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Antifatigue and Anti-Inflammatory Effects of Cervus elaphus L., Angelica gigas Nakai, and Astragalus membranaceus Bunge Complex Extracts in Physically Fatigued Mice

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Abstract:
Fatigue is a common complaint among people under stress, causing an array of negative effects on physical function. In this study, we investigated the antifatigue and anti-inflammatory effects of *Cervus elaphus* L., *Angelica gigas* Nakai, and *Astragalus membranaceus* Bunge complex extracts (CAA) using a treadmill stress test in animal models. The mice were administered various doses of CAA (50–200 mg/kg bw per day) once daily for 21 days. After exhaustive treadmill exercise, the running time of CAA-treated mice increased 1.5 times; fatigue-related biochemical parameters, including lactate dehydrogenase (∼30%), creatine kinase (∼20%), and proinflammatory cytokines interleukin (IL)-1β (∼10%), and IL-6 (∼10%) in the serum and muscle tissue were downregulated compared with those in exercised control mice. This study provides strong evidence for the prevention of CAA-induced inflammatory incidences mediated by the blockade of nuclear factor-κB activation. Collectively, our results indicate that CAA can alleviate symptoms of fatigue in mice as an effective anti-inflammatory agent.

Keywords:
Angelica gigas *Nakai*,
antifatigue,
anti-inflammatory,
Astragalus membranaceus *Bunge*,
Cervus elaphus *L.*

INTRODUCTION

Fatigue is one of the most common debilitating symptoms, with a significant economic and global burden on modern society. Fatigue commonly occurs among people who live a stressful life and can be seriously aggravated by excessive mental and physical stress. Dysfunction of the immune, endocrine, or antioxidant systems can also lead to chronic fatigue, which is an acquired multisystem disease caused by widespread inflammation, neuropathology, and immune system dysfunction.[1] Therefore, fatigue is often associated with inflammatory diseases such as cancer, anemia, and chronic fatigue syndrome.[2]

In this study, a treadmill test was used to investigate whether certain agents have an antifatigue effect in various fatigue models. Highly intensive treadmill exercise increases the expression of proinflammatory cytokines in the muscle and blood.[3] Various biochemical parameters, including blood lactate levels, lactate dehydrogenase (LDH), and creatine kinase (CK), are altered during fatigue symptoms.[4] In addition, oxidative stress induces fatigue with

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increased levels of interleukin (IL)-1β and IL-6, and alteration of mitochondrial function by exposure to reactive oxygen species (ROS) can induce muscular fatigue.[5,6]

Excessive ROS production results in oxidative damage to a variety of macromolecules and causes diverse pathological states.[7] Oxidative imbalance in skeletal muscle results in increased muscle fatigability.[8] Therefore, some antioxidants can alleviate fatigue by counteracting oxidative stress. Catalase is a powerful antioxidant enzyme that plays a significant role in ROS removal. The presence of a large amount of catalase in the body reduces free radical damage to cells, delays fatigue, and accelerates recovery after the fatigue stage.[9]

Natural resources have been applied as ergogenic agents, usually in optimal combinations. *Cervus elaphus* L. (CEL), also known as deer antler, is a well-known animal resource in oriental medicine for the treatment of various physical disorders.[10,11] CEL is a representative animal ingredient that has been reported to improve fatigue symptoms.[12] However, some reports show that CEL has side effects such as fever, diarrhea, and vomiting if it does not suit an individual's constitution.[10,13]

Our previous screening test showed that CEL, *Angelica gigas* Nakai (AGN), and *Astragalus membranaceus* Bunge (AMB) complex extracts (CAA) decreased a wide array of fatigue-associated biochemical and physiological parameters, which were further investigated in an optimal combination in a forced exercise model with mice.[14] It was confirmed that the synergistic effect was better when used in combination with herbal medicine than when CEL was used alone. This study aimed to verify the efficacy of various concentrations at the optimal combination ratio based on previous screening results.

MATERIALS AND METHODS

Materials

CAA extracts were obtained from Kwangdong Pharmaceutical Co., Ltd. (Seoul, Republic of Korea). In brief, the materials (which were recombined by applying the extraction yield of individual substance; CEL:AGN:AMB, 1:2:1) were extracted in boiling water for 8 h, filtered, evaporated, and spray dried. The dry residue was stored at −80°C.[14]

Animal model

The 48 male ICR mice (6 weeks old) were procured from Raon Bio, Inc. (Yong-in, Republic of Korea). The mice were allowed to adapt to their surroundings for 1 week before starting the experiments under controlled lighting (12-h light/12-h dark cycle), humidity (50–60%), and temperature (20–23°C). The experimental procedures and animal care were approved by the animal care committee of Korea University (KUIACUC-2022-0035).

Mice were assigned to six groups ($n = 8$/group), including the negative control (CON, saline), exercise control (E-CON, saline), positive control (P-CON, fermented porcine placenta extract at 66.7 mg/kg bw per day), and various doses of CAA groups [CAA-L (50 mg/kg bw per day); CAA-M (100 mg/kg bw per day); CAA-H (200 mg/kg bw per day)], according to previous reports.[14] Mice were orally administered the treatments once daily for 21 days.

Treadmill exercise test

The treadmill exercise test was performed as previously described.[14] In brief, all mice (except CON mice) were familiarized with the treadmill for 1 week before the treadmill test. All mice (except CON mice) ran on the treadmill at 10 m/min for 10 min, followed by 16 m/min for 10 min and 21 m/min for 10 min once a week for 3 weeks. The time to exhaustion from exercise (10 m/min for 5 min, followed by 16, 18, 21, 24, 26, 29, 32, 34, and 37 m/min for 3 min each, and 40 m/min until exhaustion) was determined on the 21st day.

The time of exhaustion during treadmill running was defined as the time between the commencement and the first failure to maintain treadmill exercise for >3 min. At the point of exhaustion, the mice were removed from the treadmill and immediately euthanized with avertin (200 mg/kg bw per day), and blood, liver, and skeletal muscle were collected.[15]

Serum parameters analysis

Serum was separated by centrifugation at 12,000 *g* for 20 min at 4°C. The levels of serum LDH and CK were determined using an AU480 chemistry analyzer (Beckman Coulter, Brea, CA, USA).

ELISA

The levels of catalase (serum/liver), IL-1β (serum/muscle), IL-6 (serum/muscle), LDH (muscle), and CK (muscle) were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed as previously described.[14] In brief, proteins in the cell lysates were separated using 10% sodium dodecyl salt-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Boston, MA, USA). The membrane was then blocked with 5% skim milk solution and incubated with the target primary antibodies phospho-nuclear factor kappa B and nuclear factor kappa B (NF-κB) (Cell Signaling, Danvers, MA, USA) at 1:1000 dilutions with agitation at 4°C. The blots were incubated with a secondary antibody for 1 h at 25°C, visualized using enhanced chemiluminescence reagents (Bio-Rad Laboratories), and examined using an Image Quant LAS-4000 chemiluminometer (GE Healthcare, Chicago, IL, USA).

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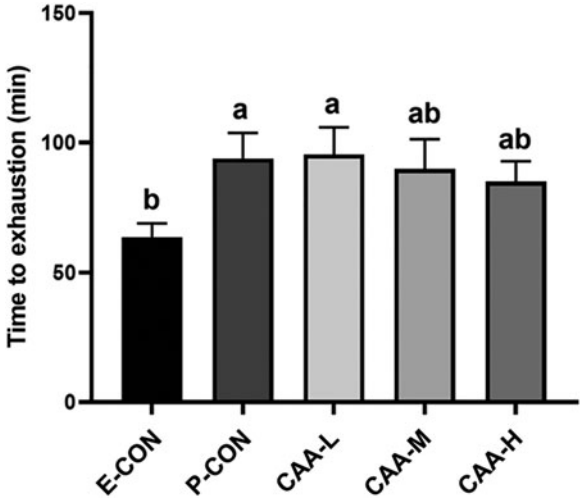

FIG. 1. Effects of CAA administration on time to exhaustion in exercised mice. Data are expressed as means ± standard errors. A *P*-value <.05 was considered statistically significant. CAA-L, CAA low dose at 50 mg/kg bw per day; CAA-M, CAA middle dose at 100 mg/kg bw per day; CAA-H, CAA high dose at 200 mg/kg bw per day; E-CON, exercise control; P-CON, positive control.

Statistical analysis

The experimental data were processed using IBM SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). Unless otherwise specified, the data are expressed as the mean of three independent experiments. The differences between the groups were analyzed with one-way analysis of variance followed by Dunnett's multirange test, where $*P < .05$, $**P < .01$, and $***P < .001$ were considered to indicate a significant difference.

RESULTS

Effect of CAA administration on the time to exhaustion in mice

To evaluate the effects of CAA on physical fatigue induced by the treadmill, a running exhaustion test was carried out on the 21st day of the study. Exhaustion times are shown in Figure 1. In this study, the time to exhaustion was the shortest in the E-CON group. The groups treated with samples took a longer time to reach exhaustion P-CON (1.25-fold) and CAA (1.10–1.16-fold, respectively) than the E-CON group. These results suggest that CAA supplementation significantly enhanced endurance exercise capacity.

Effect of CAA administration on fatigue-associated biochemistry

Serum and skeletal muscle tissues were collected immediately after the exhaustive exercise test, and analyzed to investigate the effects of CAA administration on the antifatigue-associated indices. Compared with the CON group, long-term exercise fatigue significantly increased LDH levels in both the serum (1.67-fold) and muscle tissue (1.38-fold) in the E-CON group (Fig. 2). Elevated levels of LDH were significantly suppressed in the CAA-H (200 mg/kg bw per day) group compared with those in the E-CON group in both serum and muscle tissue ($P < .05$).

In addition, the level of CK in skeletal muscle tissue (2.95-fold) was significantly higher in the E-CON group than in the CON group, although only an increasing trend was observed in the serum (1.38-fold). CAA treatment showed a similar declining pattern as CK treatment in both serum and muscle tissue (Fig. 3).

Effect of CAA administration on fatigue-related proinflammatory cytokines

We studied whether CAA would regulate the levels of IL-1β and IL-6 in the serum and skeletal muscle tissue after treadmill exercise. Compared with the CON group, exhaustive exercise seemed to significantly increase IL-1β and IL-6 levels in the serum (1.67-fold) in the E-CON group (Fig. 4). Administration of CAA-M (100 mg/kg bw per day) and CAA-H (200 mg/kg bw per day) significantly downregulated IL-6 levels in the serum compared with those in the E-CON group (Fig. 4) ($P < .05$).

However, there were no significant changes in the CAA-treated groups compared with those in levels of IL-1β, the P-CON group (Fig. 4) ($P < .01$). In addition, it was confirmed that the levels of IL-1β in muscle tissue increased significantly in the E-CON group, with a similar tendency to serum ($P < .001$), and were then suppressed by CAA treatment (Fig. 5). In addition, there was no noticeable change according to sample treatment in IL-6 levels (Fig. 5).

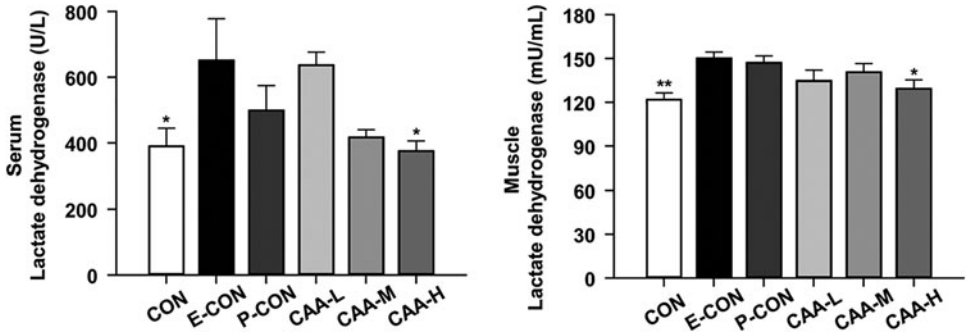

FIG. 2. Effects of CAA administration on levels of LDH of mice serum and muscle tissue. Data are expressed as means ± standard error. A *P*-value <.05 was considered statistically significant. * and **indicate statistical significance compared with the E-CON group. CON, normal control; LDH, lactate dehydrogenase.

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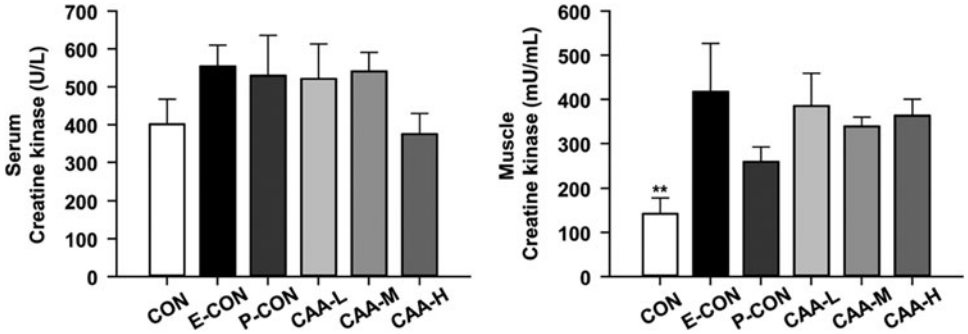

FIG. 3. Effects of CAA administration on levels of CK of mice serum and muscle tissue. Data are expressed as means ± standard error. A *P*-value <.05 was considered statistically significant. **Indicates statistical significance compared with the E-CON group. CK, creatine kinase.

Effect of CAA administration on fatigue-induced oxidation index

Preventive antioxidants represent the first line of defense during heavy exercise and thus inhibit the production of free radicals and ROS through antioxidant enzymes. As shown in Figure 6, the levels of catalase in the serum and liver tissues were evaluated. The E-CON group showed the lowest catalase levels in both serum and liver tissue, whereas the CAA-treated groups showed significantly increased catalase activity in comparison with the E-CON group.

Since NF-κB is an important transcriptional regulator that modulates proinflammatory cytokines, we examined whether the inhibitory action of CAA administration on inflammatory mediators was linked to the regulation of NF-κB activation. As shown in Figure 7, Western blot analysis revealed highly increased protein levels of NF-κB after exhaustive exercise. Meanwhile, CAA treatment significantly inhibited the upregulation of NF-κB in a dose-dependent manner. Thus, our findings suggest that CAA exerts its anti-inflammatory effects by attenuating NF-κB activation.

DISCUSSION

In our study, we observed the antifatigue and anti-inflammatory effects of CAA on several biochemical markers in exercised mice. CAA treatment improved treadmill exercise-induced exhaustion time by enhancing catalase activity and decreasing serum and muscle levels of LDH, CK, IL-1β, and IL-6. These results indicate that CAA may inhibit proinflammatory cytokine production by reducing NF-κB activation. Comprehensively, CAA could alleviate fatigue and be a viable candidate for antifatigue and anti-inflammatory treatment.

The association between physical exercise and fatigue can be investigated by examining muscle metabolites. Exercise causes the accumulation and production of metabolic products, such as LDH and CK.[16] An increase in LDH levels with CK is an index of cellular necrosis and tissue damage in skeletal muscles.[17] Lactate is considered an active metabolite that plays a primary role in the induction of muscle fatigue and subsequent conversion to LDH. CK in the body is generally present in the muscles, and an increase in CK in the blood causes muscle damage.[18]

Many natural medicines such as CEL, AGN, and AMB can improve animal exercise capacity by prolonging swimming time, running time, and increasing forelimb grip strength.[19] For instance, Sripirom and Srisawat confirmed that Rusa deer velvet antler can enhance the exercise endurance of mice through a weight-loaded forced swimming test, and alleviate serum creatine and LDH levels.[20] In addition, Kim et al reported that fermented antlers recovered stamina and muscle strength by inhibiting muscle proteolysis and promoting muscle synthesis in exercised mice.[21] Go et al also confirmed that polysaccharides from AMB prolonged running time to exhaustion and inhibited oxidative stress induced by exhaustive exercise.[22]

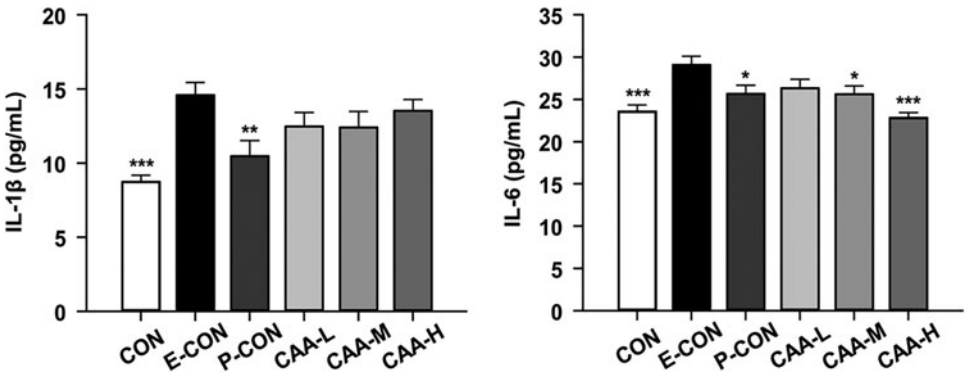

FIG. 4. Effects of CAA administration on levels of proinflammatory cytokine in mice serums. Data are expressed as means ± standard errors. A *P*-value <.05 was considered statistically significant. *, **, and ***indicate statistical significance compared with the E-CON group.

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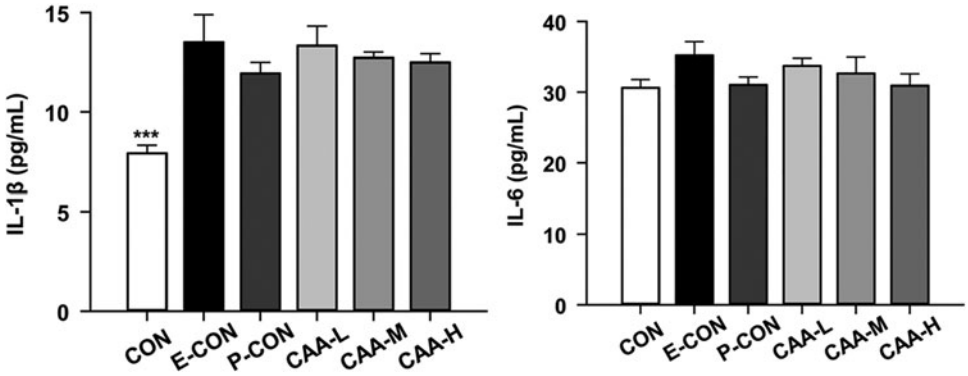

FIG. 5. Effects of CAA administration on levels of proinflammatory cytokine in mice muscle tissue. Data are expressed as means ± standard errors. A *P*-value <.05 was considered statistically significant. ***Indicate statistical significance compared with the E-CON group.

In our study, significant increases in LDH and CK levels were observed in the specimens obtained after the treadmill test compared with those in the CON group. Our results showed that LDH and CK levels were positively regulated by CAA treatment in the serum and/or muscle after treadmill exercise, indicating that CAA may exhibit antifatigue effects. Similar to Lee's research, we used a biopharmaceutical complex and confirmed its synergistic effect when using the complex.[23] Furthermore, Hong et al also confirmed the antifatigue properties of Gongjin-Dan, a representative traditional oriental herb complex, by regulating the secondary metabolites of serum and muscle.[24] This can be evidence to support many studies that can expect greater synergy in using complexes rather than single substances.

Strenuous exercise induces the secretion of proinflammatory cytokines in muscle.[25,26] The levels of IL-1β and IL-6 are increased in the serum and skeletal muscles of patients with chronic fatigue syndrome.[26] The production of proinflammatory cytokines can be suppressed by blocking NF-κB activation.[27] NF-κB is a transcription factor that regulates the expression of inflammatory genes. Activation of cells by diverse extracellular stimuli causes phosphorylation of IkappaB kinase.[28] Liberated NF-κB then reaches the nucleus and interacts with the κB motif in the promoter region of target genes, such as inflammatory cytokines.[28]

Our results showed that CAA decreased the release of proinflammatory cytokines and effectively inhibited NF-κB activation, suggesting that the antifatigue activity of CAA is mediated by suppressing the inflammatory reaction through the blockade of NF-κB. According to Cheng, deer velvet antler extracts exert anti-inflammatory effects by reducing the release of IL-1β and IL-6 in arthritic mice.[29] CEL also exhibits anti-inflammatory efficacy by inhibiting NF-κB activation in mice with lipopolysaccharide-induced lung injury.[30] In addition, AGN and AMB extracts have been reported to modulate NF-κB activation under inflammatory conditions.[31,32] Thus, the NF-κB signaling pathway is a potential mechanism for the regulation of inflammation by CAA.

Better antioxidant enzyme activities can ameliorate fatigue because the antioxidant defense becomes weaker during fatigue.[33] Antioxidants have been considered as antifatigue agents because they increase catalase activity in the liver.[9] Our results showed that CAA increased catalase levels in the serum and liver tissues. Our results were consistent with a previous study in which Go et al reported high activities of catalase in an exhaustive exercise animal model to confirm the anti-inflammatory effects of AMB.[22]

Kwon et al also investigated the antioxidant and antifatigue effects of a standardized fraction from AGN and other herb complexes, which significantly restored depleted antioxidant enzymes (*e.g.*, superoxide dismutase and catalase).[34] Thus, consistent with previous reports, this research supports that CAA can improve the antioxidant defense of mice after extensive exercise and confer antifatigue capability.

In conclusion, our study demonstrated that CAA regulates fatigue-related secondary metabolites and suppresses

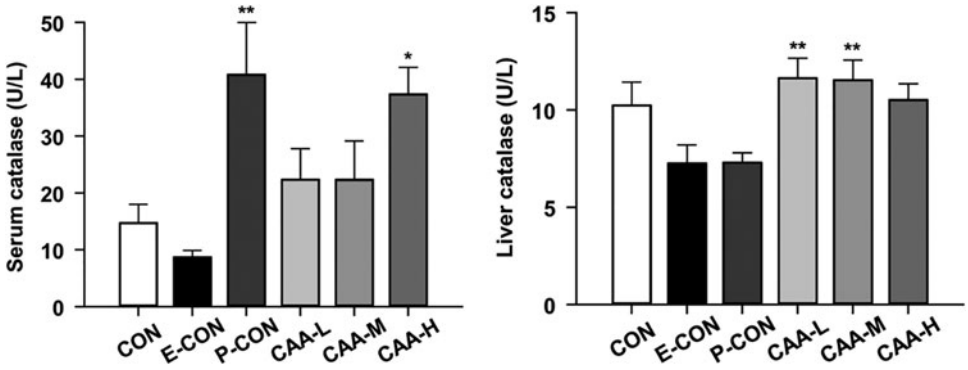

FIG. 6. Effects of CAA administration on catalase activities of mice serums and liver tissues. Data are expressed as means ± standard errors. A *P*-value <.05 was considered statistically significant. * and **indicate statistical significance compared with the E-CON group.

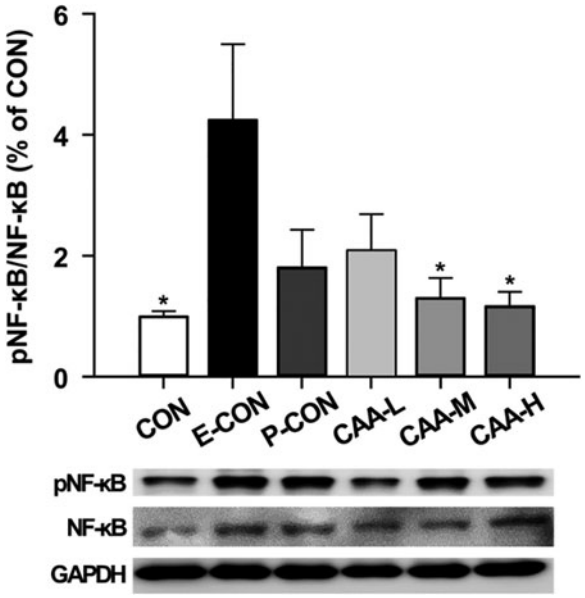

FIG. 7. Effects of CAA administration on expression of NF-κB in mice muscle tissues. Data are expressed as means ± standard errors. A *P*-value <.05 was considered statistically significant. *Indicates statistical significance compared with the E-CON group. NF-κB, nuclear factor kappa B.

inflammatory mediators by attenuating NF-κB activation. Based on these findings, CAA could be useful in fatigue treatment as a potential antifatigue and anti-inflammatory agent.

AUTHORS' CONTRIBUTIONS

Conceptualization was contributed by Y.J.K. and Y.T.K.; investigation was done by W.Y.H., S.J.O., Y.G.H., and M.S.K.; data curation was carried out by W.Y.H. and I.J.; original draft preparation was carried out by W.Y.H., J.H.P., and Y.J.K.; methodology was taken care by W.Y.H., J.H.P., and I.J.; formal analysis was done by W.Y.H., S.J.O., Y.G.H., and M.S.K.; reviewing and editing were carried out by B.K.H., J.H., S.K., J.K.K., E.C.S., and Y.J.K.; funding acquisition and project administration were carried out by Y.T.K., K.W.L., B.J., and D.N.; and supervision was taken care by Y.J.K.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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