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RESEARCH

Investigation of the biochemical and histopathological effects of vitamin C, selenium, and therapeutic ultrasound on muscle damage in rats

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Abstract

Muscle injury is a common type of soft tissue injury. Increased oxidative damage has been reported after muscle injuries. Therapeutic ultrasound is commonly used for such injuries. This study compared the efficacy of therapeutic ultrasound treatment and various antioxidant agents in experimental muscle injuries. For this purpose, some serum enzymes, oxidative stress, and inflammatory markers were evaluated together with histopathological examinations. Six groups were formed with 6 male Wistar albino rats in each group. These groups were control, only injury (OI), ultrasound (U), vitamin C (Vit C), selenium (S), and mixture (M). Muscle injury was caused by a laceration of the gastrocnemius muscle in all groups except the control group. No treatment was performed in the OI group. At the end of the 6-day application, all rats were sacrificed. As for serum enzymes, CK, ALT, and AST levels returned to control values in almost all treatment groups. Total oxidative status (TOS) and oxidative stress index (OSI) increased in the OI group, while they decreased in the S and M groups. In addition, the decrease in MPO activity in the blood tissue of the Vit C group was statistically significant. There were no significant changes between groups in terms of serum inflammatory markers and histological findings. This study has shown that the ingestion of vitamin C and selenium may contribute to the treatment of muscle injury in addition to therapeutic ultrasound treatment. However, further studies are needed to support these results.

Keywords Wistar albino rats · Muscle injury · Therapeutic ultrasound · Vitamin C · Selenium · Oxidative stress

Introduction

Muscle injuries are common injuries in daily life. Nevertheless, they are a bigger problem for athletes (Barroso and Thiele 2015). Physical therapy and rehabilitation are performed in both acute and chronic phases of muscle injuries. In rats with gastrocnemius muscle contusion injury, therapeutic pulsed ultrasound (TPU) treatment positively contributed to myoregeneration in the early stages of muscle damage (Rantanen et al. 1999). The use of ultrasound for 5 min daily was found to be more effective in repairing muscle damage caused by contusion (Shu et al. 2011). In a study in which a laceration injury was created in the gastrocnemius muscle, TPU treatment was found to have a positive effect on the regeneration of the damaged muscle (Piedade et al. 2008).

In fact, the effectiveness of ultrasound on the mechanism of muscle recovery is still not confirmed in the current studies. However, the use of ultrasound is popular in sports medicine and soft tissue injuries. In Europe, Japan, and the

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USA, ultrasound therapy is routinely used to treat muscle damage in athletes (Warden 2003).

In a study involving experimental muscle injury with low-intensity pulsed ultrasound and dimethyl sulfoxide treatment, inflammatory markers such as tumor necrosis factor-alpha (TNF-α), IL-1β, nuclear factor-kappa B protein levels, and c-jun n-terminal kinase were shown to be reduced (Engelmann et al. 2012). Some studies have reported that TPU reduces some oxidative stress parameters and serum creatine kinase levels in muscle injury (Freitas et al. 2007).

On the other hand, other studies have reported that muscle injury causes oxidative damage (Freitas et al. 2007; Saborido et al. 2011). Free radical formation and activation of the inflammatory process are the two main pathways that come into play after a traumatic lesion (Ostjen et al. 2019). It has been observed that reactive oxygen species (ROS) also play an important role in driving the inflammatory cascade, which is important in trauma models (Toufektsian et al. 2001; Cuzzocrea et al. 2004). Cytokines promote migration, proliferation, and survival of different cell types at the site of injury (Serrano et al. 2008). The importance of tumor necrosis factor-α (TNF-α), a cytokine produced by activated macrophages, including IL-6, in muscle repair is currently under investigation (Li 2003). Infiltrating neutrophils secrete ROS via myeloperoxidase (MPO) due to the biological effects of TNF-α, such as neutrophil activation and increased infiltration of muscle by inflammatory cells (Aggarwal 2003; Barker et al. 2009). ROS have been reported from human and animal blood and tissues to play a role in the initial and ongoing process of wound healing (Silva et al. 2011). Increased ROS production (Scicchitano et al. 2018) results in the release of important enzymes such as creatine kinase and lactate dehydrogenase into the extracellular environment (Silveira et al. 2012; Kozakowska et al. 2015). To compensate for this oxidative increase, intrinsic or extrinsic antioxidant defenses must be activated.

Melatonin, considered a potent antioxidant and anti-inflammatory agent, has been shown to be effective for muscle recovery in an experimental model of induced muscle trauma in rats (Ostjen et al. 2019). Quercetin has been reported to reduce oxidative stress biomarkers after skeletal muscle injury. Similarly, TPU also reduced the levels of these oxidative biomarkers (Sousa Filho et al. 2021). Vitamin C, a primary antioxidant in plasma and cells that reduces oxidative stress (Frei et al. 1990; Judge et al. 2008), is highly concentrated in neutrophils, which helps protect against inflammatory respiratory bursts (Levine et al. 1996) and may reduce infiltration of these cells into skeletal muscle (Kearns et al. 2004; Judge et al. 2008).

Among antioxidant micronutrients, selenium is important because of its anti-inflammatory activity (Rayman 2000) and its cofactor function in antioxidant selenoenzymes that prevent ROS-induced intracellular damage (Berger and Chioléro 1995; Arbogast and Ferreiro 2010). Selenium is required for the intracellular antioxidant system as a structural component of the active site of glutathione peroxidase and other antioxidant selenoproteins (Burk et al. 2003). Some antioxidants have been associated with TPU for the treatment of muscle lesions, suggesting that TPU may improve drug delivery. In this context, it showed a positive reduction in oxidative stress markers (Filho et al. 2018; Santos et al. 2020).

In the current study, vitamin C and selenium were selected as two potent antioxidants to evaluate their protective effects against oxidative stress caused by muscle injury. In the light of this literature, for the first time in this study, based on the hypothesis that the treatment model of TPU and vitamin C, known as antioxidant, and selenium on blood muscle tissue can be successful, an attempt was made to investigate the effects of these applications in the retrospective repair of experimental muscle injury in rats.

Materials and methods

The analyses of this study were performed at the Faculty of Medicine, Van Yüzüncü Yıl University, Department of Medical Biology, Laboratory of Enzymology. In addition, the experiments were performed at the Department of Laboratory Animals of Van Yüzüncü Yıl University.

Equipment

Male Wistar albino rats with an initial weight of 250–300 g were used for the study. These animals were obtained from the Laboratory Animal Department of Van Yüzüncü Yıl University. The rats were kept in steel cages during the study, with 6 animals in each group. During this time, the animals could drink and eat as much water as they wanted. The room in which they are housed is set up so that there are 12 h of light and 12 h of darkness. In addition, the room temperature of the laboratory was measured with a thermometer and the humidity with a hygrometer. The laboratory was constantly ventilated with a fan so that the temperature does not exceed 23 ± 1 °C.

Formation of experimental groups

The subjects were divided into control (C), only injury (OI), therapeutic ultrasound (U), vitamin C (Vit C), selenium (S), and mixed groups (M). The total 6 groups to be formed in the study were subjected to the following procedures, with 6 rats in each group. All groups received normal rat chow (protein 24%, lysine 1%, methionine 0.6%, cysteine 0.4%, cellulose 7%, NaCl 1%, calcium 1–2%, and energy 2650 kcal/kg) and tap water. Since the rats in the

1st group were administered intraperitoneally (i.p.) to the other groups, physiological water was administered i.p. to avoid differences in the results. The rats in the 2nd group were only injured and received physiological water i.p. as in the 1st group. The rats in the 3rd, 4th, 5th, and 6th groups were treated three times within 2 days, 24 h after muscle injury. Therapeutic ultrasound was applied to the rats of the 3rd group, and physiological water was administered i.p. after the ultrasound applications. Vitamin C was injected i.p. to the rats of the 4th group, and selenium was injected i.p. to the rats of the 5th group. The rats of group 6 were injected with vitamin C + selenium i.p., and therapeutic ultrasound was applied before the injection. The physiological feature of peritoneal cavity provides useful access to the body for various pharmacological agents (Chaudhary et al. 2010).

Muscle damage formation

The muscle injury model created by direct incision into the muscle was used as the basis. The rats were anesthetized by i.p. injection of ketamine (100 mg/kg). After the calves of the rats were shaved, a blunt incision was made longitudinally. The subcutaneous layer was penetrated, and the gastrocnemius muscle was reached. The gastrocnemius muscle was incised from distal to proximal to 60% of its length, 75% of its width, and 50% of its depth (Menetrey and Kasemkijwattana 2000; Chan et al. 2003). All rats were sacrificed after 6 days of application.

Application of molecules whose efficacy has been studied

Vitamin C: 3 doses of vitamin C were administered every 2 days 24 h after muscle injury. Each dose was administered as 500 mg/kg i.p. (Khassaf et al. 2003).

Selenium: 3 doses of selenium were administered every 2 days 24 h after muscle injury. Each dose was administered as 1.5 mg/kg i.p. (Özdil et al. 2004). Thus, the application lasted for a total of 6 days.

Application of therapeutic ultrasound

Therapeutic ultrasound was applied for 6 min at a frequency of 1.0 MHz (Silveira et al. 2012). Ultrasound was applied with an intensity dose of 1 w/cm2 (Freitas et al. 2007). In addition, the ultrasound probe was moved in a circular fashion during the treatment (Saliba et al. 2007). Ultrasound was applied to the posterior calf of the randomly selected left leg of the rat at an adjusted dose with ultrasound gel.

Kit, chemicals, and devices used in the analysis

TAS measurement kit (Rel Assay Diagnostics), TOS measurement kit (Rel Assay Diagnostics), IL-6 measurement kit (Rat IL-6 Platinum ELISA eBioscience BMS625), TNF-α measurement kit (Rat TNF-alpha Platinum ELISA eBioscience BMS622), Tris-hydroxy methyl amino methane, EDTA-Na_2, o-dianizidine-HCl, hydrogen peroxide (H_2O_2), potassium dihydrogen phosphate (KH_2PO_4), HTAB (MA: 364.5; hexadecyl trimethyl ammonium bromide), sodium selenite Na_2SeO_3 (Sigma-Aldrich 214,485), vitamin C/L-ascorbic acid $C_6H_8O_6$ (Sigma-Aldrich A7506).

Radwag AS 220/X precision balance, TTS 3 digital yellow line vortex, Orion 3-star pH meter, Aurigo surgical kit, Bandelin Sonopuls ultrasonic homogenizer, Hettich Universal 320R refrigerated centrifuge, Socorex Swiss Brand automatic pipette, Siemens refrigerated refrigerator, Genesys 10 UV Scanning spectrophotometer, ELISA reader and DAS washer.

Preparation of blood and tissue samples

Collection of blood and tissue samples All subjects in the group were anesthetized by administration of 100 mg/kg ketamine i.p. 24 h after the injections and ultrasound applications. Using injectors, blood and tissue samples were collected from the hearts of the animals under general anesthesia and transferred to EDTA tubes for whole blood samples and biochemical tubes for serum collection. The collected muscle tissue samples were washed in 0.9% physiological water and stored in a freezer at − 75 °C until examination in the laboratory. TAS, TOS, OSI, and MPO enzyme activities were then measured in the tissue supernatant by spectrophotometric method. For histopathological examination, the muscle tissue samples from the animals were fixed in 10% buffered formalin and sent to the pathology laboratory.

Preparation of serum samples Serum samples were prepared by centrifuging the blood transferred to biochemistry tubes for 5 min at 3000 rpm and + 4 °C. Some sera were sent to the biochemistry laboratory to measure enzyme levels of creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). The remaining serum samples were stored in a freezer at − 75 °C until the cytokines IL-6 and TNF-α were determined by the ELISA method.

Preparation of whole blood samples Whole blood samples collected in EDTA tubes were first hemolyzed with distilled water in a 1:1 ratio. The resulting hemolysate was centrifuged at 3000 rpm and + 4 °C for 5 min, and the clear supernatant remaining at the top was separated from the

precipitate and transferred to another tube. These whole blood samples were used for the analyses.

Preparation of tissue homogenates After treatment, 0.1 g of tissue was removed from muscle tissue samples stored in the freezer and 1900 µl of cold phosphate buffer (PBS) was added. The resulting mixture was homogenized in an ultrasonic homogenizer (2 min) provided it was kept cold. Subsequently, the obtained homogenates were centrifuged for 30 min at 3500 rpm at + 4 °C in a refrigerated centrifuge in suitable test tubes. The supernatants obtained from the muscle tissue were used for analysis (Xia et al. 1994; Marklund 1990).

Determination of the activity of the enzyme myeloperoxidase (MPO) (EC 1.11.1.7)

MPO enzyme activity in supernatants of gastrocnemius muscle tissue and whole blood samples was measured using a spectrophotometer at 460 nm. Absorbance values were recorded every 15 s for 3 min. In the part where the absorbance decrease is linear, the absorbance decrease per minute was determined, and the U was calculated using the following formula. Results are expressed as U/g tissue × dilution for tissue and U/mL × dilution factor for whole blood according to the modified method of Bradley et al. (1982) (Çelik and Tülüce 2007; Tuluce et al. 2011).

Determination of TAS, TOS levels, and calculation of the oxidative stress index

Total antioxidant content (TAS) in gastrocnemius muscle tissue supernatants and whole blood samples was determined by measuring at 660 nm in a spectrophotometer as indicated in the kit procedures. Trolox, an antioxidant, water-soluble vitamin E analog used in the standard method, is used to calibrate the reaction rate. The unit of TAS content in the samples is expressed as Trolox equivalent/L.

The total oxidant status (TOS) in the supernatants of the samples was determined by measuring at 530 nm with the spectrophotometer according to the instructions of the kit. The unit of TOS content in the samples is expressed as µmol H_2O_2 equivalent/L.

The oxidation stress index (OSI) was calculated by dividing the TOS/TAS value. Its unit was taken as arbitrary unit (AU) (Tülüce et al 2017, 2019).

Determination of IL-6 and TNF-α cytokine levels

Measurements were made at 450 nm with an ELISA reader as indicated in the procedures of the rat IL-6 and TNF-α Platinum ELISA kits used for the measurement of the cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) in serum. Both cytokine units are pg/ml.

Histopathological examinations

After tissue samples were fixed in 10% buffered formalin, they were embedded in paraffin blocks, and 4 µm sections were prepared using a microtome. The sections were stained with a hematoxylin–eosin stain and examined under a research microscope. Fibrosis tissue and inflammation in the muscle tissue were checked. Images of the gastrocnemius muscle taken with the × 10 objective of the microscope.

Statistical analysis

Descriptive statistics for the characteristics discussed were reported as median, mean, standard deviation, minimum, and maximum values. The Kolmogorov–Smirnov test was applied to the data as a normality test. The Levene test was used as a homogeneity test. The Kruskal–Wallis test was performed to compare the groups in terms of these characteristics. The Dunnett test was performed to identify different groups. The statistical significance level was set at 5% in the calculations, and the SPSS statistical program was used.

Results

Results of serum enzyme analysis

Biochemical methods were used to investigate the enzyme activities of CK, LDH, ALT, and AST in serum. The results obtained and statistical analyses are shown in Fig. 1 A, B, C, and D.

In all treated groups, serum CK levels were significantly lower than in the OI group. Vitamin C was found to be more effective in lowering serum CK levels than other treatment groups (Fig. 1A).

A significant decrease in LDH levels was observed in the Vit C, S, and M treatment groups compared to the OI group. In addition, a significant decrease was observed in the S group compared with the C group (Fig. 1B).

Serum ALT level, which increased significantly in the OI group compared with the C group, showed a nonstatistical decrease in all treatment groups (Fig. 1C).

While AST level significantly increased in the OI group compared with the C group, a significant decrease was

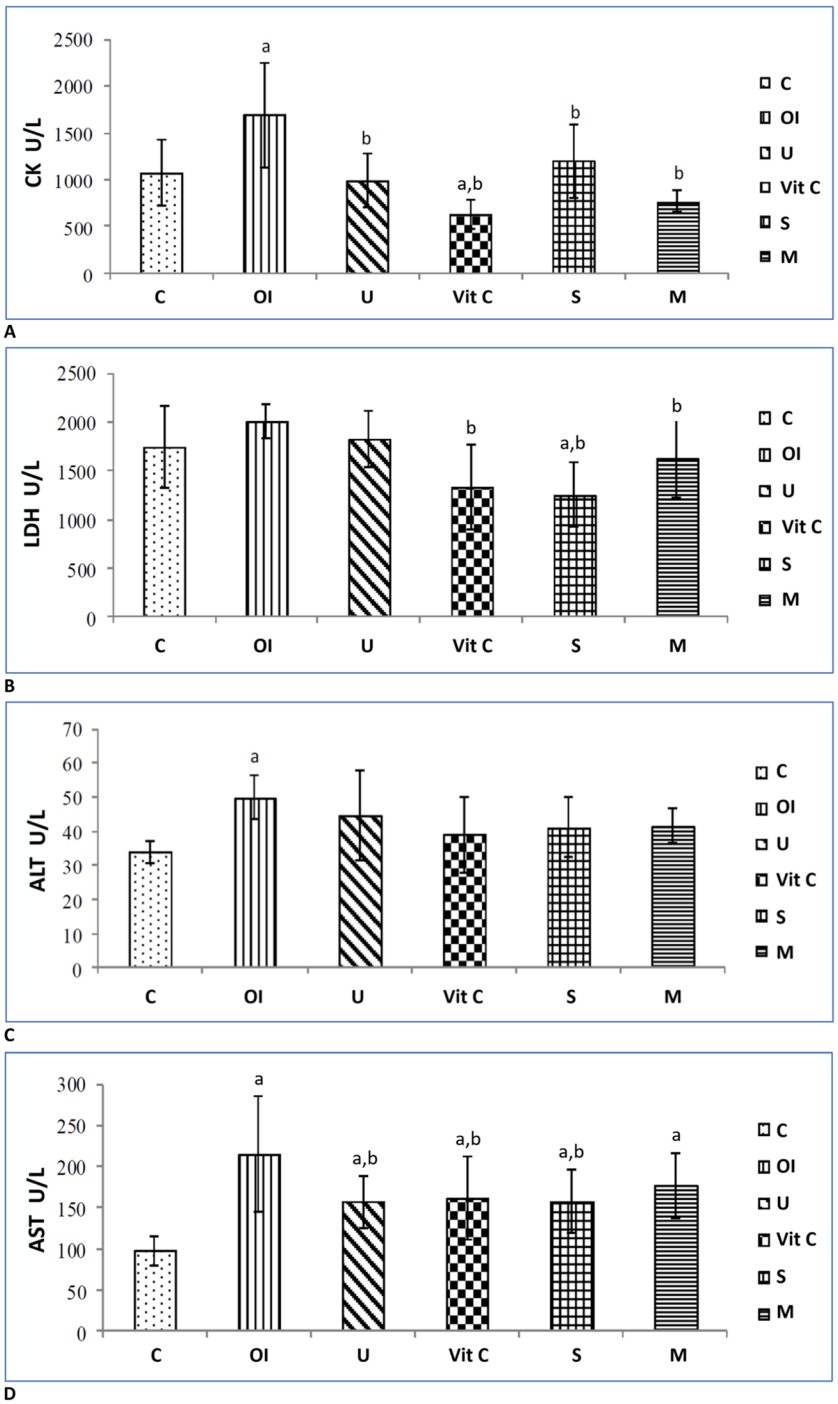


Fig. 1 Distribution of serum enzyme results according to groups and statistical data. Graph of serum CK (**A**), LDH (**B**), ALT (**C**), and AST (**D**) enzyme activities. The difference between groups receiving different letters was significant ($p < 0.05$). [a]The difference is significant compared to the C group. [b]The difference is significant according to the OI group

observed in all treatment groups compared with the OI group, except in the M group (Fig. 1D).

Results of oxidative stress and neutrophil infiltration markers

TAS, TOS, and OSI markers of oxidative stress are measured in gastrocnemius muscle tissue and blood. Descriptive statistics and comparative results on MPO, a marker of neutrophil infiltration, are shown in Figs. 2 A–D and 3 A–D.

There was no statistically significant difference in TAS and OSI measurement results in muscle tissue between groups. However, for TOS measurement results in muscle tissue, a statistically significant decrease was observed in groups S and M compared to group OI.

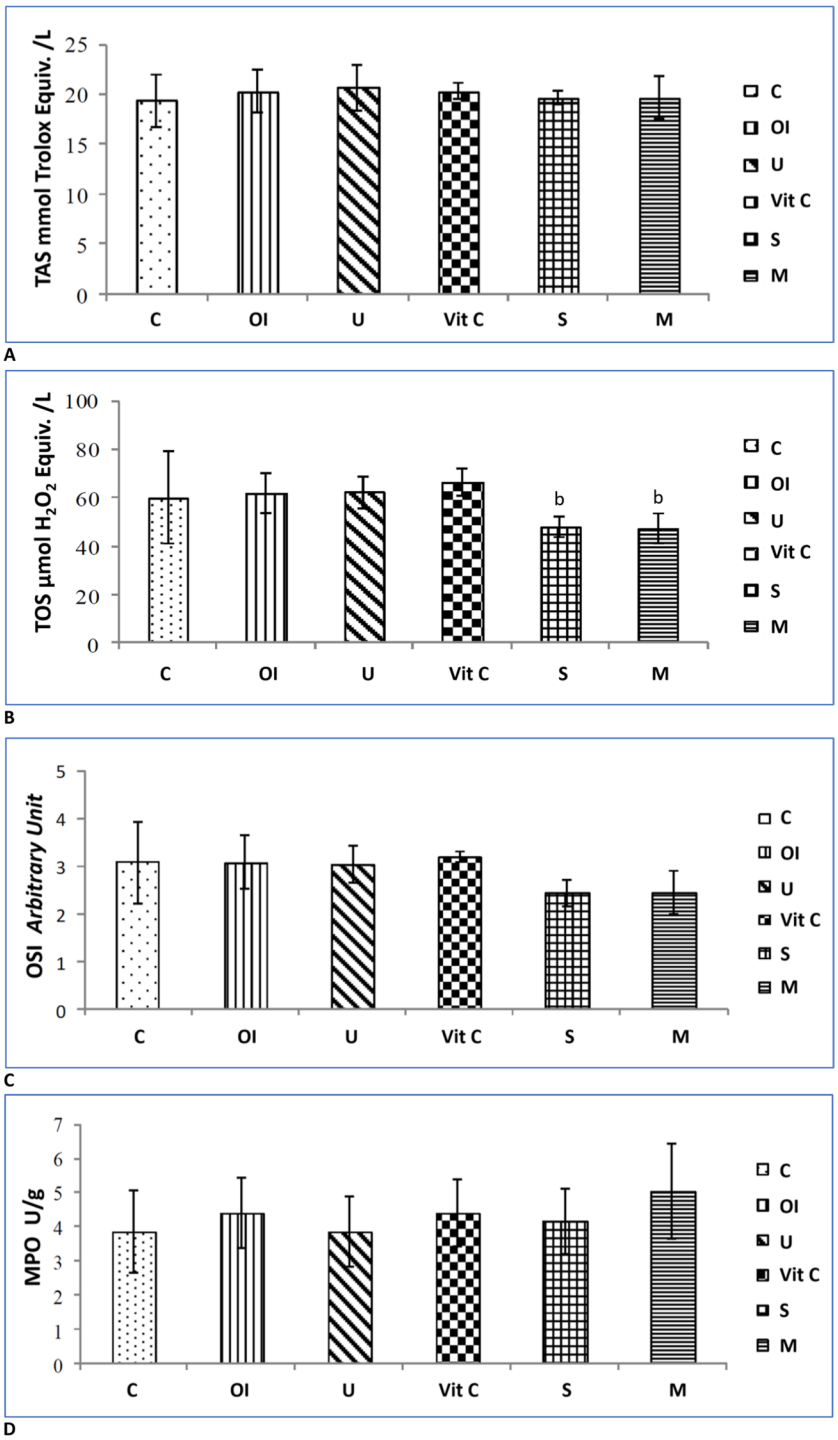


Fig. 2 Distribution of TAS, TOS, OSI, and MPO results measured in muscle tissue by groups and statistical data. Graph of muscle tissue TAS (**A**), TOS (**B**), OSI (**C**); and MPO (**D**) results. The difference between groups receiving different letters was significant ($p < 0.05$). [a]The difference is significant compared to the C group. [b]The difference is significant according to the OI group

In blood tissue, there were no statistically significant differences between groups in TAS, TOS, and OSI measurements. However, in the blood tissue MPO measurement results, a statistically significant decrease was observed in the Vit C group compared to the OI group.

Serum cytokine results

The analyses of serum cytokines IL-6 and TNF-α were performed by the ELISA method. The results obtained from the measurements and their comparative statistics are shown in Fig. 4 A and B.

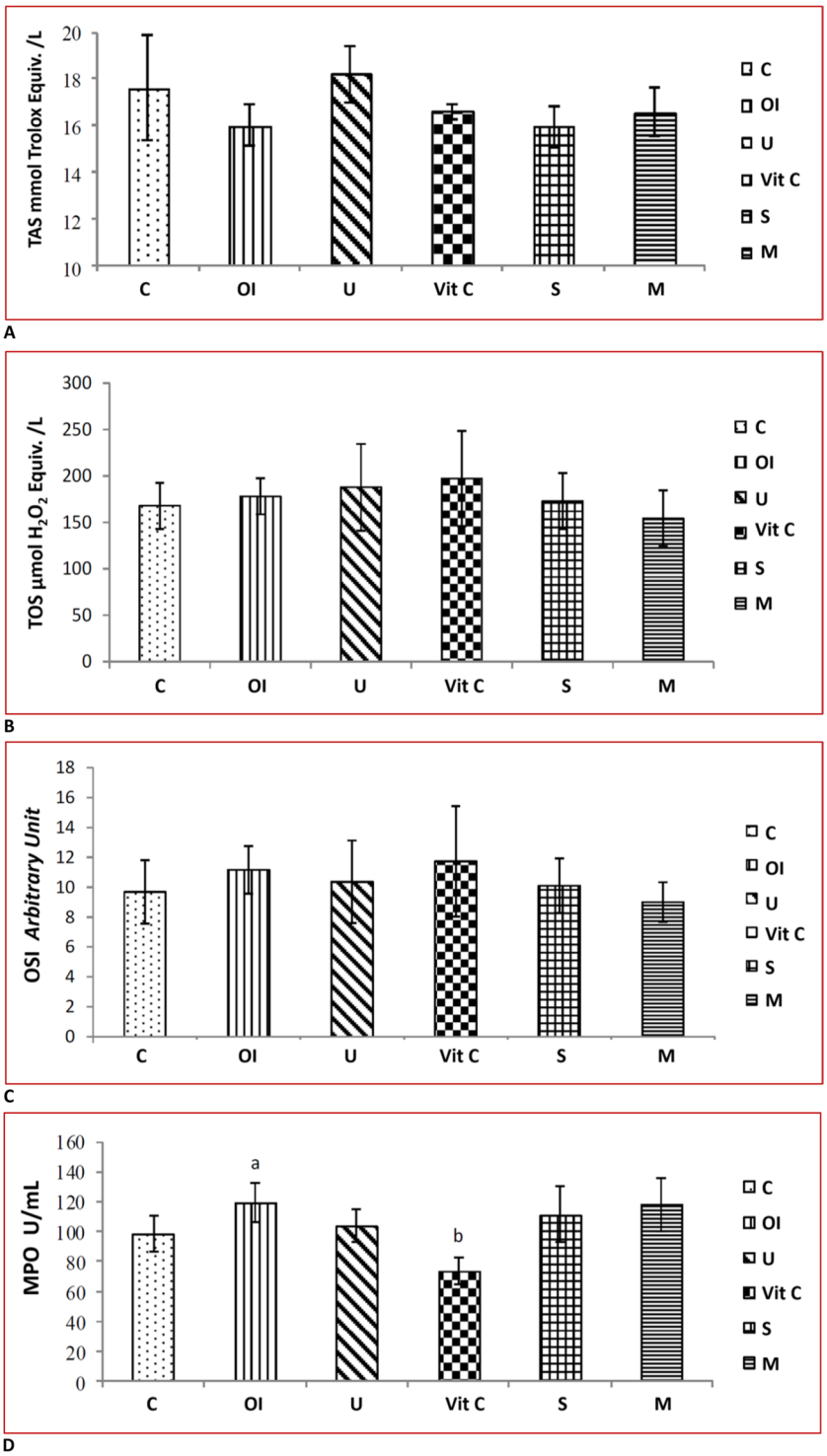


Fig. 3 Distribution of TAS, TOS, OSI, and MPO results measured in blood tissue according to groups and statistical data. Graph of blood tissue TAS (**A**), TOS (**B**), OSI (**C**), and MPO (**D**) results. The difference between groups receiving different letters was significant ($p < 0.05$). [a]The difference is significant compared to the C group. [b]The difference is significant according to the OI group

There was no statistically significant difference between the groups in terms of serum IL-6 and TNF-α cytokines measured.

Histopathological results

Microscopic examination revealed no significant difference between the C, OI, U, Vit C, S, and M groups.

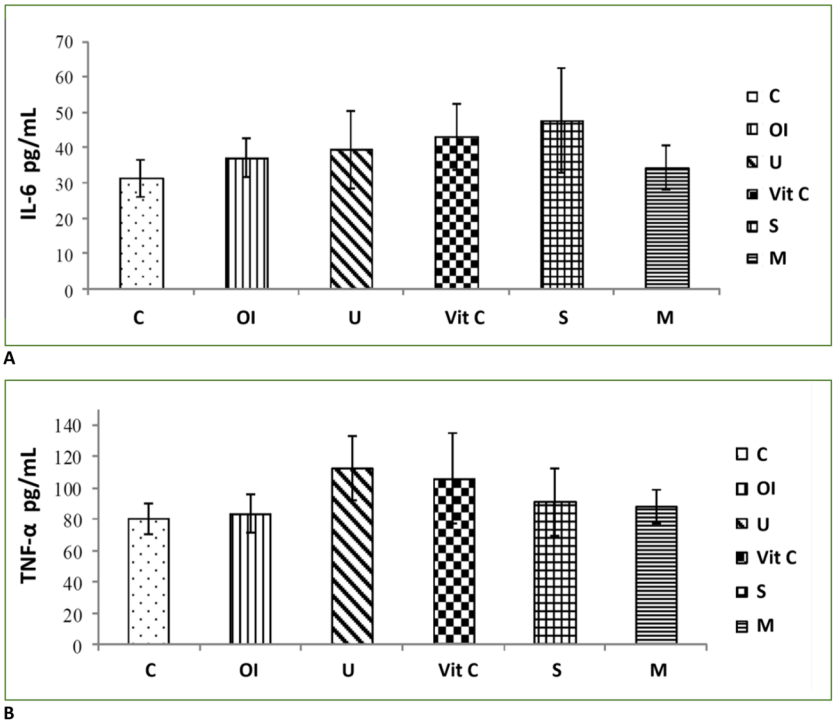


Fig. 4 Distribution of IL-6 and TNF-α cytokines results by groups and statistical data. Graph of serum IL-6 (**A**) and TNF-α (**B**) cytokines results

In addition, a normal histological structure of the gastrocnemius muscle was observed in all groups (Fig. 5 A–F).

Discussion and conclusion

Muscle injuries are one of the skeletal system problems that are common in both daily life and sports. Muscle

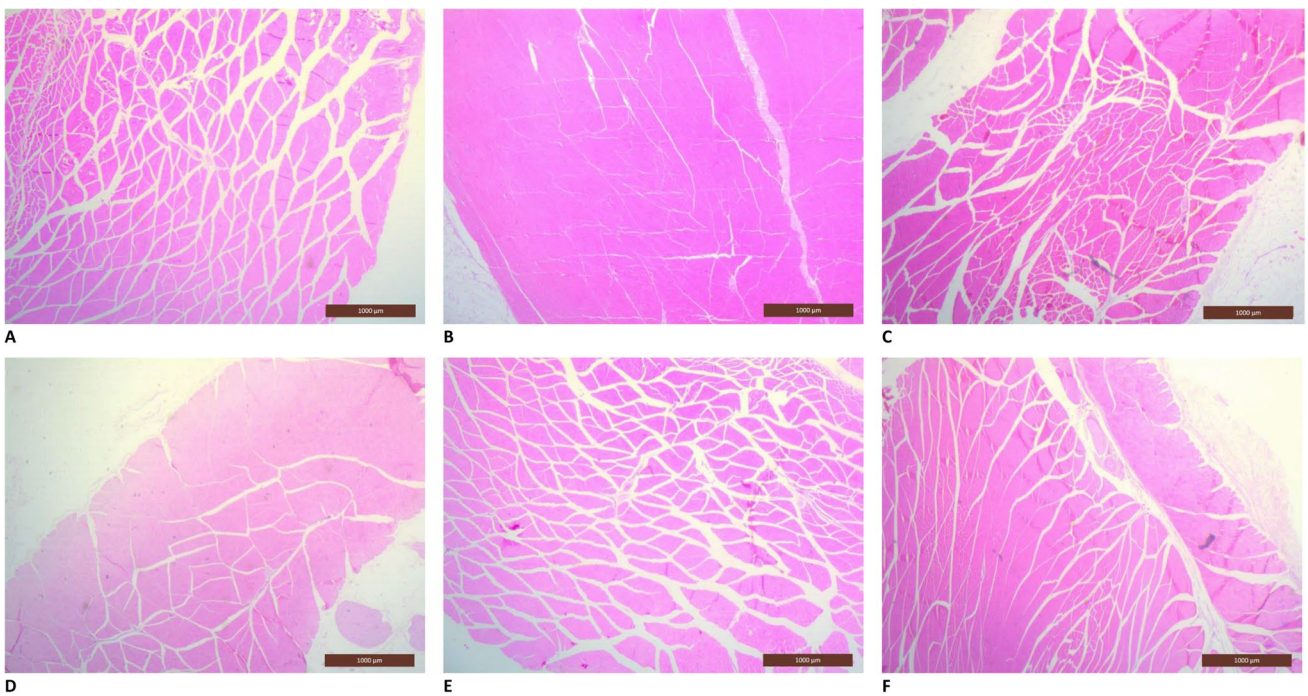


Fig. 5 Normal histological structure (HxE) of the gastrocnemius muscle in the control group (**A**), OI group (**B**), ultrasound group (**C**), Vit C group (**D**), selenium group (**E**), and mix group (**F**). Bar = 1 mm. Imaged by × 10 lens

injuries account for 10–55% of sports injuries. Muscle injuries can lead to dysfunction because the muscle healing mechanism is slow (Shu et al. 2011). Mild muscle injuries may heal without treatment, while more severe injuries require treatment. Therapeutic ultrasound is commonly used to treat muscle injuries (Warden 2003). In this study, rats with muscle injuries received therapeutic ultrasound treatment and vitamin C and selenium, which are known to have antioxidant effects. The efficacy of these treatments alone and in combination with control and incision-only groups was investigated. The rat was preferred as a model in the study because of the reported rapidity of tissue healing in these animals (Van Zutphen et al. 2001). Serum levels of CK, LDH, ALT, AST, IL-6, and TNF-α in rats, as well as TAS, TOS, MPO, and OSI parameters of blood and muscle tissues, were examined, and the results were evaluated.

CK is an enzyme in skeletal muscle, cardiac muscle, and brain. CK in the brain rarely crosses the blood–brain barrier and enters the bloodstream. Therefore, the source of CK levels measured in the bloodstream is predominantly skeletal or cardiac muscle. Injury or necrosis of skeletal or cardiac muscle increases the circulating level of this enzyme. Therefore, when CK levels are elevated, damage to skeletal or cardiac muscle should be sought first (Lawrence and Amadeo 1996; Carl and Edward 1999; Acques 2000). In this study, serum CK levels were significantly elevated in the OI group, which had only muscle injury and received no treatment, compared with the C group, which received neither incision nor treatment (Fig. 1A). Serum CK levels were elevated in the muscle-injured animals, indicating that the animal model with the cut injury was successful. This result is consistent with the literature cited above. In all treated groups (U, Vit C, S, and M), serum CK levels were significantly lower than in the OI group (Fig. 1A). One study showed that ultrasound treatment lowered serum CK levels in mice with skeletal muscle damage caused by *Bothrops jararacussu* venom (Saturnino-Oliveira et al. 2012).

Therapeutic ultrasound not only accelerates blood flow (Alfredson et al. 2002), but also has a therapeutic effect by increasing the movement of interstitial fluid (Alfredson 2005). In this study, it can be argued that ultrasound promotes tissue healing and lowers serum CK levels based on these properties and the above literature. Studies have shown that in muscle damage, the intracellular oxidant–antioxidant balance is disturbed, and oxidative damage occurs (Freitas et al. 2007; Saborido et al. 2011). Vitamin C prevents the harmful effects of ROS (Akkuş, 1995). In one study, it was observed that vitamin C decreased serum CK levels in mice with recurrent myonecrosis of the diaphragm muscle. This decrease was attributed to the fact that vitamin C reduces ROS due to its antioxidant properties (Tonon et al. 2012).

In the present study, the decrease in serum CK level of vitamin C can be attributed to the fact that vitamin C reduces ROS due to its antioxidant properties and thus accelerates the healing of damaged tissue. Selenium is present in the structure of the enzyme glutathione peroxidase. Glutathione peroxidase plays a role in reducing or preventing oxidative damage. Moreover, in selenium deficiency, cell membranes are damaged by the accumulation of free radicals (Hill et al. 2009). In a study of Steinert's muscle disease, it was suggested that selenium lowers serum CK levels and reduces muscle damage (Rossi et al. 1990).

In this study, it can be found that selenium reduces ROS and lowers serum CK level by contributing to the healing of damaged tissue due to its effect on antioxidant mechanism. The serum CK level is significantly lower in the vitamin C group than in the S group. This suggests that vitamin C is more effective than selenium in lowering serum CK levels. In addition, the serum CK level in the vitamin C group was significantly lower than that in the C group. The serum CK level in the M group, which also received vitamin C, was lower than in the C group but not statistically significant. One study argues that vitamin C improves endothelial barrier function by reducing endothelial cell permeability (May and Qu 2010). From the literature, the decrease in serum CK level by vitamin C in the control group is due to the decrease in serum CK level by decreasing the cell membrane permeability of vitamin C.

LDH has 5 isoenzymes, which are referred to in the circulation as LDH 1, 2, 3, 4, and 5. Because of the wide distribution of isoenzymes in tissues, total LDH activity is increased in many tissue and organ diseases. LDH levels also increase in acute muscle injury (Acques 2000; Sacks 2001). This study showed an increase in LDH levels in the OI group compared with the C group, but this was not statistically significant. On the other hand, there was a statistically significant decrease in LDH levels in the vitamin C, S, and M treatment groups compared with the OI group (Fig. 1B). A study on ethanol-induced liver injury showed that the combination of vitamins E, C, and selenium decreased serum LDH levels. From this study, it can be concluded that vitamin C and selenium reduce ROS, contribute to the healing of damaged tissues, and lower serum LDH levels due to their antioxidant properties. There was a statistically significant decrease in the S group compared with the C group (Fig. 1B). One study has shown that selenium increases membrane stability in colorectal cells (Ghadi et al. 2012). In this study, it can be assumed that the antioxidant property of selenium increases the membrane stability and maintains the serum LDH level at a lower level compared with the control group.

Although ALT is an enzyme mainly used to indicate liver damage, it also increases muscle damage (Lawrence and Amadeo 1996; Acques 2000). Consistent with the reported literature, in this study, serum ALT levels increased

significantly in the OI group compared with the C group (Fig. 1C). There was a decrease in all treatment groups compared with the OI group, but it was not statistically significant.

Although AST enzyme is most commonly used to detect liver injury, it may be elevated in cases such as muscular dystrophies, intramuscular injections, and muscle trauma (Lawrence and Amadeo 1996; Acques 2000; Johns 2002). In this study, AST levels were significantly increased in the OI group compared with the C group. With the exception of the M group, a significant decrease was observed in the other treatment groups compared with the OI group (Fig. 1D). It was found that therapeutic ultrasound treatment in the U group accelerated tissue healing by accelerating blood flow (Alfredson et al. 2002). Another study showed that ultrasound reduced edema by a micromassage effect that increased the movement of interstitial fluid in the treated area (Alfredson 2005). Ultrasound treatment decreased serum CK levels in rats with muscle injury (Freitas et al. 2007), but serum AST levels were not measured. In this study, it can be said that serum AST level decreased thanks to these features of ultrasound therapy. A study of ethanol-induced liver injury showed that the combination of vitamins E, C, and selenium decreased serum AST levels. In this study, it can be assumed that the antioxidant properties of vitamin C and selenium can lower the serum AST level by contributing to the reduction of ROS and the healing of damaged tissue. The combined treatment of the M group also resulted in a decrease in serum AST level compared to the OI group, but it was not statistically significant. According to this result, it can be said that the mixed treatment is not very effective in lowering the serum AST level.

The MPO enzyme is abundant in the cytoplasmic granules of neutrophils. This enzyme is released into the extracellular space upon neutrophil activation and converts H_2O_2 to hypochlorous acid. MPO is an important enzyme in the modulation of infection and inflammation. Indeed, it is associated with both the microbicidal and proinflammatory activity of neutrophils (Reynolds et al. 2006). In a study with a muscle injury, they proposed the MPO enzyme assay method to assess neutrophil infiltration (Schneider and Tiidus 2007). In this study, MPO enzyme activity in blood tissue was significantly increased in the OI group compared to the C group. Among the treatment groups, MPO enzyme activity in blood tissue decreased significantly only in the Vit C group compared to the OI group. There was also a decrease in the other treatment groups compared with the OI group, but it was not statistically significant (Fig. 3D). MPO is an enzyme that increases due to the activation of neutrophils that reach the site of inflammation by chemotaxis.

In this research, the decrease of MPO enzyme activity in blood by vitamin C can be explained by the inhibition of the harmful effects of cellular damage and inflammation by ROS (Saborido et al. 2011), which are increased by muscle damage (Akkuş, 1995). In a study of ischemia–reperfusion injury in skeletal muscle, it was suggested that oral intake of vitamin C reduces infiltration by neutrophils by reducing ROS produced by neutrophils (Kearns et al. 2001). The literature evidence supports the results of this study. The changes between groups in MPO enzyme activity in blood tissue were statistically significant (Fig. 3D). However, the changes in muscle tissue MPO enzyme activity between the groups were insignificant (Fig. 2D). The possible reason for this difference can be explained as follows: Antioxidants can stimulate healing of injured muscle tissue by increasing the integrity of the cell membrane (Qiao et al. 1993; Kearns et al. 2010). As healing progresses, the number of neutrophils reaching these cells also decreases. However, the decrease in neutrophil number in blood may not coincide in time with that in muscle tissue, and the decrease in blood tissue may last longer than that in muscle tissue. In one study, muscles were damaged by having trained and untrained rats perform high-intensity swimming exercises. In trained rats, plasma MPO enzyme activity was significantly lower than in untrained rats. However, there was no statistical significance between groups in MPO activity in muscle tissue (Morozov et al. 2006). The results in the exemplary literature also support the findings of this study.

In this study, no statistical difference was observed between the measurement results of TAS in muscle tissue (Fig. 2A) and TAS in blood tissue (Fig. 3A). Although the OSI measurement results in muscle tissue were not statistically significant, a significant decrease was observed in groups S and M (Fig. 2C). OSI results in blood were also decreased in groups S and M (Fig. 3C). This decrease in OSI results was due to the decrease in TOS in these groups. TOS measurements of muscle tissue showed a statistically significant decrease in the S and M groups compared with the OI group (Fig. 2B). The results of our M group are consistent with the studies of Santos et al. and Sousa Filho et al. who concluded that the administration of antioxidants in combination with therapeutic ultrasound reduces oxidative stress after skeletal muscle injury (Santos et al. 2020; Sousa Filho et al. 2021). Selenium is an important cofactor for the enzyme activity of glutathione peroxidase, which is thought to destroy peroxides derived from unsaturated fatty acids and necessary to prevent free radical formation (Fox 1992). In one study, selenium was found to reduce ROS by increasing the activity of some enzymatic antioxidants such as glutathione peroxidase (Wojewoda et al. 2010). In another study, it is suggested that selenium reduces oxidative stress in muscle tissue thanks to a yet unknown physiological function (Betancor et al. 2012). In this study, it can be assumed that selenium causes a decrease in TOS by reducing oxidative damage in muscle tissue. A decrease was observed in

the results of TOS measurements in blood tissue in the S and M groups compared to the OI group. This decrease is consistent with the TOS measurements in muscle tissue but is not statistically significant (Fig. 2B).

Histopathological examination in the present study revealed no significant differences between the groups (Fig. 5 A–F). In the muscle injury model used as an example in this study (Chan et al. 2003), the incision made with a scalpel was smooth, and the muscle fibers were cut longitudinally. The sacrificial process was performed on the 6th day after injury, allowing the tissue to heal to the greatest extent possible. This finding may explain why there was no significant difference between the groups on histopathological examination.

Some studies have shown that IL-6 (Choi et al. 2013) and TNF-α (Enos et al. 2013) increase exercise-induced muscle damage. Considering these studies, it is expected that IL-6 and TNF-α will increase with the increase of inflammation after muscle injury. In the muscle injury performed in the present study, there was an increase in IL-6 and TNF-α cytokines in the injured groups compared with the control group, but no statistical significance was found (Fig. 4 A, B). The reason why the TNF-α and IL-6 cytokines were statistically insignificant was that the sacrifice process was performed on the 6th day of muscle injury and the metabolism of the rats was very fast (Van Zutphen et al. 2001), because the rats recovered strongly during this period, which can be explained by the disappearance of the differences between the groups. A limitation of this study is that we did not perform confocal microscopy or TEM sections to identify the ultrastructure of the gastrocnemius muscles after muscle injury.

Conclusions

In conclusion, this study revealed that vitamin C and selenium treatments are also beneficial in muscle injuries besides ultrasound therapy. Therefore, considering the results of this study, it can be said that there is a significant improvement in the level of injured muscle tissue within 6 days.

Abbreviations AU: Arbitrary units; ROS: Reactive oxygen species; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; EDTA: Ethylenediaminetetraacetic acid; TPU: Therapeutic pulsed ultrasound; MuSCs: Muscle satellite cells; TBARS: Thiobarbituric acid reactive substances; CK: Creatine kinase; LDH: Lactate dehydrogenase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 β; H_2O_2: Hydrogen peroxide; KH_2PO_4: Potassium dihydrogen phosphate; MPO: Myeloperoxidase; C: Control; OI: Only injury; U: Ultrasound; Vit C: Vitamin C; S: Selenium; M: Mix

Author contribution Yasin Tülüce: conceptualization, methodology, supervision, writing—reviewing and editing, project administration, funding acquisition, formal analysis, resources, methodology. Necati Muhammed Tat: conceptualization, conducting, writing—original draft preparation, validation, data curation. Mehmet Kara: supervision, visualization, investigation. Ayşe Merve Tat: formal analysis, data curation. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethical approval This study was approved by Van Yüzüncü Yıl University Animal Experiments Local Committee. Approval number: 2012/04–11.

Conflict of interest The authors declare no competing interests.

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