

Oral *in vivo* Bactofection in Dextran Sulfate Sodium Treated Female Wistar Rats*

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Salmonella typhimurium SL7207 carrying Cu-Zn superoxide dismutase and an N-terminal deletion mutant of monocyte chemoattractant protein-1 genes was applied to dextran sodium sulfate treated female Wistar rats. Stool quality, food and water intake were monitored. Markers of oxidative stress, interleukin 1, interleukin 6 and tumor necrosis factor alpha were quantified. No differences were found in body weights, markers of oxidative stress in plasma and inflammatory markers in colon homogenates. Plasma concentrations of IL1, IL6 were lower in the treatment groups than in the dextran sodium sulfate group. However, dextran sodium sulfate induced inflammation could not be confirmed by plasma levels of IL1, IL6 and TNFalpha. Although some parameters showed a tendency to improve, the inflammation caused by administration of 4% dextran sodium sulfate during 7 days was low and contradictory to other studies. Results showed the potential synergic effect of combined bacteria-mediated antioxidative and anti-inflammatory gene therapy.

Key words: Bactofection, dextran sulfate sodium induced colitis, superoxide dismutase, monocyte chemoattractant protein-1, plasmid gene therapy.

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Two main forms of inflammatory bowel diseases (IBD) are ulcerative colitis and Crohn's disease (CD). Their pathogenesis is highly similar at the level of genetics, environmental factors, bacterial communities and autoimmune/immune reaction (SCALDAFERRI & FIOCCHI 2007). The most widely used murine IBD model is the dextran sulfate sodium (DSS) induced colitis model with a similar response to human therapeutics as in IBD patients (MELGAR *et al.* 2008). DSS induced colitis is a model with simple feeding in drinking water, with lower mortality than the trinitrobenzene sulfonic acid induced model. The DSS induced colitis model is associated with modified epithelial architecture (NAKANO *et al.* 1999) and with increased levels of inflammatory cytokines and che-

mokines. The effects of DSS are dependent on the genetic background of the strain of experimental animals (MELGAR *et al.* 2005).

Levels of the inflammatory chemokine monocyte chemoattractant protein 1 (MCP-1) correlate with the degree of mucosal inflammation in patients with CD (HERFARTH *et al.* 2003). Therefore, we hypothesize that blocking the receptor of this chemokine might lower inflammation. The group of prof. Egashira repeatedly used the gene encoding an anti-inflammatory protein called 7ND (seven amino acids are deleted) in different disease models. This protein is able to bind to the MCP-1 receptor without activating it (EGASHIRA *et al.* 2002; INOUE *et al.* 2002; WADA *et al.* 2004).

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Inflammation is tightly connected with reactive oxygen species and oxidative stress. Antioxidative status in patients with IBD is lower than in healthy controls (KOUTROUBAKIS *et al.* 2004; PHYLACTOS *et al.* 2001). In addition, a decreased activity of copper zinc containing superoxide dismutase (SOD1) was found in inflamed bowel mucosa (MULDER *et al.* 1991). In this context, intake of low molecular weight antioxidants (vitamin C and E) lowered the levels of oxidative stress markers, although the disease course was not altered (AGHDASSI *et al.* 2003). We hypothesize that increasing the level of SOD1 might reduce oxidative stress, which is one of the main factors causing damage in the inflamed mucosa.

Application of therapeutic proteins targeting colonic mucosa is often ineffective and costly. Therefore we decided to use gene therapy using bacteria – bactofection. Bactofection has advantages over protein therapy or viral gene therapy in terms of costs and efficiency, as bacterial vehicles improve plasmid uptake by intestinal cells and protect the plasmids from degradation in the gastrointestinal tract (PÁLFFY *et al.* 2006). Vectors for intestinal bacterial gene therapy can be derived from standard bacteria for bactofection or can be prepared from bacteria with established protective effects on colon mucosa (HUDCOVIC *et al.* 2007; HUDCOVIC *et al.* 2008).

As a vector for bactofection we used attenuated *Salmonella typhimurium* SL7207 (DARJI *et al.* 1997). This bacterial strain was shown to be an efficient tool for bactofection (GRILLOT-COURVALIN *et al.* 2002; JIANG *et al.* 2007; PAGLIA *et al.* 1998). In this study we analyzed the effects of salmonella-mediated gene therapy using SOD1 and 7ND genes in DSS-induced colitis in rats.

Material and Methods

Animals

Sixty female Wistar rats (Dobrá Voda, Slovak Academy of Science) of average weight 190g were used in the experiment*. Rats were kept in separate cages in a controlled room with 12h light-dark cycle. Fifty animals (groups DSS, GFP, 7ND, SOD1 and 7ND/SOD1; n=10 per group) received 4% DSS (MP Biomedicals, MW 36 000-50 000; #160110) in drinking water ad libitum for 7 days according to the acute colitis protocol of Wirtz *et al.* (WIRTZ *et al.* 2007). The control group (CTRL; n=10) received drinking water. After DSS treatment animals received tap water for 7 days. On the day 14 application of bacteria was started. In all treatment groups (GFP, 7ND, SOD1 and 7ND/SOD1)

the animals received daily 5×10^7 colony forming units (CFU) of SL7207 carrying the appropriate plasmid in 1 ml Luria-Bertani Medium (LB) via gastric gavage. Animals in CTRL and DSS groups received 1ml of LB medium only. On day 21 the animals were sacrificed and samples were taken. Body weight, stool consistency (0-normal stool, 1-soft formed, 2-soft formed + bleeding, 3-watery stool with blood), vaginal temperature, food and water consumption as well as thiobarbituric acid reactive substances (TBARS) in stool homogenates were monitored every four days. The research on animals was approved by the ethical committee of the Institute of Pathophysiology, Comenius University.

Bacterial strains and plasmids

Bacterial strain *Salmonella typhimurium* SL7207 (DARJI *et al.* 1997) was transformed with plasmids pcDNA3-GFP, pcDNA3-SOD1, and pcDNA3-7ND using standard Ca^{2+} heat shock transformation protocol. The pcDNA3-SOD1 plasmid was obtained from Dr. Larry W. Oberley and Dr. Yuping Zhang from the University of Iowa, Iowa, USA (ZHANG *et al.* 2002). The pcDNA3-7ND plasmid originated from Prof. Kensuke Egashira from Kyushu University, Fukuoka, Japan. Bacteria SL7207 were grown in standard LB medium in the presence of ampicillin and streptomycin at 37°C. After over-night growth the concentration of bacteria was measured and adjusted to appropriate values.

Collection of colon samples

The whole colon was cut longitudinally and washed with phosphate buffered saline. Two approximately 100mg pieces were taken for cytokine assay and TBARS measurement. The remaining colonic mucosa was stored in 4% formaldehyde for histological examination.

The concentration of proteins in the samples was measured using the Lowry assay.

Thiobarbituric acid reactive substances measurement

TBARS were measured in colon and stool homogenates (10%) by a spectrophotometric assay after derivatization with 0.67 % thiobarbituric acid in acidic solution of acetic acid (95°C, 45 min). After derivatization, the colored product was extracted to n-butanol and measured on a microplate spectrophotometer ($\lambda = 535\text{nm}$). The TBARS concentration

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in colon homogenates was expressed on the basis of the calibration curve using 1,1,3,3-tetramethoxypropan as a standard in $\mu\text{mol/g}$ of proteins and concentration in stool homogenates in $\mu\text{mol/g}$ stool.

Cytokine assay

Colon tissue samples stored in protease inhibitor solution were homogenized using a rotor-stator homogenizer. After centrifugation of colon homogenates and plasma samples the levels of proinflammatory cytokines (IL-1beta, IL-6, TNFalpha) were measured in supernatants using commercially available ELISA kits (Bender MedSystems). Cytokine concentrations were expressed in pg/mg of proteins.

Statistical analysis

Data were analyzed using one-way ANOVA. A Bonferroni *t*-test was used to evaluate the differ-

ences between groups. P-values less than 0.05 were considered significant. The calculations were performed with XLstatistics and Microsoft Excel 2007. Data are presented as means with standard deviation.

Results

Clinical monitoring

We monitored the clinical status, stool quality, food and water intake. There were no significant differences in food and water intake, vaginal temperature or stool TBARS levels during the study (data not shown). A significant difference between the CTRL group and groups with 4% DSS in stool consistency was found, but only at the end of the DSS treatment (measured at day 9). During the recovery phase (days 7-14) the stool consistency re-

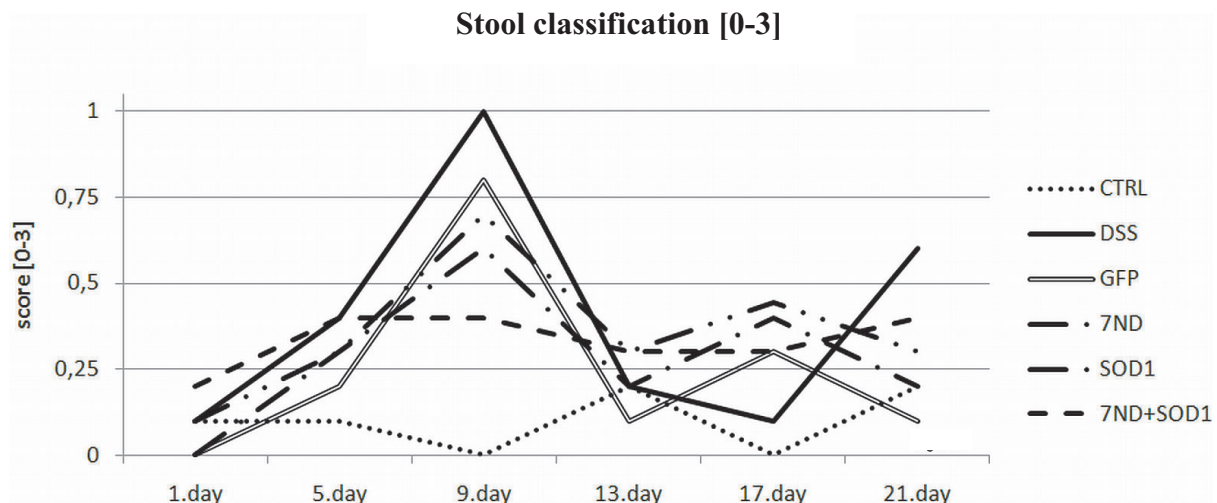


Fig. 1. Stool consistency was classified as follows: 0-normal stool, 1-soft formed, 2-soft formed + bleeding, 3- watery stool with blood. Stool samples were monitored every 4 days. Significant differences were found between DSS treated groups and control group at the end of DSS treatment (day 9).

TNFalpha concentration in plasma (NS)

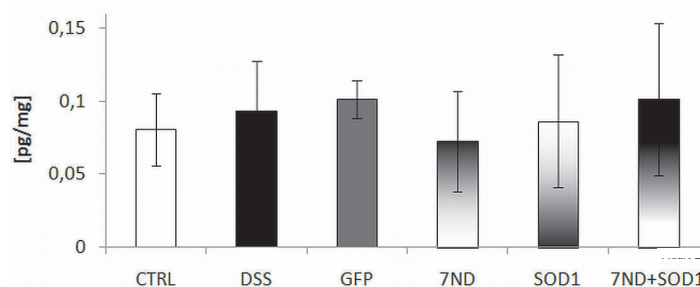


Fig. 2. TNFalpha levels in plasma samples. The values are expressed in pg/mg of plasma proteins. No significant differences between the groups were found.

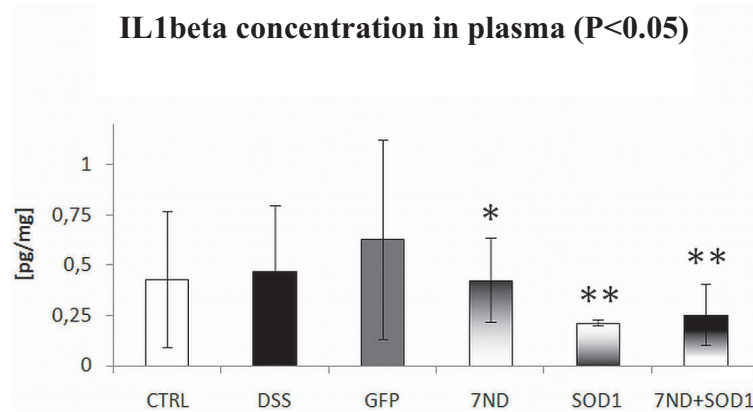


Fig. 3. IL1beta levels in plasma samples. The values are expressed in pg/mg of plasma proteins. Significant differences were found between all treatment groups and DSS group (* – $P < 0.05$; ** – $P < 0.01$).

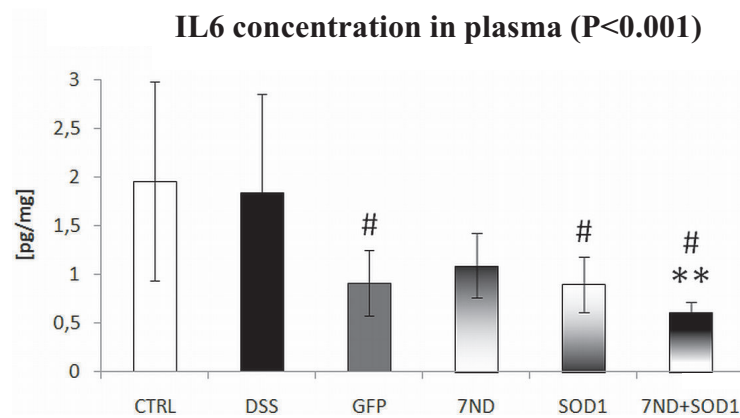


Fig. 4. IL6 levels in plasma samples. The values are expressed in pg/mg of plasma proteins. Significant differences were found between 7ND+SOD1 treatment group and DSS group (** – $P < 0.01$) and between GFP, SOD1, 7ND+SOD1 groups and CTRL group (# – $P < 0.05$).

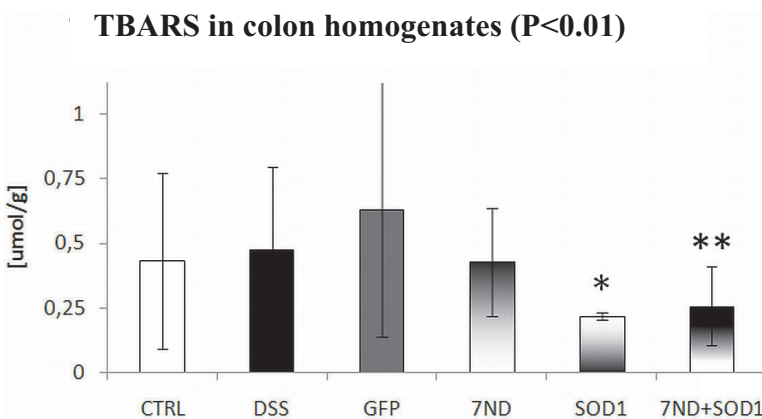


Fig. 5. TBARS levels in colon homogenates. The values are expressed in umol/g of proteins. Significant differences were found between SOD1, 7ND-SOD1 treatment groups and DSS group (* – $P < 0.05$; ** – $P < 0.01$).

turned to normal values (similar to CTRL group) in all DSS treated groups (Fig. 1).

Effects of DSS treatment and bactofection

After 21 days there were no significant differences between the CTRL group and DSS group in macroscopic and microscopic scores after histo-

logical examination. Furthermore, no effect of DSS treatment on levels of inflammatory cytokines (IL1beta, IL6, TNFalpha) in colon homogenates was found. The TNFalpha levels in plasma samples were not significantly changed by either DSS treatment or bactofection (Fig. 2); however, IL1beta and IL6 levels were lowered in all treatment groups in comparison to the DSS group. The levels of IL1beta were improved by all treatments

including antioxidative, anti-inflammatory and combined therapy (Fig. 3); combined therapy with 7ND and SOD1 decreased plasma levels of IL6 significantly when compared with the DSS group (Fig. 4). In addition, the combined therapy reduced the TBARS levels in colon homogenates significantly (Fig. 5).

Discussion

The aim of our study was to analyze the effects of anti-inflammatory, antioxidative and combined therapy with SOD1 and 7ND in a DSS induced colitis. The strategy of gene delivery was based on bactofection using attenuated *Salmonella* strain SL7207. The effects of salmonella mediated gene therapy in colon were described for the first time. In addition, in our study SOD1 and 7ND were used for the first time in a form of plasmid therapy in IBD.

The approach using live attenuated bacteria that are able to deliver plasmid DNA into the cells of colon mucosa was described previously (CASTAGLIUOLO *et al.* 2005). In that study attenuated *Escherichia coli* carrying gene *inv* encoding invasins from *Yersinia pseudotuberculosis* and gene *hly* encoding listeriolysin O (LLO) from *Listeria monocytogenes* were applied (GRILLOT-COURVALIN *et al.* 1998). As a therapeutic gene mouse TGFbeta-1 under control of the CMV promoter was used. Bacteria were administered orally to 2, 4-dinitrobenzen sulfonic acid treated mice. Bacterial therapy significantly decreased the levels of oxidative stress markers as well as the macroscopic score of colonic mucosa injury (CASTAGLIUOLO *et al.* 2005).

In bacterial gene therapy the use of prebiotics might be beneficial due to better surveillance of bacteria in the murine gastrointestinal tract and due to additional attenuation of DSS induced injury (KANAUCHI *et al.* 2003). The plasmid gene therapy with pressurized local microinjection of the vector with cationic lipids represents an alternative way to insert therapeutic genes into the colonic mucosa. In a study with T-cell receptor á knockout mice, IL22 gene therapy ameliorated intestinal inflammation (SUGIMOTO *et al.* 2008). Extracellular (EC)-SOD treatment using an ex vivo gene therapy approach lowered the mortality in models of both mild and severe DSS induced colitis in mice. Parameters of oxidative stress and the levels of pro-inflammatory cytokines also improved (OKU *et al.* 2006).

The inflammation caused by administration of 4% DSS during 7 days in our experiment was very low which stands in contrast to other studies using this model (GAUDIO *et al.* 1999 ; (STEIDLER *et al.*

2000; WIRTZ & NEURATH 2007). The recovery phase in our design might have been too long. Another possible explanation of our findings may be a lower sensitivity of the rat strain to DSS (Wistar, Dobrá Voda, Slovak Academy of Sciences) used in this study. However, whether our experimental animals were less sensitive to the DSS treatment or showed accelerated regeneration cannot be extrapolated from the results.

The efficiency of the tested therapy was difficult to evaluate because of the low impact of DSS application. The best response was achieved in IL1beta in plasma, in which all treatment groups had lowered levels of this inflammation marker when compared to the DSS group. In general, the combined treatment using anti-inflammatory and antioxidative gene therapy showed the best response in most analyzed parameters, as it lowered the plasma levels of IL1beta, IL6 and also the level of TBARS in colon homogenates significantly. However, in contrast to other studies using DSS where the treatment significantly increased TBARS in colon (DAMIANI *et al.* 2007), in our study this was not the case.

Our study provides a rationale for more rigorous choice of experimental model and animal strain used for studies concentrating on therapeutic modulation of IBD, as it has been shown for mice strains (BILLEREY-LARMONIER *et al.* 2008; STEVCEVA *et al.* 1999). On the other hand, our results might point towards a potential synergic effect and relevance of combined approaches based on application of at least two distinct therapeutic agents also in the field of gene therapy. Further studies are needed to support the hypothesis of combined anti-inflammatory and antioxidative gene therapy in IBD.

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