Protective effects of hesperidin in gastric damage caused by experimental ischemia-reperfusion injury model in rats

Filiz Ozyigit[1]* , Ayse Nur Deger[2] , Fatma Emel Kocak[3] , Mehmet Fatih Ekici[4] , Hasan Simsek[5] , Ozlem Arık[6]

1. Bandirma Onyedi Eylul University ROR – Faculty of Medicine – Department of Pharmacology – Bandirma, Turkey.
2. Kutahya Health Sciences University ROR – Faculty of Medicine – Department of Pathology – Kutahya, Turkey.
3. Kutahya Health Sciences University ROR – Faculty of Medicine – Department of Medical Biochemistry – Kutahya, Turkey.
4. Kutahya Health Sciences University ROR – Faculty of Medicine – Department of General Surgery – Kutahya, Turkey.
5. Aksaray University ROR – Faculty of Medicine – Department of Physiology – Aksaray, Turkey.
6. Kutahya Health Sciences University ROR – Faculty of Medicine – Department of Biostatistics – Kutahya, Turkey.

ABSTRACT

Purpose: This study evaluated the protective effect of hesperidin on injury induced by gastric ischemia-reperfusion. **Methods:** Fifty male Sprague Dawley rats (250–300 g) were divided into five groups: control (C), sham (S), ischemia (I), ischemia-reperfusion (I/R) and hesperidin + ischemia-reperfusion (Hes + I/R). Hesperidin was injected intraperitoneally at the dose of 100 mg/kg one hour before the experimental stomach ischemia-reperfusion. Celiac artery was ligated. After 45 minutes ischemia and 60 minutes reperfusion period, blood samples were obtained under anesthesia. Then, animals were sacrificed, stomach tissues were excised for biochemical, and histopathological analyses were performed. Malondialdehyde levels and superoxide dismutase, glutathione peroxidase activities and total antioxidant status (TAS), total oxidant status (TOS), protein, total thiol parameters were measured in plasma, and tissue homogenate samples. H + E, periodic acid–Schiff, hypoxia inducible factor, terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end-labeling (TUNEL), and proliferating cell nuclear antigen (PCNA) for cell proliferation as immunohistochemical parameters were determined. **Results:** Upon biochemical and histopathological assessment, hesperidin decreased stomach tissue changes in comparison with IR group. Ischemia-reperfusion injury led to a considerably increase in malondialdehyde, protein, and TOS levels (p < 0.001) in stomach tissue. Hesperidin treatment significantly decreased malondialdehyde, protein, and TOS levels (p < 0.001). Hesperidin increased superoxide dismutase, TAS, total thiol and glutathione peroxidase activities in comparison with IR group. Hesperidin reduced damage and also increased TUNEL and PCNA immunoreactivity in stomach tissue. **Conclusions:** Hesperidin was able to decrease I/R injury of the stomach tissue due to inhibition of lipid peroxidation and protein oxidation, duration of antioxidant, and free radical scavenger properties. Consequently, hesperidin can provide a beneficial therapeutic choice for preventing stomach tissue ischemia-reperfusion injury in clinical application.

Key words: Hesperidin. Ischemia. Reperfusion. Wounds and Injuries.

◼ Introduction

Ischemia/reperfusion (I/R) injury of the gastrointestinal tract has been linked to a high rate of morbidity and mortality in patients suffering from hemorrhagic shock, gastric ulcer bleeding, microvascular dysfunction, and gastrointestinal disorders. Due to oxidative stress, ischemia followed by reperfusion causes gastric lesions[1].

Endothelial damage increases permeability, and cellular edema, and impairs blood flow regulation, and lysis are seen in tissues following ischemia-reperfusion (I/R)[2]. Gastric I/R leads to significant damage to the gastric mucosa[3,4].

In the pathology of acute stomach lesions generated by I/R injury, free radicals, lipid peroxidation, and neutrophil infiltration play an important role. Increased levels of reactive oxygen species (ROS) promote a breakdown in cell membrane integrity, resulting in ulcer development[5]. Excessive formation of ROS, neutrophil adherence to endothelial cells, synthesis and release of pro-inflammatory mediators, and a change in gastric acid output are all factors in the pathophysiology of I/R-induced gastric mucosal injury. These events result in cellular damage, and increases vascular permeability, tissue necrosis, and organ failure[6].

Free radical scavengers or antioxidants can help to protect the gastric tissue from free radical damage[7]. Hesperidin [(2S)-3′,5-Dihydroxy-4′-methoxy-7-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]flavan-4-one], a powerful antioxidant flavanone glycoside found in the rinds of citrus fruits, has antioxidant properties similar to vitamin E[8]. In the literature, it has been reported that hesperidin can eliminate free oxygen radicals[9]. In-vitro and in-vivo studies have shown that hesperidin has antibacterial, antiviral, antihypertensive, hypolipidemic, antiulcerogenic, antineoplastic, anti-inflammatory, and antihepatotoxic effects in addition to its antioxidant action[10,11]. Since no studies on the protective effect of hesperidin in the gastric I/R injury model have been published, the purpose of this study was to investigate the protective effect and mechanism of action of hesperidin in an experimental gastric I/R injury model in rats.

◼ Methods

This experimental study was carried out at Kutahya Health Sciences University's Experimental Animal Center in Kutahya, Turkey, after receiving approval from the Animal Experiments Local Ethics Committee (Decision Number: 2014.08.08). Rats were purchased from Kutahya Health Sciences University's Experimental Animal Center. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council[12].

Materials

Hesperidin (Hesperetin 7-rhamnoglucoside; Cirantin; Hesperetin-7-rutinoside, $C_{28}H_{34}O_{15}$, MW: 610.56, purity ≥ 95%, CAS Number: 520-26-3) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, United States of America). The compound was dissolved in a saline solution containing 0.5% Tween-80. Hesperidin was administered intraperitoneally (i.p.) at doses of 100 mg/kg. Drug administration was performed 60 minutes before the surgical procedure. Hesperidin doses were preferred based on previous experiments[13].

Animal model

Adult male Sprague Dawley rats weighing 250 to 300 g were used. Rats were taken to the laboratory one week prior to starting work to ensure compliance with the medication. They were fed pellet feed and drank water during the experiment. All animals were housed 12 hours in darkness, 12 hours in light, and at optimal room temperature. They were placed into five transparent polycarbonate cages in groups of five. The rats were randomly divided into five groups (n = 10 each group) as follows:
- Group 1: control (C) group, in which the stomach was removed without any surgical treatment protocol and I/R;
- Group 2: sham (S) group, in which the rats were subjected to identical surgical procedure, but no I/R. Sisty minutes before the laparotomy, 1 mL of saline was given;
- Group 3: gastric ischemia injury (I) group;
- Group 4: gastric ischemia / reperfusion injury (I/R) group;
- Group 5: hesperidin + I/R (H + I/R) group, in which the rats were given 100 mg/kg hesperidin i.p. 60 minutes before the surgical procedure.

Preparation of tissue homogenates

For biochemical analysis, tissue samples were mixed with a cold working solution (50 mM phosphate buffer, pH 7.40), and homogenized with a mechanical homogenizer (Analytik Jena speedmill plus, Jena, Germany). The mixtures

were then centrifuged at 10,000 × g for 15 min at 4°C, and the supernatants were preserved for biochemical analysis by storing on ice.

Surgical procedures

The animals were weighed and then anesthetized with i.p. injections of 70-mg/kg ketamine (Ketalar; Pfizer, Istanbul, Turkey) and 10-mg/kg xylazine hydrochloride (Rompun; Bayer, Istanbul, Turkey). During the surgery, the body temperature was maintained at 37°C with a homeothermic dissection table. The I/R injury model was carried out as previously described[14]. The surgical procedure was performed after the surgical incision site was cut, and the skin was cleaned with povidone iodine. Experimental animals underwent laparotomy with an anterior medial incision of the anterior abdominal wall under anesthesia. The celiac artery was clamped with an atraumatic vascular clamp (Vascu Stop, Bulldog Clamp) for 45 minutes. Ischemia was evaluated by the absence of pulses or the pale color of the stomach. Following ischemia, the clamp was removed, and a 60-minute reperfusion was performed. The start of the beats and the return of the red color were accepted as signs of stomach reperfusion.

Laboratory analysis
Sample collection

After 60 min of reperfusion, the experiment was terminated. The animals were sacrificed under anesthesia, and the stomach was carefully excised. The gastric tissue samples were rinsed with a cold heparinized saline solution to remove any red blood cells or clots. A portion of the stomach samples were fixed with 10% buffered formalin for histopathological and immunohistochemical analyses. Another portion of gastric tissue samples were placed into Eppendorf tubes and immediately stored at -80°C until biochemical analysis.

Preparation of tissue homogenates

For biochemical analysis, tissue samples were mixed with a cold working solution (50 mM phosphate buffer, pH 7.40), and homogenized with a mechanical homogenizer (Analytik Jena speedmill plus, Jena, Germany). The mixtures were then centrifuged at 10,000 × g for 15 min at 4°C, and the supernatants were preserved for biochemical analysis by storing on ice.

Measurement of tissue total antioxidant status, total oxidant status, and total thiol levels

Tissue total antioxidant status (TAS), total oxidant status (TOS), and total thiol (TT) levels were measured on the Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, United States of America) using commercial reagents (Rel Assay Diagnostic, Gaziantep, Turkey) based on novel automated measurement methods developed by Erel[15–17]. TAS levels were expressed as mmol Trolox Eq/mg protein. TOS levels were expressed as mmol Trolox Eq/mg protein. TOS levels were expressed as µmol H_2O_2 Eq/mg protein. TT levels were expressed as µmol/mg protein.

Calculation of the oxidative stress index

The percent ratio of TOS to TAS was accepted as the oxidative stress index (OSI), an indicator of the degree of oxidative stress. To calculate OSI, the unit of TAS, mmol of Trolox equivalent per mg of protein, was converted to mol of Trolox equivalent per mg of protein, and the Eq. 1[18] was used:

$$OSI = [(TOS, \text{mol } H_2O_2 \text{ Eq/mg protein})/(TAS, \text{mol Trolox Eq/mg protein}) \, 100] \qquad (1)$$

Measurement of tissue superoxide dismutase and glutathione peroxidase activities

Tissue superoxide dismutase (SOD) activities were measured on the Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, United States of America) using the Ransod kit (Randox Laboratories Ltd., Crumlin, UK). SOD activities were expressed as U/mg protein. Tissue glutathione peroxidase (GSH-PX) activities were measured on the Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, United States of America) using the Ransel kit (Randox Laboratories Ltd., Crumlin, United Kingdom). GSH-PX activities were expressed as U/mg protein.

Measurement of tissue malondialdehyde (MDA) levels

Tissue malondialdehyde (MDA) levels were measured based on the thiobarbituric acid reactive substances method using commercial enzyme-linked immunosorbent assay (ELISA) kits (Cayman Inc., Ann Arbor, MI, United States of America) on a microplate reader (BMG Labtech Spectrostar Nano, GmbH, Ortenberg, Germany). MDA levels were expressed as μM/mg protein.

Measurement of tissue protein level

Tissue protein levels were measured based on the Bradford method on the Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, United States of America)[19].

Histopathologic examinations

Gastric mucosa samples were fixed in 10% formaldehyde solution, embedded in paraffin, and 4-micron thick sections were prepared. Hematoxylin-eosin (H&E) staining was applied to the sections after deparaffinization and graded hydration and studied under a light microscope (Olympus BX41; Tokyo, Japan) by a pathologist who was blinded to the study groups. The scales used by Zhang et al. were used to evaluate H&E stained sections[20]. Mucosal epithelium was graded from 0 to 3 according to the injury (including point erosion, ulcer, and point hemorrhage):

- 0: no ulcer and no erosion;
- 1: erosion in the length of ≤ 1 mm;
- 2: > 1-mm and ≤ 2-mm long erosion;
- 3: > 2-mm and ≤ 3-mm long erosion.

If the width of the lesion were > 1 mm, the scale was folded to 2. Muscle loss in stomach epithelium with periodic acid–Schiff (PAS) was graded from 0 to 3[20,21].

Immunohistochemical examinations

Hypoxia inducible factor-1α (HIF-1α) and proliferating cell nuclear antigen (PCNA) were examined immunohistochemically. Immunohistochemical staining was performed using commercially available kits (HIF-1α, Santa Cruz sc-10790; PCNA abcam ab18197) according to the manufacturer's instructions. Gastric tissue was paraffinized and rehydrated. Four–five-micron thick sections were prepared for immunohistochemical evaluation with PCNA and HIF-1α. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. Primer antibodies to PCNA and HIF-1α were applied for 60 minutes. Peroxidase activity was elevated with DAB. Hematoxylin was applied for contour staining. Positive-responding cells were counted at 400x magnification. All the histomorphological and immunohistochemical evaluations were performed using the Olympus CX 41 light microscope. In all, seven randomly selected counties were included in the immunohistochemical assessment. The percentage of cells giving positive reactions was calculated.

In-situ detection of apoptosis

We used an in-situ terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay to determine the degree of stomach apoptosis. For the TUNEL, coloring Roche 11684817910 kit was used. The sections prepared from paraffin embedded tissues were deparaffinized with xylene. It was then passed through 95% ethanol, 85% ethanol, 75% ethanol, and 70% ethanol. The sections were washed with phosphate buffered saline (PBS) for 5 minutes. Proteinase K was incubated at room temperature for 20 minutes. After washed twice with PBS, the tissues were subjected to TUNEL at 37°C for 60 minutes. TUNEL positive cells were evaluated under 400x magnification under a light microscope. Apoptotic cells were counted[22].

Statistical analysis

The data were analyzed by Statistical Package for the Social Science version 16.0 (SPSS Co., Chicago, IL, United States of America) for Windows. Numerical data were tested for normality using the Shapiro-Wilk's test. While normally distributed data were expressed as mean ± standard deviation (SD), not normally distributed data were expressed as the median and interquartile range (IQRs).

The comparisons of numerical variables among groups were tested using analysis of variance (ANOVA) and Kruskal-Wallis' tests according to the distribution of data. Homogeneity test of variances of variables was performed with the Levene's statistic to select the post-hoc test. The multiple comparisons of examined variables were tested using the Bonferroni multiple comparisons post-hoc test for variables that passed the homogeneity of variances test and using the Dunnett T3 multiple comparisons post-hoc test for variables that no passed homogeneity of variances test. Statistical significance was established at the level of $P < 0.05$.

■ Results

Tissue total antioxidant status, total oxidant status, oxidative stress index, and thiol levels

Differences in tissue TAS, TOS, OSI, and TT levels between assay groups are represented in Table 1. Significant difference was observed among groups for TAS, TOS, OSI, and TT values ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively). I/R caused significant increases in TOS and OSI levels ($P < 0.05$) and significant decreases in TAS and TT levels ($P < 0.05$) in comparison with those caused by the control and sham groups. Hesperidin decreased TOS and OSI levels and increased TAS and TT levels compared to I/R group ($P < 0.05$).

Table 1 – Differences in tissue TAS, TOS, OSI, TT, MDA levels, and tissue SOD and GSH- PX activities between assay groups.

Parameters group 1 (mean ± SD) (Control) (n = 10)	Group 2 (Sham) (n = 10)	Group 3 (Ischemia) (n = 10)	Group 4 (I/R) (n = 10)	Group 5 (H + IR) (n = 10)	P-value
TAS (mmol Trolox 0.11 ± 0.013 Eq/mg protein)	0.11 ± 0.019	0.072 ± 0.013[ab]	0.042 ± 0.01[abc]	0.076 ± 0.015[abd]	< 0.0001
TOS 0.022 ± 0.015 (μmol H_2O_2 Eq/mg protein)	0.027 ± 0.013	0.058 ± 0.014[ab]	0.078 ± 0.015[ab]	0.049 ± 0.019[abd]	< 0.0001
OSI (Arbitrary 0.02 ± 0.013 Unit)	0.024 ± 0.01	0.083 ± 0.021[ab]	0.19 ± 0.031[abc]	0.067 ± 0.021[abd]	< 0.0001
SOD (U/mg 1.39 ± 0.22 protein)	1.25 ± 0.15	0.82 ± 0.10[ab]	0.53 ± 0.14[abc]	1.07 ± 0.26[acd]	< 0.0001
GSH-PX (U/mg 7.69 ± 1.73 protein)	7.34 ± 1.34	4.02 ± 1.12[ab]	2.60 ± 0.65[abc]	4.58 ± 1.42[abd]	< 0.0001
MDA (μM/mg 0.23 ± 0.06 protein)	0.24 ± 0.08	0.70 ± 0.20[ab]	0.76 ± 0.14[ab]	0.48 ± 0.13[abcd]	< 0.0001
TT (μmol/mg 22.5 ± 3.34)	21.5 ± 4.07	10.31 ± 2.33[ab]	7.08 ± 1.15[abc]	11.46 ± 2.83[abd]	< 0.0001

IR: ischemia/reperfusion; H: hesperidin; TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative stress index; SOD: superoxide dismutase; GSH-PX: glutathione peroxidase; MDA: malondialdehyde; TT: total thiol. Results are expressed as the mean ± standard deviation (SD). Since all data were normally distributed, the comparisons of variables among groups were analyzed with the one-way analysis of variance; p-value shows the differences among all groups. Since all data were passed the homogeneity of variances test, the multiple comparisons between two groups were analyzed with the Bonferroni-post-hoc test; (a) compared to control group; (b) compared to sham group; (c) compared to ischemia group, (d) compared to I/R group. Source: Elaborated by the authors.

Tissue superoxide dismutase and glutathione peroxidase activities

Differences in tissue SOD and GSH-PX activities between assay groups are represented in Table 1. Significant difference was observed among groups for SOD and GSH-PX activities ($P < 0.0001$, $P < 0.0001$, respectively). SOD and GSH-PX activities in the I/R group were significantly lower compared to the control and sham groups ($P < 0.05$). Hesperidin increased SOD and GSH-PX activities when compared to the I/R group ($P < 0.05$).

Tissue malondialdehyde levels

Differences in tissue MDA levels between assay groups are represented in Table 1. Significant difference was observed among groups for MDA levels ($P < 0.0001$). The group subjected to IR showed significantly increased in MDA levels compared to those of the control and sham groups ($P < 0.05$). Hesperidin decreased MDA levels compared to the I/R group ($P < 0.05$).

Histopathological and immunohistopathologic results

Comparisons of scored histopathological and immunohistochemical values in gastric tissue samples among assay groups are represented in Table 2. In the histopathological examinations, our results demonstrated that I/R injury caused a marked increase

in the total histopathological gastric damage score compared with the control and sham groups (P < 0.05, P < 0.05, respectively). A dose of 100 mg/kg of hesperidin significantly reduced the gastric damage scores compared to the I/R group (P < 0.05).
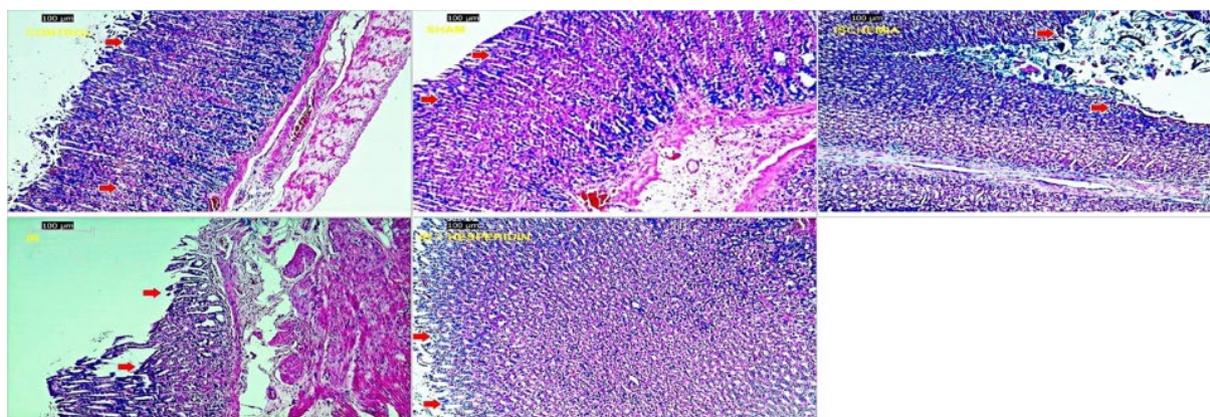
Compared with the control and sham group, significant surface ulcers were observed in the I/R group (grade 2). However, improvement in the epithelial ulceration was seen in the H + I/R group (grade 1) (Figs. 1 and 2). When apoptosis in the gastric tissue was examined using the TUNEL method, we observed that I/R injury caused a significant increase in the apoptotic index compared with the control and sham groups (P < 0.05, P < 0.05, respectively). A dose of 100 mg/kg of hesperidin significantly reduced the apoptotic index compared with the I/R group (P < 0.05) (Fig. 3). The effect of hesperidin on cell proliferation was examined by PCNA immunostaining. Study results demonstrated that I/R injury caused a significant decrease in the number of PCNA positive cells compared with the control and sham group (P < 0.05, P < 0.05, respectively).

However, hesperidin increased the number of PCNA positive cells compared to I/R group (P < 0.05) (Fig. 4). HIF-1α was examined using immunohistochemical staining. Study results demonstrated that I/R injury caused a significant increase in HIF-1α compared with the control and sham group (P < 0.05, P < 0.05, respectively). Hesperidin pre-treatment reduced the number of HIF-1α positive cells compared to I/R group (P < 0.05) (Fig. 5).

Table 2 – Comparisons of scored histopathological and immunohistochemical values in gastric tissue samples among assay groups.
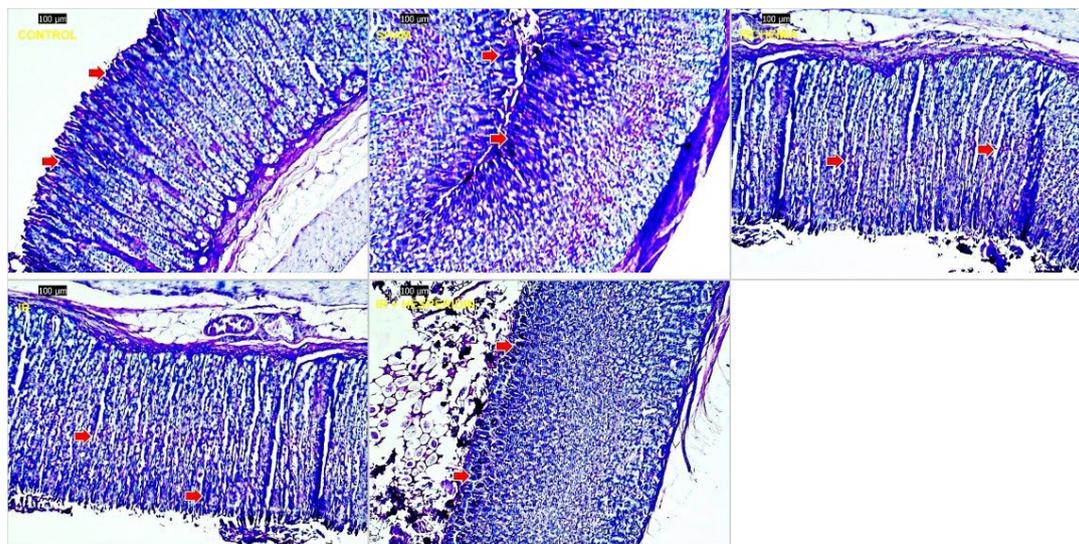
Parameters (mean ± SD)	Group 1 (Control)	Group 2 (Sham)	Group 3 (Ischemia)	Group 4 (I/R)	Group 5 (H + IR)	P-value
Gastric damage	(n = 10) 0.0 (0.0–0.0)	(n = 10) 0.0 (0.0–0.0)	(n = 10) 2.0 (1.0–2.0)[ab]	(n = 10) 2.0 (2.0–2.0)[ab]	(n = 10) 1.0 (1.0–1.0)[abcd]	< 0.0001
(H&E)						
Apoptotic index	2.2 ± 1.2	2.3 ± 1.0	11.1 ± 3.5[ab]	14.8 ± 3.9[ab]	6.1 ± 1.2[abcd]	< 0.0001
HIF-1α	8.8 ± 3.4	7.9 ± 4.2	14.8 ± 5.8[b]	16.9 ± 6.3[ab]	9.2 ±. 5.6[d]	< 0.0001
PCNA	41.3 ± 14.2	33.0 ± 8.1	19.9 ± 2.1[ab]	15.7 ± 5.3[ab]	31.0 ± 5.8[cd]	< 0.0001
PAS	0.0 (0.0–0.0)	0.0 (0.0–0.0)	2.0 (1.0–2.0)[ab]	2.0 (2.0–2.3)[ab]	1.0 (1.0–1.3)[abd]	< 0.0001

I/R: ischemia/reperfusion; H: hesperidin; HIF-1α: hypoxia-inducible factor-1α; PCNA: proliferating cell nuclear antigen; PAS: periodic acid-Schiff; H&E: hematoxylin and eosin. Normally distributed data were expressed as mean ± standard deviation (SD), and not normally distributed data were expressed as the median and interquartile range (IQRs). The comparisons of variables among groups were tested using analysis of variance and Kruskal-Wallis' tests according to the distribution of data; p-value shows the differences among all groups. The multiple comparisons between two groups were analyzed with the Bonferroni post-hoc test for HIF-1α that passed the homogeneity of variances test and with the Dunnett T3 post-hoc test for the other variables that no passed homogeneity of variances test; (a) compared to control group; (b) compared to sham group; (c) compared to ischemia group, (d) compared to I/R group. Source: Elaborated by the authors.
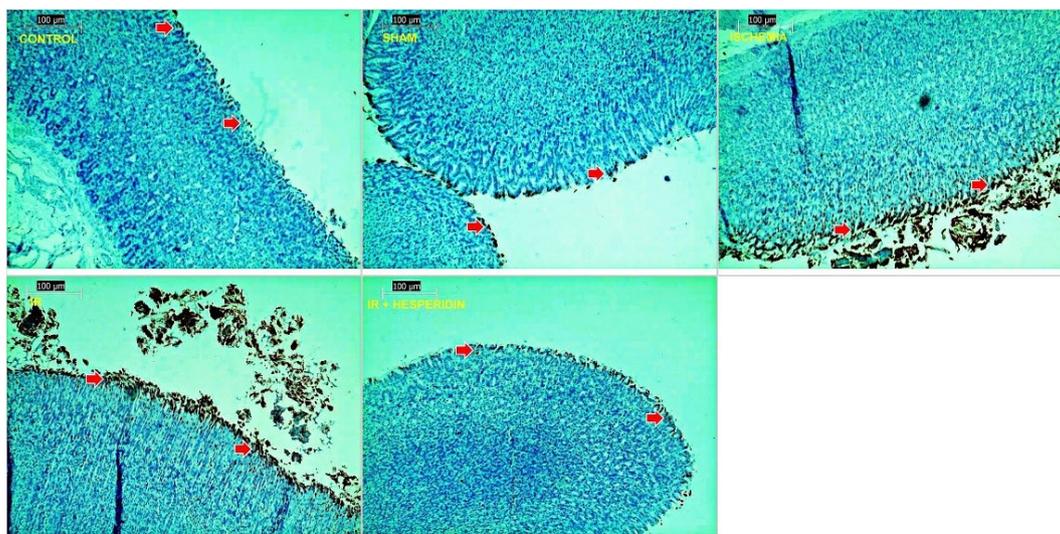

Source: Elaborated by the authors.

Figure 1 – Histopathological examination of gastric tissues in assay groups, hematoxylin and eosin (H&E) × 100. The control and sham groups contained regular stomach mucosa, epithelium. Ischemia group ulcer surface epithelium, grade 2. ischemia/reperfusion group, ulcer surface epithelium grade 2. Hesperidin + ischemia/reperfusion group, improvement in epithelial ulceration, ulcerated epithelium grade 1.
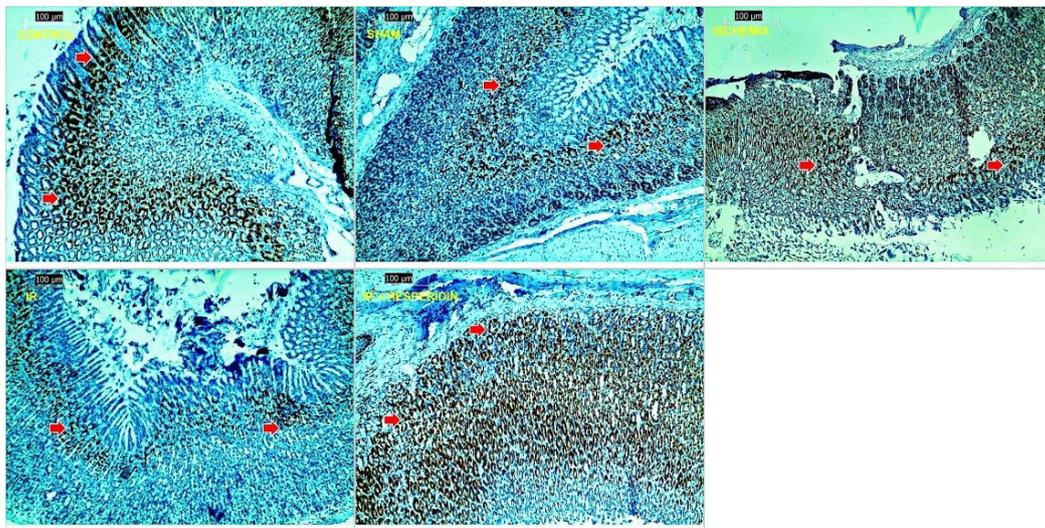
Figure 2 – Histopathological examination of stomach tissues in assay groups, periodic acid–Schiff (PAS) × 100. The control and sham groups contained regular stomach mucosa, mucin-containing epithelium. Ischemia group, decreased mucin grade 2 in epithelium. Ischemia/reperfusion group, decreased mucin grade 3 in epithelium. Hesperidin + ischemia/reperfusion group, improvement of epithelial mucin loss, cells containing mucin (PAS indicated by a red arrow).
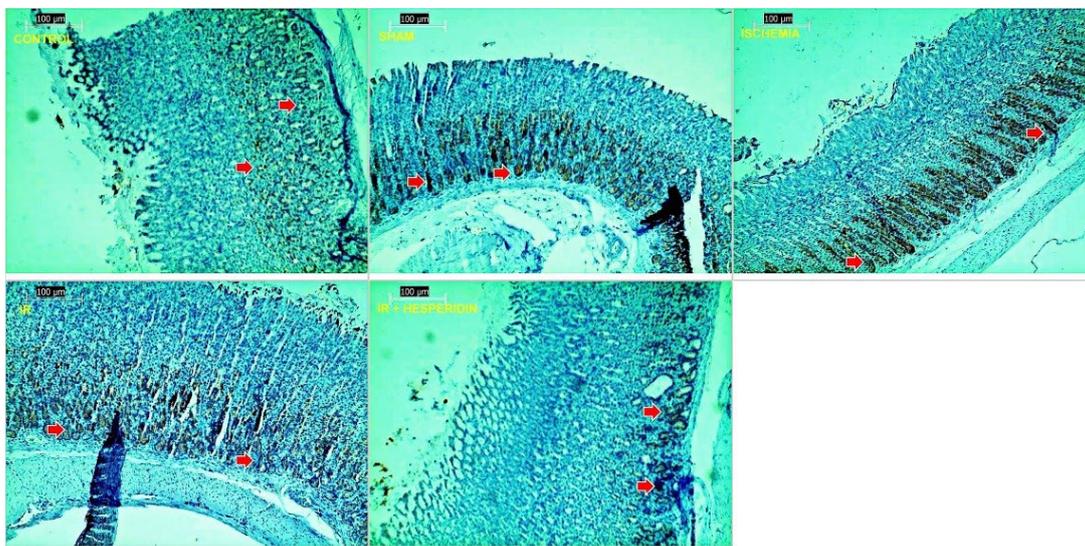
Figure 3 – Examination of apoptosis in stomach tissues in assay groups, terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end-labeling (TUNEL) × 400. The number of TUNEL positive cells per 100 was calculated from randomly selected fields. The apoptotic index was calculated as the percentage of apoptotic (TUNEL-positive stained) cells. The control and sham groups, TUNEL positive few cells, ischemia group, positive reaction with more number of cells, ischemia/reperfusion (I/R) group, positive reaction with more number of cells with more number of cells, I/R-group, positive reaction with more number of cells with TUNEL, I/R injury caused a significant increase in the apoptotic index, hesperidin + I/R group, positive reaction with fewer cells compared with ischemia and I/R groups with TUNEL. Hesperidin significantly decreased the apoptotic index. TUNEL-positive stained indicated by a red arrow.

Source: Elaborated by the authors.

Figure 4 – Examination of proliferating cell nuclear antigen (PCNA) expressions in stomach tissues in assay groups, PCNA × 400. The control and sham groups diffuse positive reaction with PCNA, ischemia group, PCNA positive reaction in a small number of cells, ischemia/reperfusion group, positive reaction with PCNA in a small number of cells. The percentage of PCNA positive cells was evaluated. Ischemia/reperfusion injury caused a significant decreased in the number of PCNA positive cells, hesperidin + ischemia/reperfusion group, positive reaction with PCNA in a larger number of cells compared with ischemia and ischemia/reperfusion groups. Hesperidin increased the number of PCNA positive cells compared with ischemia/reperfusion group. PCNA is indicated by a red arrow.

Source: Elaborated by the authors.

Figure 5 – Examination of hypoxia inducible factor-1α (HIF-1α) expressions in stomach tissues in assay groups, HIF-1α × 400. The control group contained a small number of positive cells, sham group, a few cells positive, ischemia group, positive reaction with more number of cells, ischemia/reperfusion group, positive reaction in more cells. When compared to the control and sham groups, ischemia/reperfusion damage induced a significant increase in the amount of HIF-1α positive cells, but hesperidin reduced the amount of HIF-1α positive cells. HIF-1α is indicated by a red arrow.

■ Discussion

Gastrointestinal mucosa is one of the most sensitive tissues to the ischemia. Cytotoxic effects of free oxygen radicals occurring after I/R and lipid peroxidation (LPO) play an important role in the formation of gastric mucosal lesions[23]. Increased free radicals and acidity of the I/R result in erosion and ulceration of the gastric mucosa. As a result, secretion and absorption functions deteriorate, and mucosal necrosis develops[24,25]. Gastric I/R is a complicated situation that limited information can be reached even though there are so many investigations on the damage[26,27]. Decreased or cut off blood flow in the stomach and reperfusion that occurs after it causes tissue damage at various grades. The I/R model can be created experimentally, as well as in many other organs. I/R injury increases free radical formation in the gastric mucosa, leading to LPO.

However, many studies have shown that antioxidants and free radical scavengers have gastroprotective effects by inhibiting LPO. Many antioxidant substances have the gastroprotective effect in experimental models of stomach I/R injury, which has recently been confirmed by different studies, even though there is no study of this model with hesperidin[28,29].

Degradation of oxidant and antioxidant balance in tissues is caused by oxidative stress. This causes serious damage to the gastric mucosa and negatively affects normal functions during I/R. Oxidative stress causes an increase in the products of LPO in the cellular membranes[30]. Our results indicate that the mechanisms underlying the protective actions of hesperidin against gastric I/R may be related to its capability to decrease the MDA levels. In this study, we observed an increase in MDA levels in the I/R group compared to the control and sham groups. Administration of hesperidin prior to I/R provided a protective effect against gastric I/R injury by reducing MDA levels. These results were consistent with a previous study in which 100 mg/kg hesperidin was administered i.p. Consequently, hesperidin pretreatment may demonstrate protective effects against gastric I/R injury by reducing LPO[31].

Increased free oxygen radicals (SOR) plays an important role in functional disorders in gastric I/R injury. Although the cells can protect themselves against oxidative damage, the antioxidant system is deficient in I/R damage. Endogenous antioxidant enzymes such as SOD and GPx in organism play an important role in reducing SOR's harmful effects. However, the levels of these enzymes fall during reperfusion in studies on I/R models. This is probably the result of an increase in free radicals[32–34]. During ischemia, the acceleration in the inactivation of enzymes such as SOD, GPX is observed due to the oxidants in the ischemic tissue. During the reperfusion process, cells are more susceptible to the effects of oxygen radicals. The improvement of I/R damage depends on the ability of the gastric mucosa to renew itself[35]. In this study, we found a significant decrease in antioxidant enzyme activities (SOD, GPx) in the I/R group compared with control and sham groups. In addition, we found that hesperidin at doses of 100 mg/kg appeared to increase antioxidant enzyme activities.

Our results were consistent with previous studies in which a 100 mg/kg dose of hesperidin was given[32–34,36]. The protective effect of hesperidin against gastric I/R injury may have been achieved through the antioxidant defense system. After 45 minutes of ischemia and 60 minutes of reperfusion, we observed that TOS and OSI levels, which are indicators of damage caused by free oxygen radicals, increased in the I/R group. Hesperidin pre-treatment reversed all these results.

Hesperidin has been reported to play a protective role in many experimental models with different doses, routes of administration, and administration times. Korthuis and Gute reported that damage to their work by inducing intestinal I/R injury in mice can be greatly reduced by hesperidin[37]. Dorkina demonstrated the hepatoprotective effect of hesperidin at a 100-mg/kg dose in their study of acute hepatitis by giving CCL4 to guinea pigs[38].

HIF-1 is one of the regulators which play a key role in ischemia. It consists of two subunits, α and β. The α subunit is closely related to cellular oxygen sensitivity. HIF-1α increases in sensitivity to hypoxia during ischemia[39]. In our study, the percentages of HIF-1α-positive cells were significantly increased in the I/R group when compared to the control and sham groups. The ratio of HIF-lα positive cells was lower in the hesperidin-treated group than in the I/R group. Therefore, hesperidin may be protective against gastric I/R injury by lowering HIF-1α protein levels.

In Qiao et al.'s study, although the gastric mucosa is mildly affected during ischemia, it has been found that more severe damage occurs, especially during the first hour of reperfusion, and that the ability of the stomach mucosa to repair itself was extremely high and that it was completed on the third day of complete repair. They argued that this was achieved by inhibiting gastric mucosal apoptosis and increasing the number of proliferative cells[40].

There are limited studies on gastric mucosal apoptosis and proliferation. Some studies contain different results. In the stomach mucosa, apoptotic cells, and proliferative cells are pointed out together[41].

Stomach I/R damage is associated with increased gastric mucosal injury, apoptosis, and decreased gastric mucosal cell proliferation. PCNA is present at various concentrations within the cell throughout the cell cycle. PCNA is an accessory protein that is secreted during the maximum S phase of cell cycle. The effect of hesperidin on cell proliferation was confirmed by histopathological analysis and PCNA immunostaining[42].

Hesperidin at the dose of 100 mg/kg caused an increase in cell proliferation. The TUNEL method was used to determine the apoptotic cells in the tissues. According to the TUNEL method results, the number of cells stained positive in the ischemia and I/R groups was higher than the control group, and this difference was statistically significant. These findings are similar to the protective effect of hesperidin in experimental I/R models. TUNEL for apoptosis and PCNA for cell proliferation in our study were similar to these findings[43,44]. Hesperidin caused a decrease in apoptosis and an increase in cell proliferation. Our study findings suggest that oxidative stress plays an important role in gastric I/R injury and that hesperidin pre-treatment may be useful against this damage.

■ Conclusion

This study suggests that hesperidin may have protective effects in gastric I/R injury. The mechanism underlying the protective effects of hesperidin in preventing damage due to gastric I/R injury is related to the ability to reduce lipid peroxidation, increase endogenous antioxidant enzyme activities, reduce apoptosis, and increase proliferation.

■ Conflict of interest

Nothing to declare.

■ Data availability statement

All data sets were generated or analyzed in the current study.

■ Authors' contribution

Substantive scientific and intellectual contributions to the study: Ozyigit F and Deger AN; **Conception and design:** Ozyigit F and Deger AN and Kocak FE; **Acquisition of data:** Ozyigit F, Ekici MF and Simsek H; **Analysis and interpretation of data:** Ozyigit F and Arik O; **Technical procedures:** Ozyigit F, Deger AN and Kocak FE; **Histopathological examinations:** Deger AN; **Statistics analysis:** Arik O; **Manuscript preparation:** Ozyigit F and Kocak FE; **Manuscript writing:** Ozyigit F, Kocak FE and Simsek H.

■ Funding

■ About the authors

Ozyigitis F and Ekici MF are medical doctors and associate professors.

Deger AN and Kocak FE are medical doctors and professors.

Simsek H is a physiologist and associate professor.

Arık O is a biostatistician and associate professor.

■ Acknowledgements

Not applicable.

■ References

1. Pena-Mercado E, Garcia-Lorenzana M, Arechaga E, la Rosa CH, Beltran NE. Gastric mucosa injury quantification in an ischemia-reperfusion experimental model. In: 38th Annual International Conference of the IEEE Engineering in Medicine and Biology Society. 2016. p. 2303–6. https://doi.org/10.1109/EMBC.2016.7591190

2. Gryglewski RJ, Palmer RMJ, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. Nature. 1986;320(6061):454–6. https://doi.org/10.1038/320454a0

3. Villegas I, Martín AR, Toma W, de la Lastra CA. Rosiglitazone, an agonist of peroxisome proliferators-activated receptor gamma, protects against ischemia-reperfusion damage in rats: Role of oxygen free radicals generation. Eur J Pharmacol. 2004;505(1-3):195–203. https://doi.org/10.1016/j.ejphar.2004.10.020

4. Kitahora T, Guth PH. Effect of aspirin plus hydrochloric acid on the gastric mucosal microcirculation. Gastroenterology. 1987;93(4):810–17. https://doi.org/10.1016/0016-5085(87)90444-6

5. Andrews FJ, Malcontenti-Wilson C, O'Brien PE. Polymorphonuclear leukocyte infiltration into gastric mucosa after ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol. 1994;266(1 Pt 1):G48–54. https://doi.org/10.1152/ajpgi.1994.266.1.g48

6. Konturek PC, Duda A, Brzozowski T, Konturek SJ, Kwiecien S, Drozdowicz D, Pajdo R, Meixner H, Hahn EG. Activation of genes for superoxide dismutase, interleukin-1beta, tumor necrosis factor-alpha, and intercellular adhesion molecule-1 during healing of ischemia-reperfusion-induced gastric injury. Scand J Gastroenterol. 2000;35(5):452–63. https://doi.org/10.1080/003655200750023697

7. Derin N, Izgut-Uysal VN, Agac A, Aliciguzel Y, Demir N. L-carnitine protects gastric mucosa by decreasing ischemia-reperfusion induced lipid peroxidation. J Physiol Pharmacol. 2004;55(3):595–606.

8. Guardia T, Rotelli AE, Juardez AO, Pelzer LE. Anti-inflammatory properties of plant flavonoids. Effect of rutin, quarcetin and hesperidin on adjuvant arthritis in rat. Farmaco. 2001;56(9):683–7. https://doi.org/10.1016/s0014-827x(01)01111-9

9. Koyuncu H, Berkarda B, Baykut F, Soybir G, Alatli C, Gül H, Altun M. Preventive effect of hesperidin against inflammation in CD-1 mouse skin caused by tumor promoter. Anticancer Res. 1999;19(4B):3237–41.

10. Javed H, Vaibhav K, Ahmed ME, Khan A, Tabassum R, Islam F, Safhi MM, Islam F. Effect of hesperidin on neurobehavioral, neuroinflammation, oxidative stress and lipid alteration in intracerebroventricular streptozotocin induced cognitive impairment in mice. J Neurol Sci. 2015;348(1-2):51–9. https://doi.org/10.1016/j.jns.2014.10.044

11. Ciftci O, Ozcan C, Kamisli O, Cetin A, Basak N, Aytac B. Hesperidin, a citrus flavonoid, has the ameliorative effects against experimental autoimmune encephalomyelitis (EAE) in a C57BL/J6 mouse model. Neurochem Res. 2015;40(6):1111–20. https://doi.org/10.1007/s11064-015-1571-8

12. Institute for Laboratory Animal Research National Research Council. Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press; 2010. 248 p.

13. Martínez AL, González-Trujano ME, Chávez M, Pellicer F, Moreno J, López-Muñoz FJ. Hesperidin produces antinociceptive response and synergistic interaction with ketorolac in an arthritic gout-type pain in rats. Pharmacol Biochem Behav. 2011;97(4):683–9. https://doi.org/10.1016/j.pbb.2010.11.010

14. Wada K, Kamisaki Y, Kitano M, Kishimoto Y, Nakamoto K, Itoh T. A new gastric ulcer model induced by ischemia-reperfusion in the rat: role of leukocytes on ulceration in rat stomach. Life Sci. 1996;59(19):PL295–301. https://doi.org/10.1016/0024-3205(96)00500-0

15. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem. 2004;37(2):112–9. https://doi.org/10.1016/j.clinbiochem.2003.10.014

16. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005;38(12):1103–11. https://doi.org/10.1016/j.clinbiochem.2005.08.008

17. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. Clin Biochem. 2014;47(18):326–32. https://doi.org/10.1016/j.clinbiochem.2014.09.026

18. Eren Y, Dirik E, Neselioglu S, Erel O. Oxidative stress and decreased thiol level inpatients with migraine: cross-sectional study. Acta Neurol Belg. 2015;115(4):643–9. https://doi.org/10.1007/s13760-015-0427-y

19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248–54. https://doi.org/10.1006/abio.1976.9999

20. Zhang YM, Wei EQ, Li L, Qiao WL, Wang L, Zhang JF. Extracellular signal-regulated kinase pathways may mediate the protective effect of electrical stimulation of the paraventricular nucleus against ischaemia-reperfusion injury of the gastric mucosa. Clin Exp Pharmacol Physiol. 2007;34(8):742–52. https://doi.org/10.1111/j.1440-1681.2007.04652.x

21. Wang T, Zhou YT, Chen XN, Zhu AX, Wu BH. Remote ischemic postconditioning protects against gastric mucosal lesions in rats. World J Gastroenterol. 2014;20(28):9519–27. https://doi.org/10.3748/wjg.v20.i28.9519

22. Cai Y, Wang W, Liang H, Sun L, Teitelbaum DH, Yang H. Keratinocyte growth factor improves epithelial structure and function in a mouse model of intestinal ischemia/reperfusion. PLoS One. 2012;7(9):e44772. https://doi.org/10.1371/journal.pone.0044772

23. Zimmerman BJ, Granger DN. Reperfusion injury. Surg Clin North Am. 1992;72(1):65–83. https://doi.org/10.1016/s0039-6109(16)45628-8

24. Tanaka J, Yuda Y. Role of lipid peroxidation in gastric mucosal lesions induced by ischemia-reperfusion in the pylorus-ligated rat. Biol Pharm Bull. 1993;16(1):29–32. https://doi.org/10.1248/bpb.16.29

25. Yasue N, Chan ET, Kaplowitz N, Guth PH. Effect of phorone and allopurinol on ischemia-reperfusion injury in gastrointestinal mucosa of the rat. Pharmacology. 1992;44(6):334–43. https://doi.org/10.1159/000138938

26. De La Lastra CA, Cabeza J, Motilva V, Martin MJ. Melatonin protects against gastric ischemia-reperfusion injury in rats. J Pineal Res. 1997;23(2):47–52. https://doi.org/10.1111/j.1600-079x.1997.tb00334.x

27. Wada K, Kamisaki Y, Kitano M, Nakamoto K, Itoh T. Protective effect of cystathionine on acute gastric mucosal injury induced by ischemia-reperfusion in rats. Eur J Pharmacol. 1995;294(2-3):377–82. https://doi.org/10.1016/0014-2999(95)00558-7

28. Liu Y, Gou L, Fu X, Li S, Lan N, Yin X. Protective effect of rutin against acute gastric mucosal lesions induced by ischemia-reperfusion. Pharm Biol. 2013;51(7):914–9. https://doi.org/10.3109/13880209.2013.771375

29. Mard SA, Mojadami S, Farbood Y, Gharib Naseri MK. The anti-inflammatory and anti-apoptotic effects of gallic acid against mucosal inflammation- and erosions-induced by gastric ischemia-reperfusion in rats. Vet Res Forum. 2015;6(4):305–11.

30. Váli L, Taba G, Szentmihalyi K, Fébel H, Kurucz T, Pallai Z, Kupcsulik P, Blázovics A. Reduced antioxidant level and increased oxidative damage in intact liver lobes during ischaemia-reperfusion. World J Gastroenterol. 2006;12(7):1086–91. https://doi.org/10.3748%2Fwjg.v12.i7.1086

31. Bayomy NA, Elshafey SH, ElBakary RH, Abdelaziz EZ. Protective effect of hesperidin against lung injury induced by intestinal ischemia/reperfusion inadult albino rats: Histological, immunohistochemical and biochemical study. Tissue Cell. 2014;46(5):304–10. https://doi.org/10.1016/j.tice.2014.05.009

32. Ekinci Akdemir FN, Gülçin İ, Karagöz B, Soslu R, Alwasel SH. A comparative study on the antioxidant effects of hesperidin and ellagic acid against skeletal muscle ischemia/reperfusion injury. J Enzyme Inhib Med Chem. 2016;31(Suppl. 4):114–8. https://doi.org/10.1080/14756366.2016.1220378

33. Gandhi C, Upaganalawar A, Balaraman R. Protection against in vivo focal myocardial ischemia/reperfusion injury-induced arrhythmias and apoptosis by hesperidin. Free Radic Res. 2009;43(9):817–27. https://doi.org/10.1080/10715760903071656

34. Celik E, Oguzturk H, Sahin N, Turtay MG, Oguz F, Ciftci O. Protective effects of hesperidin in experimental testicular ischemia/reperfusion injury in rats. Arch Med Sci. 2016;12(5):928–34. https://doi.org/10.5114/aoms.2015.47697

35. Marubayashi S, Dohi K, Ochi K, Kawasaki T. Protective effects of free radical scavenger and antioxidant administration on ischemic liver cell injury. Transplant Proc. 1987;19(1 Pt 2):1327–8.

36. Gaur V, Kumar A. Hesperidin pre-treatment attenuates NO-mediated cerebral ischemic reperfusion injury and memory dysfunction. Pharmacol Rep. 2010;62(4):635–48. https://doi.org/10.1016/s1734-1140(10)70321-2

37. Korthuis RJ, Gute DC. Postischemic leukocyte/endothelial cell interactions and microvascular barrier dysfunction in skeletal muscle: cellular mechanisms and effect of Daflon 500 mg. Int J Microcirc Clin Exp. 1997;17(Suppl. 1):11–7. https://doi.org/10.1159/000179261

38. Dorkina EG. Izuchenie gepatozashchitnogo deĭstviia prirodnikh flavonoidnykh soedeniniĭ [Investigation of the hepatoprotector action of natural flavonoids]. Eksp Klin Farmakol. 2004;67(6):41–4.

39. Choi IY, Kim SJ, Jeong HJ, Park SH, Song YS, Lee JH, Kang TH, Park JH, Hwang GS, Lee EJ, Hong SH, Kim HM, Um JY. Hesperidin inhibits expression of hypoxia inducible factor-1 alpha and inflammatory cytokine production from mast cells. Mol Cell Biochem. 2007;305(1-2):153–61. https://doi.org/10.1007/s11010-007-9539-x

40. Qiao WL, Wang L, Zhang JF, Zhang YM. Effects of gastric ischemia-reperfusion on gastric mucosal cellular apoptosis and proliferation in rats. Sheng Li Xue Bao. 2006;58(3):237–43.

41. Xia HH, Talley NJ. Apoptosis in gastric epithelium induced by Helicobacter pylori infection: implications in gastric carcinogenesis. Am J Gastroenterol. 2001;96(1):16–26. https://doi.org/10.1111/j.1572-0241.2001.03447.x

42. Foley JF, Dietrich DR, Swenberg JA, Maronpot RR. Detection and Evaluation of Proliferating Cell Nuclear Antigen (PCNA) in Rat Tissue by an Improved Immunohistochemical Procedure. J Histotechnol. 1991;14(4):237–41. https://doi.org/10.1179/his.1991.14.4.237

43. Kamaraj S, Ramakrishnan G, Anandakumar P, Jagan S, Devaki T. Antioxidant and anticancer efficacy of hesperidin in benzo(a)pyrene induced lung carcinogenesis in mice. Invest New Drugs. 2009;27(3):214–22. https://doi.org/10.1007/s10637-008-9159-7

44. Kara M, Türkön H, Karaca T, Güçlü O, Uysal S, Türkyılmaz M, Demirtaş S, Dereköy FS. Evaluation of the protective effects of hesperetin against cisplatin-induced ototoxicity in a rat animal model. Int J Pediatr Otorhinolaryngol. 2016;85:12–8. https://doi.org/10.1016/j.ijporl.2016.03.019