Cytokeratin 18 and h-FABP levels in intestinal ischemia–reperfusion injury: role of coenzyme Q10

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Aim: The objective of this study was to investigate whether coenzyme Q10 (CoQ10) supplementation affects M30 and heart-type fatty acid binding protein (h-FABP) levels in intestinal ischemia–reperfusion injury-induced rats.

Materials and methods: Thirty-two male Wistar rats were randomly divided into 4 groups. 1) Sham: Animals exposed to laparotomy without clamping the superior mesenteric artery (SMA); 2) ischemia–reperfusion (IR): rats exposed to laparotomy with occlusion of the SMA for 45 min, followed by 120 min of reperfusion period; 3) CoQ10 plus sham: 10 mg kg body weight^{-1} CoQ10 was administered via intraperitoneal injection for 20 days, and thereafter animals were exposed to laparotomy without clamping the SMA; 4) CoQ10 plus IR: 10 mg kg body weight^{-1} CoQ10 was administered via intraperitoneal injection for 20 days and thereafter animals were exposed to laparotomy with occlusion of the SMA for 45 min, followed by 120 min of reperfusion period. Blood samples were taken by cardiac puncture for the analysis of serum h-FABP and M30 levels.

Results: There was no difference among the groups for serum h-FABP and M30 antigen levels.

Conclusion: Neither intestinal ischemia–reperfusion injury nor CoQ10 supplementation affect serum h-FABP and M30 levels in rats.

Key words: Ischemia–reperfusion injury, intestine, coenzyme Q10, M30, h-FABP

1. Introduction

Cessation of the blood supply causes ischemic injury, and later restoration of the blood supply initiates a cascade of events that may result in additional cell injury known as reperfusion injury (1–3). Parks et al. (4) reported that tissue lesions produced during reperfusion were greater than those produced during ischemia, in mesenteric ischemia–reperfusion (IR). Intestinal IR injury is an important problem in many situations, such as abdominal aortic aneurysm surgery, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing enterocolitis, intestinal transplantation, and hemorrhagic or septic shock (5). IR of the small intestine can cause the disruption of the mucosal barrier, bacterial translocation, and the activation of inflammatory responses, as well as hydroelectrolytic and acid–alkaline equilibrium disturbances, which are manifested in distant organs (1). Therefore, intestinal IR can induce injury to secondary organs such as the liver (6), heart (7), kidneys (8), and lungs (9).

Fatty acid-binding protein (FABP) is a small (15 kDa) cytoplasmic protein that is expressed in tissues with an active fatty acid metabolism (10). The primary function of FABP is the facilitation of intracellular long-chain fatty acid transport (11). The cellular expression of FABPs is responsive to changes in lipid metabolism as induced by pathophysiological stimuli like ischemia, endurance training, and diabetes. Heart-type FABP (h-FABP) is mainly expressed in cardiomyocytes, but to a lesser extent also in skeletal muscle, distal tubular cells of the kidney, specific parts of the brain, lactating mammary glands, and placenta (10). h-FABP is a sensitive and early marker for myocardial injury (12).

Cytokeratin 18 (CK18) is a member of the intermediate filament family of cytoskeletal proteins and is widely expressed in epithelial and endothelial cells (13). Cytokeratins are mainly released from tumor cells; they are also released into the circulation where they may comprise partly degraded intermediate filament complexes (14). M30 and M65 antibodies can be used in the detection of CK18 fragments in the circulation (15). The M30 apoptosense detects a neoepitope specific to apoptosis caused by caspase cleavage of CK18 at aspartate 396. The

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M65 measures total soluble CK18 (both caspase cleaved and intact forms), which are released during apoptotic and nonapoptotic forms of cell death (16).

Coenzyme Q10 (CoQ10) is a lipid soluble endogenous provitamin naturally found in the mitochondria. CoQ10 carries out 2 major biochemical functions in the cells; the first is to serve as an electron and proton carrier for energy coupling and the second is to serve as an antioxidant, acting as a primary scavenger of free radicals (17,18). Disruption of the mitochondrial respiratory chain after IR results in overproduction of reactive oxygen species, opens the mitochondrial permeability transition pore, and activates and leaks apoptosis mediators, such as cytochrome c and caspase 9, out of mitochondria into cytosol. These proapoptotic factors activate downstream events that eventually lead to cell apoptosis (19). Numerous studies have suggested that CoQ10 can scavenge free radicals and are therefore beneficial against IR injury in several models of rat IR injury such as the limb (20), brain (21), and liver (22). CoQ10 may therefore be a logical choice as a therapy for intestinal ischemia. However, CoQ10 has never been used as a treatment for intestinal IR injury.

In the present study we hypothesized that since intestinal IR induces an injury to secondary organs like the heart, and since recent reports have emphasized the role of apoptotic cell death in the pathogenesis of IR injury, intestinal IR may affect M30 and h-FABP levels. CoQ10 supplementation may have a therapeutic role in the treatment of intestinal IR injury. Based on the current reports, the present study was designed to determine the effect of CoQ10 supplementation on M30 and h-FABP levels in intestinal IR injury-induced rats.

2. Materials and methods

2.1. Animals
Thirty-two male Wistar rats (aged 16 weeks) weighing between 300 and 350 g were used in the present study. Rats were housed in polycarbonate cages and maintained on a 12:12-h light/dark cycle in a temperature (22 ± 2 °C) and humidity (50%) controlled room. All animals were given standard rat chow and tap water ad libitum. Animals' care and all surgical procedures were approved by the Animal Ethics Committee of the Experimental Medicine Research and Application Center of Selçuk University. The experiment was performed in compliance with the Principles of Laboratory Animal Care formulated by the National Institute of Health.

2.2. Experimental design
Thirty-two rats were randomly assigned to 4 experimental groups, 8 rats in each: sham (S), intestinal IR, CoQ10 plus sham (CoQ10+S) and CoQ10 plus intestinal IR (CoQ10+IR). The rats in sham groups underwent laparotomy only, without superior mesenteric artery (SMA) occlusion. The rats in intestinal IR groups underwent a laparotomy and intestinal IR. The rats in CoQ10 groups received a pretreatment by intraperitoneal injection with CoQ10 at 10 mg kg body weight^{-1} for 20 days before operation.

2.3. CoQ10 treatment and surgical procedures
Rats were fasted for 12 h with free access to water before operation. The CoQ10 supplemented groups (CoQ10+S, CoQ10+IR) received an intraperitoneal administration of CoQ10 (C9538, Sigma Chemical Co., St. Louis, MO, USA) at doses of 10 mg kg body weight^{-1} per day dissolved in corn oil (C8267, Sigma Chemical Co.) for 20 days before the operation. The rats were anesthetized by intramuscular injection of ketamine hydrochloride (50 mg kg body weight^{-1}) and xylazine (10 mg kg body weight^{-1}). The abdomen was opened with a midline incision. Intestines were exteriorized and the SMA was dissected. After identifying the SMA, the small intestine was subjected to ischemia by occluding the SMA with a nontraumatic microvascular clamp. Adequate occlusion was confirmed by pallor and the absence of pulsation in the mesenteric vessels of the small intestine. The clamp was removed 45 min later and reperfusion was provided for 120 min. Reperfusion was confirmed by the restoration of pulsation and color prior to closing the incision. During the reperfusion phase, the abdominal cavity was closed with sutures. In the present study, we chose a model of 45-min occlusion of the SMA because the effectiveness of this duration was demonstrated in a previous study (2). Rats in the sham group were exposed to the abdominal incision but not the intestinal IR. After the reperfusion state, blood samples were taken by cardiac puncture and the rats were sacrificed by cervical dislocation.

2.4. Biochemical analysis
Blood samples were transferred into tubes containing no additive and allowed to clot at room temperature for 30 min, and then serum was separated by centrifugation at 3000 rpm for 15 min. Serum aliquots were stored at –80 °C immediately until analysis. Serum h-FABP levels were measured by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (Cusabio Biotech Co., Ltd., Newark, DE, USA). Briefly, standards or samples were added to the precoated plate with an antibody specific to h-FABP, and avidin conjugated to horseradish peroxidase was added to each microplate well and incubated. A 3,3',5,5'-tetramethyl-benzidine substrate solution was then added to each well. The enzyme–substrate reaction was terminated by the addition of a sulfuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. h-FABP levels were expressed as ng mL^{-1}. Serum levels of M30 antigen were determined with a commercially available ELISA kit (Peviva AB, Bromma, Sweden) according to the

manufacturer's instructions. M30-Apoptosense ELISA is based on the M30 monoclonal antibody, which specifically recognizes a neoepitope formed after cleavage of CK18, a process specifically occurring during apoptosis of CK18-containing cells. Briefly, samples were placed into wells coated with a monoclonal antibody as a catcher. After washing, a horseradish peroxidase-conjugated antibody was used for detection. Reference concentrations of M30 antigen were used to prepare assay calibration. The absorbance was determined with an ELISA reader at 450 nm. M30 levels were expressed as U L^{-1}.

2.5. Statistical analysis

Statistical analyses were carried out using SPSS for Windows, version 15.0. Data were presented as mean ± standard deviation (SD) and median (min-max) for normally distributed and not normally distributed variables, respectively. Data within groups were initially analyzed using the Shapiro–Wilk test for normality. For normally distributed variables (M30), Levene's test was used to test whether variances among the groups were homogeneous. If Levene's test indicated homogeneous variances, the groups were compared by one-way analysis of variance for equal variances. h-FABP levels that were not normally distributed were analyzed with the nonparametric Kruskal–Wallis test. The Mann–Whitney test was used for paired comparisons. P < 0.05 was considered statistically significant.

3. Results

Serum h-FABP levels of the groups are demonstrated in the Table. There was no statistically significant difference among the groups (P > 0.05) (Figure 1).

Serum M30 levels of the study groups are demonstrated in Figure 2. M30 levels were not different among the groups (S: 196.53 ± 45.84 U L^{-1}, IR: 229.20 ± 96.03 U L^{-1}, CoQ10+S: 181.27 ± 58.31 U L^{-1}, CoQ10+IR: 202.64 ± 48.58 U L^{-1}).

Table. Effects of intestinal IR and CoQ10 supplementation on serum h-FABP levels. Data are expressed as medians (minimum-maximum). S: Sham, IR: ischemia–reperfusion, CoQ10+S: coenzyme Q10 plus sham, CoQ10+IR: coenzyme Q10 plus ischemia–reperfusion.

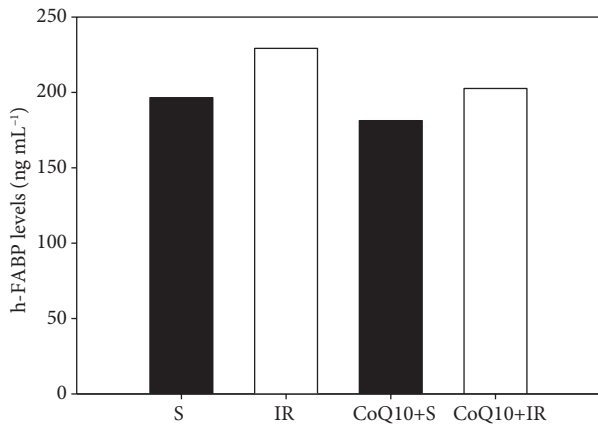| Groups	n	h-FABP (ng mL^{-1})
S	8	0.2696 (0.08–3.36)
IR	8	0.0800 (0.08–1.92)
CoQ10+S	8	0.0800 (0.08–0.90)
CoQ10+IR	8	0.0800 (0.08–0.21)

Figure 1. Effects of intestinal IR and CoQ10 supplementation on serum h-FABP levels. Data are expressed as medians. S: Sham, IR: ischemia–reperfusion, CoQ10+S: coenzyme Q10 plus sham, CoQ10+IR: coenzyme Q10 plus ischemia–reperfusion.
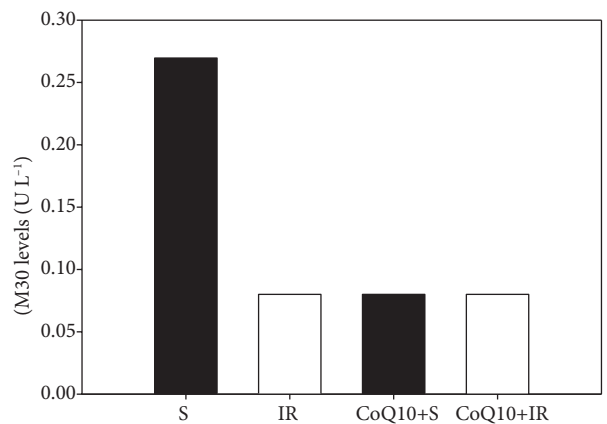

Figure 2. Effects of intestinal IR and CoQ10 supplementation on serum M30 levels. S: Sham, IR: ischemia–reperfusion, CoQ10+S: coenzyme Q10 plus sham, CoQ10+IR: coenzyme Q10 plus ischemia–reperfusion.

4. Discussion

This study is the first to evaluate the effect of CoQ10 supplementation on M30 and h-FABP levels in intestinal IR injury-induced rats. The evidence from this study indicates that neither intestinal IR nor CoQ10 supplementation affected serum M30 and h-FABP levels in rats. This study provides a new insight into intestinal IR injury. We demonstrated that 45 min of intestinal ischemia with 120 min of reperfusion may not affect intestinal barrier integrity and epithelial lining.

In the present study, serum h-FABP levels were not affected by intestinal IR and CoQ10 supplementation. In previous studies (7,23), a marked deficit of cardiac performance with dysfunction in myocardial contraction and relaxation was reported as a consequence of intestinal IR in experimental animals. Horton and White (23) demonstrated that one of the mechanisms that promote cardiac dysfunction after intestinal IR in rats is the production of ROS in cardiac cell membranes. It has been shown that although cardiac high-energy phosphate levels are preserved during experimental intestinal IR injury, fatty acid oxidation is impaired (24). As fatty acids are the preferred fuel of the heart, altered substrate selection and reliance on alternative fuels could lead to cardiac impairment (25). However, there is no report evaluating the effects of intestinal IR on h-FABP levels. Based on the current findings, we suggest that our ischemia period was too short to elevate h-FABP levels in the circulation. It has been suggested that when intestinal ischemia is limited to a period of less than 2 h, only the villi are affected while the crypt cells remain intact, and there is a rapid recovery of function (26). In the current study we applied 45 min of ischemia and this duration was not sufficient to affect h-FABP levels. Since intestinal and liver fatty acid-binding proteins are mainly expressed in the villi and not in the crypt, both proteins may be early and sensitive plasma markers of intestinal ischemia (10). Previous studies (27,28) described the applicability of intestinal fatty acid-binding protein for the detection of rat intestinal injury. In the present study, since we investigated distant organ damage, we measured h-FABP levels. h-FABP is more specific for heart muscle than other types of fatty acid-binding protein (29) and it is an early marker of myocardial injury (12). Additionally, CoQ10 supplementation did not affect h-FABP levels in intestinal IR induced or sham-operated rats. To our knowledge, no study to date has investigated the effects of CoQ10 on h-FABP levels. However, in previous reports the effects of CoQ10 supplementation were investigated in many different IR models, such as hepatic (22), urinary bladder (30), and limb (20) ischemia, and in those studies the protective effect of CoQ10 against IR-induced oxidative stress was demonstrated. In the present study, these findings suggest that mechanisms other than oxidative stress may affect h-FABP levels. Therefore, CoQ10 supplementation may not affect h-FABP levels.

In the present study, neither intestinal IR nor CoQ10 supplementation affected serum M30 levels. In many studies (15,31,32) it has been demonstrated that serum M30 levels increased in several types of cancers, and it has been also found that M30 levels were correlated with tumor volume (33). Previous reports (34,35) have suggested the role of apoptotic cell death in the pathogenesis of IR injury. Apart from necrotic cell death, immunohistochemistry for M30 revealed that apoptotic cell death occurred particularly during reperfusion (36). In the study by Grootjans et al. (36), M30 arteriovenous concentration differences remained low during reperfusion, suggesting that M30 remained within the apoptotic bodies and was not released into the circulation. In addition, leaking M30 (from apoptotically dying enterocytes undergoing secondary necrosis) was minimally exposed to lamina propria vasculature, because M30-positive cells were mainly detected in the detached enterocytes, thereby limiting release into the circulation (36). Based on this current information, unchanged M30 levels might be related to the duration of ischemia. As we mentioned above, in intestinal ischemia that is limited to a period of less than 2 h, only the villi are affected while the crypt cells remain intact. In a study by Topaloglu et al. (35), it was suggested that hepatocyte apoptosis may play a role in the development of IR injury of the liver. In another study, Ulukaya et al. (37) showed that CK18 seems to be useful to discriminate and quantitate the type of cell death during early ischemia and reperfusion periods of liver transplantation. Additionally, CoQ10 supplementation did not affect serum M30 levels in intestinal IR induced or sham-operated rats. To our knowledge, there is no report to date demonstrating the effects of CoQ10 on M30 levels. Therefore, we suggest that CoQ10 supplementation may not affect M30 levels in intestinal IR-induced or sham-operated rats.

In conclusion, the results of the present study suggest that intestinal ischemia–reperfusion injury with a short ischemic period and/or coenzyme Q10 supplementation does not affect serum h-FABP and M30 levels in rats. However, further and detailed studies are needed in this area to clarify the mechanism of action, the alterations that occur during the intestinal ischemia–reperfusion, and the role of coenzyme Q10 in this process.

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