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Short Communication

Intestinal ischemia-reperfusion injury mediates expression of inflammatory cytokines in rats

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Abstract. The small intestine is an organ with very well developed immunological activity, responsible for synthesis of specific inflammatory mediators that participate in causing the systemic inflammation that can occur after ischemia-reperfusion injury. The aim of our work was to study mRNA expression and protein concentration of inflammatory cytokines IL-10 and TNFα in the jejunal wall after intestinal ischemia-reperfusion injury (IRI). Cytokine concentration levels confirmed the direct effect of IRI on the inflammation process. The results refer to the changes in balance between pro-inflammatory and anti-inflammatory mediators and indicate that the predominant disturbance of homeostasis after intestinal IRI is present after 1 hour of reperfusion.

Key words: Intestinal ischemia-reperfusion — Cytokines — Rat

Ischemia-reperfusion injury (IRI) of the small intestine is a very important process associated with the induction of inflammation and tissue injury. Ischemia is followed by restoration of the blood flow/reperfusion. Paradoxically, reperfusion injury is more severe and causes more damage through the production of inflammatory and apoptotic mediators and free oxygen species than ischemia itself. During IRI activated neutrophils in the blood stream cause changes in homeostasis of the organism by the administration of inflammatory mediators that could lead to multiple organ failure (MOF) (Moore et al. 2005) or even to MODS (Multiple organ dysfunction syndrome). During shock, the specific cells in the small intestine undergo extensive cell death and intestine itself is the source of the inflammatory factors (Penn and Schmid-Schonbein 2008). Reperfusion of the small intestine causes the release of the cytokines, pro-inflammatory lipids and proteins into the extravascular area. These pro-inflammatory mediators are subsequently transported by the lymph and reabsorbed back to the blood stream, where they affect the polymorphonuclear neutrophils (Moore et al. 2005; Senthill et al. 2006).

Several clinical studies point to the fact, that increased levels of circulating cytokines like TNFα, IL-10, IL-6 or IL-1β that are upregulated by pro-inflammatory cytokine TNFα are associated with organ dysfunction, inflammation, and are causes of death (Pellegrini et al. 1996; Struber et al. 1996; Kriegel and Amiji 2011). TNFα has an important role in inflammatory processes because through the activation of its antagonists the processes of acute and chronic inflammation phase could be blocked (Ferenčík et al. 2009). Stimuli for the expression of TNFα can be bacterial, viral, parasital, or acute ischemic origin (Huang et al. 2009; Lula et al. 2009; Roman-Campos 2009). After IRI, TNFα is synthetized by macrophages, lymphatic cells, mast cells, endothelial cells, fibroblasts and even nerve cells (Gilles et al. 2003). IL-10 is the anti-inflammatory agent, or antagonist which downregulates TNFα (Meisel et al.

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1996) and it is produced by a wide variety of cells such as macrophages, B-cells, T-lymphocytes, or monocytes. The regulation of cytokine release is carried out through a series of specific negative and positive feedback loops. Increased concentration of TNFα is involved in upregulation of IL-10 (Meisel et al. 1996). Nussler et al. (2003) stated the surprising result that the expression of IL-10, a well-known anti-inflammatory cytokine, led to increased tissue injury in the small intestine and liver.

The purpose of this study was to explore the changes in specific inflammatory cytokines, their specific roles in affecting the inflammation process and homeostasis of the organism, considering the early phases of IRI (1 hour) and comparing the results with longer reperfusion periods (24 hours and 30 days).

This study was approved by the Committee for Ethics on Animal Experiments at the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia, and the experimental protocol was approved by decision of the State Veterinary and Food Administration of the Slovak Republic No. 2843/08-221a

45 adult male Wistar rats, 3–4 months old, with approximate weight about 250–350 g, were randomly assigned into three experimental groups which underwent ischemia by complete occlusion of the mesenteric artery for one hour followed by 1 h (R1, $n = 10$), 24 h (R24, $n = 10$) and 30 days (R30, $n = 10$) of reperfusion periods. Animals in the control group (C, $n = 15$, 5 in each control group) underwent laparotomy, without ischemic attack. Surgical procedure was thoroughly described by Tóth et al. (2012). After the reperfusion injury the animals were sacrificed, and two 3 cm long jejunal samples for RNA isolation and ELISA were taken approximately10 cm from the *Ligamentum Trietzi,* washed with cold saline, dried and frozen thoroughly in liquid nitrogen.

Total RNA was isolated from the complete wall of the small intestine using the Trizol separation method (Invitrogen, Carlsbad, CA) and Qiagen RNeasy mini kit (Qiagen, Germany) according to the manufacturer´s protocol. RNA concentration and purity were determined by spectrophotometry (A_{260}/A_{280} ratio) using a Biophotometer plus (Eppendorf, Germany). Total mRNA was transcripted to cDNA by using Revert Aid H minus FirstStrand cDNA Synthesis Kit (Fermentas, Germany). Inflammatory cytokine expression in the jejunal wall was measured using PCR amplification for 30 cycles. For TNFα, an initial denaturation step at 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 64.2°C for 60 s and 72°C for 45 s. For IL-10, an initial denaturation step at 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 52.5°C for 60 s and 72°C for 45 s. GAPDH was used as a reference gene, initial denaturation step at 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 55.4°C for 60 s and 72°C for 45 s. The primers had the following sequences (Tab. 1). RT-PCR products were separated electrophoretically on 0.8% agarose gels, stained with EvaGreen (Fermentas, Germany), and DNA band intensities were measured using G:BOX Chem, SyngeneTools Software. Each sample was done three times. For protein assay, tissue with approximate weight of 30–80 mg was homogenized in PBS, with 30 mg of tissue homogenize in 400 µl of PBS + 2 µl proteinase inhibitor (Sigma, Germany), and right after homogenization another 400 µl of PBS + 2 µl of proteinase inhibitor was added. After centrifugation (8000 rpm/8 min/4°C), the supernatant was collected and diluted 1:4 in sample diluent present in the kit (Platinum ELISA rat IL-10 and TNFα, eBioscience, Bender Medsystem, Austria). Cytokine assays were performed as described in the manufacturer´s protocol. Each reaction in the kit was done in duplicate. Data analyses were performed using µQuant Reader (Biotech) operating Gen5 software. For quantification of total protein in tissue, Bradford Assays were performed. Data are presented as mean ± standard error of the mean (SEM). One way analysis of variance (ANOVA) and Student-Newmann-Keuls tests were used for comparison of the experimental groups. The significance level was established as $p \leq 0.05$ for all the tests. Statistical analysis was evaluated using the GraphPadInSTAT programme.

Table 1. Sequences of primers used for PCR

Gene	Sequences of primers	Size (bp)
TNFα	FP: GAGCCCCCAATCTGTGTCCTTCTA RP: CCCCGGCCTTCCAAATAAATACAT	454
IL-10	FP: TTCCATCCGGGGTGACAATA RP: CAGTAGATGCCGGGTGGTTC	490
GAPDH	FP: TGGGGCCAAAAGGGTCATCATCTC RP: GCCGCCTGCTTCACCACCTTCTT	456

In this experiment, we focused on observing the early stages of intestinal IRI with regard to cytokine release. We observed the direct effect of the early phases of IRI on the mRNA levels of inflammatory cytokines TNFα and IL-10. We studied the levels of cytokines after 1 hour, 24 hours and 30 days of reperfusion comparing with control groups. We observed significantly increased mRNA expression of anti-inflammatory cytokine IL-10 (Fig. 1) which correlated with the concentration of proteins in the tissue (Tab. 2) in experimental groups after 1 hour and 30 days of reperfusion. As regards pro-inflammatory cytokine TNFα, significant changes in mRNA expression (Fig. 2) had similar tendencies as changes in protein concentration (Tab. 2) after 1 hour of reperfusion. We can assume that the highest differences in expression of both cytokines were present after 1 hour of reperfusion probably as a direct reaction to the ischemic attack. As it could be seen in the results (Tab. 2) the protein concentration in control group 1 hour after reperfusion

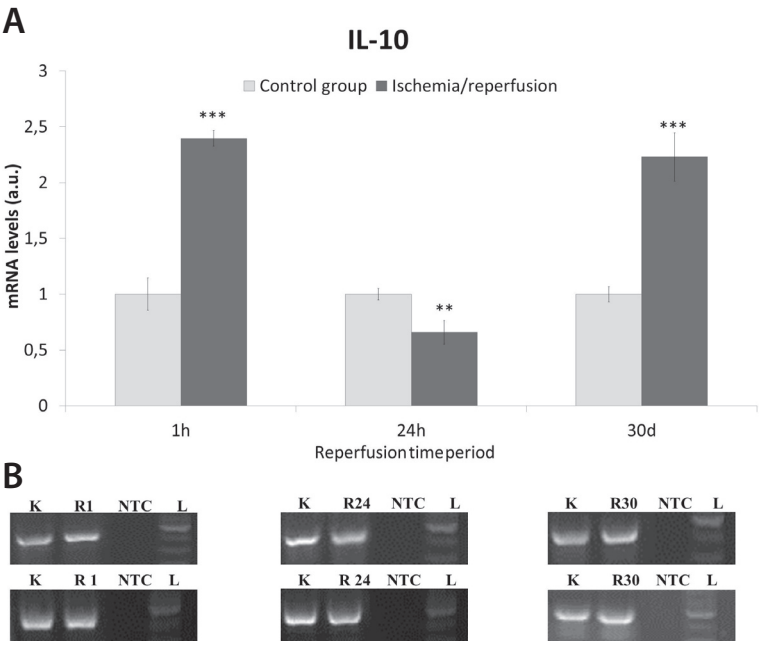

Figure 1. mRNA levels of IL-10 in rat small intestine after ischemia/reperfusion injury. **A.** Semiquantification graph of gene expression. Results are presented as mean ± SEM. *** $p < 0.001$; ** $p < 0.01$ significantly different as compared to controls. C1, C24, C30, control groups underwent laparotomy without ischemic attack, 1 hour, 24 hours and 30 days after laparotomy organs were harvested; R1, R24, R30, experimental groups underwent 1 hour ischemia with following reperfusion period of 1 hour, 24 hours and 30 days. **B.** Detected bands for IL-10 and GAPDH. For PCR reaction was used negative control (NTC) and band position was determined against the ladder (L), experimental groups (R) compared to controls (K).

increased in comparison to control groups after 24 h and 30 days of reperfusion, the laparotomy itself probably caused the increase in pro-inflammatory TNFα protein concentration. These results correspond with several other studies demonstrating the role of IRI in cytokine expression (Deitch et al. 1994; Nussler et al. 2003; Souza and Teixeira 2005). Souza et al. stated that TNFα plays an essential role in promoting tissue injury after IRI, and they also indicated that the degree of tissue injury is determined by the balance between TNFα and its antagonist IL-10. It is believed that the expression of anti-inflammatory cytokine IL-10 increases after IRI, which corresponds with our results. Several studies have shown that there were increased levels of TNFα and IL-10, not only in the jejunal wall but also

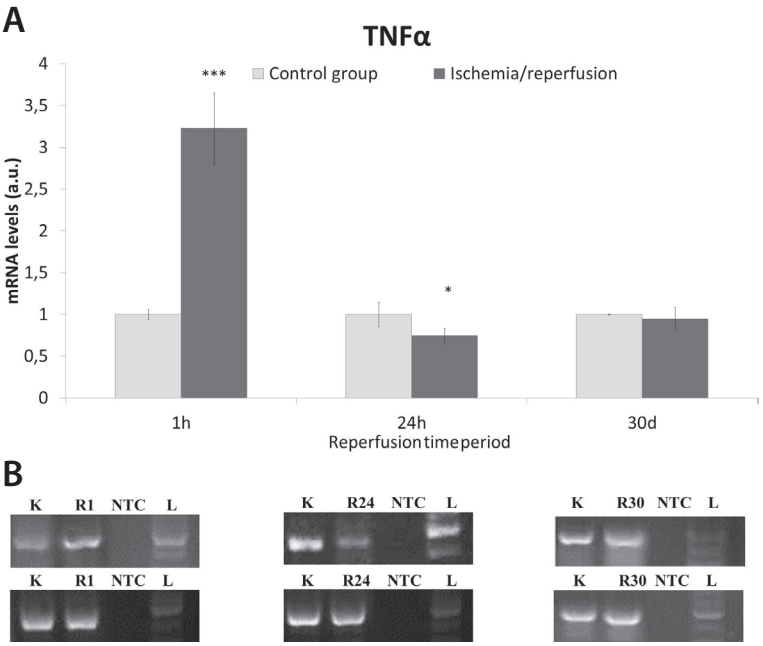

Figure 2. mRNA levels of TNFα in rat small intestine after 1 hour ischemia and subsequent reperfusion injury. **A.** Semiquantification graph of gene expression. Results are presented as mean ± SEM. *** $p < 0.001$; * $p < 0.05$ significantly different as compared to controls. C1, C24, C30, control groups underwent laparotomy without ischemic attack, 1 hour, 24 hours and 30 days after laparotomy organs were harvested; R1, R24, R30, experimental groups underwent 1hour ischemia with following reperfusion period of 1 hour, 24 hours and 30 days. **B.** Detected bands for TNFα and GAPDH. For PCR reaction was used negative control (NTC) and band position was determined against the ladder (L), experimental groups (R) compared to controls (K).

Table 2. Concentration of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNFα (pg/ml) after various periods of reperfusion

	C1	R1	C24	R24	C30	R30
IL-10	88.6 ± 10.5	154.86 ± 2.7***	84.95 ± 5.9	152.96 ± 18.5*	51.26 ± 0.12	160.34 ± 14.5***
TNFα	178.92 ± 9.9	513.42 ± 55.4***	88.76 ± 10.06	294.69 ± 12.68***	92.82 ± 4.56	228.06 ± 12.12***

*** $p < 0.001$, * $p < 0.05$ *vs.* control groups. C1, C24, C30 – control groups underwent laparotomy without ischemic attack, 1 hour, 24 hours and 30 days after laparotomy organs were harvested; R1, R24, R30 – experimental groups underwent 1 hour ischemia with following reperfusion period of 1 hour, 24 hours and 30 days.

in distant parenchymatous organs such as lungs, liver and kidneys (Oltean et al. 2009; Park et al. 2011). We can conclude that IRI directly affects the levels of pro-inflammatory cytokine TNFα as well as anti-inflammatory cytokine IL-10. The antagonistic effect of IL-10 and TNFα is well known, since through increase in IL-10 level the synthesis of TNFα is inhibited (Zingarelli et al. 2001). However, Yuan et al. (2011) observed increased levels of serum TNFα 2 hours after reperfusion, although the levels of serum IL-10 decreased in comparison to control. The concentration of pro-inflammatory TNFα on protein level in our study was significantly highest 1 hour after reperfusion, similarly to Mondello et al. (2010), whereas after 24 hours and 30 days of reperfusion it showed decreasing tendency probably because of the started regeneration processes. As regards anti-inflammatory IL-10, we observed increased mRNA expression as well as protein concentration 1 hour after reperfusion, probably due to its ability to partially suppress the inevitable inflammatory reaction. In fact, 30 days after reperfusion the mRNA levels of IL-10 were lower, but concentration of particular protein in the tissue significantly increased. We suppose, the mRNA expression of IL-10 was inhibited because of the started regeneration and reparative processes. The concentration of released active protein in intestinal tissue was still high, even though the mRNA expression was probably silenced. We can assume that significant increase in the levels of both cytokines could be understood as a result of the effort of maintaining the balance in the small intestinal wall.

In conclusion, our results proved that intestinal IRI has a direct effect on the production of inflammatory mediators, mostly in the early stages after the procedure. However, after a longer reperfusion period (30 days) the regeneration processes in the small intestine have started and the expression levels of inflammatory mediators are decreased. It is necessary therefore to study efficient protective strategies with regard to the early phases of IRI. Increased understanding of IRI molecular mechanisms can result in better, more detailed and efficient therapeutic processes leading to the protection of the organism.

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