reported in patients with rheumatoid arthritis (Dunaeva et al., 2015; Laukova et al., 2017). EcDNA is being considered as a possible prognostic marker in patients with sepsis (Clementi et al., 2016; Schneck et al., 2017) or multiple trauma (Ahmed et al., 2016). It is not completely clear yet how ecDNA is metabolized and degraded. A possible explanation could be via the action of endogenous deoxyribonucleases (DNases), enzymes with the ability to cleave DNA. DNases, although they potentially may play a role in IBD, do not at present belong to extensively studied concepts.

The aim of our study was therefore to determine whether DSS-induced colitis is associated with higher concentration of ecDNA in mice and whether this increase is related to altered DNase activity in tissues and contents of organs of the gastrointestinal tract.

Received August 27, 2018. Accepted December 28, 2018.

This study was supported by the Ministry of Education of the Slovak Republic (grant VEGA 1/0204/17) and the Slovak Research and Development Agency (grant APVV-17-0505).

Corresponding author: Roman Gardlik, Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, Sasinkova 4, Bratislava 811 08, Slovakia. Phone: (+421) 2 59357 296; Fax: (+421) 2 59357 631; e-mail: romangardlik@gmail.com

Abbreviations: DNase – deoxyribonuclease, DSS – dextran sulphate sodium, ecDNA – extracellular DNA, IBD – inflammatory bowel disease, PBS – phosphate-buffered saline.

Material and Methods

Animals

Eleven male 8–10 week-old mice of the C57/BL6 strain (Velaz, Prague, Czech Republic) were used. The animals were housed 5–6 per cage, at a temperature of 23 °C, 50% humidity, with 12/12-h light/dark cycle and provided with *ad libitum* access to standard chow and water. The animal protocol was designed to minimize pain or discomfort to the animals. The animals had been acclimatized to laboratory conditions for two weeks prior to experimentation. All animals were anesthetized by isoflurane and euthanized by cervical dislocation for tissue and body fluid collection. The study was approved by the institutional Ethics Committee.

Induction of colitis

Before the experiments, mice were randomly distributed into two groups, the control group (N = 5) and DSS group (N = 6). Mice from the DSS group were exposed to water containing 2% DSS (molecular weight = 40,000, AppliChem, Darmstadt, Germany) for 7 days, while control mice received tap water (Wirtz et al., 2007).

Assessment of colitis

Bodyweight and water intake were monitored daily. Stool consistency was scored on a scale from 0 to 3, representing as follows: 0 = thick, formed stool, 1 = soft stool, 2 = watery stool, 3 = soft or watery stool with the presence of blood. Mice were also macroscopically assessed for the presence of altered behaviour. After 7 days of DSS treatment, bristled fur could be seen, and mice preferred to stay in the corner of the cage with limited movement during this time.

Isolation of samples

On day 7 of the experiment, mice were anesthetized with isoflurane and euthanized by cervical dislocation for the collection of samples. Prior to sample collection and authentication, blood was collected via retroorbital puncture using EDTA and heparin-coated tubes (Sarstedt, Nűmbrecht, Germany). Plasma was obtained by centrifugation of blood samples at 1,600 × *g* for 10 min. Tissues were washed thoroughly with fresh cold 0.9% PBS. Samples were harvested from the stomach, duodenum, jejunum, ileum and colon. The samples were flash-frozen in liquid nitrogen immediately after collection and stored at –20 °C until further use. Before the collection of colon samples, colon length was measured after excision from ileocecal junction to proximal rectum.

EcDNA isolation and measurement

EcDNA was isolated from the plasma samples using the QiaAmp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the protocol of the manufacturer. The concentration of isolated ecDNA was measured using a Qubit 3.0 fluorometer and Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA). EcDNA was kept frozen at –20 °C until further use.

Determination of DNase activity

The 10 ml of the reaction mixture was prepared from 4 ml of 10 mM $CaCl_2$, 4 ml of 10 mM $MgCl_2$, 800 µl of 0.5 M Tris HCl pH 7.5, 800 µl of distilled water, and 320 µl of isolated DNA was pipetted into each fluorescence microtitre plate. The thoroughly mixed and stirred reaction mixture was pipetted to 96 wells of the microtitre plate. One column (8 wells) was left for the calibration. In this column, 95 µl was pipetted.

The calibrator sample of known DNase activity was made using DNase I (RNase-free DNase set, Qiagen, Hilden, Germany). This enzyme was activated by mixing it with RDD buffer right before the measurements. To make the calibration, 5 µl of RNase-free DNase I was added to 35 µl of RDD buffer and stirred with a pipette in the first of the eight calibration tubes. To avoid enzyme function disruption, vortexing was avoided. Next, the first calibration value (the contents of the first tube) was diluted in seven 2-fold dilutions. All the calibration tubes were prepared in this way. The last point consisted of only RDD buffer representing the negative control. These reference values were pipetted to the fluorescence microtitre plate wells in the amount of 5 µl per well.

After that, 10 µl of the homogenates of tissues and their respective contents were added to 90 µl of the reaction mixture. Each homogenate was measured in duplicate, and to prevent inter-assay variability, the contents and tissues of the same organs were always measured in the same fluorescence microtitre plate. Next, the fluorescence microtitre plate was incubated for 15 min at 37 °C. To stop the incubation and enhance its fluorescence when bound to dsDNA, 3 µl of Goodview dye (Ecoli, Bratislava, Slovakia) was added into each well. This Goodview mixture was prepared by diluting 3 µl of Goodview in 10 ml of distilled water, and 100 µl of the mixture was added to each well in the microtitre plate. According to the dye description sheet obtained from the manufacturer, excitation maximums were assessed. The maximum at 268 nm was chosen as the most suitable according to the protocol. Finally, the fluorescence intensity was measured at 530 nm.

DNase activity in the plasma was measured using 1% agarose gel (4 mM Tris-HCl pH 7.5, 20 ml $CaCl_2$, 20 ml $MgCl_2$ and 7 ml DNA (5 mg/ml)) by the single radial enzyme diffusion (SRED) method (Nadano et al., 1993). One µl of each sample was pipetted into the holes of the agarose gel and incubated overnight at 37 °C in the dark. Then the diameters of the circles were measured using ImageJ software (NIH, Bethesda, MD). Concentration of proteins in the samples was measured using a bicinchoninic acid kit (Sigma-Aldrich, Steindheim, Germany).

Statistical analysis

Data were analysed using one- or two-way ANOVA. Data are presented as mean ± standard deviation. P values less than 0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 6 Software (GraphPad Software, La Jolla, CA).

Results

Model of colitis

Mice from the DSS group exhibited an average 8% weight loss on the last day of the experiment. This weight loss started on day 4 and lasted until the end of the experiment. In contrast, mice from the control group gained on average 3 % of their initial bodyweight (Fig. 1A). Stool consistency indicates the severity of IBD based on the density and presence of blood in the stool. The score rose from the second day to the fifth day of the experiment for the DSS group while remaining on the baseline level for the control group (Fig. 1B). Since DSS was dissolved in water, differences in water consumption could be partially responsible for any observed effects. The amount of consumed water was not significantly different between the tested groups. Colon length was higher in the DSS group compared to the control group (on average 7.65 cm and 6.55 cm, respectively), but this difference was not significant (Fig. 1C). Relative weight and colon length showed a positive correlation (Fig. 1D). These data suggest that the model of murine colitis was successfully established.

Concentration of ecDNA

The average concentration of ecDNA in the DSS group was significantly higher (51 ng/ml) compared to the control group (21.1 ng/ml, t = 3.639, P = 0.0054) (Fig. 2A). The correlation between relative weight of mice and concentration of plasma ecDNA was not significant but showed a clear trend (Fig. 2B).

DNase activity in plasma

The DNase activity was measured in the plasma. There was no difference between the groups (P = 0.97).

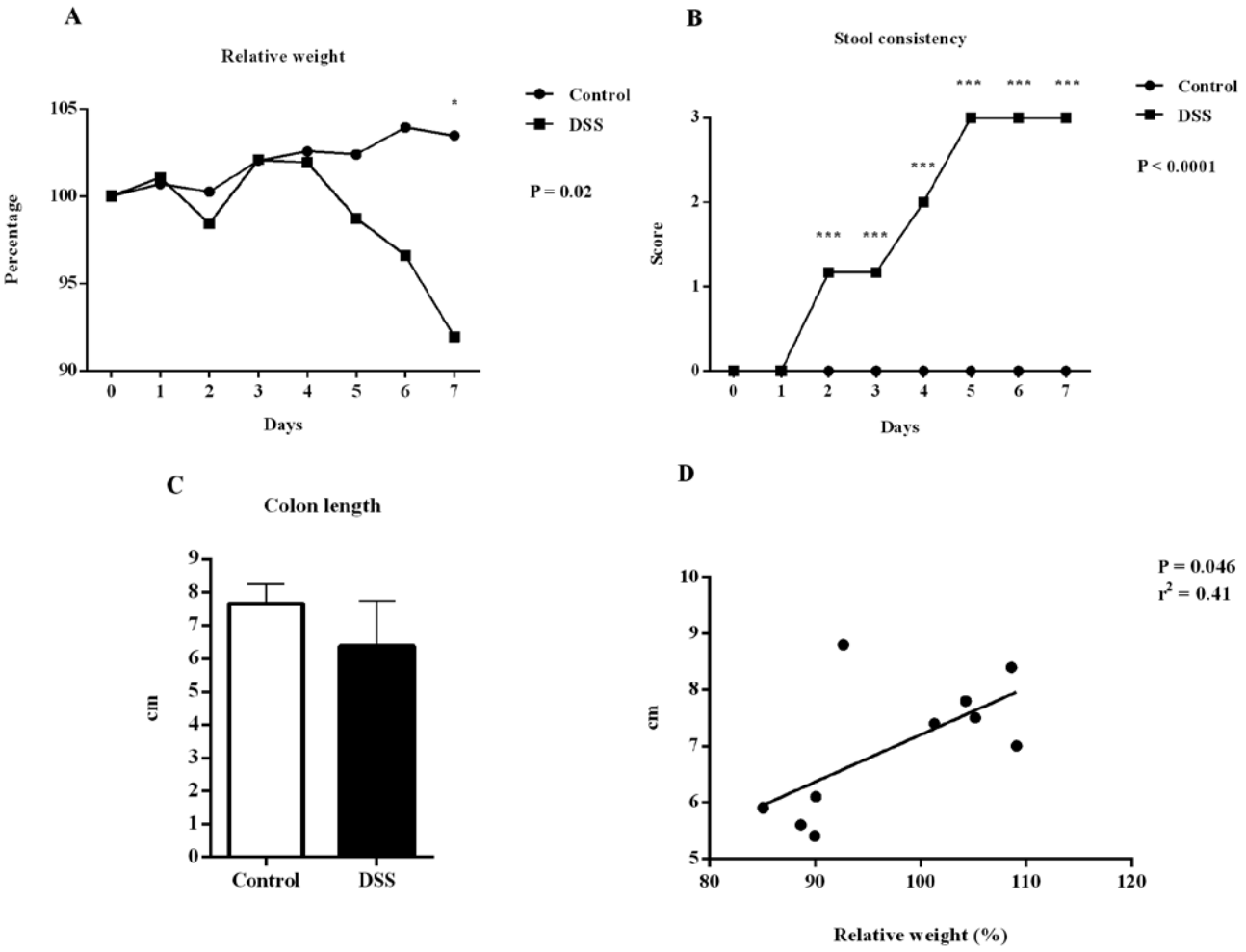

Fig. 1. Markers of colitis
A – percentage of initial weight of both groups during the experiment, B – stool consistency score of both groups during the experiment, C – the control group showed longer colon than the DSS group, D – positive correlation between relative weight and colon length of the tested groups; * P < 0.05, *** P < 0.001

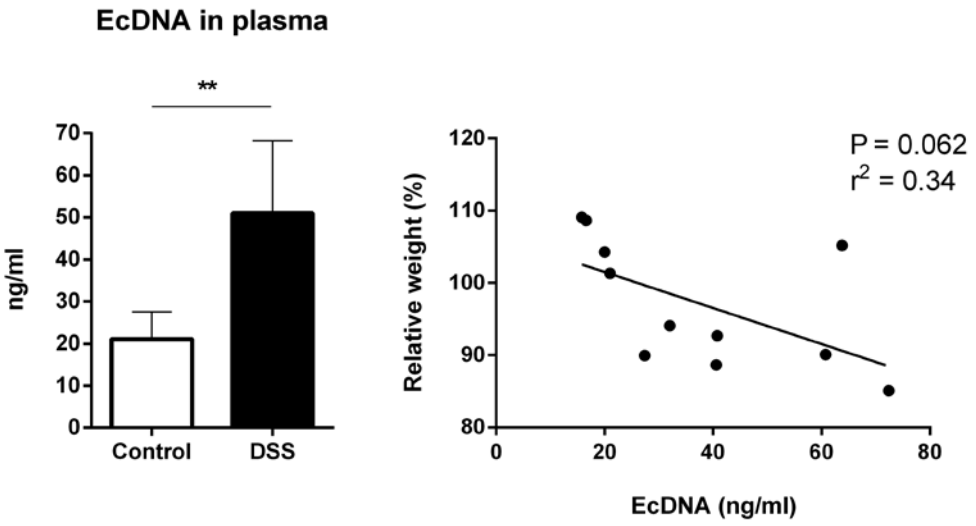

Fig. 2. EcDNA in the plasma and its correlation with relative weight
A – ecDNA in the plasma of the DSS group was significantly higher compared to the control group; B – positive correlation between relative weight and ecDNA in the plasma; *** $P < 0.001$

DNase activity in gastrointestinal tract

The DNase activity was measured in the whole gastrointestinal tract from the oesophagus to the distal colon (Fig. 3A). The highest activity was observed in the pancreas (5.7-fold higher in the control group, Ku/mg of protein) and duodenum (1.7-fold higher in the control group, Ku/mg of protein). The lowest activity was measured in the samples of stomach (2-fold higher), caecum (6.1-fold higher), proximal (2.6-fold higher) and distal colon (4.8-fold higher in the control group, Ku/mg of protein). The DSS group exhibited a significantly lower level of DNase activity in the colon compared to the control group (Fig. 3B). There was a positive correlation between plasma ecDNA and DNase activity in the colon (Fig. 3C).

Discussion

In our study, we measured the concentration of plasma ecDNA and DNase activity in organs of the gastrointestinal tract in a mouse model of DSS-induced colitis. EcDNA was significantly higher in the DSS group compared to the control group. The result of higher ecDNA concentration in colitic mice is in agreement with a previous study, which measured ecDNA in human UC samples and found it to correlate with the severity of the disease (Koike et al., 2014). However, the presented findings are in contrast with another study of our research group, where no change of ecDNA concentration in a mouse model of colitis was observed (Bábíčková et al., 2018). Despite this discrepancy, our results contribute to the growing amount of evidence that points toward a pro-inflammatory role of ecDNA. The effect of ecDNA on the severity of inflammation may depend on the environment in which ecDNA is present. There is evidence that ecDNA coming from colitic mice exhibits a beneficial effect when compared to ecDNA from healthy mice. This may suggest a potential positive role of ecDNA during colitis, acting in a protective manner (Muzes et al., 2017).

Based on the pro-inflammatory signalling of ecDNA, it may seem logical that removing excessive ecDNA from the circulation might help alleviate or even eliminate the inflammation completely. This could be done by administration of DNases. Under physiological conditions, they may be responsible for rapid degradation of ecDNA in order to prevent induction of inflammation. Malickova et al. (2011) showed that the activity of DNase I is lower in the patients suffering from IBD. However, to our knowledge, our study was the first to focus on measuring the DNase activity in gastrointestinal tissues of mice with induced colitis. The amount of DNase activity varied dramatically among organs of the gastrointestinal tract. With the exception of samples from the liver and jejunum, the activity was lower in the DSS group. In the case of pancreas, ileum and caecum, it was significant. The highest activity was detected in the pancreas and duodenum.

Since DNases cleave DNA, we hypothesize that this could be caused by a higher presence of substrate for DNases such as ecDNA. It is not clear, however, whether the substrate that leads to the increase of DNase activity in these organs originates from the digestion process or it is a product of some defence mechanism during the inflammation. The lowest DNase activity was observed in the samples of stomach, caecum and colon. While the dynamics of DNase activity is not yet understood, a trend of decreasing activity could be seen in both groups starting from the ileum and duodenum for the control and DSS group, respectively. A possible explanation for this discovery could be the presence of a DNase inhibitor distal from the caecum. Since DNase activity was lower in the control group as well, the potential inhibitor could

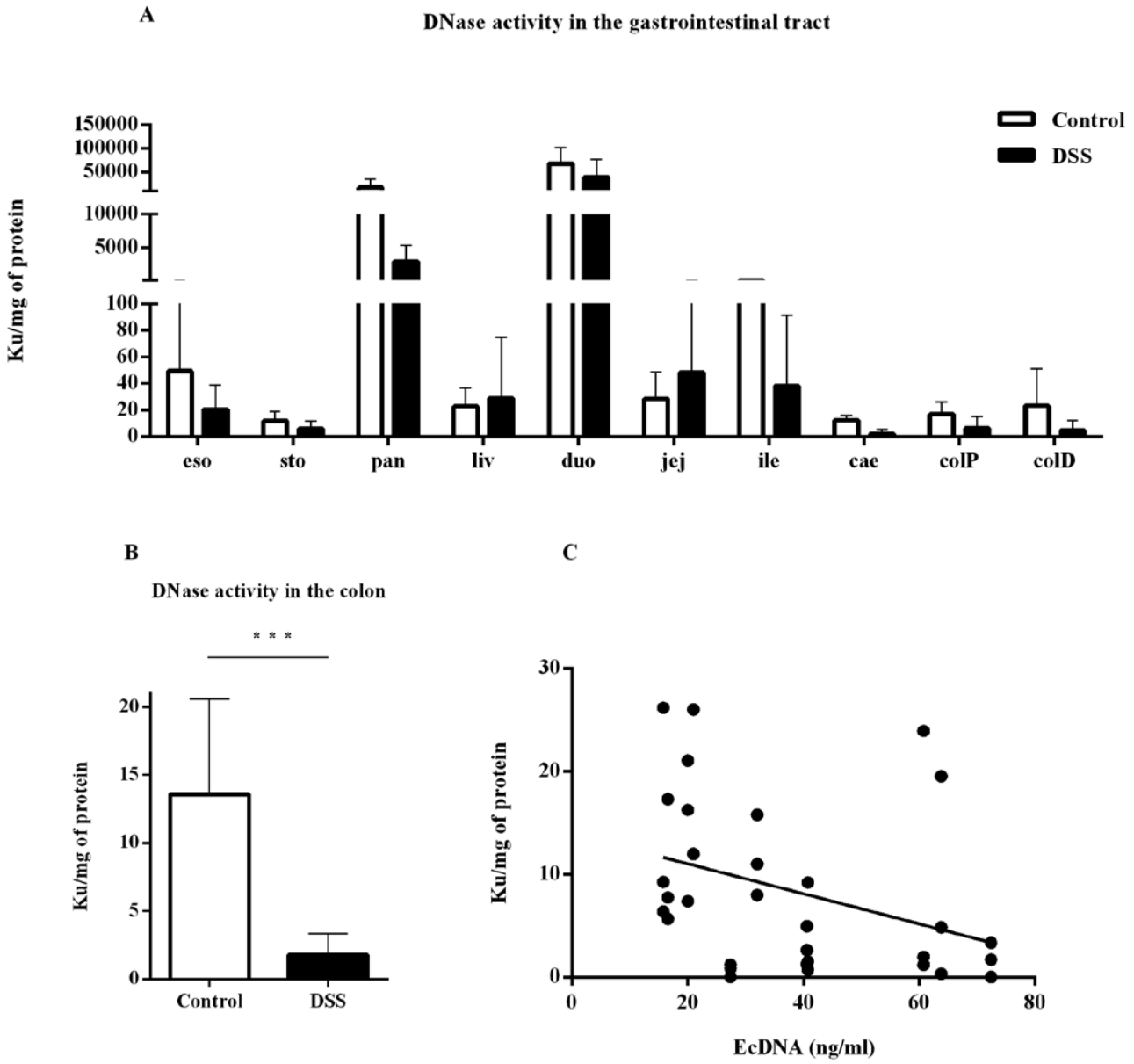

Fig. 3. DNase activity and its correlation with ecDNA
A – DNase activity was highest in the pancreas and duodenum and lowest in the samples of stomach, caecum and colon; eso – oesophagus, sto – stomach, pan – pancreas, liv – liver, duo – duodenum, jej – jejunum, ile – ileum, cae – caecum, colP – proximal colon, colD – distal colon; B – DNase activity was significantly higher in the control group compared to the DSS group; C – positive correlation between ecDNA in the plasma and DNase activity in the colon; *** $P < 0.001$

also be present in the colon at physiological conditions, affecting the healing of the intestinal mucosa only in case of long-term or recurrent inflammation.

Further, since there were no differences in plasmatic DNase I activity between the groups, the increase in plasma ecDNA concentration was likely not caused by decreased DNase I activity in the plasma. This indicates that ecDNA might play a role in the pathogenesis of IBD. This, however, has not been proved by the results of the present study.

Despite the importance of our findings, our research has some limitations. Since ecDNA can come from the nuclei, mitochondria or bacteria, in future studies it is important to determine the exact composition and origin of ecDNA. This could provide an answer to the question whether it is self or non-self ecDNA to which the immune system reacts. Moreover, it is not yet clear how the concentration of ecDNA changes during the colitis. This may also be an aspect that could provide us with further understanding of the role of ecDNA during the inflammation. The high variability could also be viewed as a limitation, likely due to the number of the used animals. It would therefore be essential to acquire larger numbers of animals and different strains to test whether this observed phenomenon occurs in C57BL/6 mice only or is independent on the mouse strain. There are

also a few limitations considering the DNase activity. Since DNases represent a group of enzymes, it is important to assess precisely which enzymes are responsible for the activity or lack thereof. Similarly, as with the ecDNA, studying the dynamics of the DNase activity before, during and after induction of colitis could also prove useful.

In conclusion, it will be the subject of future studies to determine whether the higher concentration of ecDNA during colitis is caused by the inflammation or the inflammation is induced by the increased ecDNA content. Likewise, it remains to be established whether DNase treatment could prevent or ameliorate intestinal inflammation caused by the colitis. It is therefore vital to continue research in this field, which could lead to a novel therapeutic approach for patients suffering from IBD.

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

References

Ahmed, A., Soliman, R., Samir, S. (2016) Cell free DNA and procalcitonin as early markers of complications in ICU patients with multiple trauma and major surgery. *Clin. Lab.* **62**, 2395-2404.

Bábíčková J., Čonka, J., Janovičová L., Boris, M., Konečná, B., Gardlík, R. (2018) Extracellular DNA as a prognostic and therapeutic target in mouse colitis under DNase I treatment. *Folia Biol. (Praha)* **64**, 10-15.

Clementi, A., Virzi, G. M., Brocca, A., Pastori, S., de Cal, M., Marcante, S., Granata, A., Ronco, C. (2016) The role of cell-free plasma DNA in critically ill patients with sepsis. *Blood Purif.* **41**, 34-40.

Click, B., Ramos Rivers, C., Koutroubakis, I. E., Babichenko, D., Anderson, A. M., Hashash, J. G., Dunn, M. A., Schwartz, M., Swoger, J., Baidoo, L., Barrie, A. 3rd, Regueiro, M., Binion, D. G. (2016) Demographic and clinical predictors of high healthcare use in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* **22**, 1442-1449.

Dunaeva, M., Buddingh', B. C., Toes, R. E. M., Luime, J .J., Lubberts, E., Pruijn, G. J. M. (2015) Decreased serum cell-free DNA levels in rheumatoid arthritis. *Auto Immun. Highlights* **6**, 23-30.

Koike, Y., Uchida, K., Tanaka, K., Ide, S., Otake, K., Okita, Y., Inoue, M., Araki, T., Mizoguchi, A., Kusunoki, M. (2014) Dynamic pathology for circulating free DNA in a dextran sodium sulfate colitis mouse model. *Pediatr. Surg. Int.* **30**, 1199-1206.

Krieg, A. M. (2002) CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**, 709-760.

Laukova, L., Konecna, B., Babickova, J., Wagnerova, A., Meliskova, V., Vlkova, B., Celec, P. (2017) Exogenous deoxyribonuclease has a protective effect in a mouse model of sepsis. *Biomed. Pharmacother.* **93**, 8-16.

Lui, Y. Y., Woo, K. S., Wang, A. Y., Yeung, C. K., Li, P. K., Chau, E., Ruygrok, P., Lo, Y. M. (2003) Origin of plasma cell-free DNA after solid organ transplantation. *Clin. Chem.* **49**, 495-496.

Malickova, K., Duricova, D., Bortlik, M., Hruskova, Z., Svobodova, B., Machkova, N., Komarek, V., Fucikova, T., Janatkova, I., Zima, T., Lukas, M. (2011) Impaired deoxyribonuclease I activity in patients with inflammatory bowel diseases. *Autoimmune Dis.* **2011**, 945861.

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., Benchimol, E. I., Panaccione, R., Ghosh, S., Barkema, H. W., Kaplan, G. G. (2012) Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* **142**, 46-54.

Muzes, G., Kiss, A. L., Tulassay, Z., Sipos, F. (2017) Cell-free DNA-induced alteration of autophagy response and TLR9-signaling: Their relation to amelioration of DSS-colitis. *Comp. Immunol. Microbiol. Infect. Dis.* **52**, 48-57.

Nadano, D., Yasuda, T., Kishi, K. (1993) Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. *Clin. Chem.* **39**, 448-452.

Nguyen, D. N., Stensballe, A., Lai, J. C., Jiang, P., Brunse, A., Li, Y., Sun, J., Mallard, C., Skeath, T., Embleton, N. D., Berrington, J. E., Sangild, P. T. (2017) Elevated levels of circulating cell-free DNA and neutrophil proteins are associated with neonatal sepsis and necrotizing enterocolitis in immature mice, pigs and infants. *Innate Immun.* **23**, 524-536.

O'Driscoll, L. (2007) Extracellular nucleic acids and their potential as diagnostic, prognostic and predictive biomarkers. *Anticancer Res.* **27**, 1257-1265.

Sharma, S., Fitzgerald, K. (2011) Innate immune sensing of DNA. *PLoS Pathog.* **7**, e1001310.

Schneck, E., Samara, O., Koch, C., Hecker, A., Padberg, W., Lichtenstern, C., Weigand, M. A., Uhle, F. (2017) Plasma DNA and RNA differentially impact coagulation during abdominal sepsis - an explorative study. *J. Surg. Res.* **210**, 231-243.

Wirtz, S., Neufert, C., Weigmann, B., Neurath, M. F. (2007) Chemically induced mouse models of intestinal inflammation. *Nat. Protoc.* **2**, 541-546.

Zhong, X. Y., Burk, M. R., Troeger, C., Kang, A., Holzgreve, W., Hahn, S. (2000) Fluctuation of maternal and fetal free extracellular circulatory DNA in maternal plasma. *Obstet. Gynecol.* **96**, 991-996.