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Effects of intestinal ischemia reperfusion injury on the level of specific genes in rats

Wpływ niedokrwienno-reperfuzyjnego uszkodzenia jelita na poziom określonych genów u szczurów

Abstract:

The small intestine is an organ with very well developed immunological activity. There are specific cells in the mucosa of the small intestine responsible for releasing the inflammatory mediators that can lead to Multiple Organ Dysfunction Syndrome (MODS), which is a very complex process that can occur after ischemia-reperfusion injury. The accumulation of specific inflammatory mediators in the wall of the small intestine also increases the expression of apoptotic genes. The aim of this study was to detect and analyse the changes in the expression of apoptotic genes (Bax, Bcl2) and the genes responsible for the production of cytokines (TNFα, IL1β, IL6, IL10) and tumour growth factor beta (TGFβ). Male Wistar rats underwent ischemia performed by complete occlusion of the mesenteric artery. Ischemia was followed by reperfusion periods of 1 hour, 24 hours, and 30 days. Subsequently, the total RNA was isolated from the complete wall of the small intestine and RT-PCR (real-time) was performed. There was a significant increase in the levels of specific genes (Bax, Bcl2, TNFα, IL1β, IL6, IL10, TGFβ) after one hour of reperfusion and a decreased tendency after 24 hours and 30 days.

Streszczenie:

Jelito cienkie jest narządem o bardzo dobrze rozwiniętej aktywności immunologicznej. W błonie śluzowej jelita cienkiego znajdują się specjalne komórki odpowiedzialne za uwalnianie mediatorów zapalenia, mogące wywołać zespół niewydolności wielonaczyniowej (ang. Multiple Organ Dysfunction Syndrome (MODS)), który jest bardzo złożonym procesem, jaki może wystąpić po uszkodzeniu niedokrwienno-reperfuzyjnym. Nagromadzenie swoistych mediatorów zapalenia w ścianie jelita cienkiego zwiększa również ekspresję genów apoptotycznych. Celem tej pracy było wykrycie i analiza zmian w ekspresji genów apoptotycznych (Bax, Bcl2) oraz genów odpowiedzialnych za wytwarzanie cytokin (TNFα, IL1β, IL6, IL10), a także transformującego czynnika wzrostu beta (TGFβ). U samców szczurów rasy Wistar wywoływano niedokrwienie poprzez całkowite zamknięcie tętnicy krezkowej, po czym następowały okresy reperfuzji trwające 1 godzinę, 24 godziny i 30 dni. Następnie z całej ściany jelita cienkiego izolowano RNA całkowite i przeprowadzono badanie RT-PCR (w czasie rzeczywistym). Stwierdzono istotny wzrost poziomów określonych genów (Bax, Bcl2, TNFα, IL1β, IL6, IL10, TGFβ) po jednej godzinie reperfuzji oraz trend spadkowy po 24 godzinach i 30 dniach.

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Słowa kluczowe: *uszkodzenie niedokrwienno-reperfuzyjne, cytokiny, PCR w czasie rzeczywistym, MODS, szczury*

Introduction

Multiple organ dysfunction syndrome (MODS) is a very specific and complex process that can occur after ischemia reperfusion injury (IRI). The MODS is defined as a clinical syndrome characterized by the development of physiologic dysfunction in two or more organs. The harm to the organs is not considered to be the direct result of surgery, but it is induced by changes in the homeostasis of the organism. The mechanism of MODS consists of several phases. In the early phase, there is an overproduction of the inflammatory mediators present, which leads to the activation and migration of leukocytes and the production of mostly pro-inflammatory cytokines (IL1β, TNFα). This process eventuates in endothelial damage in junctions between endothelial cells, cell swelling, apoptosis and necrosis of the cells (1). The activation of inflammatory cells in a distant organ via circulating mediators follows (2). Another process that can lead to MODS is bacterial translocation via *tunica intima* of the blood capillaries into the blood stream. By flow of the blood, bacteria can be transported to a distant organ where the inflammatory processes can cause pathological changes and induce apoptosis. In the small intestine there is a huge number of immunologically active cells. Therefore, it is one of the organs that displays a sudden response to IRI.

Apoptosis, a very complex mechanism, takes part in embryonic development, tissue differentiation, organogenesis, immune system development and malignant transformation. The appearance of an apoptotic cell is very typical. There are changes in the permeability of the cell membrane, cytoskeletal reorganisation, proteolysis of specific cytoplasmic and nuclear proteins, collapse of the nuclei and cell shrinkage. Two apoptotic stages can be distinguished: on the level of the cytoplasm and on the level of the nucleus. It is claimed that the initial levels of apoptosis induced by the p53 gene family are reversible (3). Apoptosis is triggered by many various factors, which involve caspases, mitochondria and endoplasmic reticulum (4, 5, 6, 7). One of the factors that initialize apoptotic cascade are Bcl2 and Bax. The Bcl2 regulator gene has anti-apoptotic attributes and belongs to the Bcl2 family. Bcl2 also plays a role in regulating the integrity of the mitochondrial membrane and releasing the cytochrome c (5). Bax is also a member of the Bcl2 family, but it works antagonistically to Bcl2. It accelerates apoptosis by binding and blocking Bcl2, thus triggering the apoptotic cascade.

Cytokines belong to the family of signalling molecules. They are released by specific cells of the immune system. Concerning inflammation, we can divide cytokines in two large groups – pro-inflammatory (TNFα, IL1) and anti-inflammatory (IL10) cytokines. IL6 has a pleiotropic role as both a pro (stimulating production of antibodies) and anti-inflammatory cytokine (TNFα and IL1 inhibition). Both groups play a crucial role in maintaining the homeostasis of the organism. TNFα and IL1 are both pro-inflammatory cytokines and they can start a cascade of secondary released cytokines that can attract other cells responsible for inflammation. Higher levels of cytokines can lead to several complications (8, 9). Grotz *et al.* (10) claimed the levels of TNFα are positively related to intestinal ischemia and are highest 30 minutes after the reperfusion period.

Material and methods

Experimental model

Male Wistar rats (total n =36) with an approximate body weights of ± 320g were used in the experiment. The animals were housed in standard conditions at a temperature of ± 22°C, with a 12h period of daylight, water and food were provided *ad libitum*. We created three experimental groups of animals that underwent ischemia. Ischemia was performed by complete occlusion of *a. mesenterica cranialis.* Ischemia lasted for 1 hour in all experimental groups and was followed by a reperfusion period. The reperfusion period differed from 1 hour, 24 hours and 30 days according to the particular group (R1, R24, R30 and control group K). A 2-3 cm portion of the jejunum was taken at a distance of approximately 10 cm from the *ligamentum Trietzi*. The animals were given anaesthesia by an intraperitoneal injection of ketamine 60–80 mg/kg (Narketan 10 inj. ad us. vet., Vétoquinol S.A., Lure Cedex, France), and xylazine 8-10 mg/kg (Xylariem inj. ads. vet., RiemserArzneimittel, Greifswald-InselRiems, Germany). The harvested samples were washed thoroughly in RNAse free water, weighed and stored at -80°C.

Real-time PCR

Total RNA was isolated from the complete wall of the small intestine by Trizol reagent (Invitrogen) and purified by DNase I on RNeasy Mini Elute colones (Qiagen, RNeasy Mini Kit). Complementary DNA (cDNA) was prepared by reverse transcriptase Superscirpt II (Invitrogen). cDNA preparates were purified by ethanol

percipitation. Amplification reactions were carried out in 20 μl volumes containing 0.8 μl of cDNA, 0.8 μl of appropriate primers (primer sets for the tested genes were obtained from SABiosciences, Qiagen) and 10 μl of 2x SYBR Green / ROX PCR mix (PA-012,SABiosciences, Qiagen). PCR amplifications were performed in the real–time PCR system Mx 3000P (Stratagene, La Jolla, CA) using the relative standard curve method. The mix of cDNA sample aliquots was used for the relative standard curve construction. A normalization factor calculated from three reference genes (hypoxanthine phosphoribosyltransferase- HPRT, Fp subunit succinate dehydrogenase - SDHA, beta actin -BACT) was used for the normalization of target gene quantity in each sample (GeNorm software was utilized for the calculation). Statistic analyses were performed using GraphPadPrism software (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test).

Results

The levels of mRNA in all the examined genes have a very similar tendency. mRNA expression in all specific genes is the highest in the R1 group and is decreased in the R24 and R30 groups. (**Tab. 1**).

Tab. 1. Relative quantity of specific mRNAs

Gene	R1	R24	R30	KW
Bax	1.327 (1.229-1.337)	0.9599 (0.2426-0.9996)	0.6865 (0.3534-0.8985)	***
Bcl2	1.424 (0.9443 – 2.132)	0.6333 (0.2024-1.075)	0.7996 (0.2462-1.411)	**
IL10	1.7 (0.6775-1.923)	0.6502 (0.2368-1.263)	0.9211 (0.1234-2.056)	*
TGFB	1.498 (0.9461-1.669)	0.6999 (0.4186-1.13)	0.9124 (0.1599-1.46)	**
TNFα	1.431 (0.5217-1.991)	0.8767 (0.5058-1.007)	0.7079 (0.5028-1.052)	*
IL6	1.769 (0.8483-2.399)	0.5367 (0.283-1.038)	0.6198 (0.1454-1.146)	***
IL1β	1.584 (0.9916-2.07)	0.8242 (0.2578-1.325)	0.7886 (0.5231-1.021)	**

Table 1: Values are medians (minimum and maximum in brackets). The number of animals in each experimental group was 8. Statistical analyses were performed using the Kruskal-Wallis test (KW) followed by Dunn's Multiple Comparison Test: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. One hour ischemia was performed on all the experimental animals, followed by subsequent 1 hour (R1), 24 hour (R24) and 30 day (R30) reperfusion.

The quantity of mRNA IL1β reached the highest level after 1 hour of reperfusion (R1 group). After 24h of reperfusion (the R24 group), the level of mRNA was significantly lower (** $p < 0.01$) in comparison with the R1 group. After 30 days of reperfusion, the levels of mRNA (the R30 group) were significantly decreased (** $p < 0.01$).

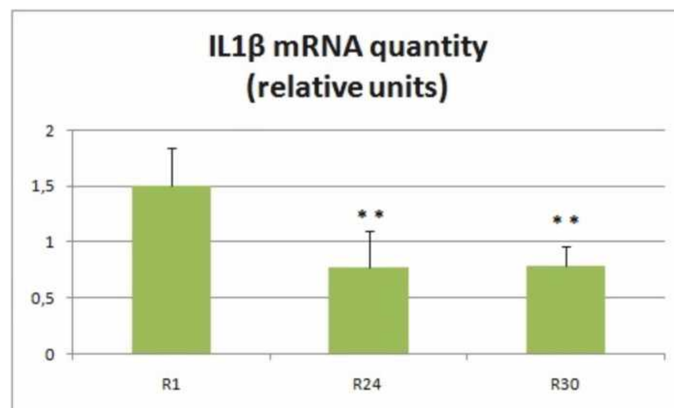

Fig. 1. IL1β mRNA quantity

Fig. 1. Level of IL1β gene expression after ischemia reperfusion injury.

n(R1) = 8, n(R24) = 8, n(R30) = 9, ** $p < 0.01$

The mRNA level of TNFα was significantly higher (* $p < 0.05$) in R1 group in comparison with groups R24 and R30.

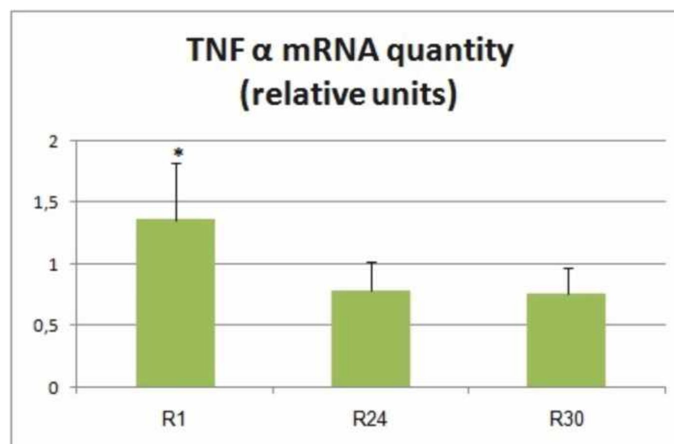

Fig. 2. TNFα mRNA quantity

Fig. 2. Level of TNFα gene expression after ischemia reperfusion injury.

n(R1) = 8, n(R24) = 8, n(R30) = 9, * $p < 0.05$

The mRNA levels of IL6, which mediates inflammation and antibody production, were significantly higher (** $p < 0.01$) in R1 group in comparison to groups R24 and R30.

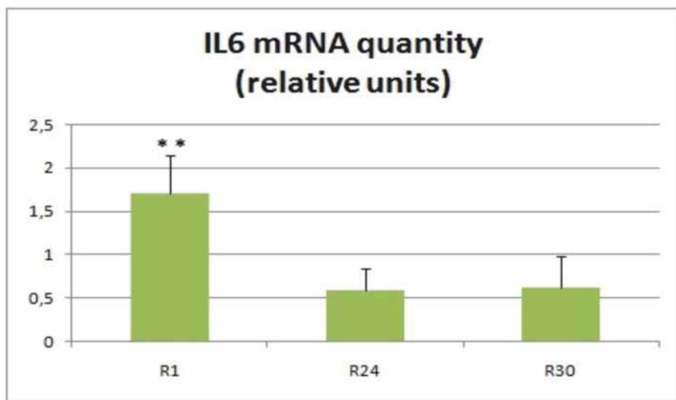

Fig. 3. IL6 mRNA quantity

Fig. 3. Level of IL6 gene expression after ischemia reperfusion injury.
n(R1) = 8, n(R24) = 8, n(R30) = 9, ** $p < 0.01$

As regards pro-inflammatory cytokine IL10, the mRNA level was significantly higher (* $p < 0.05$) in R1 group when comparing to R24 group.

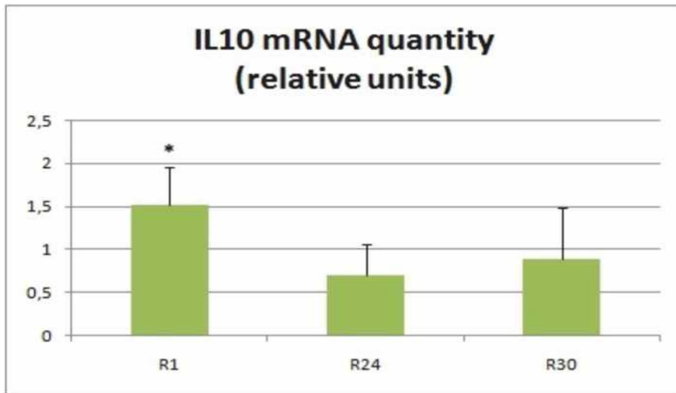

Fig. 4. IL10 mRNA quantity

Fig. 4. Level of IL10 gene expression after ischemia reperfusion injury.
n(R1) = 8, n(R24) = 8, n(R30) = 9, ** $p < 0.05$

The mRNA levels of TGFβ2 were significantly higher (** $p < 0.01$) in R1/R24 and (* $p < 0.05$) in R1/R30.

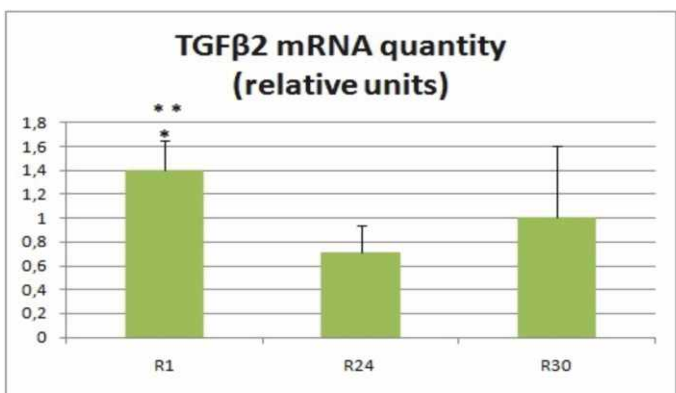

Fig. 5. TGFβ2 mRNA quantity

Fig. 5. Level of TGFβ2 gene expression after ischemia reperfusion injury. n(R1) = 8, n(R24) = 8, n(R30) = 9, ** $p < 0.01$, * $p < 0.05$

Significantly higher (** $p < 0.01$) mRNA levels of anti-apoptotic Bcl2 in R1 group relate to the fact that necrosis also played a role in cell death. In groups R24 and R30, mRNA levels were significantly lower (** $p < 0.01$). (Fig. 6).

Fig. 6. Level of Bcl2 gene expression after ischemia reperfusion injury.

n(R1) = 8, n(R24) = 8, n(R30) = 9, ** $p < 0.01$

The same effect is for the pro-apoptotic Bax gene, where we can observe an increase in the mRNA levels in R1 group and then a significant decrease (** $p < 0.01$) in groups R24 and R30.

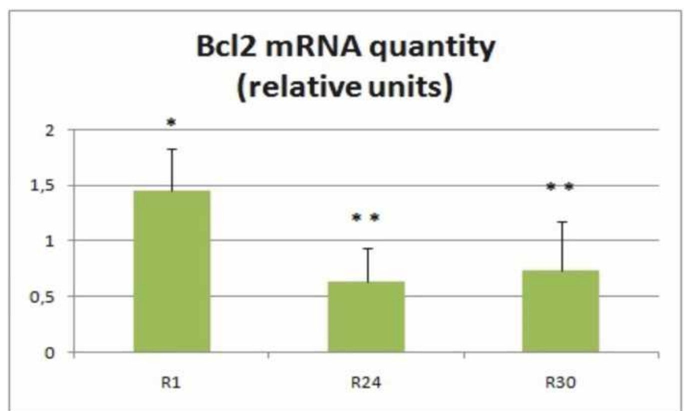

Fig. 6. Bcl2 mRNA quantity

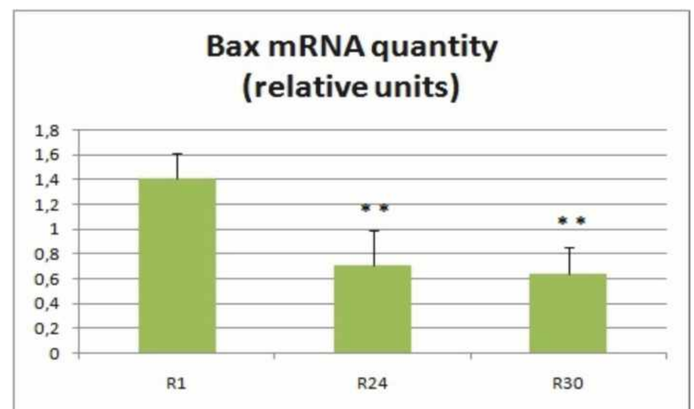

Fig. 7. Bax mRNA quantity

Fig. 7. Level of Bax gene expression after ischemia reperfusion injury. n(R1) = 8, n(R24) = 8, n(R30) = 9, ** $p < 0.01$

Discussion

The role of pro and anti-inflammatory cytokines and genes affecting apoptosis is very complex. Cytokines have a pleiotropic effect and they cause an increase in the levels

of inflammatory mediators as well as their own levels. In many studies examining cytokines and their influence on inflammatory processes, the levels of cytokines were examined directly from blood plasma mainly by protein assays [12, 13, 5]. In our work, we focused on specific genes expression directly in the site of the ischemia-reperfusion attack, which is the wall of the small intestine. Higashikawa *et al*. [9] detected the levels of specific cytokines from the intestine tissue of different mice models. They declared that cytokine expression levels of TNFα, IL6 and IL1 increased as the inflammation became advanced. On the other hand, they also stated that expression levels decreased as soon as mucosa begun to heal. In our results, the levels of all cytokines (IL1β, IL6, TNFα, and IL10) have a similar tendency, so we can conclude that the beginning of the inflammation process leads to the increase of cytokines levels but as soon as homeostasis starts to be renewed, the levels of cytokines decreased. From our results, we can conclude that the quantity of mRNA IL1β reached the highest level after 1 hour of reperfusion (R1 group). As a matter of fact, IL1β plays a role in the inflammatory process and it is responsible for higher migration and infiltration of leukocytes into the mucosa of the small intestine. Lower mRNA level suggests the beginning of the healing process 24 hours after ischemia. After 30 days, IL1β levels decreased. This is because the homeostasis of the organ is re-established. It is believed that IL1beta works synergistically with TNFα and initiate the cascade of inflammatory mediators by targeting the endothelium (11). Stallion *et al*. [14] also observed an increase in IL6 and TNFα mRNA levels after ischemia reperfusion injury in the small intestine. Apparently, TNFα mRNA levels significantly increased just after 4 hours of reperfusion and not after 1 hour, which can lead to the conclusion that, after IRI, the small intestine is the main source of IL6. As regards the levels of TGFβ 2, it is a cytokine with a specific paradoxical role in tumour development, which regulates proliferation, differentiation, adhesion and migration of many different cell types. It can inhibit cell proliferation; it stimulates apoptosis and suppresses the expression of pro-tumorigenic cytokines [15]. This means that 1 hour after reperfusion, when it is necessary to activate the apoptotic cascade as a prevention, the levels of TGFβ2 were the highest, whilst 24 hours after the reperfusion, when the healing processes started, and 30 days after reperfusion, when the mucosa had regenerated, the TGFβ2 levels decreased.

The main observation in this study was that after ischemia-reperfusion injury, the levels of all the examined genes significantly increased after 1 hour of reperfusion, while after 24 hours to a 30 day period, when the repairing and healing processes had started, the levels decreased. We can conclude that the highest mRNA levels in all specific genes in R1 group can be the result of IRI, while the decreased levels in groups R24 and R30 are the response of the subsequent regeneration process.

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