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Article

The Effect of Silymarin on Ischemia-Reperfusion Injury in Skeletal Muscles of Rats

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Abstract: Background and Aim: It is generally accepted that oxidative stress mediators play an important role in ischemia-reperfusion injury (I/R), although the exact mechanism is not fully elucidated. While the impact of silymarin on I/R injury in several tissues, including the myocardial, cerebral, gastric and liver tissue, has been examined, the present state of research on this topic remains insufficient to provide a widely accepted understanding. For this purpose, our objective was to examine the effect of silymarin on muscle tissue in rats subjected to lower extremity I/R injury. **Method:** After ethics committee approval, 18 male Wistar albino rats weighing between 225-275 g were used. The rats were divided into three groups containing six mice each, Control, Ischemia-Reperfusion and Silymarin-Ischemia/Reperfusion groups. Silymarin was administered intraperitoneally 30 minutes before the procedure. (100 mg/kg⁻¹) In the I/R groups, an atraumatic microvascular clamp was placed in the infrarenal abdominal aorta. After 120 minutes, the clamp was removed and reperfusion was achieved for 120 minutes. In muscle tissue samples taken at the end of the reperfusion period; Malondialdehyde (MDA) levels, catalase (CAT) enzyme activity levels and histopathological parameters were compared. **Results:** In the histopathological examination, no degeneration was observed in the muscle fibers in the control group, while findings of striated muscle damage such as muscle atrophy hypertrophy, muscle degeneration-congestion, internal of the muscle nucleus-oval-central nucleus, fragmentation-hyalinization and leukocyte cell infiltration were observed in the I/R group. In the S-I/R group, muscle atrophy hypertrophy, internal of the muscle nucleus-oval-central nucleus, fragmentation-hyalinization and leukocyte cell infiltration were observed to improve these damaged areas compared to the I/R group. MDA levels in the I/R group were found to be significantly higher than in the control and S-I/R groups. CAT enzyme activity was found to be significantly higher in the I/R group than in the control group. **Conclusion:** Our study revealed that the administration of silymarin at a dosage of 100 mg/kg⁻¹ by intraperitoneal injection 30 minutes before ischemia in rats effectively decreased lipid peroxidation and oxidative stress. Additionally, it successfully reduced the injury caused by ischemia/reperfusion in muscle histology.

Keywords: silymarin; malondialdehyde; catalase; ischemia-reperfusion

Introduction

Ischemia is an insufficient oxygen supply to tissues or organs due to inadequate blood flow. It can occur when there is reduced arterial or venous blood flow. Ischemia leads to cell death as it reduces the energy stored in cells and causes the accumulation of harmful substances. ⁽¹⁾ Ischemia-reperfusion (IR) injury implies the return of oxygenated blood to an area of tissue previously affected by ischemia. (proant.)It makes cell structure damaged due to cell apoptosis and necrosis.^{(2),(3)}

IR injury makes the reactive oxygen species (ROS) released. ROS mainly attacks the polyunsaturated fatty acids present in the cell membrane. Lipid peroxide radicals are generated due to the interaction between fatty acids and ROS. Metals found in the environment can act as catalysts

in reactions involving lipid peroxides, leading to the production of breakdown products such as propanal, hexanal, 4-hydroxynonenal, and malondialdehyde (MDA).⁽⁴⁾

As mentioned in previous studies, the catalase (CAT) enzyme, synthesized by many different genes in plants, is part of the enzymatic antioxidant class. It has a superior antioxidant impact owing to its capacity to effectively resolve ROS and its role in the catalysis of hydrogen peroxide.^{(5),(6)}

Many research has investigated IR injury to get an effective agent which can protect cell and its components. One of these agents; silymarin is a compound molecule isolated from *Silybum marianum*, a milk thistle plant.⁽⁷⁾ Its molecular structure is a combination of flavonolignans (silybin A and silybin B, isosilybin A, isosilybin B, silychristin and silydianin), flavonoids, fatty acids and polyphenolic compounds.⁽⁸⁾ Older studies have established silymarin's hepatoprotective effect,⁽⁹⁾ and new research examining its impact on other structures is underway. Silybin is widely acknowledged as the primary and most potent ingredient of silymarin.

Despite various research about silymarin and its effect on different tissues, to our knowledge, its relationship with lower limb skeletal muscle under IR injury is quite rare. In this study, we aimed to show the protective effect of silymarin on lower limb skeletal muscle IR injury.

Material and Method

This study was approved by our center's ethics committee (Ethic number: G.U.ET-19-044). All procedures were performed according to the accepted standards of the Guide for the Care and Use of Laboratory Animals. At the onset of experimental procedures, all rats were anesthetized with ketamine (50 mg/kg, i.p) and xylazine (10 mg/kg, i.p.). During the surgical procedure, rats were placed on a heating pad to maintain the body temperature. Rats were kept in a temperature-controlled (21±1°C) and humidity-controlled room (45–55%) maintained on a 12/12 reversed light cycle. Animals were fed with a standard pellet and allowed to drink water ad libitum.

A total of 18 Wistar albino rats weighing between 270 and 300 grams were used. Animals were equally divided into three equal groups (n= 6): the Control group (Group C), the Ischemia-reperfusion group (Group I/R), and the silymarin and ischemia-reperfusion group (Group S-I/R). Midline laparotomy was the sole surgical procedure in Group C. In Group I/R, midline laparotomy was performed similarly, and the infrarenal aorta was left clamped for 2 hours. After removing the clamp, reperfusion was established for another additional 2 hours. After following the same steps as Group I/R, silymarin was given (100 mg.kg⁻¹) intraperitoneally 30 minutes before the ischemia period in Group S-I/R.

At the end of 4 hours, gastrocnemius muscle tissue samples were collected, and subjects were sacrificed by taking intraabdominal blood and applying a lethal dose of ketamine.

The histopathological examination investigated muscle atrophy/hypertrophy, degeneration/congestion, internalization of muscle nuclei with oval-central nucleus, fragmentation/hyalinization, and leukocyte cell infiltration. Additionally, MDA levels and CAT enzyme activity were detected for biochemical analysis.

The data were analyzed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 22.0 program for Windows statistical software. The Kruskal-Wallis test was applied to assess biochemical and histological parameters. A p-value less than 0.05 was considered to be statistically significant.

Results

Gastrocnemius Muscle Tissue Histopathological Results

The muscle fiber is a multi-nucleated, syncytial-like structure that resembles a long, narrow tube. The sarcoplasm shows a subtle, light pink coloring. The sarcolemmal nuclei are elongated and thin, located in parallel with the longitudinal axis of the fiber. Group S-I/R presents a similarly light microscopic morphology to Group C, attributed to the protective properties of silymarin. Significant differences between the groups were observed in terms of muscle atrophy -hypertrophy (p = 0.015), muscle degeneration- congestion (p = 0.085), internalization of muscle nuclei -oval/central nucleus (p = 0.012), fragmentation -hyalinization (p = 0.070), and leukocyte cell infiltration (p = 0.075). The IR group exhibited a higher occurrence of muscle atrophy-hypertrophy than the control group (p = 0.002). (Table 1, Figures 1–6). Moreover, muscle atrophy-hypertrophy was markedly reduced in the S-I/R group compared to the IR group (p = 0.015). The I/R group demonstrated a higher

internalization of muscle nuclei -oval/central nucleus than the control group (p = 0.001). The S-I/R group exhibited lower internalization of muscle nuclei -oval/central nucleus than the I/R group (p = 0.047).

Figure 1-6

The muscle fiber is a multi-nucleated, syncytial-like structure that resembles a long, narrow tube. The sarcoplasm shows a subtle, light pink coloring. The sarcolemmal nuclei are elongated and thin, located in parallel with the longitudinal axis of the fiber. Group S-I/R presents a similarly light microscopic morphology to Group C, attributed to the protective properties of silymarin.

Figure 1. Skeletal muscle longitudinal section light microscopy for the Control group (H&E: hematoxylin and eosin X40). →: peripheric nucleus *: muscle fibers ►: intercellular.

Figure 2. Skeletal muscle longitudinal section light microscopy for the Control group (H&E: hematoxylin and eosin X100). →: peripheric nucleus *: muscle fibers ►: intercellular.

Figure 3. Skeletal muscle longitudinal section, group I/R (H&E: hematoxylin and eosin X40). *: muscle fibers ►: intercellular space.

Figure 4. Skeletal muscle longitudinal section light microscopy for the I/R group (H&E: hematoxylin and eosin X400). →: peripheric nucleus *: muscle fibers ►: intercellular space Dej: degeneration CN: central nucleus ON: oval nucleus H: hypertrophy Hy:hyalinization F:fragmentation inf:inflammation NF: necrotic fibrils P: Picnotic Nucleus.

Figure 5. Skeletal muscle longitudinal section light microscopy for the S-I/R group (H&E: hematoxylin and eosin X40). →: peripheric nucleus *: muscle fibers ►: intercellular h:hyalinization F:fragmentation.

Figure 6. Skeletal muscle longitudinal section light microscopy for the S-I/R group (H&E: hematoxylin and eosin X400). →: peripheric nucleus *: muscle fibers ►: intercellular ON: Oval Nucleus hy:haylinization p:picnotic nucleus f:fragmentation.

Table 1. Muscle Tissue Histopathological Data(Mean±SE).

	Group C	Group I/R	Group S-I/R	P**
	(n=6)	(n=6)	(n=6)	
Muscle atrophy-hypertrophy	0.33±0.21*	1.50±0.22	0.67±0.21*	0.015
Muscle degeneration- congestion	0.33±0.21	1.33±0.42	0.67±0.21	0.085
Internalisation of muscle nuclei - oval/central nucleus	0.17±0.17*	1.50±0.22	0.83±0.31*	0.012
Fragmentation -hyalinization	0.33±0.21	1.50±0.42	0.83±0.31	0.070
Leukocyte cell infiltration	0.50±0.22	1.17±0.31	0.33±0.21	0.075

C: Control, I/R: Ischemia/Reperfusion, S-I/R:Silymarin- Ischemia Reperfusion; P**Significance level with Kruskal Wallis test p <0.05; *p<0.05: Compared to Group I/R.

Gastrocnemius Muscle Tissue Biochemical Results

The MDA levels significantly differed between the groups (p = 0.037). The MDA levels were higher in the I/R group than in the C group (p = 0.041). Moreover, significantly reduced MDA levels were found in the S-I/R group compared to the IR group (p = 0.017) (Table 2).

Table 2. Muscle tissue MDA levels and CAT enzyme activity data [mean ± SE].

	Group C (n=6)	Group I/R (n=6)	Group S-I/R (n=6)	P**
CAT (IU/mg.protein)	499.00±49.81	687.67±60.95*	564.00±36.37	0.049
MDA (nmol/mg.protein)	1.18±0.11*	1.72±0.12	1.07±0.24*	0.037

MDA: Malondialdehyde; CAT: Catalase; P **Significance level with Kruskal Wallis test p <0.05; *p<0.05: Compared to Group I/R.

CAT enzyme activity in the gastrocnemius muscle tissues significantly differed between the groups (p = 0.049). The IR group showed higher CAT enzyme activity than the C group(p = 0.018).

Discussion

We hypothesized that silymarin has a protective effect on IR injury of lower limb muscle. Our results supported the this theory. The anti-inflammatory activity of silymarin is commonly accepted. (10),(11) Furthermore, silymarin exerts its cell-protective effects not only through its antioxidative and radical scavenging activities but also by interacting with particular receptors such as estrogen receptors, nuclear receptors, and P glycoproteins . (12)

The CAT enzyme catalyzes the reduction of hydrogen peroxide (H₂O₂) molecules in different tissues' peroxisomes. Inadequate tissue levels of catalase following IR injury might impede protection against the harmful effects of H₂O₂, resulting in cellular damage. Prior research has demonstrated that silymarin has a preventive impact on IR injury by augmenting the activity of CAT.(13)

In our study, we measured MDA levels as a lipid peroxidation marker after IR injury and found significantly decreasing MDA levels, we believe that is a possible mechanism of silymarin as an inhibitor of lipid peroxidation.

With its historical importance in an enormous field from Europe to Asia, Silybum marianum and its derivated form, silymarin is the main topic because of its therapeutic efficacy in different clinical studies. (10) (14),(15),(16)(17),(18) (Sigma-Aldrich; Merck KGaA; cat. no. SO292-50G). In our study, we used silymarin with more than 30% silybin content. However, though many studies have shown silymarin to be clinically reliable and well-tolerated, yet are also contrasting results.(16), (19), (20) The research published by Schrieber et al(19) found gastrointestinal and neurological side effects. However, other investigations in the literature have shown no adverse events, even when using equal or greater dosages. (20) ,(21)Several studies have underlined that this anti-inflammatory activity of silymarin may be dose dependent,specically while inhibits interferon-g, IL-4, and IL-10 (12),(22),(23)(24) In light of these, it is possible to accept that the effectiveness of silymarin preparations may be influenced by the chemical variations in their content. (9).

Conclusions

In summary, our research showed that silymarin has a protective effect on lower limb extremity muscle I/R injury.It augments the acitivity of CAT and decreases MDA by inhibiting lipid peroxidation.Based on previous studies, the effectiveness of silymarin may depend on dosage and types of compound.Hence, the authors of this manuscript believe that it is important to investigate the effects of silymarin on various organs and tissues in futre research prior to its application in medical conditions.

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Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions: MA, AÖ, MHZ and AK designed the study, and analyzed and interpreted data. AÖ, BK and BM performed the experiments. MA, AÖ and AK confirm the authenticity of all the raw data. AÖ, AK, MA

and BK provided scientific and technical assistance, and critically revised the article for important intellectual content. BM and BK collected samples. ŞCS and MK performed histological and biochemical experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate: Ethical approval for the study was obtained from Animal Research Committee of Gazi University (Ankara, Turkey; approval no. G.Ü.ET-19.044).

Patient consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

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