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Quercetin protects jejunal mucosa from experimental intestinal ischemia reperfusion injury by activation of CD68 positive cells

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ABSTRACT

The aim of our study was to analyse the possible protective effect of quercetin application during the jejunal ischemia-reperfusion injury (IRI) in rats. Quercetin was administered intraperitoneally 30 min before 1 h ischemia of superior mesenteric artery with following 24 h lasting reperfusion period. The male specific pathogen-free (SPF) Charles River Wistar rats were used. In the group with applied quercetin, the significantly increased ($p < 0.001$) levels of anti-inflammatory cytokine IL10 were observed both in the blood serum and jejunal tissue. The improvement of the mucosal tissue morphology and proliferating and DNA repairing cell number measured by PCNA activity were recorded by more than 30% higher in the quercetin group. Simultaneously, significant elongation of the intestinal glands ($p < 0.001$) and increase in the number of CD68-positive cells in the *lamina propria mucosae* ($p < 0.001$) in comparison with control group were found. Based on our results, the preventive application of quercetin before induction of jejunal IRI stimulates faster jejunal mucosa restoration and it seems to have immunomodulatory and anti-inflammatory effects as well. CD68-positive macrophages could have crucial role in this process since they work as both growth factor and cytokine producers.

1. Introduction

Ischemia-reperfusion injury (IRI) is a complex process occurring mostly after the surgical interventions. It results in a sequence of events leading to the cellular dysfunction or even cell death. IRI may result in distant organ damage and development of Systemic Inflammatory Response Syndrome, followed by Multiple Organ Dysfunction Syndrome. The restoration of the blood flow following the ischemic period is essential to provide oxygen and nutrients; on the other hand it triggers the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS/RNS cause disturbances in cellular membranes and in nucleic acid structures – therefore changes in gene expression and impairment of the specific cellular receptors and enzymatic complexes. Higher level of ROS/RNS activates vascular endothelium and leukocytes and triggers the adhesion molecule-cytokine cascade (Wilhelm et al., 2003). IRI of the small intestine leads to the changes in the intestinal epithelial lining and therefore to following bacterial translocation. The inflammatory mediators are expressed continuously in lower levels all over the organism, mostly by immunocompetent cells, connective tissue cells, epithelial cells and endothelium. The overproduction and changes in the upregulation and internal balance of these cytokines play the crucial role in the post-ischemic reperfusion injury of the directly attacked ischemic organ as well as distant organs. The intercellular adhesion molecule ICAM-1

Abbreviations: COX-2, cyclooxygenase-2; GCs, goblet cells; IL, interleukin; *i.p.*, intraperitoneally; IRI, ischemia-reperfusion injury; MCs, mast cells; MMS, monocyte-macrophage system; RNS, reactive nitrogen species; ROS, reactive oxygen species; SPF, specific pathogen free; TNF, tumor necrosis factor

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mediates migration of circulating polymorphonuclear neutrophils to injured tissue where they worsen the inflammatory reaction (Springer, 1990; Van Buul et al., 2007). Proinflammatory cytokine tumor necrosis factor alpha (TNFα) is synthetized mostly by macrophages, lymphocytes and endothelial cells (Ji et al., 2015), however also the other cell populations have the potential to produce the cytokines under specific conditions (e.g. IRI). Interleukin IL10 belonging to anti-inflammatory cytokine family is synthetized mostly by lymphocytes, monocytes, macrophages and mast cells, that is, by the cells responsible for inflammatory reaction in the jejunal mucosa and is known to function as a critical anti-inflammatory regulator (Saraiva and ÓGarra, 2010). Because of the overproduction of reactive oxygen species in the development of IRI, the usage of antioxidants for diminishing of the tissue damage seems to be a good choice. Quercetin (3,3′,4′,5,6-pentahydroxyflavone) is a well-known natural flavonoid providing a great variety protective effects. It is famous for its antioxidative action, ability to inhibit xanthine oxidase action, decreasing leukocytes immobilization, modulation of the expression of inflammatory mediators and interactions with other enzyme systems (Lakhanpal and Rai, 2007). After tissue injury, monocytes and macrophages undergo marked phenotypic and functional changes to play critical roles during the initiation, maintenance, and resolution phases of tissue repair (Wynn and Vannella, 2016).

IRI is a complex and multifactorial condition with a high potential to affect multiple cell populations in the jejunal mucosa that play inevitable role in inflammation, reparation and regeneration of the tissue. Recently, we published the experimental study with intraperitoneal quercetin application (preconditioning) in a model of jejunal IRI (Tóth et al., 2017). In acute phase, 4 h after the beginning of reperfusion, the quercetin induced a significant decrease in mucosal injury index accompanied by a significant decrease in cyclooxygenase-2 (COX-2) production in the epithelial lining of the intestinal villi in comparison with the control group. Based on morphology of COX-2 positive cells, the COX-2 positivity was found particularly in goblet cells of the intestinal villi epithelium and enteroendocrine cells respectively, and in the glandular epithelium. We concluded that quercetin application attenuated mucosal damage after IRI by inhibiting neutrophil infiltration which was demonstrated by a lower number of myeloperoxidase positive cells in the lamina propria during both phases of IRI and the significant decrease in the late phase after 24 h of reperfusion. The aim of presented study was to analyse the levels of selected inflammatory mediators (IL10, TNFα) in the blood serum and the jejunal wall tissue after 24 h of reperfusion. The mucosal thickness, villous height and intestinal gland depth were measured for evaluation of the morphometric parameters. The epithelial lining was analysed through presence of the goblet cells (GCs) by histochemical methods – PAS-reaction and Alcian blue staining. In the *lamina propria mucosae* were quantified the numbers of mast cells (MCs), PCNA positive cells and CD68 positive cells of monocyte-macrophage system (MMS).

2. Materials and methods

2.1. Experimental design and ethics

Adult male outbred Wistar rats – specific pathogen free (SPF) Charles River (n = 15) from Laboratory of Research Bio-models Pavol Jozef Šafárik University, Faculty of Medicine (SK PC 4013) weighing between 250 and 350 g were used in the experiment. This study was approved by the Committee for Ethics on Animal Experiment at the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia and the experimental protocol was approved by the State Veterinary and Food Administration of the Slovak Republic No Ro-3004/14-221. The animals were kept under standard conditions of monitored temperature and humidity. All animals were fed standard rat chow and water *ad libitum* and were devoid food 12 h before surgery. Animals were randomly assigned to control and experimental groups. To the control group E24 (n = 7) was applied 10% ethanol solution as a liquid solvent intraperitoneally (*i.p.*) 30 min before ischemia. Quercetin (Sigma Aldrich, Inc.; St. Louis, Missouri, United States; 1001419342Q4951-10G; 50 mg/kg) was applied to the animals from experimental group Q24 (n = 8). Quercetin was dissolved in 10% ethanol and administered *i.p.* 30 min before ischemia. After midline laparotomy, the superior mesenteric artery was isolated and jejunal ischemia was induced by its occlusion using an atraumatic microvascular clamp for 1 h. The clamp was carefully removed and the samples of tissue and blood were harvested after 24 h of reperfusion for histological and biochemical analysis.

2.2. Biochemical analysis

Concentration of inflammatory mediators in peripheral blood serum was evaluated by ELISA method (Sigma, RayBio Kit Protocol, RayBiotech; Norcross, Georgia, United States, 93289, BCBP40880). Total RNA was isolated from the wall of the small intestine using Trizol separation method (Invitrogen, Carlsbad, CA) and Qiagen RNeasy mini kit (Qiagen; Hilden, Germany, 74106, 136257856) according to the manufacturer's protocol. mRNA concentration and purity were determined by spectrophotometry (A_{260}/A_{280} ratio) using Biophotometer plus (Eppendorf; Hamburg, Germany). Total RNA was transcripted to cDNA using Revert Aid H minus Firststrand cDNA Synthesis Kit (Fermentas; Leon-Rot, Germany, K1632, 00111171). Inflammatory cytokine expression in the jejunal wall was measured by reverse transcriptase polymerase chain reaction (RT-PCR), with PCR amplification for 30 cycles. GAPDH was used as a reference gene.

2.3. Tissue specimen preparation and histological analysis

The samples of jejunum 1–2 cm long were taken 10 cm from the Trietz ligament. Biopsies were washed with cold saline and fixed in 4% paraformaldehyde. The tissue excisions were embedded in paraplast, cut into 4–5 μm thick histological sections, and mounted. After deparaffinization, the tissue sections were stained for following histological examinations. Villus height, intestinal gland depth, the thickness of mucosa and MII of the jejunum were examined. Morphometry was performed using an Olympus BX50 light microscope with Olympus Camera SP350 (Olympus, Tokyo, Japan) and QuickPHOTO Industrial 2.3 image analyzer software (Promicra, Prague, Czech Republic). Villus height (from the villus base to the tip) and crypt depth (from the villus base to the *lamina muscularis mucosae*) were measured in 12 axially-oriented villi in at least three different randomly selected quadrants of each intestinal sample. The intestinal mucosa thickness was measured in the same manner (from the villus tip to the *lamina muscularis mucosae)*. All measurements were done using magnification ×200. The number of GCs in 1 mm of the epithelial lining was detected by histochemical staining with Alcian blue (Alcian blue 8GX solution, pH 2.5, Sigma Aldrich; 05500) for detection of acidic glycoproteins. PAS-reaction, modified according to McManus, was used for detection of neutral mucopolysaccharides (Čunderlíková and Balážová, 1990). The population of MCs in 1 mm^2 of the intestinal *lamina propria mucosae* was detected using metachromatic Cresyl Fast Violet (Merck Millipore, Billerica, Massachusetts, United States; 15947) staining protocol (Cook, 1961). The result of this staining technique was distinct brilliant red/purple colouring of cytoplasmic secretory granules of MCs. The number of GCs and MCs was determined in 10 different randomly selected fields using magnification ×400. The population of cells in proliferation and DNA repair (anti-PCNA, Mouse Monoclonal Antibody, BioLegend, SanDiego, California, United States; Ms-106-P1; 1:100) and cells of MMS (anti-CD68, Mouse Monoclonal Antibody, Thermo Fisher Scientific; Waltham, Massachusetts, United States; Ms-397-P1; 1:300) in 1 mm^2 of the jejunal *lamina propria mucosae* was detected in 10 different fields by immunohistochemical analysis. Positive cell populations were visualized with diaminobenzidine, DAB (Sigma-Aldrich; 32750-1G-F)