DOI: HTTP://DOI.ORG/10.31925/FARMACIA.2018.6.15 **ORIGINAL ARTICLE**

THE ADMINISTRATION OF *TRICHURIS SUIS OVA* EXTRACT DECREASES IL-17/IL-23 LEVELS IN EXPERIMENTAL INFLAMMATORY BOWEL DISEASE

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Abstract

This study investigated the anti-inflammatory effect of *Trichuris suis ova* extract (TSOE) on four cytokine biomarkers (IL-17, IL-23, IL-6 and IL-10) and on oxidative stress (erythrocyte superoxide dismutase - SOD and whole blood glutathione peroxidase - GPx). The inflammatory bowel disease (IBD) murine model was induced in rats with dextran sulphate sodium (DSS). The experiment was performed on 20 male Wistar rats. Group 1 - control group (CG) received only water; in groups 2 (DSS2), 3 (DSS3) and 4 (DSS4), 3% DSS was administered. The groups 3 and 4 received 100 µg TSOE respectively 200 µg TSOE. The mean level of IL- 23 was significantly higher in the DSS2 group compared to the CG (0.43 pg/mL *vs* 0.023 pg/mL). After TSOE treatment, the IL-23 concentration was higher in the DSS2 group compared to the DSS3 and DSS4 groups (0.43 *vs* 0.06 and 0.08 pg/mL, respectively). IL-23 was also paralleled by a decrease in the IL-17 mean level, but without statistical significance (Brown-Forsythe test, F (6; 4.34) = 2.54, p = 0.182). TSOE reduced IL-17/IL-23 levels in the DSS- induced IBD experimental model.

Rezumat

Studiul a investigat efectul antiinflamator al extractului din ouă embrionate de *Trichuris suis* (TSOE) prin evaluarea nivelului citokinelor IL-17, IL-23, IL-6 și IL-10 și a unor biomarkeri ai stresului oxidativ (superoxid dismutaza eritrocitară - SOD și glutation peroxidaza din sângele integral - GPx). Modelul murin de inflamație intestinală a fost indus la șobolani cu dextran sulfat de sodiu (DSS). Experimentul a fost efectuat pe 20 șobolani masculi sușa Wistar. Grupul 1 - grupul de control (CG) a primit doar apă; grupurilor 2 (DSS2), 3 (DSS3) și 4 (DSS4) li s-a administrat DSS 3 %. Grupurile 3 și 4 au primit 100 µg TSOE, respectiv 200 µg TSOE. Concentrația IL-23 a fost semnificativ mai mare în grupul DSS2 comparativ cu CG (0,43 pg/mL *vs* 0,023 pg/mL). După tratamentul cu TSOE, concentrația IL-23 a fost mai mare în grupul DSS2 comparativ cu grupurile DSS3 și DSS4 (0,43 *vs* 0,06 respectiv 0,08 pg/mL). IL-23 a fost, de asemenea asociată cu o scădere a nivelului mediu al IL-17, dar fără semnificație statistică (test Brown-Forsythe, F (6, 4,34) = 2,54, p = 0,182). TSOE a redus nivelele IL-17/IL-23 în modelul experimental de boală inflamatorie intestinală indus de DSS.

Keywords: *Trichuris suis ova*, inflammatory bowel disease (IBD), inflammation, IL-17/ IL-23, cytokines, oxidative stress, rats

Introduction

Inflammatory bowel disease (IBD) has a complex pathogenesis involving immunologic changes triggered by genetic and environmental factors [14]. The incidence and prevalence of IBD increased in different regions around the world, which indicates its emergence as a global disease [9, 19]. Modern therapy uses anti-TNF alfa (tumour necrosis factor) antagonistic drugs, and small molecules such as anti-integrins [5, 6, 7, 25]. Consequently, new treatment methods based on easily accepted and cost-efficient principle have become the greatest challenge for research on IBD monitoring and therapy [14]. *Trichuris suis ova* (TSO) have been promoted for the past 10 years and helminth immune modulation seems to be phase-specific. Feeding mice with one type of intestinal worm returned their mucus-producing cells to normal and shifted the bacterial composition in their guts [15]. In this context, the helminth infection might protect against inflammation in IBD, by modulating the immune system [14, 15]. The ability of helminths to manipulate the immune response of their hosts to a state described as "anti-inflammatory tolerance" has opened up the exciting possibility that earthworm products could be used as new anti-inflammatory treatment in IBD, which led to the controlled re-introduction of helminthic therapy as a viable approach [11, 17]. Chronic inflammation is also manifested by an increase in the level of pro-inflammatory cytokines coded by genes activated by the critical NF- kB transcription factor [5, 9, 21]. Inflammatory processes are plastic as they may be shaped by adequate external interventions, which could lead to a disease-free phenotype because they are involved in targeting central components of the immune system [4, 9, 23, 18]. An absolute advantage of TSO therapy is the minimal risk of accidental colonization because of the particular life cycle of this helminth. After ingestion of TSO, the larvae hatch from eggs and colonize the human colon and caecum for a few weeks, thus periodic re-administration is required although the loss of chronic infection removes any other public health risks [11]. No adverse effects, no high costs and no risk of immunosuppression were recorded after TSO inoculation in patients with chronic inflammation, while the Th1/Th17 proinflammatory response shifted towards the more anti-inflammatory Th2 response [20]. Work on experimental models has positively established that the immunoregulatory mechanisms generated by helminths is dependent on a type 2 cytokine reply. Besides the type 2 response, evidence suggests that helminths induce complementary mechanisms including increases in regulatory T cell (Treg) total and also IL-10 and/or transforming growth factor beta (TGF-β) concentrations, thus creating a real anti-inflammatory environment [11]. Immunohistochemical studies revealed that COX (cyclooxygenase)-1 was expressed in *lamina propria* and in areas of the crypt epithelium corresponding to the proliferative zone. COX-2 is inducible by cytokines such as TNF- alpha activated through the NF-kappa B pathway [5]. In another inflammation experimental model, a direct link between oxidative stress and pro-inflammatory status was observed [2, 3]. The presence of reactive oxygen species (ROS) triggers the gene expression pathways involved in inflammation. ROS are considered secondary messengers for the mediated activation of NF- kB [4]. ROS oxidize the NF- kB subunits thus blocking DNA binding and the transcriptional activity of this factor. During inflammation, antioxidant protection decreases when the following two oxidative stress markers are affected: SOD (superoxide dismutase), which catalyses the dismutation of superoxide radical into hydrogen peroxide and oxygen or GPx (glutathione peroxidase) which plays a critical role in the reduction of lipid and hydrogen peroxides [3]. Another biomarker for this type of inflammation could be asymmetric dimethyl-arginine (ADMA), which leads to elevated serum levels, the vascular origin of ADMA and its inhibitory effect on nitric oxide synthase produced by the endothelium (eNOS) playing an essential role in IBD [16]. This study aimed to evaluate the effect of TSO antigens on some pro-inflammatory cytokines, oxidative stress and ADMA serum levels in experimental models (control and study group animals).

Materials and Methods

Study design

We have used 20 male Wistar rats weighting between 200 and 300g, which were maintained in a restricted access room, in plastic cages, under standard laboratory conditions (room temperature of 22°C, humidity 50 - 60%, on a 12/12h light/dark cycle). They had free access to standard laboratory rodent pellet formula diet. Twelve hours before the experiment, the animals underwent a fasting period with water *ad libitum*. All applicable international, national and institutional guidelines (Ethical approval no 74/ 20.02.2014) according to Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes were followed.

The solution of 3% dextran sulphate sodium (DSS) was used to induce the IBD, it was prepared fresh each day by adding DSS (MW 5000Da, Sigma, USA) to the rats' drinking water. A random number generation program was used to distribute the animals in four different groups (5 rats in each group), as follows: group 1 - CG rats received tap

water; groups 2 (DSS2), 3 (DSS3) and 4 (DSS4) received 3% DSS added to their drinking water, four cycles of seven days with a seven day break between two cycles. *T. suis* eggs were obtained by dissecting pregnant female worms collected from wild boars, under stereomicroscope. Next, the eggs were incubated in 0.5% formalin at 33°C until embryonation, i.e. around 38 days. The embryonated eggs underwent repeated washings with saline solution (0.9% NaCl) to remove the formalin. In the final suspension, a number of 76000 embryonated eggs/mL was counted (760 eggs/0.01 mL). Tissuelyzer was used to disrupt the TSO, 5 cycles of 3 minutes, followed by 20 minutes of ultrasonication. The product obtained was kept at -18°C until use. In the eight week of the experiment, the inoculation of an original *Trichuris suis ova* extract (TSOE) started using the following protocol: group 3 received 100 µg TSOE (corresponding to 7600 TSO), subcutaneous inoculation, 4 inoculations at a 3 - day interval; group 4 received a dose of 200 µg TSOE (corresponding to 15200 TSO), subcutaneous inoculation, 4 inoculations at a 3 - day interval; intra-cardiac blood samples were obtained under anaesthesia (isoflurane) at the end of the experiment. Anaesthesia was performed using an induction chamber, with 5% isoflurane (Iso-Vet, Piramal Healthcare UK Limited, Northumberland, United Kingdom) in 100% oxygen delivered at 5 L/min until loss of righting reflex. During the procedure, anaesthesia was maintained with 1.2% isoflurane in 100% oxygen with a flow of 1 L/min. The effect of chronic administration of DSS and TSOE was evaluated by the assay of the activities of whole blood GPx and erythrocyte SOD using commercially available kits as well as by the ELISA test used for cytokine dosage. Venous blood samples were drawn into lithium heparin collection tubes. GPx activity was quantified at 37°C using a RANSEL kit (Randox Labs., UK) on a Cobas Mira Plus (Roche) analyser at a wavelength of 340 nm. SOD activity assay was determined using a RANSOD kit (RANDOX Labs., UK). The haemoglobin (Hb) concentration was determined using Drabkin's method [8]. Rat IL-17, 23, 6, 10 ELISA Kits (ABBEXA, UK) were used for the quantitative detection of serum cytokines. A rat ADMA ELISA commercial kit (MyBioSource Labs., USA) was used to determine its concentration in serum.

Statistical analyses

Arithmetic mean and standard deviation were employed to summarize continuous quantitative variables in the four studied groups using the Gaussian distribution. ANOVA and Welch's ANOVA test (as well as Tukey's or Games-Howell test in the post-hoc analysis) were used to compare interleukin values and oxidative stress parameters in the four groups. Pearson's Linear Correlation Coefficient (r) was used to describe linear correlations between oxidative stress parameters and interleukins. The statistical significance level was set to $\alpha = 0.05$ for all two-sided statistical tests. The statistical data analysis was performed with R version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Descriptive statistics of the studied parameters for each group are described in Tables I-III. We found that IL-23 mean values were significant different in the studied groups (Welch's ANOVA non-parametric test, $F(3; 6.99) = 23.69$, $p = 0.0005$). The Games Howell post-hoc test revealed that there were differences in IL-23 mean values with a tendency toward statistical significance (Table II) in the mean level of IL-23 which was lower in CG compared to DSS2 (0.06 pg/mL *vs* 0.43 pg/mL). At the end of the experiment, IL-23 serum mean level was lower in DSS3 and DSS4 compared to DSS2 (0.03 and 0.08 pg/mL, respectively *vs* 0.43) (Figure 1).

Table I

IL-6, IL-10, IL-17, IL-23, GPx, Hb, SOD in the studied groups

	IL-6 (pg/mL)	IL-10 (pg/mL)	IL-17 (pg/mL)	IL-23 (pg/mL)	GPx (U/gHb)	gHb g/dL	SOD U/gHb
Groups	**Mean ± SD**						
Control	100 ± 17	283.14 ± 178.32	37.60 ± 4.66	0.06 ± 0.02	897.83 ± 132.26	12.58 ± 1.12	240.78 ± 50.99
DSS2	84 ± 19.78	1237.57 ± 235.95	55.60 ± 28.70	0.43 ± 0.14	898.45 ± 33.46	11.88 ± 0.59	273.6 ± 20.06
DSS3	118.67 ± 11.93	322.57 ± 338.63	38.00 ± 3.85	0.03 ± 0	930.04 ± 181.48	13.4 ± 0.38	264.27 ± 24.63
DSS4	104 ± 39.61	800.29 ± 952.99	38.20 ± 5.63	0.08 ± 0.02	1092.78 ± 99.95	14.2 ± 0.45	264.4 ± 24.54

IL-6, IL-10, IL-17, IL-23 represent interleukins 6, 10, 17 and 23 respectively, SOD = superoxide dismutase, GPx = glutathione peroxidase, Hb = haemoglobin

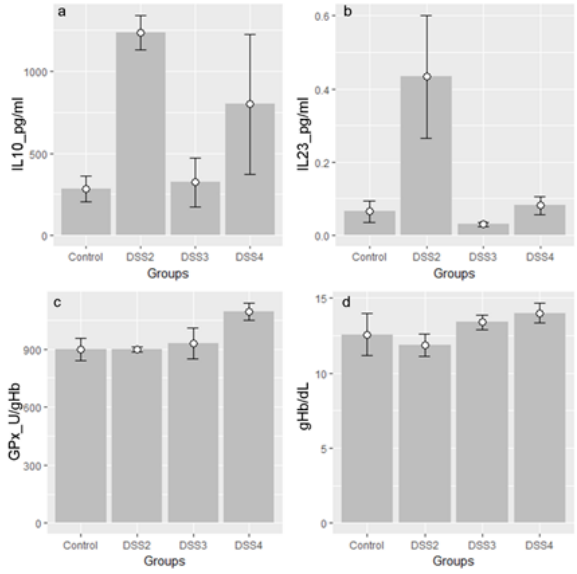

Figure 1.

The effects of TSO administration on cytokines (a and b), GPx activity (c) and Hb level (d)

Note. Each point represents a mean value. Error bars for IL-10 and GPx represent one SE (standard error) while error bars for IL-23 and gHb represent the lower and upper limits of the 95% confidence interval for mean values

Table II

Post-hoc analysis for significant differences regarding IL-10, IL-23 and Hb in the studied groups

Pairwise comparison of studied parameters by Tukey or Games-Howell post-hoc tests			
	IL10 (pg/mL)	**IL23 (pg/mL)**	**Hb (g/dL)**
Control *versus* DSS2	0.049*	0.056	0.434
Control *versus* DSS3	0.999	0.199	0.302
Control *versus* DSS4	0.431	0.664	0.028*
DSS2 *versus* DSS3	0.062	0.054	0.019*
DSS2 *versus* DSS4	0.568	0.056	0.001*
DSS3 *versus* DSS4	0.498	0.056	0.553

*adjusted p-value < 0.05 denoted statistical signification; italic values denoted a tendency toward statistical signification (p < 0.10)
IL-10, IL-23 represent interleukins 10 and 23, Hb = haemoglobin

Table III

Pearson correlation coefficients in DSS2, DSS3 and DSS4 groups

DSS2								
	ADMA	**IL6**	**IL10**	**IL23**	**IL17**	**SOD (U/gHb)**	**Hb (g/dL)**	**GPx (U/gHb)**
ADMA	1							
IL6	0.94*	1						
IL10	-0.80	-0.85	1					
IL23	0.60	0.71	-0.55	1				
IL17	0.51	0.46	-0.49	-0.29	1			
SOD (U/gHb)	-0.24	-0.39	-0.07	-0.20	-0.25	1		
Hb (g/dL)	-0.12	-0.38	0.38	-0.81	0.48	0.08	1	
GPx (U/gHb)	-0.20	-0.27	0.41	0.40	-0.90*	0.21	-0.33	1
DSS3								
	ADMA	**IL6**	**IL10**	**IL23**	**IL17**	**SOD (U/gHb)**	**Hb (g/dL)**	**GPx (U/gHb)**
ADMA	1							
IL6	-0.68	1						
IL10	0.91*	-0.60	1					
IL23	0.19	0.07	0.24	1				
IL17	0.16	0.26	0.48	0.61	1			
SOD (U/gHb)	-0.14	0.57	-0.34	-0.43	-0.29	1		
Hb (g/dL)	0.06	0.01	0.01	-0.91*	-0.38	0.61	1	
GPx (U/gHb)	0.24	-0.47	-0.04	0.44	-0.40	-0.33	-0.59	1

DSS4								
	ADMA	**IL6**	**IL10**	**IL23**	**IL17**	**SOD (U/gHb)**	**Hb (g/dL)**	**GPx (U/gHb)**
ADMA	1							
IL6	-0.56	1						
IL10	0.19	-0.57	1					
IL23	-0.83	0.36	-0.34	1				
IL17	-0.09	-0.07	0.77	-0.39	1			
SOD (U/gHb)	-0.18	-0.36	0.85	0.17	0.53	1		
Hb (g/dL)	0.01	-0.44	-0.40	0.34	-0.68	-0.31	1	
GPx (U/gHb)	-0.01	0.36	0.23	0.07	0.13	0.45	-0.71	1

*$p < 0.05$; italic values denoted a tendency toward statistical signification ($p < 0.10$).
IL-6, IL-10, IL-17, IL-23 represent interleukins 6, 10, 17 and 23 respectively, SOD = superoxide dismutase, GPx = glutathione peroxidase, Hb = haemoglobin

There was a statistically significant difference in IL-10 mean values between the studied groups (ANOVA test, $F (3; 16) = 4.72$, $p = 0.035$). Using the data post-hoc analysis, there was a significant increase in mean concentrations between CG and DSS2 (Table II). A decrease in the mean level of IL-10 with a tendency toward statistical significance ($p = 0.054$) was registered after TSO inoculation between DSS3 and DSS2 (322.57 pg/mL *vs* 1237.57 pg/mL). IL-17 cytokine level also decreased in DSS3 and DSS4 groups, compared with DSS2 group, but without statistical significance (Anova test, $F (3;16) = 1.75$, $p = 0.197$). A significant positive linear correlation was reported between ADMA and IL-6 in the DSS2 group ($r = 0.94$) and also a significant negative correlation between IL-17 and GPx ($r = -0.90$). We registered a significant positive linear correlation between IL-10 and ADMA in the third group ($r = 0.91$) and a statistically significant negative correlation between Hb level and IL-23 concentration too ($r = -0.91$). A statistically significant negative linear correlation was established between SOD and IL-17 in the TNBS5 group ($r = -0.91$).

The experimental model of IBD is a valid and reproducible one, as previously reported in other study. An increase in IL-10 serum concentration was revealed in the DSS2 group compared to CG. This level decreased significantly after treatment with 100 μg TSOE (DSS3 *vs* DSS2). The administration of 200 μg TSOE increased the mean level of IL-10 in DSS4 compared to DSS3, but without statistical significance. After TSOE administration, a decrease in the IL-6 mean level in the DSS4 group compared to DSS3 group of rats was recorded. Thus, helminth immune modulation seems to be dose-dependent. Certain studies revealed a clear dose-dependent negative association between chronic infections with helminths and inflammatory diseases mediated by pro and anti-inflammatory cytokines, especially IL-10 [12, 22]. The immuno-regulatory properties of helminths involve both innate and adaptive immune mechanisms such as the suppression of interleukin (IL)-23 expression, IL-17 and interferon (IFN)γ, exacerbated type 2 cytokine responses, with induction of the regulatory cytokine IL-10, transforming growth factor (TGFβ) and T cell responses [11]. The significant increase in IL-17 and IL-23 levels in the DSS group proved the presence of the inflammatory process. In addition, the statistically significant decrease in the IL-23 mean level in DSS3 compared to DSS2 group, paralleled with the significant increase in Hb concentration and also the decrease with statistical significance in the IL-10 level suggests that the TSOE had beneficial effects in this experimental model of IBD. Moreover, a negative and statistically significant correlation ($r = -0.91$) was observed between the Hb level and the IL-23 mean level after TSO extract administration (DSS3 group). Our data are in agreement with the fact that in IBD, anaemia is the effect of pro-inflammatory cytokines on the cells of the reticulo-endothelial system, which leads to changes in iron balance and reduces the longevity of red blood cells through the hepcidin pathway, a key regulator of the iron metabolism stimulated by inflammation [1, 13]. No statistically significant differences were registered in Hb level in DSS3 compared to CG ($p > 0.05$), which indicates the beneficial effect of the TSOE therapy. DSS administration led to a decrease with statistical significance in Hb level in the DSS2 group compared to CG. Conversely, a significant increase in Hb was registered after therapy with 100 μg TSO extract (the DSS3 group compared to the DSS2 group). The statistically significant increase in IL-23 in the DSS2 group vs. the control group decreased significantly after the administration of 100 μg TSOE (DSS3) and 200 μg TSOE (DSS4), respectively. However, IL-23 in the DSS4 group was significantly higher compared to the DSS3 group. Although the IL-23 mean level was significantly lower in DSS4 compared to DSS2 group, a significant increase in IL-23 concentration was revealed in DSS4 compared to the DSS3 group. This could suggest that the dose of 200μg TSOE has a pro-inflammatory effect. IL-17 varied similarly to IL-23 but without statistical significance. This proves once again the crucial role of the IL-

17/IL-23 pathogenic axis in IBD [5]. The activity of antioxidative enzymes (SOD and GPx) did not vary statistically significantly after the administration of DSS or TSOE treatment. Positive correlations were observed between IL-17 (r = 0.51) and IL-23 (r = 0.6) mean concentrations and ADMA in DSS2 group. A pro-inflammatory milieu with high concentrations of IL-17/IL-23 induces a prothrombotic state in endothelial cells [24]. Our results showed a connection between the proinflammatory state, oxidative stress and TSOE administration, in the inflammatory model induced by DSS.

Conclusions

This study demonstrated that TSOE reduced the pro-inflammatory cytokines, thus reducing inflammation, in the DSS experimental ulcerative colitis model corresponding to the human IBD and being reproducible with characteristics indicative of chronic inflammation in the case of the mentioned protocol.

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