Experimental Research

Evaluation of *Rheum ribes L.* Protective Effect with G Protein-Coupled Estrogen Receptor-1 (GPER-1) Levels in Experimental Liver Ischemia-Reperfusion Model

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ABSTRACT

Objective: To evaluate the relationship between the cell protective effects of *Rheum ribes L.* and G protein-coupled estrogen receptor-1 (GPER-1) levels in ischemia-reperfusion (I/R) injury.

Material and Method: The 32 male Wistar-Albino rats we used in this study were randomly divided into 4 groups of 8 rats each; control group (group 1), sham group (group 2), I/R group (group 3), I/R+*Rheum ribes L.* group (group 4). While no procedure was applied to the control group, 30 minutes of ischemia followed by 30 minutes of reperfusion was applied to the rats in all other groups. GPER-1 levels in liver tissue were measured with an ELISA reader. Histopathological examination of the tissues was performed under light microscopy.

Results: As a result of biochemical analysis; GPER-1 levels were statistically significantly decreased in the sham and I/R groups compared to control and I/R+*Rheum ribes L.* groups; in the I/R+*Rheum ribes L.* group compared to the control group ($p < 0.05$).

In the histopathological examination of the liver, necrosis and congestion observed in the Sham and I/R groups were significant when compared to the control group. While vacuolization was observed in a few experimental animals, there was a significant difference in the sham and I/R groups compared to the control group ($p < 0.05$). I/R+*Rheum ribes L.* group showed improvement in histopathological criteria in terms of vacuolization and necrosis compared to the sham group and I/R groups, and the difference was significant. ($p < 0.05$).

Conclusion: *Rheum ribes L.* can protect hepatocytes both with its antioxidant effects and GPER-1 activation.

Keywords: *GPER-1, Rheum ribes L., Ischemia Reperfusion Injury, Liver.*

ÖZ

Deneysel Karaciğer İskemi-Reperfüzyon Modelinde *Rheum Ribes L.*'Nin Koruyucu Etkisinin G Protein-Bağlı Östrojen Reseptörü 1 (GPER-1) Seviyeleri ile Değerlendirilmesi

Amaç: Karaciğer iskemi reperfüzyon (I/R) hasarında *Rheum ribes L.*'nin hücre koruyucu etkilerinin G proteini bağlı östrojen reseptörü-1 (GPER-1) düzeyleriyle ilişkisini değerlendirmektir.

Gereç ve Yöntem: Bu çalışmada kullandığımız 32 adet erkek Wistar-Albino cinsi rat randomize olarak 8'er rattan oluşan 4 gruba ayrılmıştır; kontrol grubu (grup 1), sham grubu (grup 2), I/R grubu (grup 3), I/R+*Rheum ribes L.* grubu (grup 4). Kontrol grubuna hiçbir işlem uygulamazken diğer tüm gruplardaki ratlara 30dk iskemi ardından 30dk reperfüzyon uygulanmıştır. Karaciğer dokusunda GPER-1 düzeyleri ELİSA reader ile ölçülmüştür. Dokuların histopatolojik incelemesi ışık mikroskopisinde gerçekleştirilmiştir.

Bulgular: Biyokimyasal analizler sonucu; GPER-1 düzeyleri kontrol ve *I/R+Rheum ribes L.* gruplarına göre sham ve I/R gruplarında; kontrol grubuna göre *I/R+Rheum ribes L.* grubunda istatistiksel olarak anlamalı derecede azalmıştır ($p < 0.05$).

Karaciğerin histopatolojik incelemesinde kontrol grubu ile kıyaslandığında Sham ve I/R gruplarında görülen nekroz ve konjesyon anlamlıdır. Vakuolizasyon birkaç deney hayvanında görülürken, kontrol grubuna göre sham ve I/R gruplarında anlamlı fark vardır ($p < 0.05$). I/R+*Rheum ribes L.* grubu, sham grubu ve I/R gruplarına göre vakuolizasyon ve nekroz yönünden histopatolojik ölçütlerde iyileşme göstermiştir ve fark anlamlıdır ($p < 0.05$).

Sonuç: *Rheum ribes L.* hem antioksidan etkileriyle hem de GPER-1 aktivasyonuyla hepatositleri koruyabilir.

Anahtar Sözcükler: *GPER-1, Rheum Ribes L., İskemi Reperfüzyon Hasarı, Karaciğer.*

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Decreased blood flow to the liver leads to ischemia. Reperfusion injury occurs with the restoration of blood supply. This situation affects all oxygen-dependent cells and causes deterioration of tissue and organ function. As a result, cell death occurs through differential

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apoptosis and necrosis (1, 2).

I/R injury in the liver is usually encountered during hemorrhagic shock, sepsis, liver transplantation, trauma and hepatic resection. During hepatic surgery, liver I/R may contribute to postoperative morbidity and mortality. In addition, many other distant organs are also affected by this process as a result of hepatic reperfusion injury (1, 3).

Recent studies have shown that estrogen has a new G protein-related receptor (GPER-1) in addition to its classical receptor (4). GPER are receptors located in different tissues, expressed in the plasma membrane, intracellular membranes of the endoplasmic reticulum and Golgi apparatus, and whose effects vary according to their location (4, 5). It has been reported that GPER activation in isolated rat hearts following I/R reduces infarct size (6).

Rheum ribes L., which grows in Iran, Iraq, Lebanon and Eastern Anatolia Regions of Turkey, is a perennial plant belonging to the Polygonaceae family. This plant, which is stated to contain various flavonoids in its young shoots, has many bioactivity as well as antioxidant activity. In addition to being consumed as food, it is also used in traditional treatment among the people (7, 8). Oztas et al (9) showed that *Rheum Ribes L.* had a protective effect in liver damage.

We have not come across another study in the literature investigating the interaction of *Rheum ribes L.* with GPER-1 in liver I/R injury. This study was designed to evaluate the relationship between the cell protective effects of *Rheum ribes L.* and GPER-1 levels in liver I/R injury, which is frequently encountered in clinical application and can cause serious morbidity and mortality.

MATERIAL AND METHOD

Ethics committee approval of this study was obtained from the Ethics Committee of Experimental Animals of the Faculty of Medicine of our University (Date: 22.07.2020, Session No: 2020/07, Decision No: 01). The study was carried out in Kahramanmaraş Sütçü İmam University Experimental Animals Laboratory. In all animal procedures used, care was taken to strictly comply with the "European Convention on Animal Care and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals".

Extract Preparation:

In our study, the stem part of *Rheum ribes L.* collected from Kahramanmaraş, Turkey in May 2020 was used. 200 g were taken from the stem parts of the plant and divided into small pieces. Afterwards, 2 gr KCl was added and pureed and the extract was prepared by centrifuging at 4000 rpm for 60 minutes.

Subject:

In our study, 32 adult male Wistar-Albino rats weighing between 194-250 g were used. The groups were randomly divided into 4 groups of 8 rats in each group; control group (group 1), sham group (saline 1mL) (group 2), I/R group (group 3), I/R+*Rheum ribes L.* group (*Rheum ribes L.*, 50 mg/kg/day) (group 4). All rats were kept at a room temperature of 21±1ºC for 12 hours of light and 12 hours of dark, and were fed with standard rat chow and water until the day of the experiment.

Design of experimental groups and surgical procedure:

After 12 hours of fasting, 50mg/kg Ketamine (Ketalar vial, Eczacıbaşı Turkey) was administered intramuscularly to all subjects as an anesthetic and the hairs on the anterior abdominal wall of the subjects were cut. The abdomen was sterilized with povidone-iodine solution and midline laparotomy was performed using minimal dissection.

Control group (group 1, n =8): No procedure was applied to the subjects.

Sham group (group 2, n =8): Rats were given 1mL saline (0.9% NaCl) one day before the surgical procedure. After vascular clamping of the hepatic artery and portal vein, ischemia and reperfusion procedures were applied to the liver, each lasting 30 minutes. Following reperfusion, 1mL saline was given via gavage.

I/R group (group 3, n =8): Vascular clamping was applied to the hepatic artery and portal vein. Then, ischemia and reperfusion were applied to the liver for 30 minutes each.

I/R+*Rheum ribes L.* group (group 4, n =8): *Rheum ribes L.* extract (50 mg/kg/day) was given by gavage to the rats one day before the surgical procedure. After the surgical procedure, ischemia and reperfusion procedures were applied to the liver, each lasting 30 minutes. Then, *Rheum ribes L.* extract (50 mg/kg/day) was given to the rats in this group by gavage.

All animals were sacrificed for hepatectomy. Tissues were divided into two, some of them were taken for biochemical analysis, the other part was reserved for histopathological examination (into 10% buffered neutral formaldehyde).

Biochemical Analysis:

Preparation of liver tissue homogenates:

Tissues were homogenized 1/9 (weight/volume) in cold 1.15% KCl (potassium chloride) at 13500xrpm with a homogenizer (ultra turrax) on ice. Then the homogenates were centrifuged at 14000xrpm in a cooled centrifuge at +4 ºC for 30 minutes. GPER-1 measurement was made in the supernatants obtained.

Detection of GPER-1 in liver tissue:

Rat GPER-1 level in liver tissue was measured by ELISA reader (Thermo Scientific, Finland) using commercial kit (MyBiosource, catalog number: MBS095620, USA). The kit content was adhered to throughout the experiment.

Histopathological Evaluation:

Tissues were fixed in 10% neutral buffered formaldehyde solution for 24 hours. All of the samples were

routinely followed in the tissue tracking device and paraffin blocks were prepared. Serial sections of 5 µ were prepared from these paraffin blocks with a microtome device and stained with hematoxylin-eosin (H&E) dye for each tissue sample. The study was carried out by the pathologist without knowing which tissue sample belongs to which group and by randomly selecting tissue samples. The prepared preparations were examined histopathologically by light microscopy.

The liver was evaluated for congestion, vacuolization and necrosis according to the modified Suzuki pathological scoring (10). According to this scoring system, damage; 0: None,1: Minimal degree, 2: Mild degree, 3: Moderate degree, 4: Severe degree, has been determined. Modified Suzuki scores were used to see the difference between the Sham group and the treatment group more clearly.

Statistical Analysis:

Data were evaluated with IBM SPSS Statistics for Windows version 22 program. In the evaluation of the data, the conformity of the variables to the normal distribution was examined with the Shapiro-Wilk test.

In the analysis of biochemical parameters, group comparisons of normally distributed variables One Way Anova test was used. In pairwise comparisons; Dunnett test for comparison of control group with other groups; Tukey hsd test was applied for the other pairwise comparisons except the control group. Statistical parameters were expressed as mean±standard deviation (mean±SD). Statistical significance was accepted as $p < 0.05$.

The Kruskal Wallis h test was used to compare the groups that did not comply with the normal distribution in the examination of histopathological findings. Dunn's test, one of the post hoc tests, was used for pairwise comparisons. Statistical parameters are expressed as median (min-max). Statistical significance was accepted as $p < 0.05$.

RESULTS

Biochemical findings:

A statistically significant decrease was observed in the sham, I/R and I/R+*Rheum ribes L.* groups compared to the control group in terms of GPER-1 levels (respectively; $p < 0.001$, $p < 0.001$, $p = 0.028$), A statistically significant increase was observed in the I/R+*Rheum ribes L.* group when compared with the sham and I/R groups (respectively; $p = 0.003$, $p < 0.001$) (Table 1).

Table 1. *GPER-1 levels in liver tissue.*

	Control group (Group 1, n =8)	Sham group (Group 2, n =8)	I/R group (Group 3, n =8)	I/R+*Rheum ribes L.* (50 mg/kg/day) group (Group 4, n =8)	p*
GPER-1 (ng/ml)	36.53±7.27	17.79±5.09[a]	10,23±1,76[a]	28.80±5.19[abc]	<0.05

**p: One Way Anova test, [a]p: compared with the control group (Dunnett test), [b]p: compared with the sham group (Tukey HSD test), [c]p: compared with the I/R group (Tukey HSD test), GPER-1: G protein-coupled estrogen receptor-1.*

Histopathological Findings:

The difference between the control group with the sham, I/R and I/R+*Rheum ribes L.* groups in terms of congestion scores; the difference between the control group with the sham and I/R groups in terms of vacuolization and necrosis values was statistically significant (Table 2, Figure 1).

Table 2. *Histopathological analysis findings.*

	Control group (Group 1, n =8)	Sham group (Group 2, n =8)	I/R group (Group 3, n =8)	I/R+*Rheum ribes L.* (50 mg/kg/day) group (Group 4, n =8)	p*
Congestion Median (min-max)	0,00 (0,00-1,00)	3,50 (2,00-4,00)[a]	2,50 (2,00-4,00)[a]	2,00 (2,00-3,00)[a]	<0.001
Vacuolization Median (min-max)	0,00 (0,00-0,00)	1,50 (1,00-2,00)[a]	2,00 (0,00-2,00)[a]	1,00 (0,00-2,00)	0.001
Necrosis Median (min-max)	0,00 (0,00-1,00)	3,00 (2,00-4,00)[a]	2,00 (2,00-3,00)[a]	2,00 (0,00-2,00)	<0.001

**p: Kruskal Wallis H Test, [a]p: compared with the control group (Post-hoc: Dunn test).*

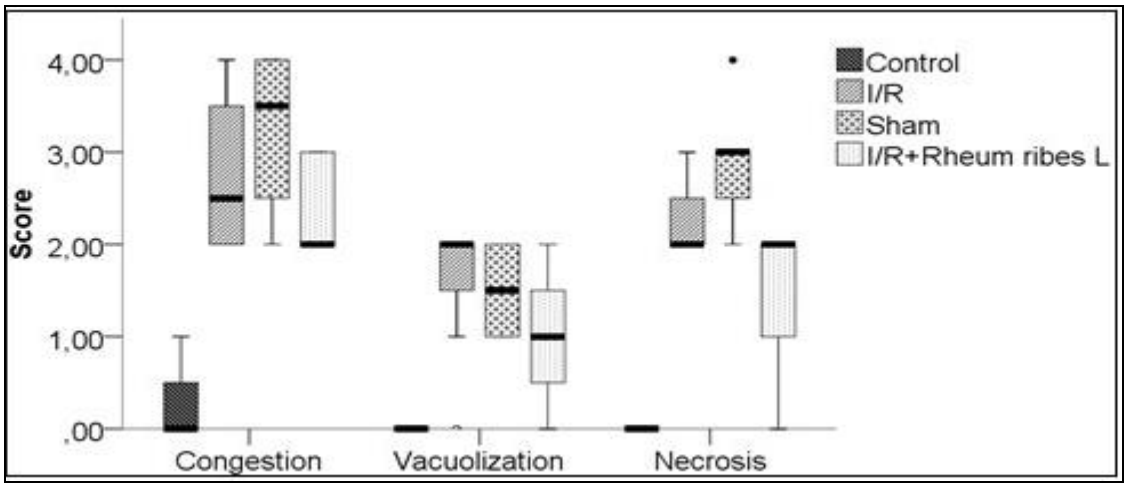

Figure 1. *Congestion, vacuolization and necrosis scores of the groups.*

In the histopathological examination of the liver, necrosis and congestion in the liver were significant in the sham and I/R groups compared to the control group. Vacuolization was seen in a few experimental animals and the difference between the control group sham and I/R group was significant. Compared to the sham group and I/R groups, the I/R+*Rheum ribes L.* group showed improvement in histopathological criteria in terms of vacuolization and necrosis. As seen in figures 2, 3 and 4, significant congestion and inflammation were observed in the livers of the sham group and the I/R groups (Figures 2, 3 and 4).

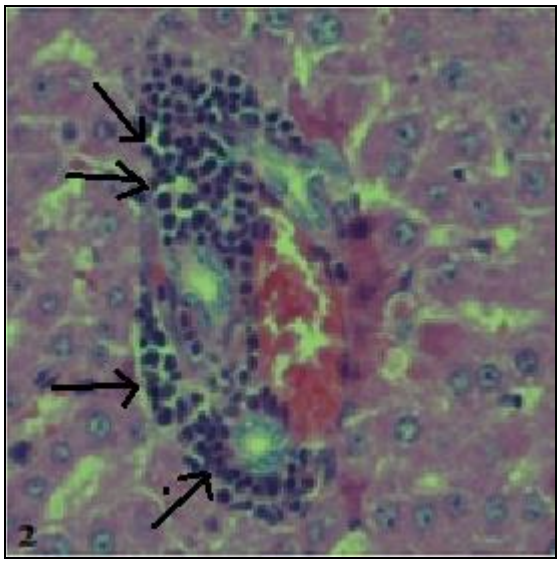

Figure 2. *Inflammation in the portal area (arrowhead) (I/R group).*

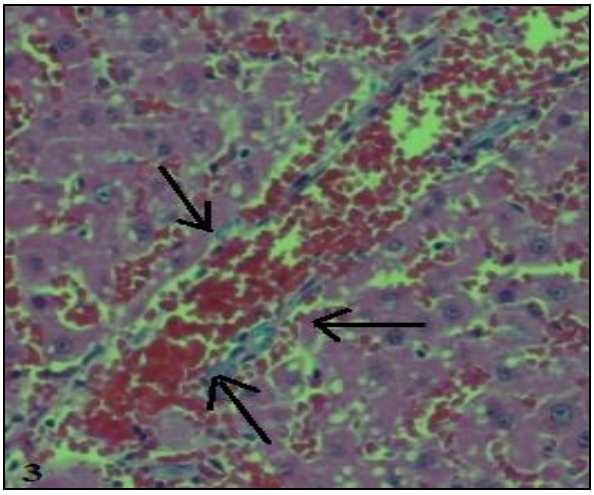

Figure 3. *Siusoidal congestion (arrowhead) (I/R group).*

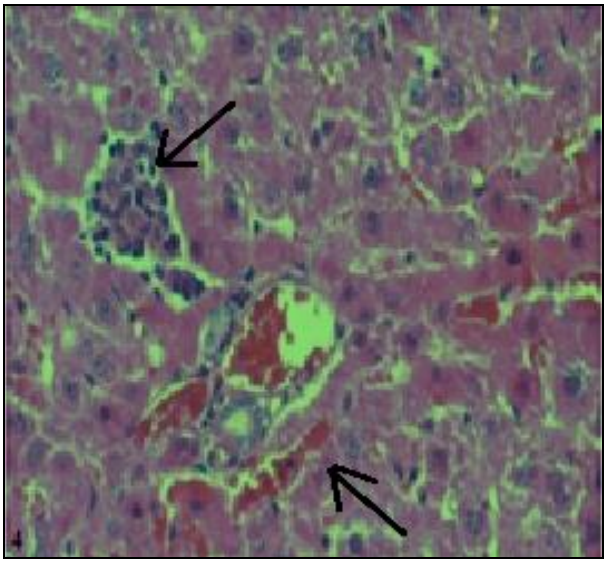

Figure 4. *Decreased sinusoidal congestion and decreased inflammation in the portal area (arrowhead) (I/R+Rheum ribes L. group).*

DISCUSSION

Loss of blood flow (ischemia) in the liver impairs the oxygenation of tissues and organs. The reperfusion that occurs to prevent hypoxic cellular damage after ischemia also damages the liver. Causes of liver I/R injury include long-term surgical liver resection (eg, Pringle maneuver), sepsis, trauma, shock, bleeding, heart failure, respiratory failure, or liver transplantation (1, 11). Liver I/R damage can cause liver dysfunction and even failure, and it can also lead to failure in distant organs such as the heart, lung, and kidney (1, 11). Liver I/R injury is a therapeutic problem that needs an urgent solution because it affects the prognosis of the disease, the success rate of the surgical procedure and patient survival (12).

It is thought that the early phase of liver I/R injury is caused by the change in the redox state of the liver tissue, while the late phase is caused by the production of cytokines and chemokines and the infiltration of leukocytes into the liver tissue (1). Depending on I/R, metabolic acidosis, increase in intracellular calcium, mitochondrial damage, Kupffer cell activation, oxidative stress develops, inflammatory response is activated and eventually necrotic or apoptotic cell death occurs (11).

Estrogens are steroid sex hormones that are particularly effective in the female reproductive system (13). They are also necessary for the development and function of the male reproductive system (14). They also play an important role in non-reproductive biological functions and pathological processes, cell proliferation, growth, migration, aging, and regulation of many disease states (14-16). In particular, 17β-estradiol, which is the dominant and strongest endogenous estrogen, plays a role in reducing the incidence of many diseases in premenopausal women (14). Estrogens exert their effects through the classical and at the same time nuclear estrogen receptors ERα and ERβ and besides these receptors, GPER-1 (14-16).

GPER-1, also known as G protein-coupled receptor 30 (GPR30) or 7-transmembrane domain G protein-associated receptor (GPCR), is a novel membrane-anchored estrogen receptor capable of inducing rapid kinase signaling in a variety of cells (12,16-19) . GPER-1 can be activated by many stimuli, including estrogen (12). GPER-1 is implicated in both transcriptional regulation and rapid, non-genomic signaling. GPER-1 is expressed everywhere in the body (14-16). GPER plays a role in reproductive, nervous, endocrine, immune and cardiovascular systems and in various diseases including cancer (14). In addition, GPER-1 signal has been shown to have a protective effect against I/R damage (19).

Estrogens show rapid effects such as calcium influx or nitric oxide (NO) release via GPER (13). NO is a short-lived gas that plays a role in protection from atherosclerosis and inflammation (20). Reduction in NO levels is one of the most important factors in the pathogenesis of I/R injury. Exogenous NO is effective in reducing oxidative stress, cytokine release, leukocyte endothelial adhesion and hepatic apoptosis (21). In the study by Meyer et al (20), deletion of GPER increased the progression of atherosclerosis and decreased vascular NO bioactivity in mice with intact ovaries. G-1 is the selective agonist of GPER, and G15 is the selective antagonist (14). Chronic treatment with G1 reduced postmenopausal atherosclerosis and inflammation without uterotrophic effects (20). It was observed by Deschamps et al (6) that G1 administration after myocardial infarction in female and male rodents reduces the damage and abnormal contractions caused by reperfusion. Weil et al (22) showed that G1 administration reduced the levels of proinflammatory cytokines.

Opening the mitochondrial permeability pore (mPTP) after I/R is effective in cell death. mPTP remains closed in myocardial ischemia, but in this case, these pores open shortly after reperfusion with the excessive increase in Ca^{2+} in mitochondria, oxidative stress and decrease in the amount of ATP. After I/R, infarct size was significantly reduced in G1-treated hearts and the Ca^{+2} load needed to induce mPTP opening increased compared with controls. Based on these results, it is stated that GPER activation provides a cardioprotective effect after I/R by inhibiting mPTP opening (23).

The clinical role and mechanism of GPER in hepatic I/R is still unclear (12). Estrogen has been shown to significantly reduce liver damage after I/R (24). In the study by Li et al (25), it was seen that estrogen has a protective effect on the mouse hepatic I/R model and administration of G15, a specific antagonist of GPER, before estrogen prevents this beneficial effect. 17β-estradiol (E2) is effective in cell cycle induction, hepatocyte proliferation and increase in liver size in larval zebrafish. GPER-1 mediates these effects. It is stated that in vivo chemical inhibition of GPER-1 in males significantly reduces E2-mediated tumor progression after chemical carcinogenesis (26). Again, in the study of Kandemir et al (16), GPER levels showed high expression in patients with chronic hepatitis B.

A prominent feature of liver I/R injury is an excessive inflammatory response. NOD-, LRR- and pyrin domain containing 3 (NLRP3) plays a role in I/R injury by activating inflammation as an important pattern recognition receptor of innate immunity. G1 pretreatment or NLRP3 silencing in hepatic I/R injury improved histological changes and hepatocyte apoptosis (12). Again, in the study of Lin et al (27), it was observed that estrogen significantly inhibited apoptosis caused by hepatic I/R damage and had a protective effect on liver I/R damage (27).

Rheum ribes L. is a perennial herbaceous plant that grows in temperate and subtropical climates, grows on rocks and stony areas, 40–150 cm tall, blooms in May-June (28). Fresh stems and petioles are consumed as vegetables, and the roots are used in the treatment of many diseases (29). *Rheum ribes L.* has an important antioxidant effect with its content, and the molecules it contains vary according to the region where it grows and the part of the plant used in the treatment (30, 31).

In the study of Bakir et al (32) it was observed that *Rheum ribes L.* had a protective effect on CCl4-induced liver toxicity.

The significant increase in GPER-1 levels in the I/R+*Rheum ribes L.* group compared to the sham and I/R groups in our current study suggests that *Rheum ribes L.* is effective in GPER-1 expression. However, the significant decrease observed in GPER-1 levels in the I/R+*Rheum ribes L.* group compared to the control group shows that the surgical intervention itself is also effective in reducing GPER-1 levels and that the treatment cannot provide a complete recovery. In this case, further studies should be conducted to determine whether a full recovery in GPER-1 levels can be achieved by re-adjusting the dose of *Rheum ribes L.*

Phytoestrogens show their physiological effects by activating ERα and ERβ as well as GPER (14). Since it is known that GPER-1 is activated by various phytoestrogens with antioxidant effect and *Rheum ribes L.* also contains molecules with antioxidant activity, based on the histopathological data we obtained from our study, we think that *Rheum ribes L.* not only increases GPER-1 levels, but also has a protective effect against liver I/R damage by activating GPER-1.

The limitation of this study is that there is not enough literature information about the effect of *Rheum ribes L.* on liver I/R damage or GPER-1 levels. This situation makes it difficult for us to interpret the mechanisms that *Rheum ribes L.* can use in the effect of GPER-1 levels in I/R injury. Again, as far as we know, the region where the plant grows and the part used for treatment cause it to contain different molecules. In our study, plants were collected from a single site and we do not know which of the molecules found in the stem of the plant is more effective in increasing GPER-1 levels. This is another limitation.

Conclusion

More studies are needed on the subject at the molecular level.

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