

Ascorbic Acid Greatly Decreases Creatine Kinase Levels in An Animal Model of Statin/Fibrate-Induced Myopathy

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Research article

Keywords: ascorbic acid, fibrate, muscular disease, rats, statin

Posted Date: January 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-151070/v1>

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Abstract

Background

Drug-induced myopathy is one of the frequent forms of muscle disease, and drugs used for hyperlipidemia, especially the statins are a common culprit, and particularly when combined with a fibrate. Clinicians usually measure plasma levels of three enzymes, creatine kinase (CK), aldolase and lactate dehydrogenase (LDH) for diagnosis of myopathy and determination of its severity. Physical exercise can aggravate statin-associated muscular disease. The question is whether antioxidants like ascorbic acid (vit. C) can prevent such myopathy.

Methods

In this experiment a combination of oral atorvastatin (ATV, 80 mg/kg/day, orally) and gemfibrozil (GMF, 1000 mg/kg/day, orally) was used for ten days plus exercise in days 8, 9 and 10 to induce myopathy in rats. To add physical exercise, the forced swimming test was applied in the last three days. Ascorbic acid (50 mg/kg/day, orally) was added to ATV/GMF plus exercise regimen throughout the 10 days in the treatment group. The mean blood levels of CK, aldolase and LDH were measured in addition to swimming tolerance times.

Results

There was a significantly lower swimming tolerance time ($P < 0.05$) and higher CK levels ($P < 0.01$) in rats receiving ATV/GMF/Vit.C plus exercise compared with rats not taking Vit.C. LDH and aldolase didn't decrease significantly.

Conclusions

A protective role of vit.C against drug-induced myopathy is suggested by the findings of this study.

Background

Drug-induced myopathy is one of the most common types of muscle disorder which can be as mild as a mild myalgia with or without slight weakness or as severe as extensive rhabdomyolysis leading to even acute renal failure (1, 2).

The statin group of drugs, are mentioned among the frequent causes of myopathies. Their wide clinical use is due to the fact that atherosclerosis can be dramatically prevented or ameliorated by regular use of them, which makes this class of medications as the first choice in controlling lipid-associated cardiovascular disease. Statins act by inhibition of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoA), while fibrates decrease very low-density lipoprotein and increase high density lipoprotein. Clinical myonecrosis or rhabdomyolysis is stated to be rare after the use of statins, around 0.1 percent of users, but milder forms are more common (3, 4). Statin intolerance has been defined as

unacceptable symptoms and/or laboratory abnormalities prompting their discontinuance, with an increased risk of future atherosclerosis, though (5, 6).

The term "statin-associated muscle symptoms" (SAMS) is nowadays used to describe the range of complaints in patients taking statins. In some patients, autoantibodies to HMGCoA are found, and said to be highly specific for autoimmune myositis or myopathy. The genes including *SLCO1B1* rs4149056 and other variants affecting pharmacokinetics and pharmacodynamics of statins include cytochrome P450 genes (such as CYP3A4, CYP3A5 and CYP2D6) which demonstrate great polymorphism, and also the vitamin D receptor gene (7, 8).

Some studies suggest that statin-induced myalgia may be due to cellular stress which is at least partially a function of genes involved in cellular metabolism in skeletal myocytes, as single-nucleotide polymorphisms in the above-mentioned genes are more common in patients with statin myalgia (9). So, it is understandable that antioxidant agents could have beneficial effects for SAMS. The increase in susceptibility to myopathy is greater in patients receiving concurrent therapy with a number of drugs, particularly those that inhibit CYP3A4 (10), as well as with fibrates (11).

Measurement of plasma level of some enzymes which have high intracellular concentration in striated muscle cells and are released into the circulation during muscle injury is the common practice for diagnosis, follow-up and monitoring of effectiveness of treatment in patients with muscle diseases. Creatine kinase (CK) is the most widely used enzyme in this regard, because it is more specific to skeletal muscle, rapidly appears in blood after muscular injury, and can be determined very conveniently by simple photometric reactions in most clinical laboratories. Physical activity may increase serum CK levels. Aldolase is found in most body tissues but mainly in skeletal muscle, liver, and brain. It is an enzyme involved in glycolytic pathway. Although increased aldolase level is not as specific or sensitive as CK levels in detection of myopathies, but it is occasionally elevated in patients with myositis who have normal CK levels (12). Another enzyme useful for diagnosis and monitoring of muscular disorders is lactate dehydrogenase (LDH) which converts pyruvate to lactate. Its drawback is that many tissues other than muscles have high concentrations of LDH which is released upon cell membrane dysfunction (13).

Ascorbic acid is an essential water-soluble vitamin that acts as a cofactor and antioxidant. As an electron donor, it is necessary for collagen hydroxylation, carnitine biosynthesis, and hormone/amino acid production, among other roles (14).

The current study tries to evaluate the effect of ascorbic acid on improvement of statin/fibrate-induced myopathy in rats, based on our previous studies (unpublished data) which showed that combination of ATV and GMF plus exercise (forced swimming test) is a reliable model for induction of myopathy.

Methods

Chemicals

The drugs used in this study included ascorbic acid and gemfibrozil (both from Darupakhsh Co., Iran) and atorvastatin calcium (Sobhan Co., Iran), which were dissolved in distilled water to prepare solutions for feeding tube gavage every morning at 9 am.

Animals

This experiment involved 30 male Wistar rats, about 56 days old and weighing 250–300 g which were sourced from the animal house of the School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. They were kept in separate cages (n=6 in each cage) with usual temperature and humidity control (20–25 °C, 50–60% relative humidity) and 12 h light/dark cycles. The rats had free access to tap water and standard food. The entire animal trial was approved by the ethics committee in Shahid Sadoughi University of Medical Sciences, Yazd, Iran, regarding care and work on laboratory animals (approval letter No. IR.SSU.MEDICINE.REC.1398.183). Rats were randomly allocated to five groups (n=6): a *control without swim* group that took vehicle (distilled water) without any swimming; *control plus swim* group that received vehicle and performed forced swimming test on days 8, 9 and 10; *ATV/GMF without swim* group which were fed gavage ATV (80 mg/kg/day) and GMF (1000 mg/kg/day) for 10 days without swimming; *ATV/GMF plus swim* group that received the same ATV and GMF doses for 10 days and had forced swim on days 8, 9 and 10; and *ATV/GMF/vit.C plus swim* group which had the same dose of ATV and GMF but were also treated with 50 mg/kg/day of ascorbic acid orally for 10 days in addition to forced swimming on days 8, 9 and 10. The above-mentioned doses were already found to be the best amounts according to our previous study (submitted to a peer-reviewed journal but not published as yet). Weights of the rats were measured at day 10 using an electronic balance.

Forced swimming test

For maximum physical activity, a previous published method (15) was a little modified, so that the animals were individually placed in a large glass cylinder containing water (25 °C). From the beginning of swimming to the point of near-drowning due to fatigue the duration of their movements was recorded by chronometer. The average time in the 3 days was used as swimming tolerance time.

Plasma enzyme activity measurements

At the 10th day of the study, the rats were sacrificed by deep anesthesia using ketamine (50 mg/kg) and xylazine (10 mg/kg), and 3 mL of blood was drawn from the heart and put in plastic tubes containing K₂-EDTA. The plasma was sent to laboratory (Khatamolania laboratory, Yazd, Iran) for quantitative measurement of the enzyme levels according to instructions of biochemical UV-spectrophotometric assay kits, all employing kinetic reactions at 37 °C. To determine CK values the CK-NAC-LQ kit (Audit Diagnostics, Ireland) was used. Measurement of aldolase was similarly by a employing a commercial kit (Biorexfars, Iran). LDH measurement was conducted using the kit manufactured by Bionik, Iran. The stated linearity ranges for measuring CK, aldolase and LDH were 2-2000 U/L, 1-28 U/L and 2-1450 U/L, respectively.

Statistical analysis

For the comparison of variables including mean of plasma enzyme levels, swimming tolerance times and weights of animals in the 5 groups we used one-way analysis of variance (ANOVA). The Tukey post hoc test was used to compare multiple groups. Statistically significant difference was defined as a P value less than 0.05.

Results

Weight of the rats

The mean weights of each group at the end of study were not significantly different (P value > 0.05) (**figure 1**).

Swimming tolerance time

Mean of the swimming tolerance times at days 8, 9 and 10 was calculated in the 3 groups which had forced swimming. It showed statistically significant difference (P value < 0.001) between control plus swim group and ATV/GMF plus swim group. Vit.C increased swimming tolerance time in ATV/GMF/Vit.C group vs. ATV/GMF (P < 0.05) while it was significantly less than control group (P < 0.01) (**figure 2**).

Levels of CK

Levels of the enzyme CK in plasma were significantly higher in control plus swim group and ATV/GMF plus swim group in comparison with the other groups (P value < 0.001). The ATV/GMF/vit.C plus swim group had significantly lower CK levels in comparison with all of the other groups (P value < 0.001). The CK levels in ATV/GMF without swim group and control without swim group were not significantly different (**figure 3**).

Levels of LDH

Plasma levels of LDH happened to be significantly more in control plus swim group and ATV/GMF plus swim group vs. the other groups which didn't swim (P value < 0.001). However, the LDH levels of ATV/GMF without swim group were not significantly different from control without swim group. LDH levels did not decrease significantly in ATV/GMF/Vit.C plus swim group in comparison with ATV/GMF plus swim group (P value > 0.05) (**figure 4**).

Levels of aldolase

Plasma aldolase activity was found to be significantly higher in control plus swim group vs. control without swim group (P value < 0.05). Plasma aldolase levels of ATV/GMF plus swim group were also significantly more than control without swim group (P value < 0.01) and again significantly above the levels in ATV/GMF without swim group (P value < 0.05). Plasma aldolase levels in ATV/GMG/Vit.C group

plus swim were a little more than ATV/GMF plus swim group but not significantly (P value > 0.05) (**figure 5**).

Discussion

Statins have a known side effect as myopathy, and this could be aggravated by simultaneous use of some other drugs, especially those which inhibit CYP3A4 as the cytoplasmic enzyme responsible for metabolism of statins like simvastatin, lovastatin and atorvastatin (10). A similar effect is seen also when statins are co-prescribed with fibrates (16). Fibrates have been associated with muscle toxicity; an effect that is more pronounced in patients also treated with a statin. The mechanism is not agreed upon. Glucuronidation, which is an important pathway for renal excretion of lipophilic statins, appears to be significantly inhibited by gemfibrozil (17).

The incidence of myopathy with the simultaneous administration of some statins and gemfibrozil is estimated 1 to 5 percent (18, 19), including rhabdomyolysis as the most severe form (4). Muscle toxicity can be reduced by changing the type of fibrate (e.g., fenofibrate is found to bring the lowest risk) and by using statins at relatively low doses because the adverse effect is dose-dependent (20, 21). Since the concomitant use of statins and fibrates is a common practice, and many patients have hypertriglyceridemia accompanied by hypercholesterolemia, this subject was a trigger for the current study.

Physical exertion has already been found to aggravate SAMS (22) leading to increase in plasma CK levels, understandably more in untrained people. So, the risk of myopathy would be lower if a gradual program of increasing exercise is followed in these individuals, letting time for metabolic clearance of drugs and adaptation. However, the muscle injury from exercise even without such adaptation is usually mild and subclinical (22).

In our project, we exposed the rats to a sudden challenge of forced maximal exercise to see the highest possible myopathy. Hence, the serum levels of CK, aldolase and LDH were significantly more in those rats which had forced physical activity vs. the rats without any enforced physical exercise. The rats receiving combined ATV and GMF showed further increase in the plasma enzyme levels. Swimming tolerance time was strikingly decreased (P value < 0.001) in rats consuming ATV and GMF (**figure 2**), supporting the synergistic effect of exertion and drug.

To compare our work with other studies in this field a few are available. In a study by Osaki et al., a model for statin-induced myopathy was created using skeletal muscle-specific HMGCoA reductase knockout mice. They showed postnatal myopathy with high serum CK levels and myonecrosis, which underlines the role of HMGCoA reductase in metabolism of statins (23). In another work, Nakahara et al induced myopathy by HMGCoA reductase inhibitors in rabbits, followed by histopathological examination of skeletal muscle and measurement of CK. The findings included light microscopic muscle fiber necrosis and degeneration, altered acid phosphatase activity in cells, and electron microscopic alterations including autophagic vacuoles, swelling of mitochondria, disruption and hypercontraction of myofibrils (24).

In the current research, the most dramatic effect of ascorbic acid was reducing elevated CK (as a main biomarker of skeletal muscle damage) levels in ATV/GMF plus swim group (**figure 3**). It increased swimming tolerance time in the drug-induced myopathy group, too (**figure 2**). Ascorbic acid could not alter elevated LDH and aldolase levels in the drug-induced myopathy group, though (**figures 4, 5**). Perhaps the molecular mechanism is that ascorbic acid provides electrons needed for reducing oxygen, the antioxidant capabilities also shared by a number of other compounds, including vitamin E and folic acid. It is also a cofactor for reduction of folate to dihydro- and tetrahydrofolate (14).

Ascorbic acid is involved in the following biologic processes: 1. Fatty acid transport: The transport of long-chain fatty acids across the mitochondrial membrane is a carnitine-dependent process, and carnitine synthesis requires ascorbic acid as an electron donor (25). 2. Collagen synthesis: Formation of collagen requires enzymatic hydroxylation of two amino acids, proline and lysine; ascorbic acid is an electron donor in reactions catalyzed by the enzymes; prolyl hydroxylase and lysyl hydroxylase, which form hydroxyproline and hydroxylysine, respectively. Failure of this step in collagen synthesis results in impaired wound healing, defective tooth formation, and deficient osteoblast and fibroblast function. 3. Synthesis of neurotransmitter norepinephrine, which involves hydroxylation of dopamine by the enzyme dopamine-beta-mono-oxygenase, where ascorbic acid is a required cofactor 4. Metabolism of prostaglandin and prostacyclin: It may be capable of attenuating the inflammatory response or even sepsis syndrome (24). 5. Nitric oxide synthesis: Ascorbic acid may promote synthesis of nitric oxide, a potent vasodilator (26, 27). 6. Mitochondrial health: Regarding the extreme dependence of muscle activity to mitochondria for ATP, it is worth mentioning that mitochondrial injuries in general result in oxidative stress, i.e., higher levels of reactive oxygen species (28, 29) leading to cell membrane damage through lipid peroxidation.

Conclusion

Ascorbic acid decreases CK levels in statin/fibrate-induced myopathy and improves skeletal muscle activity in rats. Ascorbic acid may be promising in decreasing muscle injury induced by lipid-lowering medications.

Abbreviations

ANOVA: One-way analysis of variance; CK: Creatin kinase; LDH: Lactate dehydrogenase; Vit C: Vitamine C; ATV: Atorvastatin; GMF: Gemfibrozil; SAMS: Statin-associated muscle symptoms

Declarations

Ethics approval and consent to participate

The animals were sourced from the animal house of the School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran and approved by the ethics committee in Shahid Sadoughi

University of Medical Sciences, Yazd, Iran, regarding care and work on laboratory animals (approval letter No. IR.SSU.MEDICINE.REC.1398.183).

Consent for publication

Not applicable

Availability of data and material

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

Competing interests

The authors declare that they have no conflicts of interest.

Funding

No funding

Authors' contributions

MZ and FA and MSZ conceived and designed the experiments, performed the experiments, wrote the paper. SH and MRN participated in the interpretation of data, contributed to discussions and prepared figures and tables. All authors have read and approved the manuscript.

Acknowledgements

This work was supported by Faculty of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

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Figures

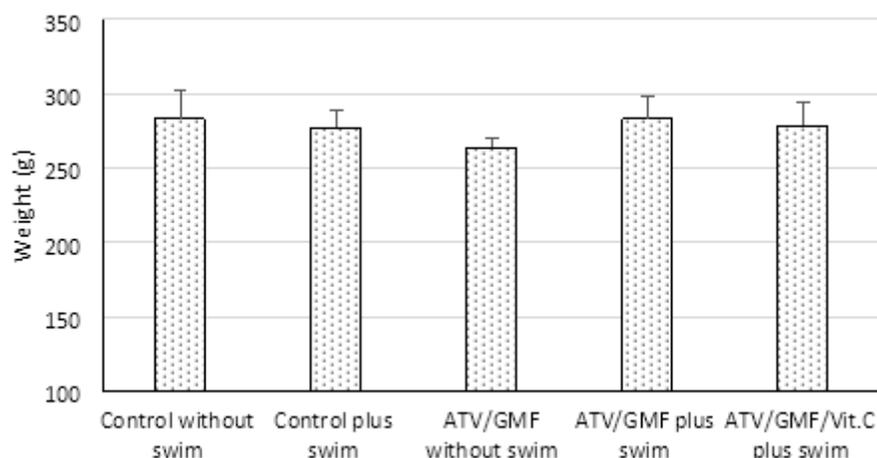


Figure 1

Weight of rats in the experimental groups at the end of study compared with the controls. ATV: atorvastatin, 80 mg/kg/day for 10 days; GMF: gemfibrozil, 1000 mg/kg/day for 10 days; Swimming was done in the days 8, 9 and 10. The numbers on each bar are mean, and the error bar is SEM (n = 6 in each group). There difference between the groups was no significant, one-way ANOVA followed by Tukey post hoc test (P value > 0.05).

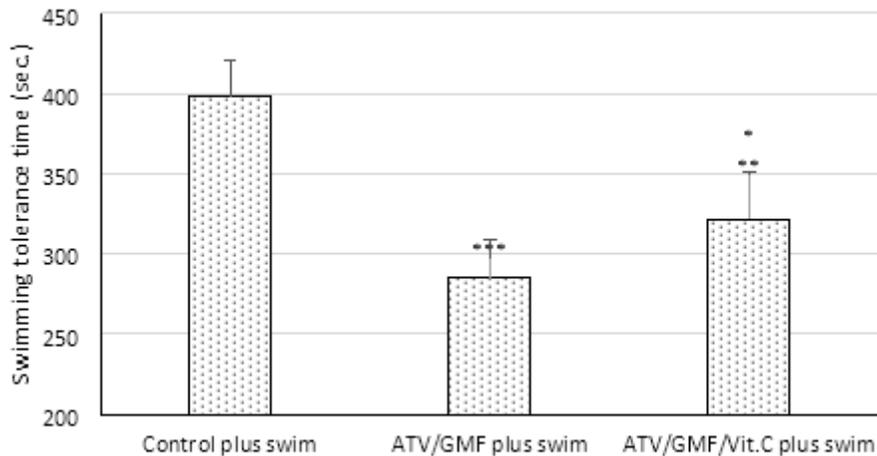


Figure 2

Mean of the swimming tolerance times of rats in 3 groups. ATV: atorvastatin, 80 mg/kg/day for 10 days; GMF: gemfibrozil, 1000 mg/kg/day for 10 days; Vit.C: ascorbic acid. 50 mg/kg/day; Swimming was done in the days 8, 9 and 10. The numbers on each bar are mean, and the error bar is SEM (n = 6 in each group). ***P value < 0.001 and **P value < 0.01 in comparison with control group; *P value < 0.05 in comparison with ATV/GMF plus swim group; One-way ANOVA followed by Tukey post hoc test.

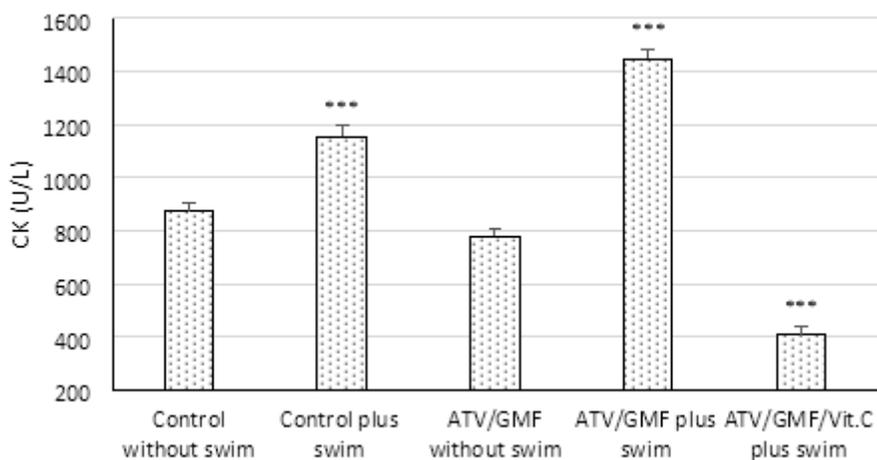


Figure 3

Plasma creatine kinase (CK) levels of rats in control and treated groups. ATV: atorvastatin (80 mg/kg/day for 10 days); GMF: gemfibrozil (1000 mg/kg/day for 10 days); Vit.C: ascorbic acid (50 mg/kg/day for 10

days); Swimming was done in the days 8, 9 and 10. The numbers on each bar are mean, and the error bar is SEM (n = 6 in each group). *** P value < 0.001 in comparison with all of the other groups; One-way ANOVA followed by Tukey post hoc test.

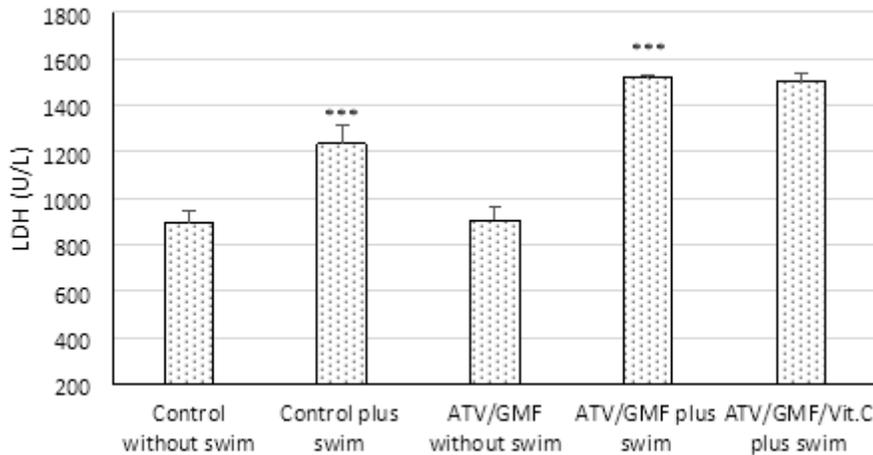


Figure 4

Comparison between control and treated groups of rats regarding plasma lactate dehydrogenase (LDH) levels. ATV: atorvastatin, 80 mg/kg/day for 10 days; GMF: gemfibrozil, 1000 mg/kg/day for 10 days; Vit.C: ascorbic acid, 50 mg/kg/day for 10 days; Swimming was done in the days 8, 9 and 10. The numbers on each bar are mean, and the error bar is SEM (n = 6 in each group). ***P value < 0.001 in comparison with the groups which did not have swimming. LDH levels did not decrease significantly in ATV/GMF/Vit.C plus swim group in comparison with ATV/GMF plus swim group (P value > 0.05); One-way ANOVA followed by Tukey post hoc test.

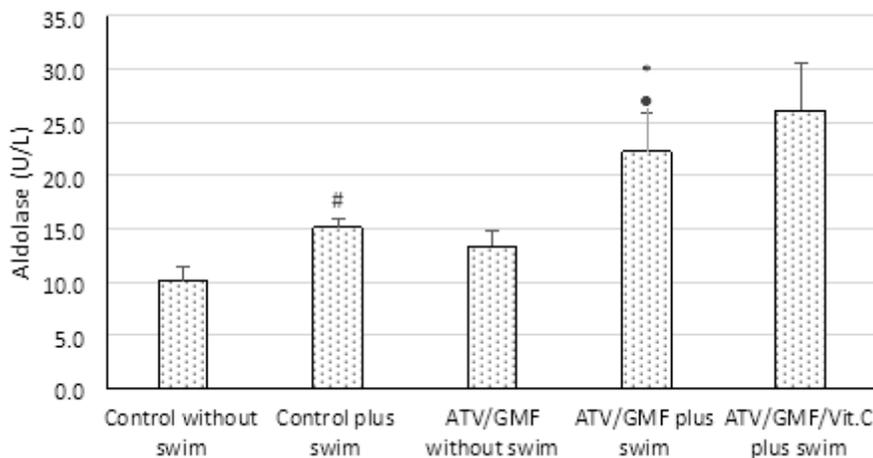


Figure 5

Aldolase levels of rats compared between control and treated groups. ATV: atorvastatin, 80 mg/kg/day for 10 days; GMF: gemfibrozil, 1000 mg/kg/day for 10 days; Swimming was done in the days 8, 9 and 10. The numbers on each bar are mean, and the error bar is SEM (n = 6 in each group). *P value < 0.01 in comparison with control without swim group, ● P value < 0.05 in comparison with ATV/GMF without

swim group, #P value < 0.05 in comparison with control without swim group; One-way ANOVA followed by Tukey post hoc test.

Supplementary Files

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