Influence of spruce needle polyprenols on rat behavior and muscle tone altered by atorvastatin

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Abstract

A lot of the biological activities of long-chain polyprenols, such as antioxidant, antistress and membranoprotective, have been described in a context of their peripheral - hepatoprotecting action and mechanisms provided via lowering of serum cholesterol level through affecting its biosynthetic pathway. Little is known about the central effects of polyprenols, however, it was found that a small amount of orally supplied polyprenols may reach the brain. The present study was focused to investigate whether and how polyprenols may influence CNS functions in female Wistar rats, as well as modify changes in behavior, muscle strength/tone, blood cholesterol level and creatine kinase activity, caused by atorvastatin. Our data demonstrated that polyprenols (1, 10 and 20 mg/kg) administered *per os* for 16 days did not alter behavior (locomotion, memory, pain perception) and blood cholesterol level, while the dose of 20 mg/kg increased muscle tone and plasma creatine kinase activity. Atorvastatin at the dose of 80 mg/kg also did not influence locomotor activity and memory, while it caused analgesic effect and a considerable decrease in muscle

strength which was protected by polyprenols at all tested doses. These data suggest usefulness of polyprenols as a complement in statin therapy to reduce muscle-related side effects.

Keywords: polyprenols, atorvastatin, behavior, muscle strength

Introduction

In eukaryotic cells, cholesterol as well as long-chain polyprenols and dolichols are synthesized in a mevalonate-dependent pathway. Therefore, these substances are considered as biogenetically related compounds with different molecule structures and functions (Surmacz and Swiezewska, 2011; Buhaescu and Izzedine, 2007). Polyprenols as linear polymers are identified in almost all living organisms, and also are found in human diet, fruits and beverages, such as tea, coffee and wine. The needles of conifer trees are one of the richest sources of polyprenols. Free polyisoprenoid alcohols and their fatty acid esters serve as structural components of cellular membranes, modulating their physico-chemical properties such as fluidity and permeability (Chojnacki and Dallner, 1988; Wang et al., 2008).

The majority of data on polyprenols and polyprenyl phosphates had been focused on their ability to prevent toxic injuries of the liver and restore disturbed hepatic functions by lowering the levels of serum cholesterol through effects on its biosynthetic pathway (Pronin et al., 2014), as well as by protecting unsaturated membrane lipids from oxidative free radicals (Bizzarri et al., 2003). Recent studies demonstrated antioxidant, antistress, hepatoprotective and membranoprotective effects of Ropren®, a commercial pharmaceutical preparation (a substance of pure

polyprenols isolated from the green verdure of *Picea abies* L.) (Fedotova et al., 2012; Pronin et al., 2014).

To understand the role of polyprenols in organism and possibilities to alter their level is particularly important because of widely used cholesterol-lowering drugs – statins – which, by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, may halt production not only of cholesterol but also of polyprenols and dolichols. In this context, the statin-induced myopathies can be explained by depletion of the isoprenoids and hence, by the impaired protein prenylation and deficiency of coenzyme Q10 (Buhaescu and Izzedine, 2007; Pronin et al., 2014; Littlefield et al., 2014). However, the overall incidence, such as myalgia, muscle aches, or cramps in clinical practice varies from 0.3% to 33% (Bays, 2006; Wilkinson et al., 2014).

Over the years, the data demonstrated the occurrence also of neuropsychiatric reactions associated with statin treatment. They include behavioral alterations, e.g., irritability, cognitive and memory impairments, sleep disturbances (Tuccori et al., 2014). A long-term treatment of mice (7 months) with the lipophilic statin atorvastatin revealed that this drug altered general behavior, as well as learning and memory, without impacting motor function in the rotarod test (Schilling et al., 2014). However, up to now, there are little data about influence of polyprenols on the CNS functions in experimental animals. Recently, Ropren® was shown to ameliorate cognitive deficiencies in an experimental animal model relevant to Alzheimer's disease (Fedotova et al., 2012).

We hypothesized that polyprenols may protect the statin-induced changes. The idea was based on the data that statin-induced depletion of dolichols could be compensated by plant polyprenols that might avert a deficiency in dolichyl phosphate

cycle. Therefore the present study aimed to investigate the effects of polyprenols (isolated from spruce needles of *Picea abies* L.) to evaluate a broad spectrum of their action in parallel with that of atorvastatin. Both substances were assessed for their influence on rat behavior, muscle tone, cholesterol level and creatine kinase activity.

Materials and methods

Animals

Female *Wistar* rats weighing 230-245 g were obtained from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. Animals were housed in plastic cages (5 per cage) with food and water *ad libitum*, and kept in a controlled laboratory environment (temperature 22° C, humidity 50-60 %, 12 h light/dark cycle). Female rats were used, because female sex is considered as a risk factor to get statin-induced adverse effects (Sathasivam and Lecky, 2008). All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were conducted in accordance with the EU Directive 2010/63/EU and local laws and policies on the protection of animals used for scientific purposes. Animal protocol for this study was approved by the Animal Ethics Committee of the Food and Veterinary Service, Riga, Latvia and approved protocol was strictly adhered to.

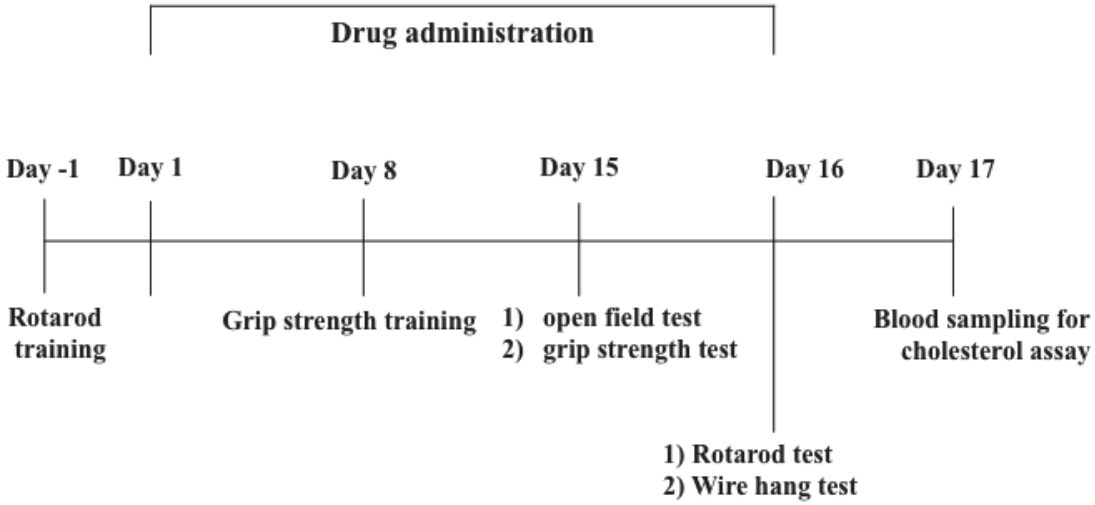
Drugs

Commercially available purified polyprenols (C55–C95) isolated from needles of *Picea abies* L. spruce were supplied by JSC BioLat, Latvia. Atorvastatin (Atoris) was used as model-drug (KRKA, Slovenia).

Experimental design

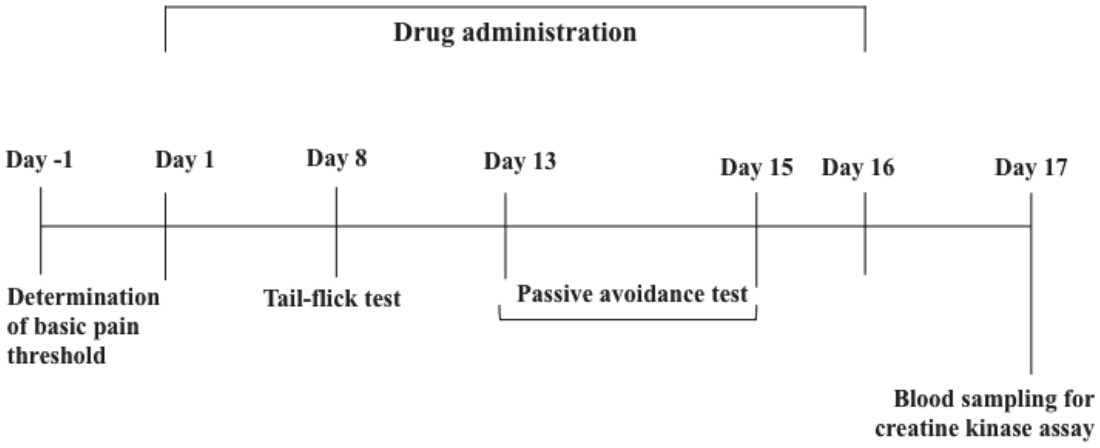
Two experimental setups (1 and 2) were designed to limit experimental procedures in experimental animals.

Setup 1



Experimental animals were treated with polyprenols (at the doses of 1, 10 and 20 mg/kg) dissolved in refined sunflower oil in a volume of 2 ml/kg. Atorvastatin tablets were grinded to powder, suspended in saline and administered at dose the 80 mg/kg in a volume of 10 ml/kg. All drugs were administrated once daily by the oral route (*per os*) via orogastric cannula for 16 consecutive days and tested in several pharmacological tests. Control animals received saline (10 ml/kg per rat) and refined sunflower oil (2 ml/kg per rat). Rats were randomly divided into 8 groups (n = 9-10 per group), treated with polyprenols and atorvastatin alone, and their concomitant administration. Atorvastatin or saline administrations were carried out in the morning, whereas polyprenols or refined sunflower in the afternoon. All behavioral tests were conducted between 9:00 and 15:00 with an interval of 2 h between them.

Setup 2



The setup 2 was carried out in a similar manner as the setup 1, however, polyprenols were administered at the dose of 20 mg/kg, which demonstrated significant effects in several tests performed in setup 1. Rats were randomly divided into 4 groups (n = 9-10 per group), treated with polyprenols (20 mg/kg), atorvastatin (80 mg/kg) and their concomitant administration.

Behavioral tests

Open field test

General locomotor activity was evaluated in an open field apparatus (round arena, 98 cm in diameter, with 40 cm high walls) using video-tracking programme with software Panlab Smart Version 2.5 (PanLab, Spain). The arena was illuminated by a light (60 W) fixed 100 cm above the center of arena. At the beginning of the test, the rat was gently placed into the center zone of the round arena and left to explore the arena freely for 5 min. Horizontal locomotor activity was quantified as the distance walked in cm and analysed in whole arena (total distance).

Passive avoidance test

A day before testing (on day 13), animals were habituated to the step-through passive avoidance apparatus (model 7550, Ugo Basile, Italy), consisting of light and dark compartments. The acquisition trial was conducted on day 14, when each rat was placed in the light compartment. As soon as the rat entered the dark compartment, the door was closed and the rat received an inescapable foot-shock (0.5 mA, 2 s) through the grid floor. The step-through latencies, i.e., time spent in the light compartment before entering the dark chamber, were measured in seconds. The retention test was carried out 24 h after the acquisition trial (i.e., on day 15). No foot-shock was applied in this trial. The differences of step through latencies between retention and acquisition days were calculated. The step-through latency maximum testing limit was 240 s for both training and retention days.

Tail flick test

Analgesic activity of the tested drugs was assessed by using an Analgesy-Meter (model LE 7106, Panlab, Spain). A day before start of the drug treatment, the individual tail flick latency was determined as a pain threshold. On day 8, 2 h after the drug administration, a concentrated burning light was directed onto the tail of the animal. The time in seconds taken for the animal to withdraw its tail after exposition to the heat was considered as the latent period. Baseline tail flick latency was from 2.5 to 4.5 s. A cut-off time of 10 s was considered to prevent any possible tissue damage. The mean values of the two tail flick latencies measured with 5 min interval were used for analysis. Each animal was used as its own control. Antinociception was quantified as the percentage of maximal possible effect (%MPE = [(postdrug latency-predrug latency)/(cut-off time (10 s) - predrug latency)] x 100).

Assessment of muscle strength and tone

Grip strength test

A rat grip strength meter (model 47105, Ugo Basile, Italy) was used to assess forelimb strength. On day 8, animals were pre-trained for six training trials to establish the reliable assessment of gripping ability, and on the day 15 the grip strength test was performed. Animals were positioned by facing the T bar of the grip strength meter and the forelimbs of rats were placed on the tension bar. When the rat grasped the bar, animal was gently pulled steadily by the root of the tail away from the T bar. The grip strength meter determined and recorded automatically the maximum force displayed by each animal in grams. The mean value of five consecutive measurements for each animal was calculated. Rats were allowed to recover for 30 s between the measurements.

Wire hang test

This test was used to assess forelimb strength. The apparatus consisted of a stainless steel wire (90 cm length, 3 mm in diameter), fixed horizontally between two vertical supports and 60 cm above a soft padded surface. On day 16, wire hang test was carried out. The rat was forced to grasp the central position of the wire with its forepaws. The latency (s) to fall from the wire on flat soft pad was measured. When the latency time was over 120 s, the rat was released from the wire, and the time was recorded as 120 s. The trial was conducted three times for each rat and the longest duration was the value used for evaluation. A resting pause between the consecutive attempts was 3 min.

Rotarod test

Locomotor coordination and balance were measured in rats on accelerating rat rotarod apparatus (model 47700, Ugo Basile, Italy). A day before the beginning of the treatment, rats were pre-trained for five trials in the rotarod test. Each time the rat fell off the rotarod at the training trials, it was immediately placed back onto the treadmill to achieve 5 min stability. On day 16, animals were tested in the rotarod task. The gradually accelerating rotor mode was used to increase the speed slowly from 4 to 40 rpm over 5 min period. The trial ended when the rat fell from the rod or after 5 min, which was used as the maximal time for the test. An hour break was given between four consecutive trials. The time (as latency in seconds to fall) and the speed on the rotarod treadmill were automatically registered. Results were expressed as mean of values of four trials.

Assessment of cholesterol level and creatine kinase activity

At the end of the study, on day 17, rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and blood for measurement of cholesterol level and creatine kinase (CK) activity was collected by cardiac puncture. Few drops of the collected blood were taken to measure the blood cholesterol level using Accutrend GCT meter (Roche Diagnostics, Switzerland) expressed in mM/l. For the determination of CK activity, the blood was collected into tubes containing heparin. The tubes were immediately centrifuged at 3000 rpm for 10 min and stored at - 80° C until CK assay. CK determination in rat blood plasma samples was performed by standard spectrophotometric analysis according to the instructions of the manufacturer (Sigma-Aldrich, USA) by using commercial kit for Creatine Kinase Activity Assay (MAK116). The absorbance was read at 340 nm at 37° C by using spectrophotometer

(INFINITE M200 PRO NanoQuant, Tecan Group Ltd., Switzerland). The data were calculated in units/l. One unit of CK activity was defined as the amount of enzyme that transfers 1.0 μmol of phosphate from phosphocreatine to adenosine diphosphate (ADP) per min at pH 6.

Statistical analysis

GraphPad Prism 6 software (GraphPad Software Inc., USA) was used for the statistical analysis. All data were expressed as the mean ± S.E.M. Differences among experimental groups were analysed by one-way ANOVA followed by Uncorrected Fisher's LSD post-test for *in vivo* tests, and by Kruskal-Wallis followed by the Dunn's Multiple Comparison test for biochemical data. In all cases, differences with p value < 0.05 were considered statistically significant.

Results

Experimental data of setup 1

Influence on general locomotor activity

Polyprenols at the doses of 1, 10 and 20 mg/kg did not influence the total distance walked by rats compared with the control group (Fig. 1). Administration of atorvastatin at dose of 80 mg/kg and also the combined administration of both polyprenols and atorvastatin did not affect the rats' locomotor activity (Fig. 1).

Influence on muscle strength/tone and coordination

In the grip strength test (Fig. 2), administration of polyprenols at all tested doses did not show effect on rat grasping strength compared with the control group. When treated with atorvastatin at the dose of 80 mg/kg, rats exhibited marked reduction in grasping strength compared with the control group. Polyprenols administered at dose of 20 mg/kg reversed the atorvastatin effect by increasing the grasping strength to the control level.

Rat hanging time (Fig. 3) was not altered by administration of polyprenols at 1 and 10 mg/kg doses, while the dose of 20 mg/kg prolonged hanging time by about 2-fold vs. control group. Atorvastatin at the dose of 80 mg/kg significantly (about by 3-fold) decreased the hanging time compared with the control group, indicating that atorvastatin reduced muscle strength. Polyprenols at all tested doses significantly prolonged rat hanging time that was reduced by atorvastatin, thereby restoring muscle strength to control level.

In accelerating rotarod test (data not shown), polyprenols at all doses, atorvastatin at the dose of 80 mg/kg and concomitant administration of polyprenols at all doses with atorvastatin showed no influence on rat falling latency compared with that of the control group.

Blood cholesterol level was not changed in any tested group (data not shown).

Experimental data of setup 2

Influence on learning/memory

Data obtained in the passive avoidance response test (Fig. 4) demonstrated no significant changes in step-through latencies in rats treated with polyprenols (20

mg/kg), or atorvastatin (80 mg/kg), or the combined administration of both drugs, compared with the control group.

Analgesic activity

In the tail flick test (Fig. 5), polyprenols at the dose of 20 mg/kg did not show analgesic activity and did not alter the maximal possible effect (MPE), compared to the control group. Atorvastatin at the dose of 80 mg/kg, however, produced a marked increase in the MPE compared with the control group. Concomitant administration of polyprenols (20 mg/kg) with atorvastatin (80 mg/kg) did not alter the atorvastatin-induced analgesic effect.

Influence on plasma creatine kinase activity

A significant increase (by about 25%) in plasma creatine kinase activity was observed after the administration of polyprenols at dose of 20 mg/kg compared with the control group (Fig. 6). The creatine kinase activity after treatment with atorvastatin (80 mg/kg) or concomitant its administration with polyprenols (20 mg/kg) was comparable to that of the control group.

Discussion

Mostly reviewed data about polyprenols and their biological effects and mechanisms are related to their peripheral action and are associated with hepatoprotective activity due to cholesterol-lowering, antioxidant, membrane-protecting properties (Cantagrel and Lefeber, 2011; Hartley and Imperiali, 2012; Pronin et al., 2014). Recent report (Milenkovic et al., 2013) demonstrated that polyprenols may interact with cellular signalling cascades regulating activity of

transcription factors and expression of genes, particularly by the influence on microRNA.

Up to now, little is known about central effects of both endogenous and exogenous polyprenols. There are some interesting data that demonstrate that the content of polyisoprenoid alcohols is greatly increased in the tissues during lifespan. For instance, a 100-fold increase in the human brain has been observed in 80 year old individuals vs. newborns. Moreover, equal amounts of dolichols and phospholipids have been noted in senile pituitary glands. It is suggested that organism tries to protect the brain from oxidative stress and lipid peroxidation by increasing the level of dolichyl phosphate and coenzyme Q10 in the brain (Surmacz and Swiezewska, 2011).

As to the bioavailability of exogenous polyprenols supplied orally, only about 0.05% of the total amount were found in rat organs (Cantagrel and Lefeber, 2011), with the highest uptake in the liver and stomach, and about 10-fold less in the brain (Jakobsson et al., 1989). However, it is suggested that the altered physiological conditions or pathological processes may enhance polyprenol uptake in the brain. These data are intriguing and provide an interest to study polyprenols' influence on the CNS functioning in more details. Only some data have been found about the neuropharmacological actions of polyprenols in animals. For instance, rats with β-amyloid peptide (25-35)-induced amnesia treated with Ropren® at a peroral dose of 8.6 mg/kg for 28 days demonstrated significant improvement of spatial learning (Fedotova et al., 2012).

The present study was aimed to examine a broad spectrum of pharmacological actions of polyprenols isolated from spruce needles *(Picea abies* L.) in rats after a 16-day treatment with a focus on behavior, muscle tone and some biochemical parameters in the blood. In addition, we wanted to clarify whether and how

polyprenols may alter these activities in statin-treated rats, considering that the mechanism of action of statins involves not only inhibition of cholesterol biosynthesis, but also that of polyprenols/dolichols (Buhaescu and Izzedine, 2007, review). We tested polyprenols at the doses of 1, 10 and 20 mg/kg *per os* for 16 days. Atorvastatin was chosen as the model compound. This statin is described as highly lipophilic substance capable to penetrate tissues, such as the brain (Sierra et al., 2011) and muscles (Rosenson et al., 2014). The atorvastatin dose of 80 mg/kg and the treatment regimen for 16 days were found as a middle dose and timing selected from literature (Madsen et al., 2008).

Our data showed that polyprenols at the doses of 1, 10 and 20 mg/kg did not influence rat locomotor activity tested in the open field test, neither did they alter the learning/memory processes in the passive avoidance response test. Our data are in a good agreement with those showing a lack of activity of polyprenol substance (Ropren®) in non-spatial tests but demonstrating improved spatial learning in Alzheimer's disease model male rats (Fedotova et al., 2012). In our experiments, the CNS functions in the open field and memory tests have also not been changed by atorvastatin. However, the data about the influence of statins on CNS functions are conflicting in human studies. On one hand, experimental studies supported links between cholesterol intake and amyloid synthesis, and the observational studies indicated that patients receiving statins had a reduced risk of dementia (Wagstaff et al., 2003). In addition, numerous data demonstrated that statins may ameliorate neurodegenerative symptoms in Alzheimer's and Parkinson's diseases, stroke and multiple sclerosis. These observations suggest that neuroprotection is dependent on cholesterol rather than isoprenoid depletion (van der Most et al., 2009). On the other hand, statins were shown to cause behavioral alterations (severe irritability, homicidal

impulses, threats to others, depression and violence, paranoia, antisocial behavior), cognitive and memory impairments, sleep disturbances and sexual dysfunction (Tuccori et al., 2014).

In the tail flick test, atorvastatin demonstrated a considerable analgesic activity by elongating the tail flick latency by about 3.5 times. Polyprenols at the dose of 20 mg/kg *per se* lacked this activity; neither did they alter the analgesic action of atorvastatin. Analgesic and anti-inflammatory activities of atorvastatin were already described in literature previously in different analgesia tests (Dwajani et al., 2012; Garcia et al., 2011; Jaiswal and Sontakke, 2012). The anti-inflammatory activity and the inhibition of the carrageenan-induced paw edema was shown in rats treated with polyprenols isolated from alcoholic extracts of *Capparis spinosa* (al-Said et al., 1988).

Unlike studies which did not show any influence of polyprenols and atorvastatin on behavior, our study shows that muscle strength was considerably increased by polyprenols at the dose of 20 mg/kg in the wire hang test, and significantly decreased by atorvastatin at the dose of 80 mg/kg in both tests (grip strength and wire hang). Particularly dramatic atorvastatin effect was observed in wire hang test, when muscle weakness was about 3-fold vs. control. In these experiments, polyprenols at all tested doses significantly protected from atorvastatin-induced alterations by restoring muscle strength. At the same time, neither atorvastatin nor polyprenols influenced muscle tone and coordination in the accelerating rotarod test.

The mechanisms of atorvastatin myopathies are mostly explained by its direct influence on mevalonate pathway leading to the inhibition of cholesterol biosynthesis and endogenous polyisoprenoid production, resulting in lowering of the concentrations of metabolites necessary for cellular processes. Thus, the deficiency of such molecules as ubiquinone (provides mitochondrial electron transport), isoprenoid

pyrophosphates (protein prenylation) and dolichol (protein glycosylation) is taken as essential for the development of statin-induced myopathy (Baker, 2005; Manoukian et al., 1990). Other data showed that atorvastatin (10 mg/kg for 2 months in rats) may induce a down-regulation of protein expression (Camerino et al., 2011).

As our experiments were carried out in normolipidemic rats, it was already predictable that atorvastatin will not influence cholesterol level. It was also not influenced by polyprenols. Atorvastatin also did not alter creatine kinase activity, and, in the light of recent data, this enzyme (namely, its activation) is not considered as a hallmark of atorvastatin-induced myopathies (Abdelbaset et al., 2014; Ballard et al., 2013). At the same time, polyprenols at the dose of 20 mg/kg caused an elevation (by about 25 %) in creatine kinase activity and increase in muscle strength. Indeed, at present, we cannot explain this phenomenon, particularly why in combination with atorvastatin (when polyprenols restored muscle tone in atorvastatin-treated animals) the activity of creatine kinase remained at control values. One of the speculations can be that polyprenols by elevation of the creatine kinase activity may intensify intracellular energy transportation and ATP generation, leading to normalization of energy processes impaired by atorvastatin.

In summary, one may conclude that polyprenols are safe substances that do not alter behavior and memory and may act as successful protectors of atorvastatin-induced muscle weakness. These data indicate that combination of polyprenols with atorvastatin may be helpful for reducing muscle-related side effects in patients receiving a long-term atorvastatin therapy.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgement

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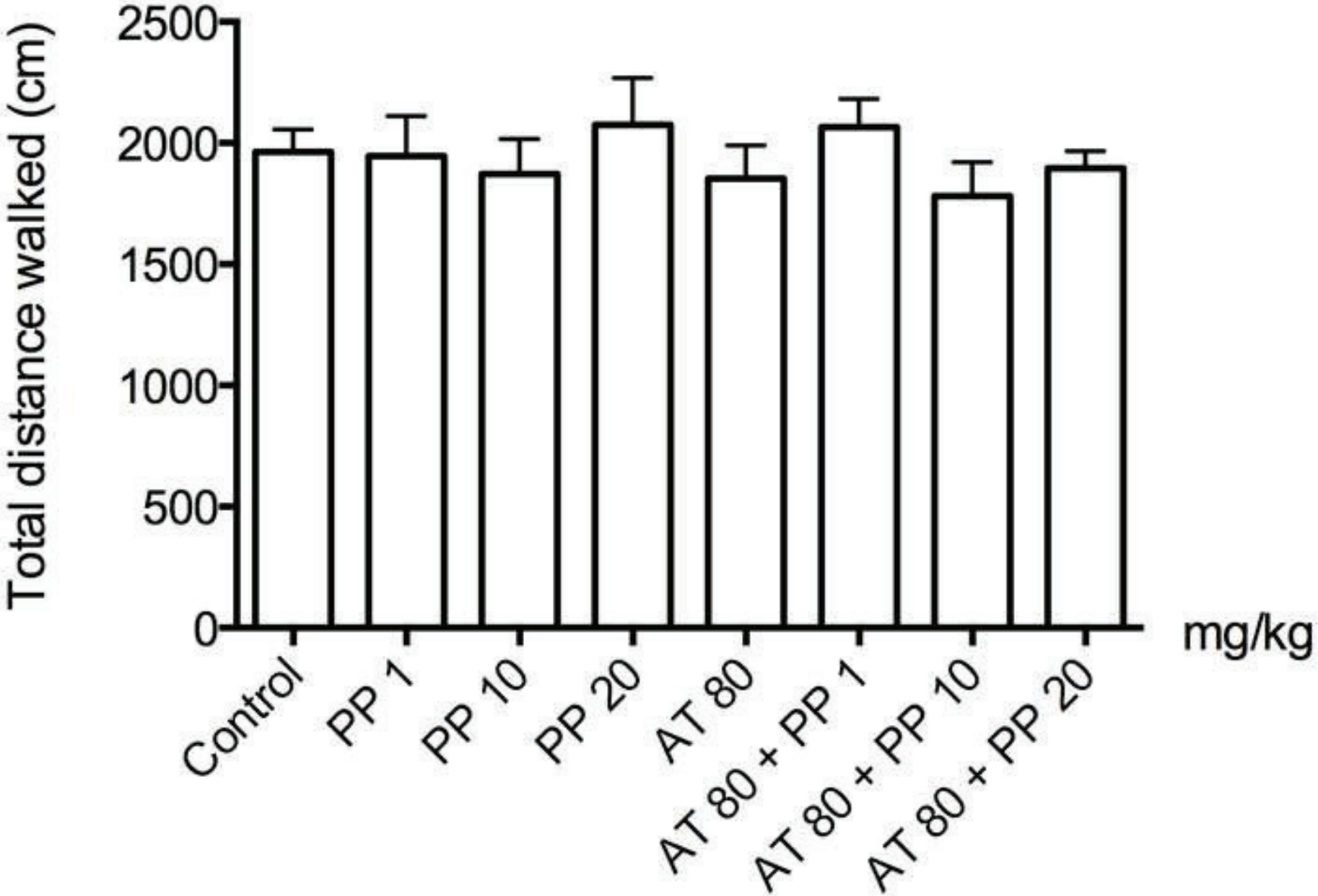

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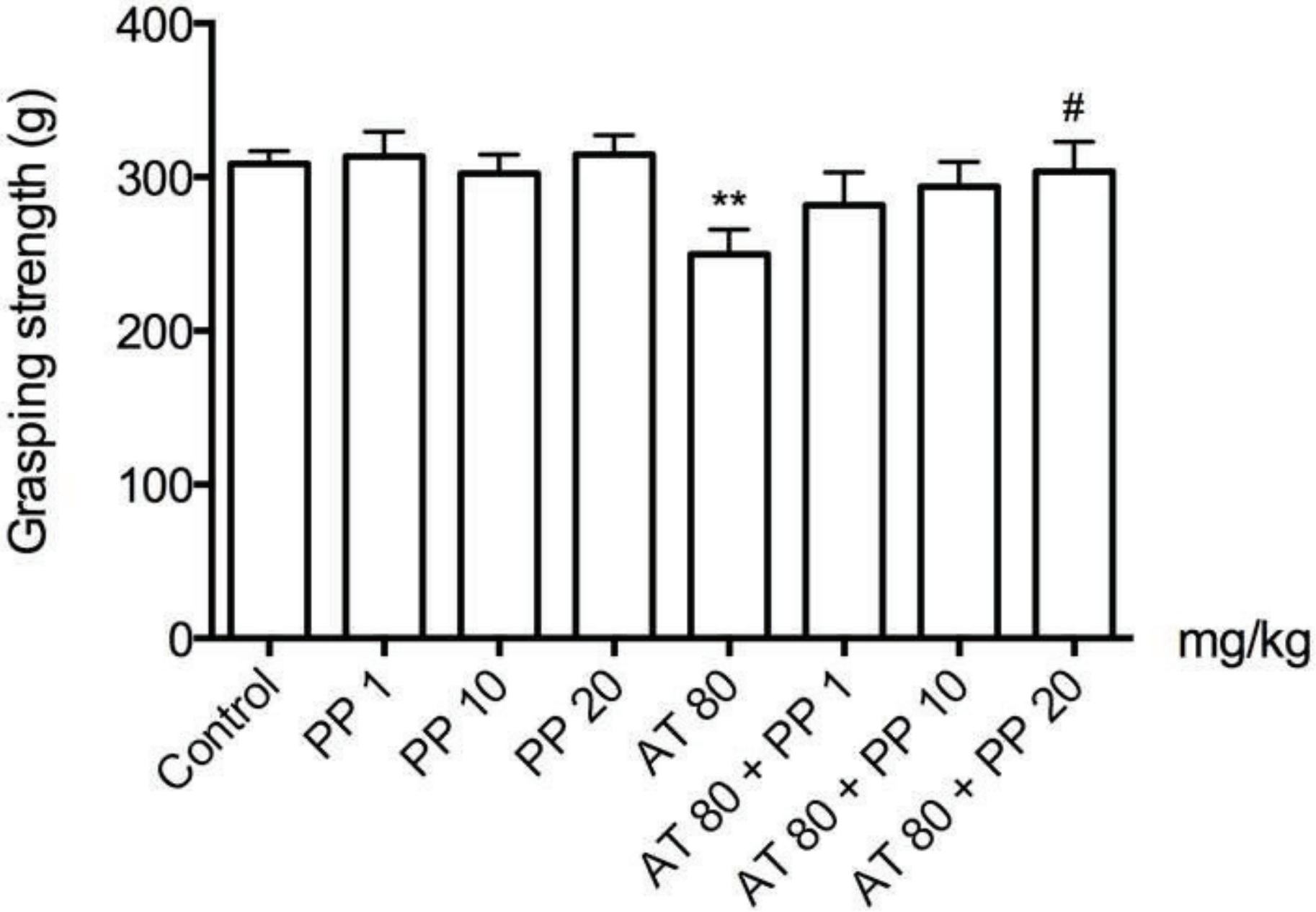

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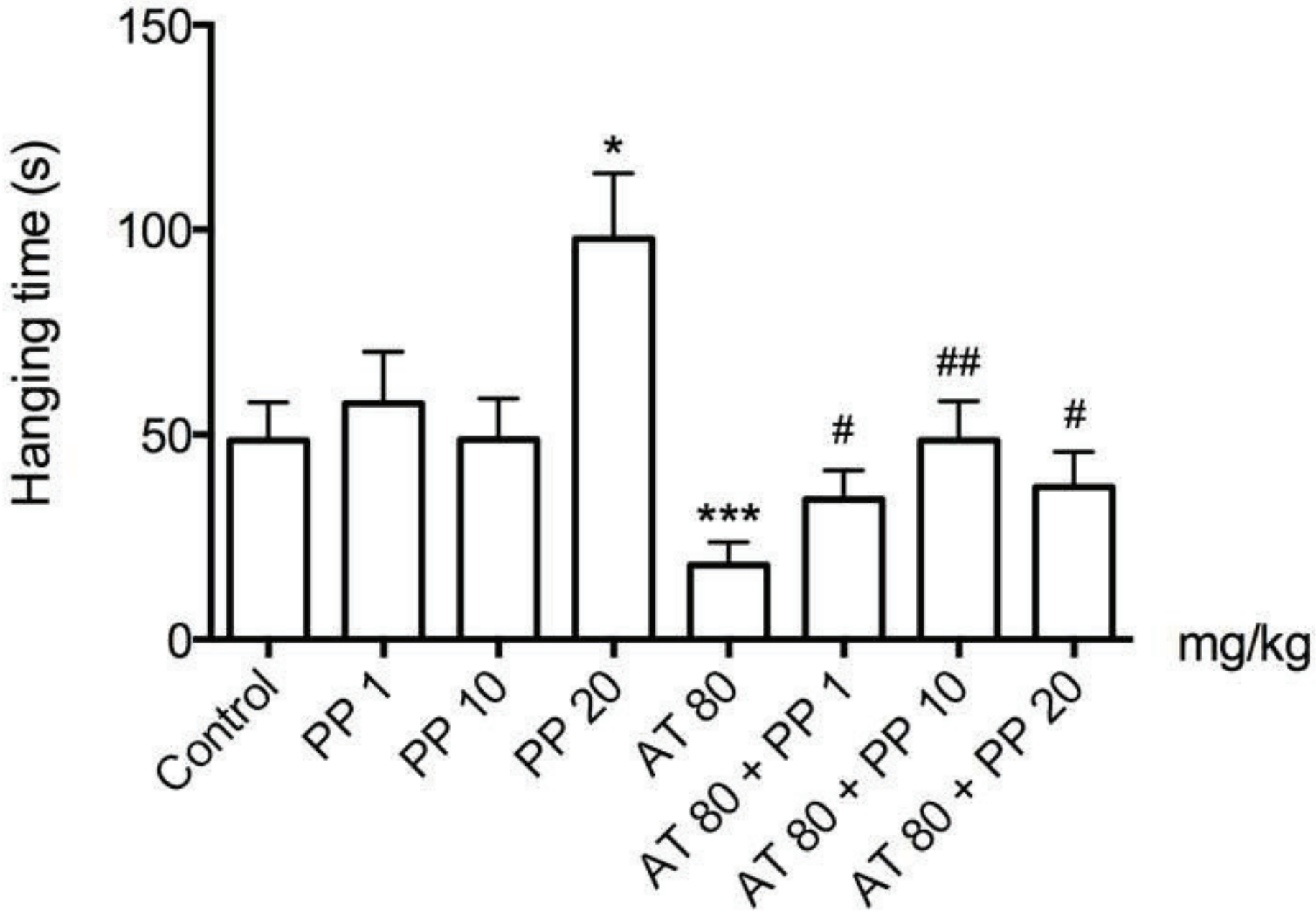

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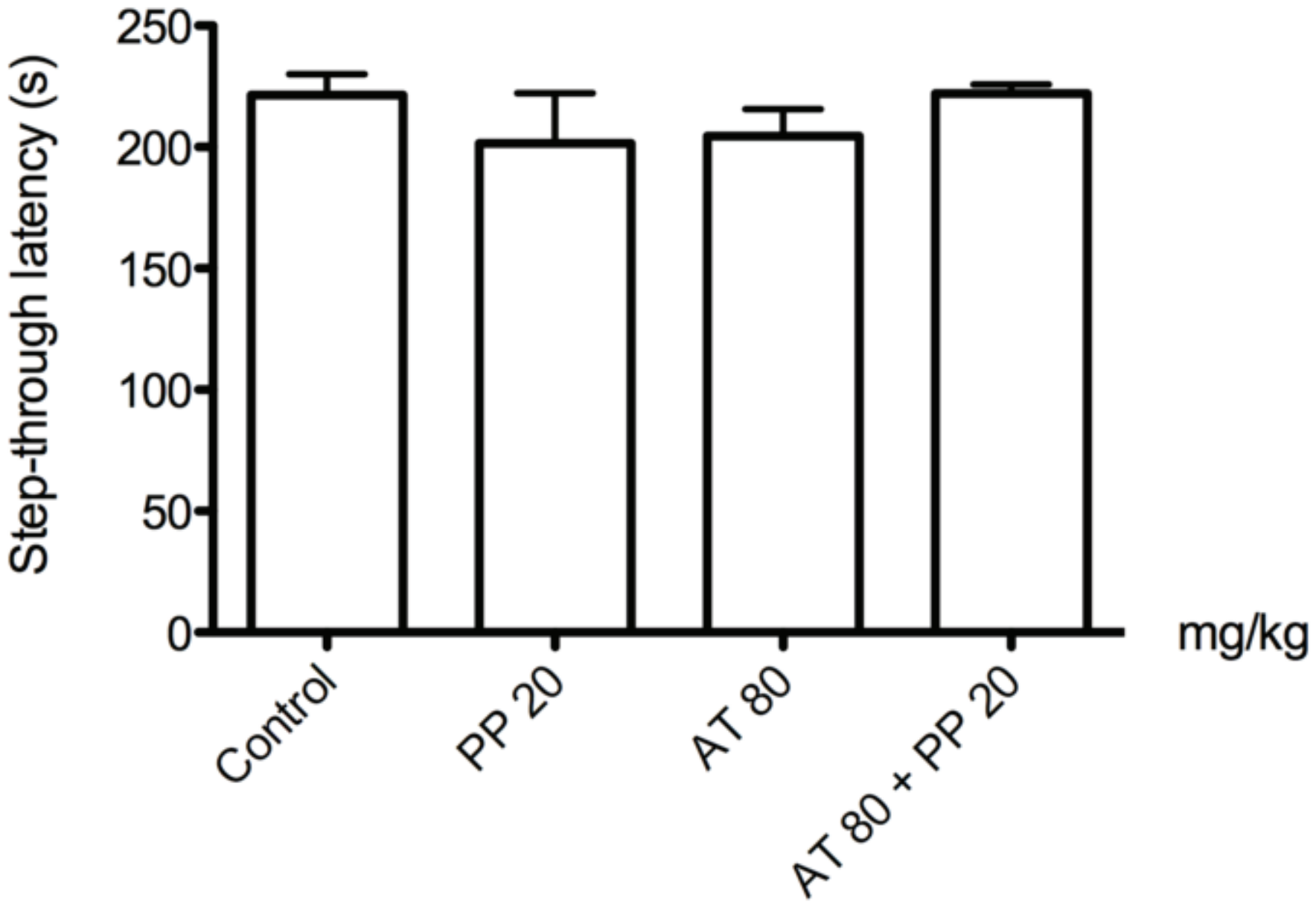

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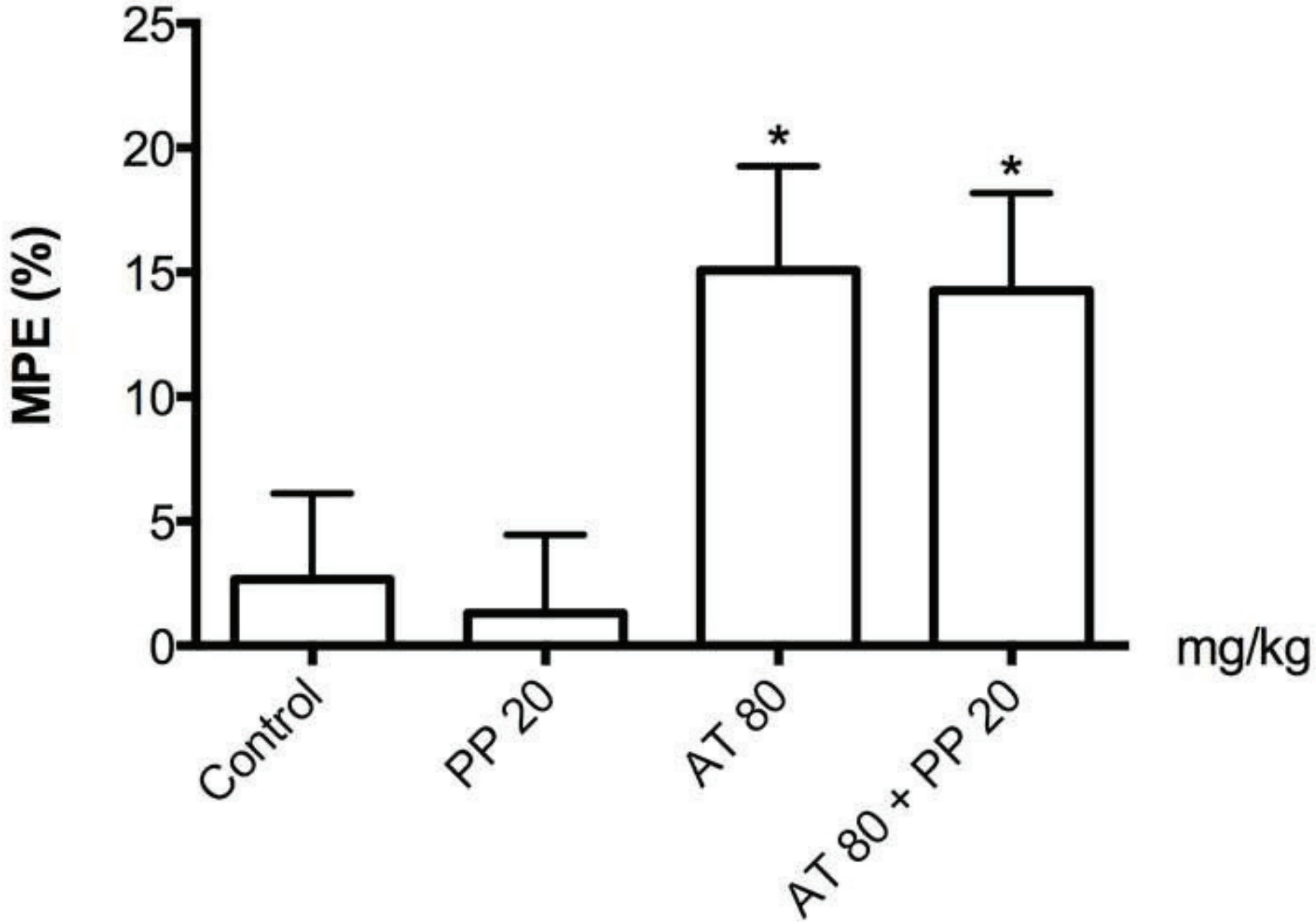

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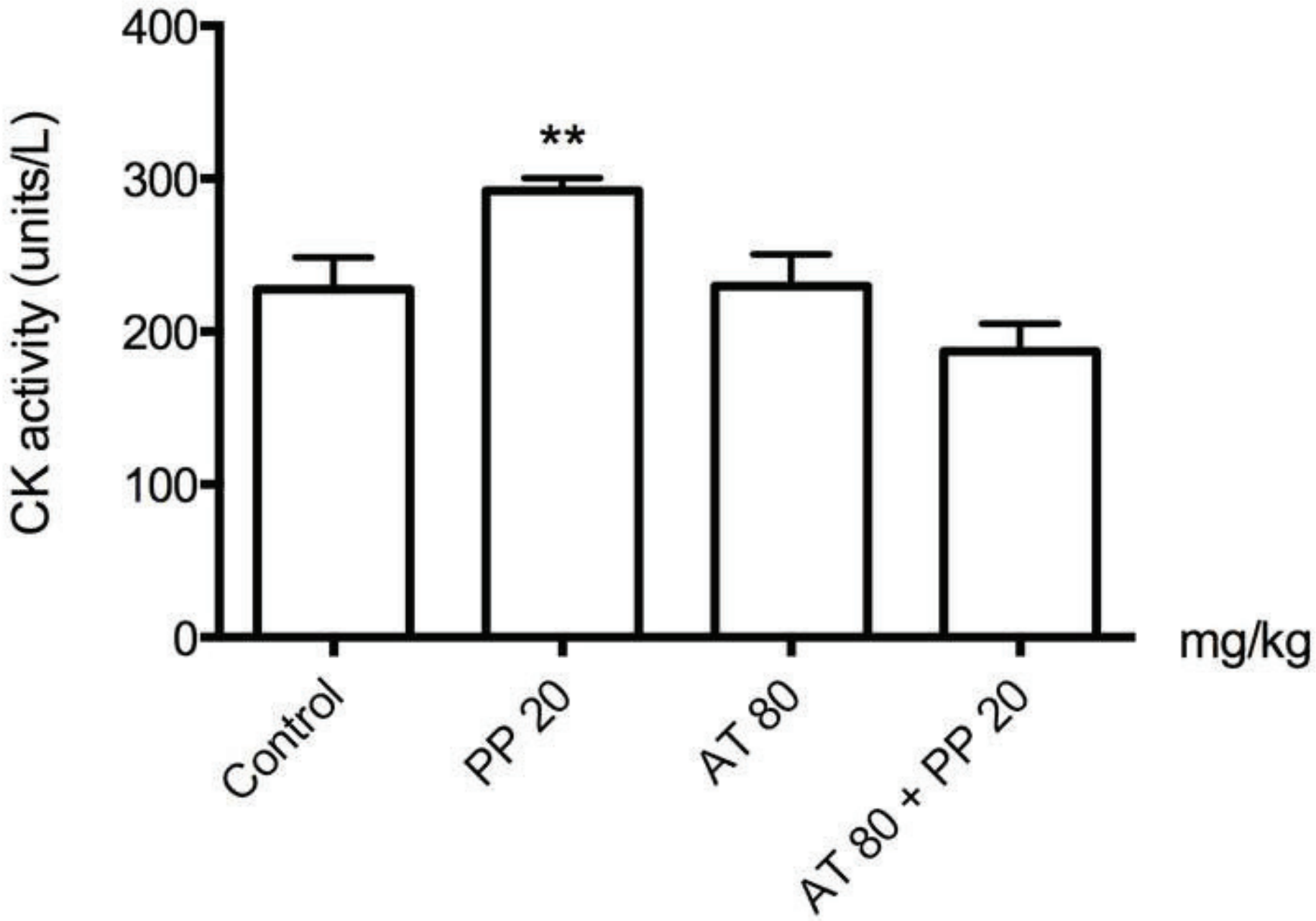

Legends

Fig. 1. Locomotor activity in the open field test in rats (n=9-10 per group). The total distance walked (cm) was measured on day 15 after peroral administration of polyprenols (PP) at 1, 10 and 20 mg/kg, atorvastatin (AT) at 80 mg/kg and their combination. Control rats were treated with vehicle (saline + oil). Values are means ± S.E.M.

Fig. 2. Muscle strength in the grip strength test in rats (n=9-10 per group). Grasping strength (g) was measured on day 15 after peroral administration of polyprenols (PP) at 1, 10 and 20 mg/kg, atorvastatin (AT) at 80 mg/kg and their combination. Control rats were treated with vehicle (saline + oil). Values are means ± S.E.M. One-way ANOVA followed by Uncorrected Fisher's LSD post-test. ** $p < 0.01$ vs. control; # $p < 0.05$ vs. AT 80 mg/kg.

Fig. 3. Muscle strength in wire hang test in rats (n=9-10 per group). Hanging time (s) was measured on day 16 after peroral administration of polyprenols (PP) at 1, 10 and 20 mg/kg, atorvastatin (AT) at 80 mg/kg and their combination. Control rats were treated with vehicle (saline + oil). Values are means ± S.E.M. One-way ANOVA followed by Uncorrected Fisher's LSD post-test. * $p < 0.05$ and *** $p < 0.001$ vs. control; # $p < 0.05$ and ## $p < 0.01$ vs. AT 80 mg/kg.

Fig. 4. Influence on memory in passive avoidance response test in rats (n=9-10 per group). Difference of step-through latency (s) was measured on day 15 after peroral administration of polyprenols (PP) at 20 mg/kg , atorvastatin (AT) at 80 mg/kg and their combination. Control rats were treated with vehicle (saline + oil). Values are means ± S.E.M.

Fig. 5. Analgesic activity in tail flick test in rats (n=9-10 per group). The maximal possible effect (MPE, %) was measured on day 8 after peroral administration of polyprenols (PP) at 20 mg/kg, atorvastatin (AT) at 80 mg/kg and their combination. Control rats were treated with vehicle (saline + oil). Values are means ± S.E.M. One-way ANOVA followed by Uncorrected Fisher's LSD post-test. * $p < 0.05$ vs. control.

Fig. 6. Creatine kinase (CK) activity (units/l) in rat plasma determined spectrophotometrically after peroral administration (for 16 days) of polyprenols (PP) at 20 mg/kg, atorvastatin (AT) at 80 mg/kg and their combination (n=9-10 per group). Control rats were treated with vehicle (saline + oil). Values are means ± S.E.M. Kruskal-Wallis test followed by the Dunn`s Multiple Comparison test. ** $p < 0.01$ vs. control.