Effect of ascorbic acid on collagen and some inflammatory mediators in rats

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ABSTRACT

Musculoskeletal injuries as a kind of trauma that the human body is exposed to, adversely affect the quality of life and workforce of individuals due to restriction of movement function. This study aimed to evaluate the effects of dose-dependent ascorbic acid (AA) administration on the repair process after gastrocnemius muscle injury in rats. In this study, 5-month-old 66 male Wistar Albino rats were used and rats were randomly divided into 6 groups of 11 each [control, muscle injury, healthy (with 5 mg/10 mg/kg/day AA-treated group), injury (with 5 mg/10 mg/kg/day AA-treated group)]. A linear incision was made in the gastrocnemius muscle of thirty-three animals included in the muscle injury groups. AA (5-10 mg/kg/day) was administered to the four groups intraperitoneally just after surgery once a day. Animals were sacrificed twenty-one days later. Blood and tissue samples were used for cytokine, collagen, and histological measurements. It was found that a dose of 5 mg/kg/day AA administration reduced serum IL-6 and muscle tissue TNF-α levels, and increased liver tissue IL-10 levels. Muscle tissue collagen levels were not statistically different between the groups in parallel with our histological results. In our study, it was demonstrated that vitamin C has effects on inflammatory mediators during muscle tissue repair to explain the mechanism detailed molecular analyzes are needed.

Introduction

Musculoskeletal injuries affect lots of people worldwide and can cause pain and physical disability (30). They adversely affect the quality of life and labor force due to the limitation of movement function (7). Wound healing is a dynamic process and involves interactions between the extracellular matrix, many various cell types, and chemical mediators (12). The tissue repair process consists of four overlapping stages: (i) hemostasis, (ii) inflammation, (iii) proliferation, and (iv) remodeling (29). The tissue repair process is affected by many factors including age, gender, systemic diseases, nutrition, medicine, oxygenation, and radiation (13).

Ascorbic acid (vitamin C, AA) is a water-soluble micronutrient that is important for immune system functions and has an antioxidant effect (8). It is also involved in the formation process of collagen, which supports bones, teeth, tendons, muscle fibers, and blood vessel walls (21). The results of previous studies have shown that AA modulates cytokine production, stimulates keratinocyte differentiation, and increases neutrophil migration and T lymphocyte proliferation (6, 9, 10, 14). The data suggest that AA may positively affect the healing of damaged tissue. On the other hand, there is no consensus on the safe and effective dose range of AA administration after tissue injuries in the literature. Some

studies have reported that high doses of AA positively affect the wound healing process (23, 27). However, studies are reporting that low-dose AA is more effective than high-dose (11, 15).

Cytokines are important regulators of the tissue repair process. IL-6 and TNF-α are cytokines involved in the early stage of the inflammatory response (2). It is known that IL-10 exerts an anti-inflammatory effect and inhibits the release of pro-inflammatory cytokines (16). A previous study by Bowie and O'Neill (5) reported that AA inhibited TNF-mediated NF-kB activation. A further animal study found that AA reduced the inflammatory response by inhibiting IL-6 and TNF-α expression (26).

In this study, we aimed to examine the effects of dose-dependent AA administration on collagen synthesis and inflammatory parameters in the case of skeletal muscle injury.

Materials and Methods

This study was approved by the Experimental Animals Local Ethics Committee at the Ankara University with decision number 2019-9-96. All experimental procedures were performed according to institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research.

In this study, 66 adult male Wistar Albino rats were used. As a result of the power analysis using the G*Power 3.0.10 software (University of Kiel, Germany), 11 animals for each group were included in the study at 80% power and 5% significance level. Since evaluations will be made by determination of the cytokine levels and due to the potentially high standard deviations of the cytokine measurements, the number of animals was decided as 11 per group. Animals were randomly divided into six groups (n = 11/group): (i) control, (ii) muscle injury, (iii) healthy rat with 5 mg/kg/day AA (Healthy 5AA), (iv) healthy rat with 10 mg/kg/day AA (Healthy 10AA), (v) muscle injury with 5 mg/kg/day AA (Injury 5AA) and (vi) muscle injury with 10 mg/kg/day AA (Injury 10AA). AA (100468 L (+)-Ascorbic Acid, Merck, Germany) was administered intraperitoneally just after surgery and then once a day until the euthanasia procedure. Distilled water was applied intraperitoneally to rats in the muscle injury group. Animals were maintained in 12-h light and dark cycle. Food and water were provided *ad libitum*. For surgery, rats were anesthetized intraperitoneally with 100 mg/kg ketamine and 10 mg/kg xylazine. A linear incision was made in the right gastrocnemius muscle of the animals with a No. 11 surgical blade, 5 mm deep and 5 mm long. The wound was closed and sutured under sterile conditions with 3/0 absorbable suture material (18).

Twenty-one days after AA administration, animals were sacrificed with an overdose of ketamine and xylazine. Blood samples, liver, and gastrocnemius muscle were taken. Blood samples were centrifuged for 10 min at 2800 rpm to obtain serum. Blood and tissue samples were stored at -80 °C until collagen and cytokine measurements were performed.

Measurement of serum and tissue cytokines and muscle tissue collagen: Tissues were weighed for cytokine measurement in tissues, homogenization solution was added according to tissue weight and the measurement was made by optimizing the amount of tissue. Protein isolation solution was prepared using RIPA lysis buffer and protease inhibitors (pepstatin, leupeptin, aprotinin, 2 µg/ml) (Sigma, USA), and tissue samples were homogenized. Muscle tissue collagen I levels, serum and tissue IL-6, IL-10, and TNF-α measurements were performed using commercially available ELISA kits (Bioassay Technology Laboratory, China) according to instruction.

Histological analysis: The gastrocnemius muscle pieces were fixed with 10% neutral buffered formalin for 1-2 days for evaluation under a brightfield microscope (Zeiss Axio Scope A1, Oberkochen, Germany), after the dissection. Following washing under the tap water and dehydration with ethanol series, the muscle pieces were incubated in xylene until the clearing. After the tissues were embedded into the paraffin mixture, transverse sections were cut (5-7 µm) by sliding microtome (RM 2125RT, Leica, Germany) and stained using Hematoxylin-Eosin (H&E) (Figure 1) (4).

Histological evaluation was based on measuring the diameter of muscle fibers under the brightfield microscope (Figure 2). Three photos were captured in each skeletal tissue section and the diameters of ten muscle fibers on each of photos were measured in all animal. All measurements on digital images were recorded using the Axiovision Rel 4.8 software (Germany) and evaluated statistically.

Statistical analysis: IBM SPSS Statistics 25 (Armonk, New York, USA) was used for statistical analysis. Data were presented as mean ± standard deviation. ANOVA was used for the analysis of homogeneous variables. Post hoc Tukey's test was used when the F values were significant. Kruskal-Wallis test was used for the data analysis without homogeneous distribution. Spearman test was used for correlation analysis and $P<0.05$ was regarded as statistically significant.

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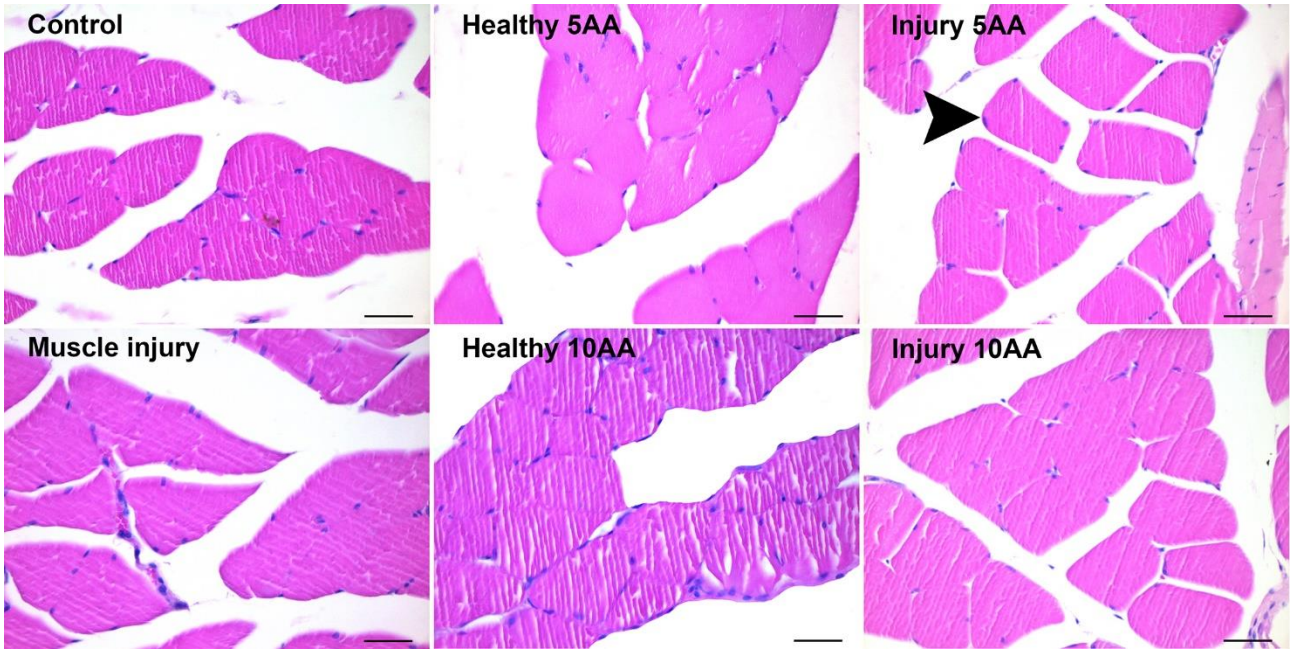

Figure 1. The muscle fibers of control, muscle injury, and the experimental groups (Arrowhead: Muscle fiber). Staining: H&E, (X40), Bar= 50 µm.

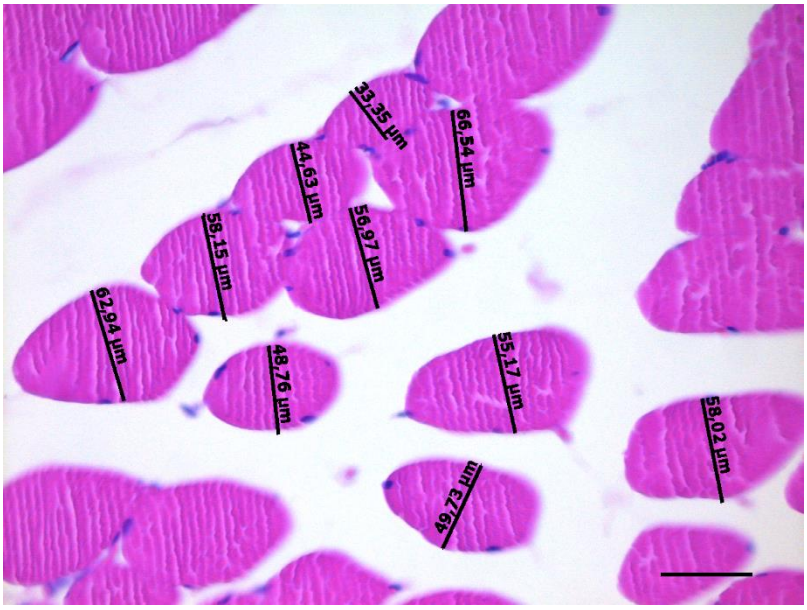

Figure 2. The measurement of muscle fibers on images. Staining: H&E, (X40), Scale bar= 50 µm.

Results

Although there was no significant difference between the groups in terms of weight gain (P>0.05), rats tended to weight gain, and the weight gain of the muscle injury group was found to be lower than other groups.

Levels of IL-6, IL-10, and TNF-α examined in serum and tissues of groups to understand inflammatory responses are shown in Table 1.

Collagen type I levels were not different between the groups in parallel with our histological results (Table 2).

Correlation analysis was performed to determine the relationship between cytokine levels and collagen type I levels in muscle tissue. According to these results, muscle IL-6 levels were correlated negatively with IL-10 levels (R = -0.350, P<0.01). There was a positive correlation between serum IL-6 levels and serum IL-10 (R = 0.325, P<0.01) and serum TNF-α levels (R = 0.638, P<0.01). Muscle tissue collagen type I levels were correlated with serum IL-6 (R = 0.377, P<0.01) and IL-10 (R = -0.248, P<0.05) levels. Our results showed that muscle tissue TNF-α levels were correlated with serum TNF-α (R = 0.544, P<0.01) and IL-6 (R = 0.327, P<0.01) levels.

The data obtained as a result of the examination of muscle fiber diameters are shown in Table 3. Muscle fiber diameters were not different between the groups.

Table 1. IL-6, IL-10, and TNF-α levels in serum and tissues (mean ± SE).

		Control (pg/ml)	Muscle Injury (pg/ml)	Healthy 5AA (pg/ml)	Healthy 10AA (pg/ml)	Injury 5AA (pg/ml)	Injury 10AA (pg/ml)
Serum	**IL-6**	0.047 ± 0.000[a]	0.055 ± 0.001[ab]	0.049 ± 0.002	0.047 ± 0.000	0.045 ± 0.000[b]	0.047 ± 0.000
	IL-10	0.272 ± 0.007	0.228 ± 0.017	0.248 ± 0.009	0.223 ± 0.021	0.198 ± 0.006	0.258 ± 0.022
	TNF-α	0.052 ± 0.001	0.050 ± 0.000	0.047 ± 0.000	0.050 ± 0.000	0.049 ± 0.000	0.048 ± 0.000
Muscle	**IL-6**	0.478 ± 0.073	0.676 ± 0.086	0.703 ± 0.027	0.511 ± 0.058	0.855 ± 0.102	0.894 ± 0.090
	IL-10	0.095 ± 0.010	0.066 ± 0.008	0.077 ± 0.007	0.068 ± 0.008	0.053 ± 0.003	0.056 ± 0.003
	TNF-α	1.576 ± 0.065	1.786 ± 0.076[c]	1.569 ± 0.023	1.576 ± 0.013	1.416 ± 0.008[c]	1.589 ± 0.104
Liver	**IL-6**	0.512 ± 0.019	0.518 ± 0.004	0.539 ± 0.038	0.525 ± 0.005	0.550 ± 0.017	0.507 ± 0.014
	IL-10	0.754 ± 0.023[d]	0.690 ± 0.009	0.833 ± 0.015[d]	0.814 ± 0.017	0.763 ± 0.023	0.731 ± 0.015
	TNF-α	0.633 ± 0.055[e]	0.723 ± 0.023	0.637 ± 0.051[f]	0.845 ± 0.035[ef]	0.754 ± 0.017	0.818 ± 0.024

Significant statistical differences between the groups were shown in letters (P<0.05).
a: Control - Muscle injury,
b: Muscle injury - Injury 5AA,
c: Muscle injury - Injury 5AA,
d: Control - Healthy 5AA,
e: Control - Healthy 10AA,
f: Healthy 5AA - Healthy 10AA.

Table 2. Collagen type I levels in muscle tissues (mean ± SD).

		Control (ng/ml)	Muscle Injury (ng/ml)	Healthy 5AA (ng/ml)	Healthy 10AA (ng/ml)	Injury 5AA (ng/ml)	Injury 10AA (ng/ml)
Muscle	**Collagen Type I**	0.100 ± 0.025	0.101 ± 0.018	0.093 ± 0.014	0.106 ± 0.025	0.095 ± 0.021	0.089 ± 0.013

Table 3. Mean muscle fiber diameter values of groups (mean ± SD).

Groups	Mean Muscle Fiber Diameter (µm)
Control (n = 11)	61.807 ± 3.881
Muscle Injury (n = 11)	58.783 ± 5.291
Healthy 5AA (n = 11)	58.893 ± 5.602
Healthy 10AA (n = 11)	57.344 ± 4.567
Injury 5AA (n = 11)	58.195 ± 3.734
Injury 10AA (n = 11)	59.171 ± 5.033

Discussion and Conclusion

In this study, we made an incisional wound model in the gastrocnemius muscle of rats and discuss the effects of dose-dependent AA administration on collagen synthesis and tissue cytokine levels.

In our study, no significant difference was found in weight gain between the groups. Similarly, in the animal study of Loizidis et al. (20) it was found that the administration of vitamin C did not have a significant effect on body weight gain, feed, and water intake. In our study, there was no adverse effect on the viability and comfort of rats, except to avoid standing on the injured leg during the first 24 hours after surgery.

It is well-known that cytokines are important regulators of the tissue repair process. Serum IL-6 levels were observed to be significantly lower in Injury 5AA compared with the muscle injury group according to our results. In the same group (Injury 5AA), the decrease in serum IL-10 level was detected but was not statistically significant. There was no significant difference in serum TNF-α levels between the groups. In a study that examined the effects of single-dose intraperitoneal vitamin C treatment (250 mg/kg) on serum cytokine levels in rats, levels of IL-6, IL-10, and TNF-α in serum were found to be lower in the vitamin C treated group (3). Different from our study, a single dose of AA was administered in the above-mentioned study. A human study concluded that vitamin C uptake (500 mg/day) did not have a significant effect on IL-6 and IL-10 concentrations in plasma and blood mononuclear cells in response to exercise (1). On the other hand, in one study, the effects of dose-dependent vitamin C uptake (500-1500 mg/day) on cytokine levels of runners after the ultramarathon were examined. Levels of IL-6 and IL-10 in plasma after the race were found to be significantly lower in the vit C-1500 group compared with the placebo and vit C-500 groups (22).

In this study, IL-6 levels in muscle tissue were not significantly different between the Injury 5AA, Injury 10AA, and muscle injury groups. TNF-α levels in muscle tissue were observed to be significantly lower in Injury 5AA compared with the muscle injury group. In a study that investigated the effects of AA on inflammatory markers in the case of multifidus muscle injury in rats, it was found that AA reduced the inflammatory response in muscle tissue. In that same study, expression of muscle tissue IL-6 and TNF-α was significantly inhibited in the

AA group on the 1st, 3rd, and 7th days after surgery (26). In another study, it was reported that AA administration (200 mg/kg) reduced TNF-α levels, myonecrosis, and inflammation in the diaphragm muscle (27). Different from our study, a single dose of AA was administered in both studies. This situation causes difficulties in interpreting the dose-dependent effects of AA on muscle tissue cytokine responses. In our study, the decrease in muscle tissue IL-10 levels in the Injury 5AA and Injury 10AA groups was not statistically significant. We were not able to find a study evaluating the effects of dose-dependent AA administration on muscle tissue IL-10 levels, searching the literature data. In one study, it was reported that vitamin C supplementation reduced IL-10 production by blood mononuclear cells (28).

In our study, liver tissue IL-6 levels were not significantly different between the groups. Liver tissue IL-10 levels were significantly higher in Healthy 5AA compared to the control group. In the Injury 5AA and Injury 10AA groups, the increase in liver tissue IL-10 levels was not statistically significant. Liver tissue TNF-α levels were observed to be significantly higher in the Healthy 10AA group compared to the control and Healthy 5AA groups according to our results. In the Injury 5AA and Injury 10AA groups, the increase in liver tissue TNF-α levels was not significant compared to the muscle injury group. In one study, it was reported that vitamin C supplementation under cyclic heat stress, significantly reduced rat liver tissue IL-6 and TNF-α expression (31). In another study, the effects of vitamin C administration on perfluorooctane sulfonate (PFOS)-induced liver steatosis in mice were examined. It was reported that TNF-α positive cells that increased PFOS exposure in the liver were reduced by vitamin C treatment (24).

In the literature, the effect of the topical AA in rats with an incisional wound model was examined in one study. It was reported that the topical application of AA accelerated the tissue repair process. In the same study, it was observed that no inflammatory symptoms were found in the AA-treated group on the 7th postoperative day. In histological evaluation, it was observed that collagen fibers in the papillary layer were thicker in the AA-treated group on the 14th postoperative day (19). In a study that investigated the effect of AA deficiency on skeletal muscle in senescence marker protein-30 knockout mice, it was found that the cross-sectional area of muscle was significantly smaller in the AA deficiency group compared with the AA-treated group (1.5 g/L) (25). In the animal study that examined the effect of vitamin C on muscle renewal in the case of cytotherapy, it was observed that vitamin C enhanced muscle tissue collagen type I expression (17). However, in our study, AA administration did not show a significant effect on collagen type I levels at the dose and time administered. In the study of Kim et al. (17) the effect of AA on collagen synthesis was examined at the level of gene expression. In our study, the measurement of collagen was performed with the ELISA method. This suggests that the effect of AA on collagen synthesis may be limited at the level of gene expression. On the other hand, the heterogeneity in AA supplementation protocols in terms of the route of administration, dose, frequency, and duration causes difficulties in interpreting the effects of AA.

In conclusion, our results showed that AA administration could affect cytokine responses in the case of skeletal muscle injury and be effective in alleviating the inflammatory state. The absence of a significant difference in muscle tissue collagen levels may either suggest that AA administration is not effective on collagen synthesis in the process of healing muscle injury or may be effective on a different level other than tissue level. However, to understand the clear relationship, examinations at the level of gene expression, at different doses, and periods will be useful. In our study, it is thought that AA administration after tissue injuries could be effective, but it is emphasized that further studies should be conducted to define the safe and effective dose range.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

SI, NY and PAA conceived and planned the experiments. SI, NY, BK, DB, SA, FTÇ and PAA carried out the experiments and contributed to sample preparation. BB carried out the statistical analysis. SI, NY, SA, FTÇ and PAA took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Experimental Animals Local Ethics Committee at the Ankara University (Decision Number: 2019-9-96).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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