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The effects of whortleberry on ischemia reperfusion-induced myocardial injury in rats

Sıçanlarda yaban mersininin iskemi-reperfüzyona bağlı miyokard hasarı üzerine etkileri

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ABSTRACT

Background: The aim of this study was to investigate the potential protective effect of whortleberry by examining the effects on heart tissue at the molecular level of ischemia-reperfusion injury caused by surgical repair of a ruptured abdominal aortic aneurysm.

Methods: Between May 2018 and February 2019, a total of 32 male Sprague-Dawley rats were randomly assigned into control, sham (ischemia-reperfusion+glycerol), ischemia-reperfusion, and ischemia-reperfusion+whortleberry groups. Hypovolemic shock was applied to the rats in the ischemia-reperfusion groups for one hour. The abdominal aorta was explored following midline laparotomy and atraumatic microvascular clamps were applied from the infrarenal level. Following one-hour ischemia, the clamps were removed, and reperfusion was established for two hours. In the sham group, intraperitoneal glycerol once daily was applied five days before surgery. In the whortleberry group, whortleberry treatment was administered via the intraperitoneal route five days before ischemia-reperfusion.

Results: The ischemia-reperfusion group exhibited a decrease in the glutathione levels and an increase in the malondialdehyde levels ($p<0.01$ and $p<0.01$, respectively). We also observed an increase in the caspase-3 positivity in cardiac myofibrils ($p<0.01$). Whortleberry administration lowered both malondialdehyde levels and numerical density of caspase-3 positive cardiac myofibrils, while increasing the heart tissue glutathione levels, compared to the ischemia-reperfusion alone group ($p<0.01$, $p=0.011$, and $p=0.011$, respectively).

Conclusion: Whortleberry may be beneficial in preventing cardiac tissue damage caused by ischemia-reperfusion in the surgical repair of ruptured abdominal aortic aneurysms.

Keywords: Heart, infrarenal occlusion, ischemia, oxidative stress, reperfusion, whortleberry.

ÖZ

Amaç: Bu çalışmada rüptüre abdominal aort anevrizmasının cerrahi onarımına bağlı iskemi reperfüzyon hasarının moleküler düzeyde kalp dokusu üzerindeki etkileri incelenerek, yaban mersininin muhtemel koruyucu etkisi araştırıldı.

Çalışma planı: Mayıs 2018 - Şubat 2019 tarihleri arasında toplam 32 adet erkek Sprague-Dawley sıçan rastgele olarak kontrol, sham (iskemi-reperfüzyon+gliserol), iskemi-reperfüzyon ve iskemi-reperfüzyon+yaban mersini gruplarına ayrıldı. İskemi-reperfüzyon gruplarındaki sıçanlara bir saat süreyle hipovolemik şok uygulandı. Abdominal aort midline laparatomi ile eksplore edildi ve infrarenal seviyeden atravmatik mikrovasküler klempler uygulandı. Bir saatlik iskemi sonrasında, klempler kaldırıldı ve iki saat süreyle reperfüzyon uygulandı. Sham grubuna cerrahiden beş gün önce, günde bir kez intraperitoneal gliserol uygulandı. Yaban mersini grubuna, iskemi-repefüzyondan beş gün önce intraperitoneal yoldan yaban mersini tedavisi uygulandı.

Bulgular: İskemi-reperfüzyon grubunda glutatyon düzeylerinde düşüş ve malondialdehit düzeylerinde artış gözlendi (sırasıyla, $p<0.01$ ve $p<0.01$). Kalp miyofibrillerinde kaspaz-3 pozitifliğinde de bir artış gözlendi ($p<0.01$). Yaban mersini tedavisi, yalnızca iskemi-reperfüzyon grubuna kıyasla, hem malondialdehit düzeylerini hem de kaspaz-3 pozitif kalp miyofibrillerinin sayısal yoğunluğunu azalttı ve kalp dokusundaki glutatyon düzeylerini artırdı (sırasıyla, $p<0.01$, $p=0.011$ ve $p=0.011$).

Sonuç: Rüptüre abdominal aort anevrizmalarının cerrahi onarımında iskemi-reperfüzyona bağlı kalp dokusu hasarının önlenmesinde yaban mersini yararlı olabilir.

Anahtar sözcükler: Kalp, infrarenal tıkanıklık, iskemi, oksidatif stres, reperfüzyon, yaban mersini.

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Ruptured abdominal aortic aneurysm (RAAA) is responsible for 1 to 2% of deaths above the age of 65 years.[1] Ischemia-reperfusion (I/R) injury occurs in the lower extremities alone as a result of clamping and declamping to the abdominal aorta during intact aneurysm surgery.[1] Hemorrhagic shock before clamping in the surgical treatment of RAAAs and consequent diffuse tissue perfusion impairment are also added to the manifestation. This complex situation results in injury in distant organs, such as the lungs and heart, leading to systemic inflammatory response syndrome and multiorgan failure, and increases mortality in the surgical treatment of RAAAs.[1]

Previous studies have shown mortality rates as high as 60% in RAAAs.[2] A significant decrease in mortality has occurred in elective cases due to recent advances in surgical and postoperative care techniques since the adoption of surgical treatment of abdominal aortic aneurysms. However, no significant progress has been recorded in the high mortality rates in RAAAs, the standard treatment of which requires emergency surgical intervention.[2] In particular, no specific treatment has been developed to reduce mortality rates associated with cardiovascular events and multiorgan failure caused by diffuse tissue perfusion disorders. The essential difference between elective cases and RAAA repair is the hemorrhagic shock period. Animal studies have shown that hemorrhagic shock and the surgical repair process is an I/R event involving the entire body.[2,3]

Although the mechanism involved in multiorgan failure due to aortic occlusion with cross-clamps has not been fully understood yet, inflammation resulting from inflammatory cytokines and reactive oxygen radicals (ROS) release has been implicated.[1-5] Although various previous studies have investigated kidney and lung tissue in the context of abdominal aortic occlusion, there have been no extensive studies focusing on the heart tissue.[6,7] The most familiar technique to identify lipid peroxidation deriving from increased ROS production leading to oxidative stress in tissue is the measurement of malondialdehyde (MDA) levels.[8,9] Malondialdehyde is one of the most easily tested end products of both enzymatic and non-enzymatic lipid peroxidation reactions.[10] Glutathione (GSH), one of the antioxidant enzymes involved in the elimination of ROS, is another important marker of oxidative stress.[11] As an antioxidant, it protects the cells against lipid peroxidation through participation in various metabolic events, such as hyperperoxide-mediated mutagenesis, the protection of unsaturated lipids in biomembranes, and the regulation of prostacyclin and prostaglandin biosynthesis.[12] Previous studies have shown that oxidative stress resulting in aortic occlusion gives rise to cascade-dependent apoptosis (particularly caspase-3), thus leading to deoxyribonucleic acid damage.[13]

Vaccinium myrtillus L. (bilberry), commonly known whortleberry (WB), contains a number of phenolic compounds including flavonoids and tannins such as quercetin, myricetin and isorhamnetin, ellagitannins, phenolic acids, and anthocyanins.[14-16] The WB, an anthocyanoside and novel free radical scavenger, has been used as an antioxidant in vascular, cardiac, and intestinal tissues.[17] It has been shown to ameliorate oxidative stress in tissues reducing MDA levels, while increasing those of antioxidant enzymes.[15-18] Recent studies have also identified no risk of interaction between WB and metabolism of therapeutic drugs.[14]

In this experimental study, we aimed to investigate the potential protective effect of WB, the flavonoid- and phenolic compound-rich antioxidant, against the deleterious effects on cardiac tissue of infrarenal aortic clamping-related I/R.

MATERIALS AND METHODS

This prospective, experimental study was conducted at Recep Tayyip Erdoğan University Medical Faculty between May 2018 and February 2019. A total of 32 male Sprague-Dawley rats aged three to four months with a mean weight of 250±35 g were obtained from the Recep Tayyip Erdoğan University Medical Faculty Animal Care and Research Unit. The animals were housed at the Recep Tayyip Erdoğan University Medical Faculty, Faculty of Medicine, Basic Medical Sciences Experimental Animal Application Unit in a 12-h light: 12-h dark cycle at a room temperature of 22±3°C and 55 to 60% humidity. All animals received humane care in accordance with the criteria of the National Academy of Sciences Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study was approved by the Recep Tayyip Erdoğan University Medical Faculty Animal Ethical Committee.

Study population

All rats were randomly assigned into control, sham (I/R+glycerol), I/R, and I/R+WB groups. The rats in the glycerol group received 1 mL glycerol via the intraperitoneal (i.p.) route once daily for five days before surgery. The rats in the WB group received 100 mL/kg/day i.p. WB for five days prior to surgery.[15,19] All rats in the study were anesthetized with 50 mg/kg i.p. ketamine hydrochloride (Ketalar®, Eczacıbaşı Parke-Davis, Istanbul, Turkey) and

10 mg/kg i.p xylazine hydrochloride (Alfazyne®, Alfasan International B.V., Woerden, Holland). Anesthesia was maintained throughout the procedure with the administration of ketamine at intervals such as to permit continuations spontaneous respiration. The rats were, then, immobilized in the supine position under a heating lamp. The right internal jugular vein was cannulated for fluid replacement and right carotid artery was cannulated with a 22-gauge branule (Novacath™, Medipro Co., Istanbul, Turkey) to monitor the mean arterial pressure (MAP). The skin was shaved as appropriate for an abdominal midline incision and stained with povidone-iodine solution for sterilization. Shock in the form of MAP ≤50 mmHg was induced in all groups, except for the control group by the collection of blood from the cannula inserted in the carotid artery using an injector containing 500 U of heparin (Nevparin, 5000 U/mL, Mustafa Nevzat Pharmaceuticals, Istanbul, Turkey), thus simulating an aneurysmal rupture. Additional blood was collected when required to induce a MAP of ≤50 mmHg, and the times and quantities were recorded. The shock phase was applied for 60 min. The collected blood was stored at room temperature for subsequent use in resuscitation. The rats in the control group underwent laparotomy alone. This was subsequently closed with 5/0 prolene sutures, and the rats were kept under anesthesia until the end of the study.

In the groups subjected to I/R, the abdomen was opened with a midline incision following completion of the shock phase. The retroperitoneum was opened, and the abdominal aorta was explored by diverting the intestines to the right. Anticoagulation was established with 100 IU intravenous (i.v.) heparin. Bulldog clamps were attached to the abdominal aorta from the infrarenal level and proximally to the iliac bifurcation to initiate the I/R phase. Half the previously collected blood stored at room temperature was returned through the cannula inserted in the jugular vein. Ischemia was applied for 60 min. At the end of the ischemic period, the other half of the blood was returned through the cannula immediately prior to removal of the clamps. Once the clamps were removed, the abdomen was closed, and the rats were reperfused for 120 min. The MAP was kept above 100 mmHg with i.v. administration of additional isotonic solution, if required. At the end of the reperfusion period, blood specimens were collected from the right ventricle through median sternotomy in all rats for biochemical analysis. Following the completion of the I/R model, all rats were sacrificed by exsanguination through the carotid artery cannula.[7,20]

Biochemical analyses

The heart tissue specimens were homogenized by adding phosphate buffer at a volume five times higher than the tissue volume weight. Homogenized tissues were, then, centrifuged for 15 min at 3,000 g for biochemical assays.[21] The Ellman method was employed to measure cardiac GSH levels.[22] Analysis was performed at 412 nm on a spectrophotometer, and the results were expressed in μmoL/L. The values obtained were divided by the tissue weight and expressed in μmoL/g tissue. The MDA levels were measured using the method described by Draper and Hadley[23] on a spectrophotometer at 532 nm, and the results were expressed in μmoL/L. The values obtained were divided by the tissue weight and expressed in μmoL/g tissue.

Histopathological analysis

The rat heart tissues were also subjected to histopathological examination. The tissues were quickly trimmed and fixed for 36 h in 10% formalin (Sigma Aldrich, St. Louis, MO, USA) for light microscopy examination. Routine procedures were applied before the tissue specimens were embedded in paraffin blocks (Merck, Darmstadt, Germany). The sections of 4 to 5 μm in thickness were taken from the paraffin blocks with the assistance of a microtome (Leica, RM2125RT, Germany) prior to staining with hematoxylin (Harris hematoxylin, Merck, Germany) and eosin (H-E) (Eosin G, Merck, Germany). Tissues were, then, examined under a light microscope (Olympus BX51, Olympus Corp., Tokyo, Japan) and photographed with an Olympus DP71 camera (Olympus Corp., Tokyo, Japan).

Immunohistochemical (IHC) analysis

Determination of caspase-3 immune activity used to identify apoptotic cells in the heart tissue involved the avidin-biotin-peroxidase method. Briefly, 2 to 3-μm thick sections were taken from the paraffin blocks and placed onto positively-charged slides (Patolab, China). Following deparaffinization, the tissue specimens were treated with 3% H_2O_2 solution for 15 min for the purpose of blocking endogenous peroxidase activity. Secondary blocking solution was applied for 20 min to prevent background staining, after which the tissues were incubated with primary antibody (caspase-3, rabbit polyclonal, Abcam Inc., United Kingdom) for 60 min. Tissue specimens were, then, incubated with secondary antibody (Goat Anti-Rabbit IgG H&L (HRP) (ab205718, Abcam Inc., United Kingdom). Diaminobenzidine chromogen (DAB Chromogen, Abcam Inc., United Kingdom) solution

was dropped onto the tissues. An image signal was, then, obtained on the light microscope. Counterstaining was applied to tissues stained with Harris hematoxylin (Merck, Darmstadt, Germany), and an appropriate blocking solution was employed (Table 1).

Statistical analysis

Statistical analysis was performed using the PASW version 18.0 software (SPSS Inc., Chicago, IL, USA). Non-parametric data were expressed in median (min-max) values, while parametric data were expressed in mean ± standard deviation (SD). Intergroup analyses were performed using the Kruskal-Wallis and Tamhane T2 tests for non-parametric data. Parametric data were analyzed using one-way analysis of variance (ANOVA) and Tukey honestly significant difference (HSD) test. A *p* value of <0.05 was considered statistically significant.

RESULTS

Biochemical results

The MDA levels in the I/R and I/R+glycerol groups were higher than those in the control group (p=0.001 and p=0.03, respectively; Table 2). In contrast, MDA levels in the WB treatment group were significantly lower than in the I/R group (p=0.005; Table 2). We observed no significant difference in the MDA levels between the I/R and I/R+glycerol groups (p=0.6334; Table 2).

There was decrease in the GSH levels in both I/R and I/R+glycerol groups, compared to the control group

Table 1. Semi-quantitative analysis

Grade		%
0	None	-
1	Mild (less than)	5
2	Moderate	6-25
3	Severe (more than)	25

Table 2. Biochemical analysis results

	MDA (µmoL/g tissue)	GSH (µmoL/g tissue)
Groups	Mean±SD	Mean±SD
Control	1.0±0.8	8.5±0.6
I/R	1.15±0.0[a,e]	6.3±0.6[f]
I/R+Glycerol	1.13±0.1[b]	6.2±0.4[g]
I/R+WB	1.0±0.0[c]	7.8±0.6[h,i]

MDA: Malondialdehyde; SD: Standard deviation; GSH: Glutathione; I/R: Ischemia-Reperfusion WB: Whortleberry; HSD: Honestly significant difference.
Tukey HSD test
[a]p=0.001 Control group versus the IR group;
[b]p=0.03 Control group versus the I/R+Glycerol group;
[c]p=0.005 I/R group versus the I/R+WB group;
[d]p<0.01 Control group versus the Glycerol group;
[e]p=0.6334 Glycerol+IR group versus the I/R group;
[f]p=0.001 Control group versus the IR group;
[g]p=0.01 Control group versus the I/R+Glycerol group;
[h]p=0.05 I/R group versus the I/R+WB group;
[i]p=0.661 Glycerol+IR group versus the I/R group.

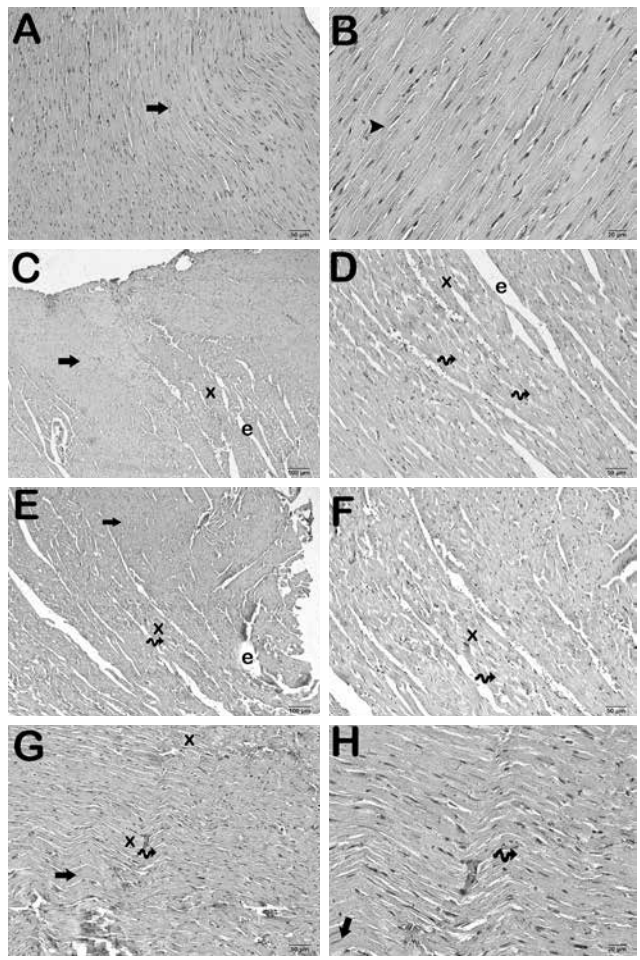

Figure 1. Light microscopy photographs of heart tissues (H-E). **(a, b)** Heart tissue sections from control group showing cardiac muscle myofibrils (arrow) with normally organized isotropic and anisotropic bands (arrow), and typical collateral branches (arrowhead). **(c, d)** I/R and **(e, f)** I/R+glycerol group sections showing swollen degenerative heart muscle cardiac myofibrils (×) among cardiac muscle cardiac myofibrils with a normal organization, and diffuse edematous areas **(e)**. Heart muscle cardiac myofibrils with lost cytoplasm content (spiral arrow). **(g, h)** I/R+whortleberry group sections showing diffuse heart muscle cardiac myofibrils with normally organized I-A bands (arrow), along with swollen heart muscle cardiac myofibrils with loss of cytoplasm contents (spiral arrow) among normally organized cardiac myofibrils.
H-E: Hematoxylin eosin; I/R: Ischemia-Reperfusion.

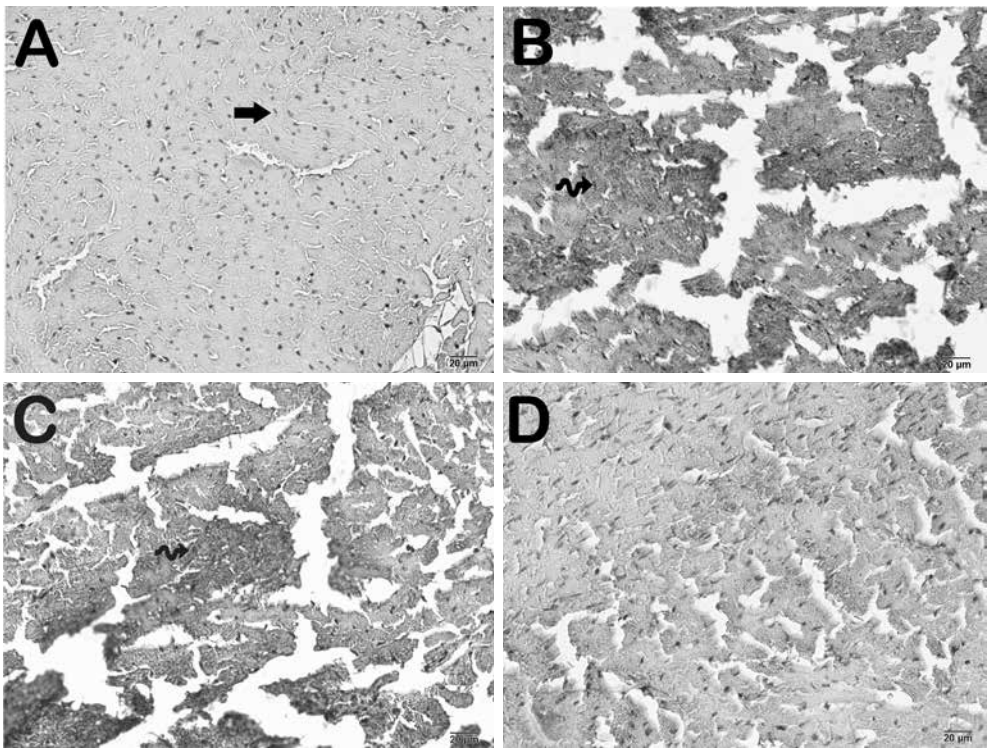

Figure 2. Whortleberry treatment reduced infrarenal aortic I/R-induced myocardial apoptosis *in vivo*. Caspase-3. **(a)** Control group: Cardiac myofibrils with a normal organization (arrow) (Caspase-3 positivity score mean: 0.0±0.4). **(b)** I/R treatment group: Apoptotic cardiac myofibrils can be seen (spiral arrow) (caspase-3 positivity score mean: 2.5±0.5). **(c)** I/R+glycerol treatment group: Caspase-3 positivity in cardiac myofibrils (spiral arrow) (caspase-3 positivity score mean: 2.5±0.4). **(d)** I/R+whortleberry treatment group: Decreased caspase-3 positivity in cardiac myofibrils (spiral arrow) (caspase-3 positivity score mean: 1.0±0.8).
I/R: Ischemia-Reperfusion.

(p=0.001 and p=0.01, respectively; Table 2). However, the GSH levels in the WB group were higher than the I/R group (p=0.05; Table 2). No significant difference in the GSH levels was observed between the I/R+glycerol and I/R group (p=0.6614; Table 2).

Histopathological results

Microfibrillar isotropic and anisotropic bands in the control group heart tissues exhibited a normal morphology, and the collateral branches were normal in structure (Figure 1a, b). In contrast, in addition to normal cardiac myofibrils, sections from the I/R treatment group exhibited diffuse degenerative cardiac myofibrils and edematous areas. The presence of swollen cardiac myofibrils in these areas was particularly evident (Figure 1c, d). On histopathological examination, the I/R+glycerol group resembled the I/R treatment group (Figure 1e, f). However, in the I/R+WB treatment group, in addition to cardiac myofibrils containing A-I bands with a typical organization, we also observed a number of swollen cardiac myofibrils (Figure 1g, h).

IHC results

The number of cardiac myofibrils exhibiting caspase-3 positivity was significantly higher in the I/R and I/R+glycerol groups, compared to the control group (p<0.01; Figure 2a-c; Table 3). However, the caspase-3 positivity in apoptotic cardiac myofibrils in

Table 3. Semi-quantitative analysis results

	Caspase-3 positivity scores
Groups	Mean±SD
Control	0.0±0.4
I/R	2.5±0.5[a]
I/R+Glycerol	2.5±0.4[a]
I/R+WB	1.0±0.8[b,c]

SD: Standard deviation; I/R: Ischemia-Reperfusion; WB: Whortleberry;
[a]p<0.01 compared to the control group;
[b]p=0.03 compared to the I/R group;
[c]p=0.03 compared to the I/R+Glycerol group;
Kruskal Wallis-Tamhane's T2 test.

the I/R+WB treatment group was significantly lower than in the I/R and I/R+glycerol groups (p=0.03 and p=0.03, respectively; Figure 2b-d; Table 3).

DISCUSSION

The number of studies investigating the effects on heart tissue of I/R injury following surgical repair of RAAA is very limited. Although no previous studies have used histopathological methods to examine cardiac injury resulting from I/R developing in association with infrarenal abdominal aortic clamping, Sharma et al.[24] observed necrotic cardiac myofibrils, cytoplasmic vacuolization in cardiac myofibrils, cardiomyocyte loss, and edematous areas. Li et al.[25] also reported cardiomyocyte vacuolization, irregularly and loosely arranged myofibrils, and inflammatory cell infiltration in heart tissues of rats subjected to I/R injury. Russ et al.[26] demonstrated myocyte enlargement in association with oxidative stress. Similarly, we observed swelling in degenerative cardiac myofibrils and edematous areas between cardiac myofibrils. However, we observed no inflammation in cardiac tissue. This can be attributed to insufficient time being available for the development of inflammation, since the experiment was concluded approximately three h following infrarenal abdominal aortic clamping.

Until now, aortic occlusion studies have reported that I/R induces lipid peroxidation and oxidative stress.[27,28] In their study, Ulus et al.[6] showed that I/R resulted in lipid peroxidation by increasing the ROS production and MDA levels in tissue. Other authors also reported that GSH reduced oxidative stress by lowering the production of ROS that otherwise increased in tissue as a result of I/R through scavenging free oxygen radicals and by increasing glutathione peroxidase activity.[29] In their study, Qiu et al.[27] showed a decrease in GSH levels in cardiac tissue following I/R. We also observed a decrease in GSH levels and an increase in MDA levels in association with I/R following surgical repair of RAAAs.

Furthermore, recent studies have demonstrated that, in addition to increased ROS levels, abdominal aortic clamping also induces apoptosis in cardiac myofibrils.[24,27] Zhang et al.[30] reported that it caused apoptosis in cardiomyofibrils by increasing the expression of tumor necrosis factor-α and interleukin-6 in cardiac tissue following I/R. Similarly, Han et al.[31] reported that I/R-induced myocardial injury caused an increase in caspase-3 expression in cardiac myofibrils. In addition, Tan et al.[32] reported an increase in the TUNEL and caspase-3 expression following I/R. We also found an increase in the caspase-3 expression in cardiac myofibrils.

To the best of our knowledge, no previous study has investigated the protective effects of WB against myocardial injury caused by abdominal aortic clamping, yet. In their study, Ozlem et al.[17] reported that WB lowered MDA levels that increased in association with I/R. Zhao et al.[29] also reported that WB exhibited cardioprotective benefits by increasing the levels of GSH and other antioxidants and reducing aortic lesions. Eren et al.[15] reported that WB reduced apoptosis by lowering caspase-3 expression. Consistent with the literature, we determined that WB reduced levels of MDA caused by I/R following surgical repair of RAAAs, while increasing GSH levels and reducing caspase-3 positivity.

This study constitutes a pilot study examining the effects of WB on cardiac injury following I/R due to abdominal aortic clamping. The WB dose and application time were determined in the light of the current literature. However, this study needs to be supported by pharmacodynamic and pharmacokinetic studies considering oxidative tissue damage following I/R and which can eliminate the existing disadvantage regarding its clinical use. In addition, this study investigated only cardiac tissue levels of the antioxidant enzyme GSH, and our findings need to be supported by a further investigation of other antioxidant molecules and enzymes, and of mitochondrial calcium levels for apoptosis and other apoptotic mechanisms.

In conclusion, ischemia-reperfusion following the surgical repair of ruptured abdominal aortic aneurysms increases reactive oxygen radical production, resulting in apoptosis in cardiac myofibrils. In addition, whortleberry exhibits protective effects on cardiac myofibrils by reducing oxidative stress and apoptosis.

Declaration of conflicting interests

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