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ORIGINAL RESEARCH

Effects of Lupeol on Intestinal Anastomosis After Experimental Intestinal Ischemia-Reperfusion Injury in Rats

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Background: Intestinal ischemia/reperfusion (I/R) injury can occur in a wide variety of diseases and surgeries. If necessary, the blood flow should be restored, including re-anastomosis by removing the intestines with impaired circulation. In this process, anastomotic strength is as important as inflammatory responses and oxidative stress. Therefore, we conducted the study to investigate the effects of lupeol on intestinal ischemia-reperfusion injury, not only biochemically and histopathologically but also on anastomotic strength and miRNAs.

Methods: Female rats were randomly divided into six groups. While only laparotomy was performed in the control group (Group C), anastomosis was performed in the sham group (Group S). In the other groups, the superior mesenteric artery was clamped for 45 minutes. In the groups I/R^{1} and L^{1}, the intestine was transected, and end-to-end anastomosis was performed at the 1st hour of reperfusion. In the groups I/R^{24} and L^{24}, this procedure was performed at the 24th hour of reperfusion. In addition, lupeol treatment was given before reperfusion and for the following 4 days in the groups L^{1} and L^{24}. All rats, except the control group, bursting pressure was measured on the 5th day of anastomosis, and then all rats including the control group were sacrificed. TNF-α, IL-6 levels in blood samples and MDA, GSH, caspase-3, miR-29b-3p, miR-34a-5p, miR-495-3p levels in intestinal tissues were measured, and intestinal histopathology was also examined.

Results: Lupeol treatment, which was statistically significant in some parameters, demonstrated positive effects by decreasing TNF, IL-6, MDA, caspase-3, histopathological damage levels and increasing GSH and bursting pressure. In addition, lupeol decreased miR-34a-5p expression and increased miR-29b-3p and miR-495-3p expression.

Conclusion: Lupeol protected the intestines from I/R damage with its antioxidant and anti-inflammatory effects. Besides, it reduced the histopathological damage and increased the anastomotic strength. Additionally, miR-29b-3p, miR-34a-5p, miR-495-3p expressions were altered by lupeol.

Keywords: lupeol, ischemia/reperfusion injury, small intestine, miR

Introduction

Intestinal ischemia/reperfusion (I/R) injury can occur in a wide variety of diseases and surgeries such as major trauma, acute mesenteric ischemia, midgut volvulus, necrotizing enterocolitis, cardiopulmonary bypass, abdominal aortic aneurysm surgery, sepsis and hypovolemic shock and is an unavoidable condition in intestinal transplantation.[1–3] Intestinal ischemia, which occurs in approximately 1 in 1000 hospital admissions, is a life-threatening abdominal emergency with a mortality rate exceeding 60%.[4,5] Intestinal ischemia is more commonly observed in older age groups, and the most important causes in this population include hypotension due to sepsis or left ventricular dysfunction, as well as hypovolemia caused by dehydration or bleeding.[6] Malrotation, which occurs in approximately 0.2–1% of the population, can rapidly lead to intestinal ischemia in infants due to midgut volvulus.[7,8]

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In ischemia caused by the interruption of blood flow to any tissue, anaerobic metabolism is activated, resulting in reduced production of adenosine triphosphate (ATP) and antioxidant substances. In addition, during this period, the continued use of ATP despite decreased production leads to the accumulation of purine metabolites such as hypoxanthine and xanthine, while xanthine dehydrogenase, which normally metabolizes hypoxanthine to uric acid, converts to xanthine oxidase. During the reperfusion period, which occurs with the restoration of blood flow, purine metabolites metabolized by xanthine oxidase cause excessive reactive oxygen species (ROS) formation. ROS is produced not only from the xanthine oxidase system but also from the mitochondrial electron transport chain, the NADPH oxidase system, and the unbound nitric oxide synthase (NOS) system.[9,10] When ROS production exceeds the antioxidant capacity, it causes oxidative stress, which not only increases the inflammatory response but also leads to cell damage and apoptosis. This can only be prevented by reducing ROS formation or increasing antioxidant defense.[11,12] Tissue hypoxia, inflammation, and cell infiltration that occur during ischemia also result in the loss of the mucosal barrier that can prevent harmful bacteria and toxins from entering the blood and distant tissues or organs. It is thought that the protection of this mucosal barrier by reducing epithelial cell damage can alleviate I/R damage and microRNAs (miR) are critical factors in this process.[13,14] Therefore, in recent years, studies have been carried out in both experimental I/R models and people with inflammatory bowel disease to investigate the effects of miRs.[15,16]

In the case of ischemia occurring in the intestine, it is necessary to restore the blood flow to protect the intestine and to safely anastomosis by removing the intestines with impaired circulation. Although various factors are effective in the healing of intestinal anastomosis, ischemia is one of the most important causes, and it has been stated that I/R damage may cause deterioration of anastomotic strength. Intestinal I/R damage, whether through resection anastomosis or recirculation, is a complex process associated with inflammation, oxidative stress and cell apoptosis caused by ROS, ultimately leading to cell death.[1,2,15] Previous studies have shown that collagen accumulation in intestinal anastomoses peaks between days 4–6 and reaches its maximum on the 6th day. However, the breaking strength of the anastomosis is significantly low in the early days, making the first 3–4 days critical for its structural integrity.[17,18] These findings support our rationale for evaluating bursting pressure on the fifth day, a period when collagen deposition begins to increase but the anastomosis remains relatively weak.

Lupeol, which has antioxidant, anti-inflammatory, anticancer and wound healing effects, is a triterpene found in various vegetables and fruits such as peppers, tomatoes, figs, mangoes, strawberries. Lupeol exerts anticancer activity effect by inducing cell apoptosis, inhibiting cell proliferation, migration and invasion, and increasing the sensitivity of cells to treatment. Studies have also revealed liver, cardiovascular protective, anti-neuroinflammatory, and antimicrobial effects. Lupeol is rapidly absorbed in animals and was determined not to cause systemic toxicity in vivo at 200 mg/kg intraperitoneal and 2000 mg/kg oral doses.[19,20] Additionally, Lupeol has been proven to inhibit the progression of osteosarcoma by regulating miR-212-3p, thereby affecting miRs.[21] It is available in the literature that miR-29b-3p, miR-34a-5p, miR-495-3p expression levels also change in studies conducted with intestinal ischemia-reperfusion models.[15,22] Therefore, we designed the study to investigate the effects of lupeol on intestinal ischemia-reperfusion injury not only biochemically and histopathologically but also its effects on previously studied miRs in intestinal tissues.

Materials and Methods

This study was carried out in accordance with the Gazi University Faculty of Medicine Animal Research Laboratory after receiving the approval of the Gazi University Animal Experiments Ethics Committee (G.U.ET-22.039). All experiments were conducted in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Lupeol (cas. no. 545–47-1, purity 99.31%) supplied by TargetMol Chemicals Inc. (Wellesley Hills, MA, USA) for use in the study was dissolved in olive oil using heat (37 °C) and sonification (3 h). In an experimental study, it was reported that the 100 mg/kg dose of lupeol was the most effective, while in another review, it was stated that lupeol administered intraperitoneally up to 200 mg/kg did not cause systemic toxicity or mortality. In addition, the oral bioavailability of lupeol was also found to be less than 1%. Therefore, we chose the 100 mg/kg dose and intraperitoneal route in our study.[20,23,24]

Experimental study was performed with 36 healthy Wistar-Albino female rats with an average weight of 220.00 ±14.63 g (200–240). In this study, only female rats were used to ensure independence and homogeneity in sex hormone

levels. Additionally, female rats were preferred in this experiment because they were more easily available and cheaper due to budget limitations. The rats to be used for the experimental study were randomly selected among the animals housed in cages at appropriate room temperature and humidity in a laboratory environment with a photoperiod of 12:12 h. All rats were observed for the last 1 week before the study, and no standard chow or water limitation was applied before the procedure, and they were randomly divided into six groups of six rats each:

Group C (control group): Only laparotomy was performed, and the abdomen was closed after the SMA was seen.

Group S (Sham group): No treatment was given in this group. Anastomosis was performed after the SMA was seen.

Group I/R^1 (Ischemia-reperfusion 1 group): After 45 minutes of intestinal ischemia, anastomosis was performed at the first hour of reperfusion. No treatment was given.

Group I/R^{24} (Ischemia-reperfusion 24 group): After 45 minutes of intestinal ischemia, anastomosis was performed at the 24th hour of reperfusion. No treatment was given.

Group L^1 (lupeol+ I/R 1 group): After 45 minutes of intestinal ischemia, anastomosis was performed at the first hour of reperfusion. Intraperitoneal 100 mg/kg lupeol treatment was given before reperfusion and for the following 4 days.

Group L^{24} (lupeol+ I/R 24 group): After 45 minutes of intestinal ischemia, anastomosis was performed at the 24th hour of reperfusion. Intraperitoneal 100 mg/kg lupeol treatment was given before reperfusion and for the following 4 days.

We did not add a separate olive oil group to our study because no significant difference was found in the olive oil groups in previous studies and due to our budget constraints.[25,26]

All interventions were performed under sterile conditions using 10% povidone-iodine after general anesthesia was given with intramuscular 50 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey) and 5 mg/kg xylazine hydrochloride (Alfazyne 2%, Ege Vet, Turkey). All surgical procedures were performed between 8 am and 14 pm to reduce the effects of diurnal hormonal changes in rats. A laparotomy was performed with a midline incision in all groups. In the control group (Group C), the wound was closed after visualizing the superior mesenteric artery. In the sham group (Group S), after visualizing the superior mesenteric artery by laparotomy, the intestine was transected 15 cm proximal to the ileocecal valve, and a single layer end-to-end anastomosis was performed with 7/0 prolene suture using individual stitches. In other groups, ischemia was performed by placing atraumatic microvascular clamps on the superior mesenteric artery and preventing the blood flow to the intestine for 45 minutes, after then clamps removed for reperfusion and the incisions were closed with sutures. In groups I/R^1 and I/R^{24}, second laparotomy was performed at the 1st and 24th hours of reperfusion, respectively, and the intestine was transected 15 cm proximal to the ileocecal valve and a single layer end-to-end anastomosis was performed with 7/0 prolene suture using individual stitches. Postoperative care and pain control of rats are performed by veterinarians at the center. In this study, pain levels of the rats were evaluated using the Grimace Scale at 2, 6, 12, 24, and 48 hours postoperatively. Feed and water consumption were also monitored closely. Since no signs of stress or discomfort were observed during these evaluations, no painkillers were administered. Additionally, we believe that excessive postoperative use of painkillers could potentially influence laboratory parameters and confound study results. In groups L^1 and L^{24}, in which the same procedure was applied, an intraperitoneal 100 mg/kg lupeol treatment was given before reperfusion and for the following 4 days.

All rats, except the control group, underwent relaparotomy on the 5th day of anastomosis under anesthesia, and the intestinal lumen was occluded with 3–0 silk 10 cm proximal and distal to the anastomosis. An 18G cannula was inserted into the lumen, and saline solution was infused with an IVAC 770 infusion pump (IVAC Corporation, USA) at a rate of 2 mL/min. Intraluminal pressure was continuously monitored while the infusion was in progress, and the intraluminal pressure value at the time of leakage from the anastomosis was considered as bursting pressure. While bursting pressures were measured, intestinal rupture or leakage developed in the anastomotic line in all subjects. Since the main purpose of the study was to examine the positive effect of lupeol on the anastomotic intestines after SMA ischemia, especially the intestinal tissue of the anastomotic site was resected and examined. In addition, there was no perforation area in the untouched intestines of the all rats. The reason why there were only 6 rats in each group in this experiment was the animal ethics committee's restrictions that kept the use of rats to a minimum and the budget limitation. Therefore, in this study, bursting pressures and other parameters were measured only in the anastomotic site of bowel, and the number of rats and budget were kept to a minimum. After the above-mentioned procedures, all rats were sacrificed, and samples were taken for examinations. The intestinal segment was resected from each rat, including the anastomosis area, and debris and intestinal contents were cleaned. Half of the excised

intestine was stored in 10% formalin for histopathological examination, and the other part was immediately frozen in liquid nitrogen and stored at −80 °C for evaluation of malondialdehyde (MDA), glutathione (GSH), caspase-3, miR-29b-3p, miR-34a-5p and miR-495-3p. Blood samples taken from each rat were centrifuged at 3000 rpm for 10 minutes, and sera were placed in Eppendorf tubes and stored at −80 °C for TNF-α and IL-6 testing.

Biochemical Parameters

IL-6 and TNF-α levels were obtained using commercial ELISA (Enzyme-Linked Immuno Sorbent Assay) kits (cat. no. E0764Ra and E0135Ra, respectively, Jiaxing Korain Biotech, Zhejiang, China) following the manufacturer's recommended instructions.

A hundred mg of frozen intestinal tissue from each rat was homogenized using Phosphate-buffered saline (pH: 7.2) with a Teflon pestle shredder at 3500 rpm for 15 minutes, and the supernatant was taken after centrifugation. The values of MDA, GSH and caspase-3 were analyzed using commercial ELISA kits (cat. no. E0156Ra, E1101Ra and E1648Ra, respectively, Jiaxing Korain Biotech, Zhejiang, China).

Histopathological Evaluation

Intestinal specimens were fixated in 10% formaldehyde solution for 24 h. Sections of 4 μm thickness were prepared from paraffin blocks stained with hematoxylin-eosin (H&E), six slices of each specimen evaluated under a light microscope (Olympus BX53, Japan). Two dedicated pathologists reviewed the slides blinded of the animal groups. Histopathological scoring was recorded by taking the average of the values given by both pathologists. Consensus of grade by the pathologists was noted.

To avoid mixing etiological and morphological terms, tissue damage was classified from zero to six degrees as indicated by Swerdlow et al.[27]

The samples were classified as;

Grade 0: No pathological changes;

Grade 1: Focal loss of surface epithelium in the mucosa;

Grade 2: Mucosal infarction, with extensive loss of surface epithelium and areas with substance loss in the mucosal lamina propria. Sparing of basal parts of glands and of the lamina muscularis mucosa;

Grade 3: Complete mucosal necrosis, variable necrosis of the submucosa, but intact muscularis mucosa;

Grade 4: Complete necrosis of both the mucosa and the submucosa, and loss of the muscularis mucosa;

Grade 5: In addition to the changes of grade 4, there are also circulatory disturbances of the inner part of the external muscular layer (lamina muscularis externa);

Grade 6: Complete necrosis of all layers of the intestinal wall.

Real-Time Quantitative PCR

Total RNA using 10 mg intestinal tissue for each rat was extracted using the Norgen Biotek Animal Tissue RNA Isolation Kit (cat. no. 25700, Norgen BioTek Corp., Thorold, ON, Canada) according to the manufacturer's protocol. MagNA Lyser Green Beads (cat. no. 03358941001, Roche Diagnostics, Germany) and MagNA Lyser (Roche Diagnostics, Germany) device were used for the homogenization of tissue samples. The microScript microRNA cDNA Synthesis Kit (cat. no. 54410, Norgen BioTek Corp., Thorold, ON, Canada) was used to convert the obtained RNAs into complementary cDNA. For each sample, the cDNA reaction was prepared with 10 μL of 2x Reaction Mix, 1 μL of microScript microRNA Enzyme Mix, 4 μL of nuclease-free water, 5 μL of RNA, with a final volume of 20 μL, and incubated at 37°C for 30 min, 50°C for 30 min, and 70°C for 15 min in Light Cycler 96 (Roche Diagnostics, Germany). Then, the samples were immediately cooled and stored at −20°C.

Expression levels were determined with rno-miR-29b-3p, rno-miR-34a-5p, rno-miR-495-3p, and U6snRNA was used as the internal reference. FastStart Essential DNA Green Master Mix (cat. no. 06402712001, Roche Diagnostics, Germany) was used to determine the expression of miRs. Real-time PCR was performed in duplicate for each miRNA and included non-template control.

Real-time PCR reaction with a final volume of 20 µL for each sample, prepared with 2 µL PCR Primer (10x conc.), 10 µL Master Mix (2x conc.), 3 µL nuclease-free water, 5µL cDNA, and procedure was performed in Light Cycler 96 (Roche Diagnostics, Germany). The reaction parameters were 95°C for 10 min preincubation, followed by 45 cycles at 95°C for 10s, 60°C for 10s and 72°C for 10s and ended with melting at 95°C for 10s, 65°C for 10s and 97°C for 1s. The relative expression levels of RNA normalized to U6snRNA was quantified by $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analysis of data was conducted using SPSS Version 22.0 for Windows (IBM Corp, Armonk, NY) on a computer. All experiments were expressed as mean ± standard deviation (SD). Quantile–quantile (Q-Q) plots and the Shapiro–Wilk test were used to control the normal distribution of the data. Homoscedasticity was evaluated, and variances are homogeneous. One-way ANOVA followed by Tukey's post hoc test was used as a statistical analysis method to test for differences between groups. The value of $P<0.05$ was considered to be statistically significant. Pathology and miRNA results, where variances were not distributed homogeneously, were evaluated with the Kruskal–Wallis test and Bonferroni post hoc tests. In the post hoc test, the new p-value was found by dividing 0.05 by the number of comparisons (The value of $P <0.0033$ was considered to be statistically significant).

Results

The MDA value, which is an indicator of lipid peroxidation, in the intestine tissue was highest in the I/R^1 group, and there was a significant difference between Group I/R^1 and Groups C & L^1 (p=0.017 and 0.032, respectively). Similarly, caspase-3 value was highest in the I/R^1 group, and there was a significant difference between Group I/R^1 and Groups C & L^1 (p=0.035 and 0.041, respectively) as observed in MDA. Non-enzymatic antioxidant GSH values were highest in the Group L^{24}, the lowest in the Group I/R^{24}. There was a significant difference between Groups I/R^1 & I/R^{24} and Groups C & L^{24} (p=0.010, 0.007, and 0.001, 0.001, respectively) (Table 1).

In the blood samples, the IL-6 values were highest in the Group I/R^{24} and the lowest in the Group L^1. There was a significant difference between Group I/R^{24} and Groups C & L^1 & L^{24} (p=0.043, 0.001 and 0.006, respectively). Although TNF-α was highest in Group I/R^{24} and there was a slight decrease in the lupeol treatment groups, there was no statistically significant difference between the groups in terms of TNF-α values ($p>0.05$) (Table 1).

In the evaluation of the histopathological damage score according to the modified Swerdlow classification, no damage was detected in the Group C, while the damage was highest in the Group I/R^1. There was a statistically significant difference between Group C and only Groups I/R^1 & I/R^{24} ($p <0.001$ and p=0.003, respectively). The level of damage was lower in the lupeol-treated groups than in the ischemia groups, but there was no statistically significant difference (Table 1) (Figures 1 and 2).

Bursting pressure was highest in the Group L^{24} with 156.78±22.43, and lowest in the Group I/R^1 with 116.88±16.01. There was a significant difference in bursting pressure between Group I/R^1 and Groups L^1 & L^{24} (p=0.027 and 0.015, respectively), and also between Group I/R^{24} and Group L^{24} (p=0.037) (Table 1).

We detected that the expression of miR-29b-3p was significantly decreased in the I/R groups compared to the groups without ischemia. There was a significant difference between Group C and Groups I/R^1 & I/R^{24} (p=0.003, $p<0.001$ respectively), and also between Group S and Group I/R^{24} ($p<0.001$). Although there was no statistically significant difference, the expression of miR-29b-3p was two times higher in the lupeol treatment groups than in the I/R groups (Figure 3A). The expression levels of miR-34a-5p were highest in the Group I/R^1 and lowest in the Group C, and there was only a statistically significant difference between these groups (p=0.001). We demonstrated a slight reduction in the lupeol treatment groups compared to the ischemia groups, but no significant differences were found between the groups (Figure 3B). When we examined the expression levels of miR-495-3p, we detected that it was highest in the Group C and lowest in the Group I/R^{24}. In the statistical analysis, there was a significant difference between Group I/R^{24} and Groups C & S & L^1 (p=0.003, p=0.003, p=0.003, respectively) (Figure 3C).

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Table 1 Distribution and Standard Deviations of Biochemical Parameters, Bursting Pressure and Histopathological Damage Values According to Groups

	Group C	Group S	Group I/R^{1}	Group I/R^{24}	Group L^{1}	Group L^{24}	P value
MDA (nmol/mL)	0.90±0.12	1.16±0.35	1.34±0.11	1.26±0.29	0.93±0.15	1.00±0.10	0.006
Glutathione (mg/L)	482.91±67.78	413.72±56.88	363.78±32.36	337.80±16.67	427.08±46.21	487.25±67.45	0.000
Caspase 3 (ng/mL)	2.45±0.65	4.02±2.08	4.80±0.60	4.38±1.92	2.50±0.60	2.85±0.52	0.008
IL-6 (ng/L)	15.13±0.84	15.46±2.24	16.72±1.89	19.96±4.78	13.03±2.16	13.90±2.09	0.002
TNF-α (ng/L)	132.96±27.34	148.46±17.30	150.60±20.01	157.90±18.83	138.93±15.35	152.25±18.47	0.340
Bursting pressure (mmHg)	–	130.32±22.54	116.88±16.01	121.56±20.11	153.83±10.19	156.78±22.43	0.004
Histopathological level of damage (Modified Swerdlow)	0.00±0.00	1.20±2.68	5.00±1.41	4.00±1.58	2.33±2.06	1.66±1.36	0.003

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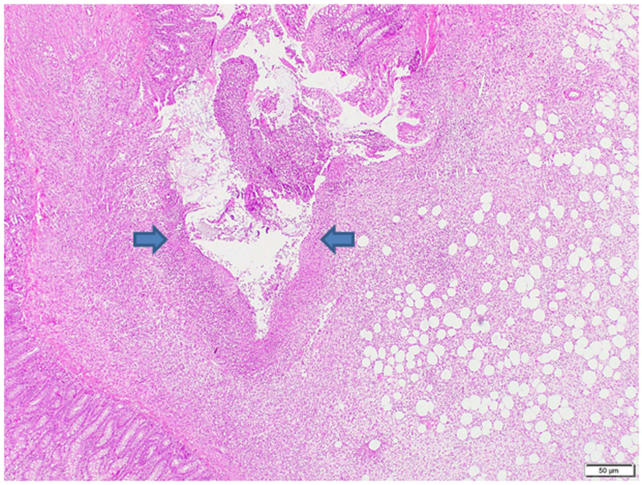

Figure 1 Complete necrosis of all layers of the intestinal wall. Blue arrows indicating area of ulceration and full thickness necrosis in the Group I/R[1] (Modified Swerdlow Grade 6) (H&Ex50).

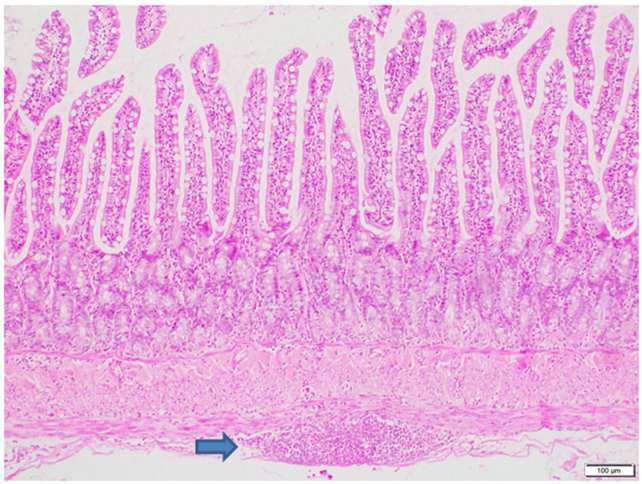

Figure 2 No severe pathological changes, only some serosal inflammation (indicated by blue arrow) secondary to surgery in the Group L[24] (Modified Swerdlow Grade 0) (H&Ex100).

Discussion

Intestinal I/R injury, which can cause local bowel or distant organ injury, is a critical condition that can occur in many diseases and surgeries. Therefore, we created an I/R model in rats and showed that ischemic damage occurred by the evaluation of intestinal tissue and blood samples. To our knowledge, lupeol was applied for the first time in intestinal I/R injury, and this study concluded that it has antioxidant, anti-inflammatory and histopathological healing effects. We also revealed that it decreased the expression of miR-34a-5p and increased the expression of miR-29b-3p and miR-495-3p.

Lupeol is a triterpene found in medicinal plants and a variety of vegetables and fruits.[25,28] Experimental studies have demonstrated the anti-inflammatory, antimicrobial, antioxidant, anticancer effects and pharmacological potential of lupeol.[19] Lupeol showed antioxidant effect in middle cerebral artery occlusion, selenite-induced cataract, myocardial ischemia and cerebral ischemia.[26,29,30] Kumari et al and Preetha et al in hepatic damage, Sudhahar et al in renal damage demonstrated that lupeol reduces MDA, which is the end product of lipid peroxidation caused by ROS, by suppressing ROS formation via antioxidant restoration and increases the non-enzymatic antioxidant GSH, which is thought to have a role in ROS scavenging.[31–33] In this current study, lupeol, which was used for the first time in the intestinal I/R model,

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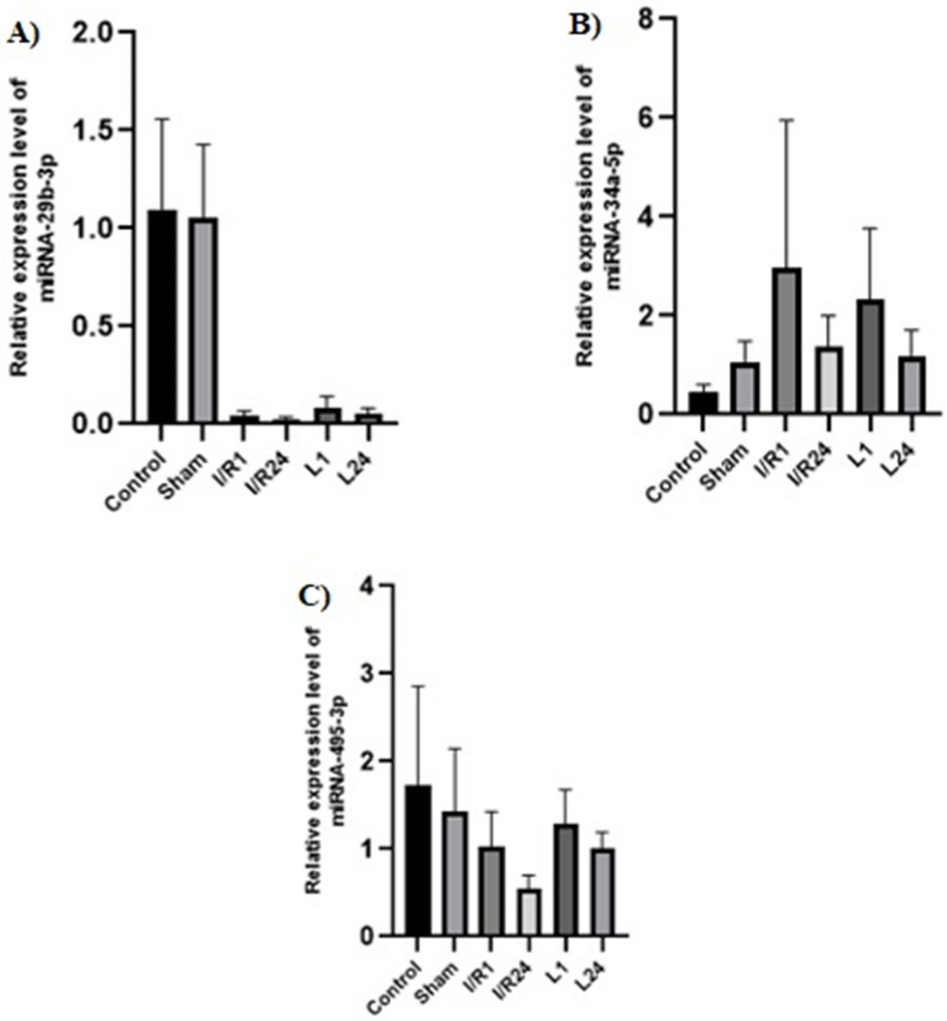

Figure 3 Effects of lupeol on expression level of miRs (**A**) miR-29b-3p, (**B**) miR-34a-5p, and (**C**) miR-495-3p.

showed its antioxidant effect by both decreasing MDA and increasing GSH. In our recently published article, we detected that lupeol similarly reduces MDA and increases GSH in renal I/R injury.[34]

Intestinal I/R damage causes excessive ROS production as well as disruption of the mucosal barrier, bacterial translocation and, accordingly, the release of inflammatory cytokines such as IL-1, IL-6 and TNF-α from neutrophils and macrophages.[12,35] Lee et al, in their experimental study on the effect of lupeol on colitis, concluded that lupeol reduced the production of IL-6, IL-12 and TNF-α through an inhibitory effect on macrophages.[36] In addition, in an experiment with Streptozotocin-induced hyperglycemic mice, it was determined that TNF-α and IL-1β were reduced by lupeol via the NF-kB pathway.[37] In our study, we found that lupeol reduced IL-6 at a statistically significant level, while there was a slight decrease in TNF-α. We have demonstrated that lupeol statistically significantly reduces TNF as well as IL in renal I/R injury.[34] The lack of a statistically significant difference in the current study may be due to the fact that we looked at the blood on the fifth day. If we had looked at the blood on the first days, perhaps the decrease would have been statistically significant. In the intestinal I/R experiment conducted by Gordeeva et al, although there was no significant change in the expression of TNF-α, which is known to activate the caspase cascade, caspase-3, which plays an important role in cell apoptosis, was increased.[3] Alongside a similar effect in the present study, we demonstrated that lupeol reduced caspase-3 levels. It was also concluded that caspase-3 is downregulated by lupeol via the PI3K/Akt signaling pathway in the experimental cerebral I/R model.[25]

In this current study, as we expected, no pathology was detected in the control group that did not undergo ischemia as a result of histopathological evaluation. We observed complete necrosis in all layers (Grade 6) according to the modified Swerdlow classification in one of the rats in the sham group that we anastomized without ischemia. In the others, there was no pathology as in the control group. In the I/R^1 and I/R^{24} groups, we detected that the mean damage level was 5 and 4, respectively. When we evaluated the treatment groups, we found that although there was no statistically significant difference, the damage level of the rats was lower with lupeol treatment. The results of our study indicated that lupeol, which was previously shown to reduce damage in histopathological examination of liver, kidney, and pancreatic tissues, and most recently in kidney tissues by our team, alleviated intestinal damage.[32,34,37]

Intestinal anastomosis, which is required due to various pathological conditions, is a procedure performed following the removal of the diseased or damaged segment.[38] In order to investigate the safety of anastomosis, there are studies in the literature in which bursting pressures are measured and both surgical techniques are compared, and various drugs are tried.[38–40] In the study by Akinci et al examining the effects of Genistein on intestinal ischemia-reperfusion, similar to our study, anastomotic bursting pressure, hydroxyproline, superoxide dismutase, and glutathione peroxidase levels and histopathological wound healing scores of all rats were measured on postoperative day 5. They opted to do a relaparotomy on the fifth postoperative day, when collagen production started to grow but the strength of the anastomosis is still weak, as anastomotic leakage is clinically evaluated on the fifth to seventh postoperative days.[41] In the study of Lu et al, in which intestinal anastomosis was performed, and early enteral feeding was applied to 898 infants, it was emphasized that the traditional belief is that at least 4–5 days are required for the reliability of anastomosis and to start enteral feding.[42] In light of these and other studies, we aimed to reveal the positive effects of the lupeol we would give by looking at the burst pressure on the fifth day, when the anastomosis was thought to be weak. We found that bursting pressures of L^1 and L^{24} groups, to which we treated with lupeol, were statistically significantly higher than the I/R^1 group. There was also a statistically significant difference when the I/R^{24} group was compared with the L^{24} group. These results indicated that lupeol had a positive effect on the intestinal anastomosis, and we attributed this to the wound healing effect of lupeol as previously shown.[43] In addition, although there was no statistically significant difference between the ischemia and treatment groups in themselves, we found that the bursting pressure was slightly higher in those who underwent anastomosis 24 hours after reperfusion. However, for the strength of the sutures and the surgical quality of the anastomosis, experiments should be planned in which these parameters are re-examined on different days such as the 8th and 12th days.

In previous studies, various miRs have been shown to play a role in the pathogenesis of intestinal I/R injury and even thought to have positive effects on intestinal barrier function.[14,44] Further, it is suggested that some miRs may play a role in the development of inflammatory bowel disease.[16] Therefore, studies on the function of miRs in intestinal I/R injury have been carried out in recent years. Zhang et al stated that overexpression of miR-29b-3p increased apoptosis by decreasing the Bcl2/Bax ratio and decreased cardiomyocyte viability.[45] In contrast, Yang et al demonstrated that miR-29b-3p decreases with ischemia and its overexpression reduces cell apoptosis in an oxygen–glucose deprivation and reoxygenation-induced cerebral I/R model. In the same study, they stated that miR-27a-5p increased in ischemia, and cell apoptosis decreased with its inhibition.[46] In intestinal I/R injury, Dai et al found that miR-29b-3p expression was significantly decreased, as in our study, and miR-29b-3p mimics decreased damage via inhibiting TNF receptor-associated factor 3 signaling by increasing Bcl2.[15] In the present study, although it was not significant, lupeol increased miR-29b-3p compared to the ischemia groups. Studies with miR-34a-5p have shown that its inhibition reduces ROS accumulation and cell apoptosis through regulation of Notch Receptor 1 signaling in myocardial I/R damage and via activation of Sirtuin 1 signaling in intestinal I/R damage.[22,47] On the other hand, Li et al reported that miR-34a-5p mimics improve intestinal barrier function and reduce damage in Caco-2 cells.[48] In our study, we detected that lupeol slightly decreased the expression levels of miR-34a-5p, which increased due to ischemia, and that miR-34a-5p levels were lower in the groups with delayed anastomosis. We also evaluated miR-495-3p expression levels and found that lupeol increased I/R-induced decreased expression levels. Luo et al previously stated that Nuclear Enriched Abundant Transcript 1 (NEAT1) downregulates miR-495-3p by activating mitogen-activated protein kinase 6 (MAPK6), and TNF-α, IL-1β, IL-18 protein expressions were suppressed by miR-495-3p mimics.[49]

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Our study examined the difference between early and delayed anastomoses and the effects of lupeol. Studies with miRs in the literature are generally designed with a single miR and the pathways it can affect. The limitations of this article are the small number of samples in the groups, examining the data only in the anastomotic site intestines, not examining the reliability of early and late anastomosis, and not conducting studies on collagen in the intestinal tissue for wound healing. The reason for these limitations is due to insufficient budget and restrictions on animal use. Therefore, more comprehensive studies should be conducted in the future.

Lupeol, which has antioxidant, anti-inflammatory, anticancer and wound healing effects, the molecular mechanisms include inducing apoptosis, inhibiting migration and invasion of cancer cells, and suppressing cell proliferation. This study concluded that it has antioxidant, anti-inflammatory and histopathological healing effects. We also revealed that it decreased the expression of miR-34a-5p and increased the expression of miR-29b-3p and miR-495-3p. We believe that it is a product that can be used therapeutically and supportively in clinical practices such as mesenteric embolism, intussusception, colitis, and intestinal anastomosis. One of the primary objectives of this study was to perform anastomosis on ischemic intestinal tissue at 1 and 24 hours post-reperfusion to evaluate the protective effects of lupeol during both the early and late phases of ischemia-reperfusion injury. The results demonstrated that lupeol treatment significantly improved the evaluated parameters in both the 1-hour and 24-hour anastomosis groups compared to the untreated ischemic groups. The decision to perform intestinal anastomosis without resecting the ischemia-affected bowel was intentional, as the aim was to preserve intestinal integrity and avoid potential complications such as short bowel syndrome. This approach allowed us to better evaluate the direct protective effects of lupeol on the ischemic intestinal tissue. Lupeol is bioactively found in various edible vegetables and fruits, and experimental studies have shown that it is rapidly absorbed and has no toxic effects even at a dose of 2000 mg/kg. On the other hand, low oral bioavailability and water solubility are its disadvantages. However, considering its broad pharmacological effect, it is likely to be beneficial in the treatment of many diseases. Therefore, studies must be conducted to increase its water solubility and oral bioavailability.

Conclusion

Despite financial limitations that restricted pathway analysis, this study demonstrated the protective effects of lupeol on I/R-induced damage by improving antioxidant and inflammatory parameters, enhancing bursting pressure, and promoting histopathological healing. Additionally, changes in miR expression patterns were observed, highlighting lupeol's potential as a therapeutic agent in I/R injury. These findings provide valuable insights into lupeol's protective effects and its role in future miR-related research.

Data Sharing Statement

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Ethical approval for this study was obtained from the Gazi University Experimental Animal Ethics Committee (G.U.ET-22.039).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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