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Full paper

Peptides obtained by enzymatic decomposition of mackerel induce recovery from physical fatigue by enhancing the SIRT1-mediated antioxidant effect in the soleus muscle of mice

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ABSTRACT

Fatigue is a serious health problem, and long-term fatigue can lead to mental illnesses and accelerated aging. Oxidative stress, which causes excessive production of reactive oxygen species, is generally thought to increase during exercise and is an indicator of fatigue. Peptides obtained by enzymatic decomposition of mackerel (EMP) contain selenoneine, a strong antioxidant. Although antioxidants increase endurance, the effects of EMP on physical fatigue are unknown. The present study aimed to clarify this aspect. We investigated the effects of EMP on changes in locomotor activity, expression levels of silent mating type information regulation 2 homolog peroxisome 1 (SIRT1), proliferator-activated receptor-γ coactivator-1α (PGC1α), and antioxidative-related proteins including superoxide dismutase 1 (SOD1), SOD2, glutathione peroxidase 1, and catalase in the soleus muscle following EMP treatment before and/or after forced walking. Treatment with EMP before and after forced walking, and not only at one or another time point, improved the subsequent decrease in the locomotor activity and enhanced the levels of SIRT1, PGC1α, SOD1, and catalase expression in the soleus muscle of mice. Moreover, EX-527, a SIRT1 inhibitor, abolished these effects of EMP. Thus, we suggest that EMP combats fatigue by modulating the SIRT1/PGC1α/SOD1-catalase pathway.

1. Introduction

Fatigue has become a serious health concern that requires urgent attention. It is defined as the inability in sustaining or initiating voluntary activities, resulting from hard physical or mental work and severe stress.[1,2] Long-term fatigue increases the risk of psychiatric disorders, Parkinson's disease, and cancer and promotes aging.[3] Fatigue is a complex and encompassing physiological phenomenon without clear etiology and may require long-term medication. Anti-fatigue drugs, such as modafinil and caffeine, often have several deleterious effects.[4] Thus, it is important to find novel anti-fatigue agents or formulations with clear efficacy and fewer side effects.

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; EMP, Peptides obtained by enzymatic decomposition of mackerel; FOXO, forkhead box O; FW, forced walking; GPx1, glutathione peroxidase 1; i.p., intraperitoneally; LH, liver hydrolysate; p, phospho; p.o., per os; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1α; ROS, reactive oxygen species; SOD, superoxide dismutase; SIRT1, silent mating type information regulation 2 homolog 1; t, total.

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Physical exercise-induced fatigue is associated with the production of reactive oxygen species (ROS). Antioxidants delay or promote recovery from fatigue by preventing the accumulation of ROS.[5] Furthermore, activated silent mating type information regulation 2 homolog 1 (SIRT1) increases the levels of antioxidant-related proteins like glutathione peroxidase 1 (GPx1), superoxide dismutase (SOD) 1, SOD2, and catalase by enhancing peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), resulting in anti-fatigue effects.[6–8] These findings suggest that components with antioxidant properties exert anti-fatigue effects.

Selenoneine, the selenium isolog of the natural antioxidant ergothioneine, has strong antioxidative effects.[9,10] Moreover, the antioxidant effects of ergothioneine are associated with SIRT1 activation.[11] Peptides obtained by enzymatic decomposition of mackerel (EMP) are abundant in selenoneine. However, it is unknown whether EMP has an anti-fatigue effect.

Here, we examined the effects of EMP on fatigue in mice, by performing the forced walking test, which is useful for evaluating physical fatigue.[12] Furthermore, we investigated the underlying molecular mechanisms of this effect.

2. Materials and methods

All experiments were performed with the approval of the Ethics Committee of Animal Experiments in Tohoku Medical and Pharmaceutical University (approval numbers: 20051-cn, 21035-cn, and 22061-cn) and according to the provisions of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize suffering and reduce the number of used animals.

2.1. Animals

We used male ddY mice (age: 6–7 weeks, weight: 26–28 g; Japan SLC, Shizuoka, Japan) for all experiments (total: n = 221; behavioral test: n = 197; western blotting analysis: n = 24). The mice were housed 5–6 per cage, under controlled environmental conditions (23 ± 1 °C, 55 ± 5%, 12/12 h light-dark cycle with lights on at 7:00 a.m.), and provided free access to food or water. The behavioral tests were performed between 09:00 h and 17:00 h. All mice were earmarked for individual identification and randomly divided into four groups using a table of random numbers.

2.2. Compounds

EMP, supplied by LS Corporation Co. Ltd., Tokyo, was dissolved in drinking water, and doses of 100, 200, 250, and 500 mg/kg were administered per os (p.o.) in a volume of 0.1 mL/10 g mouse body weight using a 1-mL syringe with an oral probe. EX-527 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), a SIRT1 inhibitor, was dissolved in 0.5% Tween 80 and a dose of 10 mg/kg was administered intraperitoneally (i.p.) 1 h before the first administration of EMP. The dose for EX-527 used was chosen based on a previous report.[13] EX-527 is a potent and highly selective SIRT1 inhibitor and exhibits much lower or no inhibitory activity against other SIRTs.[14–16]

2.3. Forced walking test

The protocol for forced walking has been described previously.[17,18] Briefly, forced walking was imposed on 10 mice in a cylindrical cage (diameter: 37 cm) at 23 ± 1 °C, which was rotated on the horizontal axis at 2.0 rpm by an electric motor, giving a walking speed of 2.3 m/min for 3 h. Control mice (n = 10) were placed in a cylindrical cage without rotation. EMP was dissolved in water and administered before and/or after forced walking. Following forced walking for 3 h, mice were individually placed in a multichannel Supermex activity box (Muromachi Kikai, Co., Tokyo) and allowed to adapt for 15 min before injection of EMP. The locomotor activity was measured every 15 min for 90 min. The protocol for the measurement of locomotor activity has been described previously.[19–24] This instrument can monitor movements per minute in all three planes of motion (sagittal, coronal, and horizontal) as one movement, owing to its infrared sensor with multiple Fresnel lenses that can be moved close enough to the cage to capture multidirectional locomotor alterations. The Supermex instrument was connected to a behavioral analyzing system (CompACT AMS, Muromachi Kikai), which could interpret each movement as one count. Therefore, vertical movements such as jumping, as well as horizontal movements such as walking and running, were counted. Measurements were performed between 12:00 h and 16:00 h, during the light phase.

2.4. Western immunoblotting study

Twenty-four mice were divided into four groups [forced walking 3 h (−)/water, forced walking 3 h (−)/EMP (250 mg/kg), forced walking 3 h (+)/water, and forced walking 3 h (+)/EMP (250 mg/kg)] or two groups [vehicle/forced walking 3 h (+)/EMP (250 mg/kg) and EX-527/forced walking 3 h (+)/EMP (250 mg/kg)]. Mice that had not undergone any behavioral tests were sacrificed by decapitation 15 min after the last administration of the vehicle or EMP. Protein extraction from soleus muscle and Western blot analysis were performed as previously described.[17,25–28] After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes and incubated with blocking solution [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.01% Tween 20, and 5% skim milk]. The membranes were treated overnight at 4 °C with antibodies against phospho-(p-) SIRT1 (1:1000; Bioss Antibodies, Boston, USA, bs-3393R), total (t) -SIRT1 (1:10000; Millipore, Burlington, USA, #07-131), p-adenosine monophosphate-activated protein kinase (AMPK; 1:1000; Cell Signaling Technology, Danvers, USA, #2531), t-AMPK (1:1000; Cell Signaling Technology, Danvers, USA, #2532), PGC1α (1:2000; Abcam Ltd., Cambridge, UK, ab54481), SOD1 (1:5000; Abcam, ab13498), SOD2 (1:5000; Abcam, ab13533), GPx1 (1:000; Abcam, ab22604), catalase (1:2000; Abcam, ab16731) and β-actin (1:1000; Cell Signaling Technology, #4967). The membranes were then washed with blocking solution without 5% skim milk, incubated with horseradish peroxidase-conjugated secondary antibody [1:5000 except for SOD1 (1:2000), SOD2 (1:20000), and GPx1 (1:50000); Cell Signaling Technology, #7074)] for 2 h, and visualized using an enhanced chemiluminescence western blotting detection reagent (Amersham Life Science, Amersham, UK). The band density was measured using densitometry (Image-J 1.43μ, National Institute of Health).

2.5. Statistical analysis

The results of experiments are expressed as mean ± standard error of the mean. The statistical significance of differences was determined using Student's *t*-test for two-group comparisons. The statistical significance of the differences was determined using one- or two-way analysis of variance (ANOVA), followed by the Tukey–Kramer post hoc test for multiple group comparisons using GraphPad Prism 7 (GraphPad Software, California, USA). The criterion of significance was set at $p < 0.05$. In some cases, when a main effect was significant without an interaction effect, an exploratory and limited pairwise post hoc comparison consistent with our a priori hypothesis was performed. All statistical analyses

were performed by investigators other than the experimenters to avoid bias and to ensure blinding.

3. Results

3.1. EMP showed an anti-fatigue effect in the forced walking test

Mice showed significantly reduced locomotor activity after the forced walking test, while treatment with EMP before and after the forced walking test improved this change [One-way ANOVA, $F(4, 42) = 10.65$, $p < 0.0001$, Fig. 1 (D)]. However, this effect was not observed when administered either before or after the forced walking test [One-way ANOVA, $F(2, 27) = 7.715$, $p = 0.0022$, Fig. 1 (B); and $F(3, 66) = 7.677$, $p = 0.0002$, Fig. 1 (C)].

3.2. EMP can activate SIRT1 in the soleus muscle

The two-way ANOVA revealed a significant interaction between group and treatment, as shown in Fig. 2 (B and C), while this result, as shown in Fig. 2 (D), was unchanged for the interaction effect [group: $F(1, 19) = 0.2367$, $p = 0.6322$, treatment: $F(1, 19) = 1.056$, $p = 0.3171$, group × treatment: $F(1, 19) = 8.244$, $p = 0.0098$, Fig. 2 (B); group: $F(1, 19) = 1.189$, $p = 0.2891$, treatment: $F(1, 19) = 3.396$, $p = 0.0810$, group × treatment: $F(1, 19) = 5.037$, $p = 0.0369$, Fig. 2 (C); group: $F(1, 19) = 0.3307$, $p = 0.5720$, treatment: $F(1, 19) = 11.65$, $p = 0.0029$, group × treatment: $F(1, 19) = 2.317$, $p = 0.1445$, Fig. 2 (D)]. Thus, we focused on the effects of EMP in mice, as shown in Fig. 2 (D).

As shown in Fig. 2, EMP administration significantly increased the phosphorylation level of SIRT1 folded by β-actin and the level of t-SIRT1 expression in the soleus muscle compared to the vehicle treatment in the forced walking groups [Fig. 2 (C and D)], while phosphorylation level of SIRT1 folded by t-SIRT1 was unchanged among all groups [Fig. 2 (B)].

3.3. EMP enhanced PGC1α, SOD1, catalase expression levels in the soleus muscle

The two-way ANOVA analysis revealed a significant interaction between group and treatment in Fig. 3 (B and F), while these results in Fig. 3 (C, D, and E) were unchanged about interaction effect [group: $F(1, 20) = 3.783$, $p = 0.0660$, treatment: $F(1, 20) = 6.941$, $p = 0.0159$, group × treatment: $F(1, 20) = 4.841$, $p = 0.0397$, Fig. 3 (B); group: $F(1, 20) = 0.0002503$, $p = 0.9875$, treatment: $F(1, 20) = 18.88$, $p = 0.0003$, group × treatment: $F(1, 20) = 0.3417$, $p = 0.5654$, Fig. 3 (C); group: $F(1, 20) = 0.2696$, $p = 0.6093$, treatment: $F(1, 20) = 1.013$, $p = 0.3262$, group × treatment: $F(1, 20) = 0.1131$, $p = 0.7401$, Fig. 3 (D); group: $F(1, 20) = 0.05706$, $p = 0.8136$, treatment: $F(1, 20) = 7.529$, $p = 0.0125$, group × treatment: $F(1, 20) = 0.679$, $p = 0.4197$, Fig. 3 (E); and group: $F(1, 20) = 2.947$, $p = 0.1015$, treatment: $F(1, 20) = 2.49$, $p = 0.1302$, group × treatment: $F(1, 20) = 5.521$, $p = 0.0292$, Fig. 3 (F)]. Thus, we focused on the effects of EMP in mice in Fig. 3 (C, D, and E).

As shown in Fig. 3, the expression level of PGC1α in the soleus muscle was reduced by forced swimming, while EMP treatment

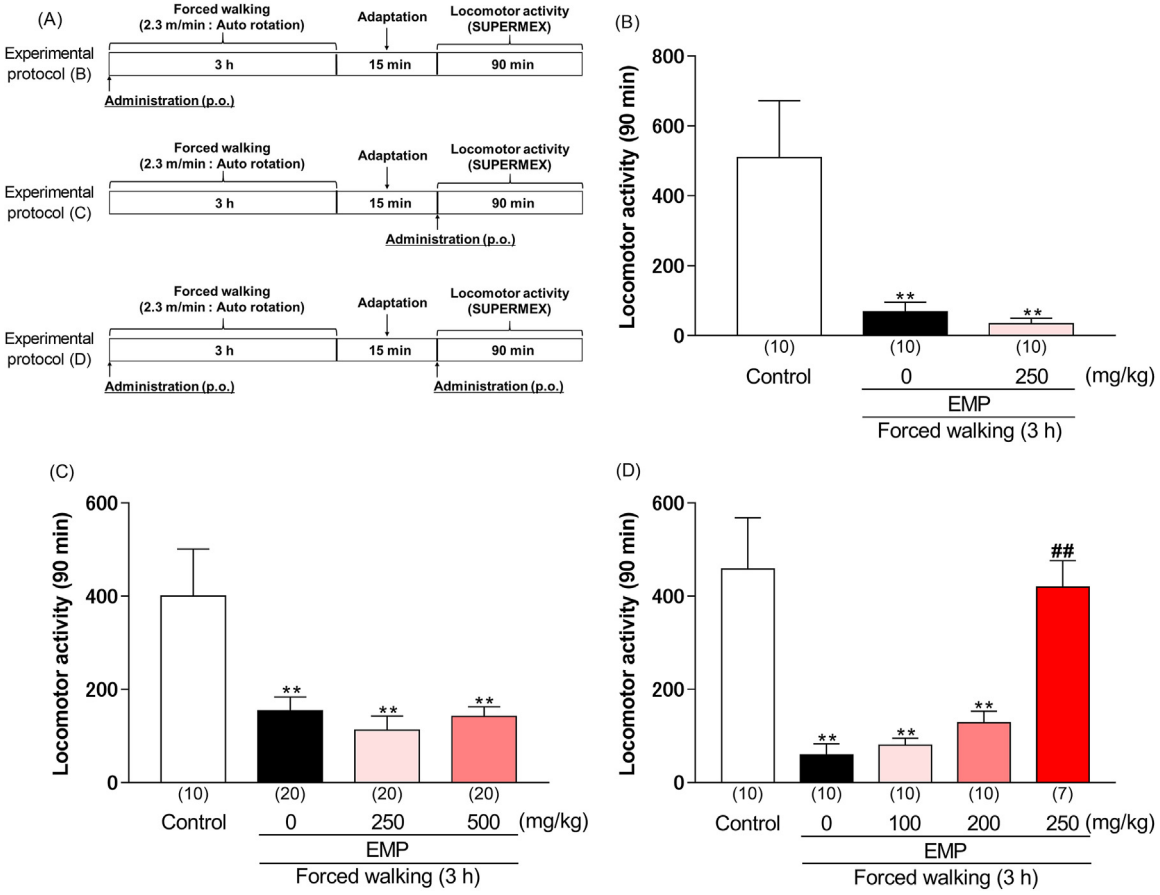

Fig. 1. Effect of EMP on locomotor activity after forced walking for 3 h. (A) Timeline of each experimental protocol. (B–D) Locomotor activity. Numbers in square brackets indicate the number of animals in each group. Bars represent mean ± standard error mean. **: $p < 0.01$ vs. control group. ##: $p < 0.01$ vs. vehicle-treated mouse group.

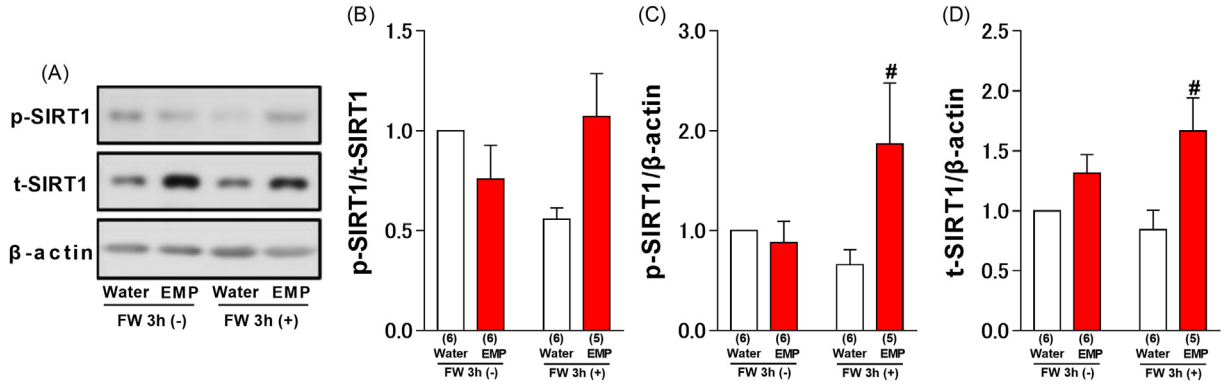

Fig. 2. Effect of EMP administration on the levels of p-SIRT1/t-SIRT1 (B), p-SIRT1/β-actin (C), and t-SIRT1/β-actin (D) in the soleus muscle after forced walking (FW). A: Representative immunoblots probed with antibodies against p-SIRT1, t-SIRT1, and β-actin, as indicated. Quantification of the normalized values of p-SIRT1 (C) and t-SIRT1 (D) levels with β-actin and p-SIRT1 (B) with t-SIRT1, respectively. Numbers in square brackets indicate the number of animals in each group. Bars represent means ± standard error mean. #: $p < 0.05$ vs. control with the forced walking group.

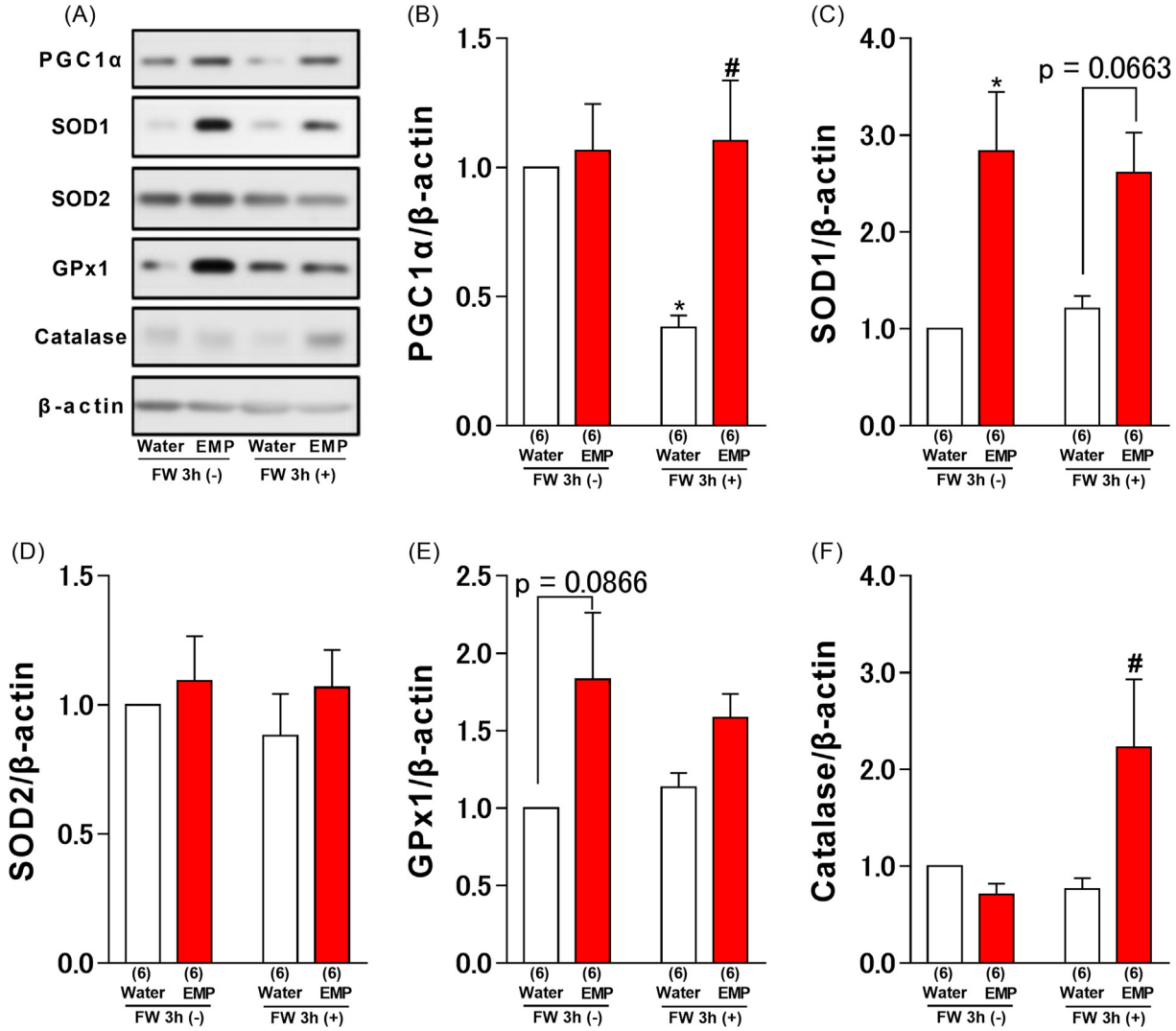

Fig. 3. Effect of EMP administration on the levels of PGC1α (B), SOD1 (C), SOD2 (D), GPx1 (E), and catalase (F) in the soleus muscle after forced walking (FW). A: Representative immunoblots probed with antibodies against PGC1α, SOD1, SOD2, GPx1, catalase, and β-actin, as indicated. Quantification of values for PGC1α, SOD1, SOD2, GPx1, and catalase normalized with β-actin levels in the soleus muscle. Numbers in square brackets indicate the number of animals in each group. Bars represent means ± standard error mean. *: $p < 0.05$ vs. control without forced walking group. #: $p < 0.05$ vs. control with the forced walking group.

improved this change [Fig. 3 (B)]. Moreover, EMP slightly elevated SOD1 ($p = 0.0663$) expression, and did not change the levels of SOD2 and GPx1 expression in the soleus muscle compared to the vehicle treatment in the forced walking groups. In the groups not subjected to forced walking, EMP enhanced SOD1 and GPx1 levels in the soleus muscle compared to the vehicle administration group, while it did not affect PGC1α expression level [Fig. 3 (C and E)].

3.4. EX-527 inhibited the EMP-induced anti-fatigue effect

To determine whether activation of SIRT1 was involved in the EMP-induced anti-fatigue effect in mice, we observed the changes in locomotor activity at the time of co-administration of the SIRT1 inhibitor EX-527 and EMP. The EMP-induced anti-fatigue effect in mice was attenuated by treatment with EX-527 [Two-way ANOVA, treatment: $F(1, 46) = 4.19$, $p = 0.0464$, inhibitor: $F(1, 46) = 6.598$, $p = 0.0135$, treatment × inhibitor: $F(1, 46) = 5.883$, $p = 0.0193$, Fig. 4 (B)].

3.5. EX-527 prevented EMP-induced enhanced levels of SIRT1, PGC1α, SOD1, and catalase expression in the soleus muscle

We investigated whether administration of EX-527 inhibited EMP-induced enhancement in the levels of SIRT1, PGC1α, SOD1, and catalase in the soleus muscle. Treatment with EX-527 attenuated the levels of SIRT1, PGC1α, SOD1, and catalase expression in the soleus muscle compared to the vehicle group after forced walking [Student's t-test: $t = 3.513$, $df = 10$, $p = 0.0056$, Fig. 5 (A); $t = 3.664$, $df = 10$, $p = 0.0044$, Fig. 5 (B); $t = 4.418$, $df = 10$, $p = 0.0013$, Fig. 5 (C); $t = 9.547$, $df = 10$, $p < 0.0001$, Fig. 5 (D)].

4. Discussion

In this study, EMP showed recovery of the forced walking-induced fatigue-like behavior in mice following EMP treatment before and after forced walking. EMP significantly increased the levels of SIRT1, PGC1α, SOD1, and catalase expression in the soleus muscle of mice after forced walking. These effects of EMP were inhibited by the SIRT1 inhibitor, EX-527. This shows that EMP may facilitate recovery from physical fatigue and this effect may involve an increase in the antioxidative-related proteins via the activation of the SIRT1-PGC1α pathway.

Oxidative stress causes the overproduction of ROS which generally increases during physical exercise.[29] Antioxidants might delay fatigue through the removal of ROS.[5] The present study showed that administration of EMP before and after, and not only at one or another timepoint, improved the forced walking-induced decrease in locomotor activity (Fig. 1D), suggesting that the effect of EMP on forced walking-induced fatigue behavior is restorative rather than preventive in mice. EMP contains selenoneine, which has strong antioxidative activity. Thus, we hypothesized that the anti-fatigue effect of EMP may be associated with its antioxidative activity.

Activation of SIRT1 enhances PGC1α activity and improves muscle fatigue.[6,7] PGC1α is required for the induction of many ROS-clearing enzymes, including SOD1, SOD2, GPx1, and catalase.[8] In the present study, the level of t-SIRT1 expression increased significantly when EMP was administered to the forced walking group (Fig. 2D), and no significant change was observed when p-SIRT1 was corrected for t-SIRT1 (Fig. 2B). Therefore, the increase in p-SIRT1 corrected for β-actin when EMP was administered to the forced walking group (Fig. 2C), was likely due to the increase in t-SIRT1. Moreover, we found that in the forced walking groups, compared with the vehicle, EMP significantly increased PGC1α, and catalase in the soleus muscle expression levels, slightly elevated SOD1 expression levels, and did not interfere with SOD2 and PGx1 expression levels (Fig. 3). However, in the groups not subjected to forced walking, compared with vehicle, EMP enhanced SOD1 and GPx1 levels in the soleus muscle, whereas it did not influence PGC1α level [Fig. 3 (C) and (E)]. In the groups not subjected to forced walking, compared with vehicle, EMP enhanced the activation of AMPK in the soleus muscle, while in those subjected to forced walking, no differences were observed in terms of AMPK activation (Supplementary Figure S1B). Activation of AMPK enhances SOD1 and GPx1 levels by increasing Forkhead box O (FOXO) 3a activity.[30] Thus, we consider that in mice not subjected to forced walking, EMP treatment increased antioxidant defense by upregulating the AMPK/SOD1-GPx1 pathway. Moreover, we found that in mice, EX-527 abolished the EMP-induced anti-fatigue effect (Fig. 4B) and enhanced the levels of t-SIRT1, PGC1α, SOD1, and catalase expression in the soleus muscle (Fig. 5). These results indicate that in mice subjected to forced walking, EMP enhanced antioxidant defense via activation of the SIRT1/PGC1α/SOD1-catalase pathway in the soleus muscle (Fig. 6).

The anti-fatigue effect of EMP was only observed when it was administered before and after forced walking. We have previously reported that liver hydrolysate (LH) exerts an anti-fatigue effect when administered twice before and after forced walking,[17] a schedule similar to that used in this study. As LH also has anti-oxidant properties,[31] anti-fatigue substances with anti-oxidant effects may be more effective when administered before and after forced walking.

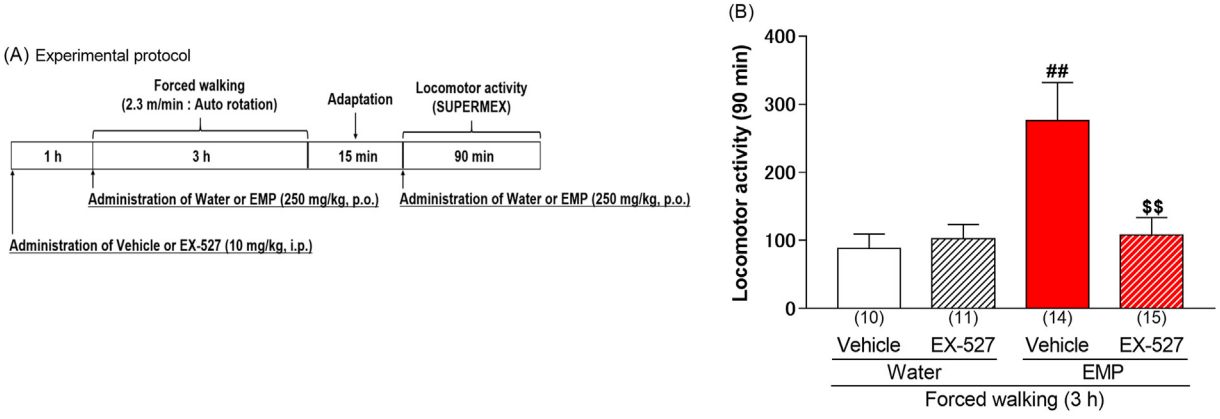

Fig. 4. Effect of EX-527 on EMP-induced anti-fatigue effect in mice. A: Time course of each experimental protocol. Numbers in square brackets indicate the number of animals in each group. Bars represent mean ± standard error mean. ##: $p < 0.01$ vs. water with the forced walking group. $$: $p < 0.01$ vs. EMP with the forced walking group.

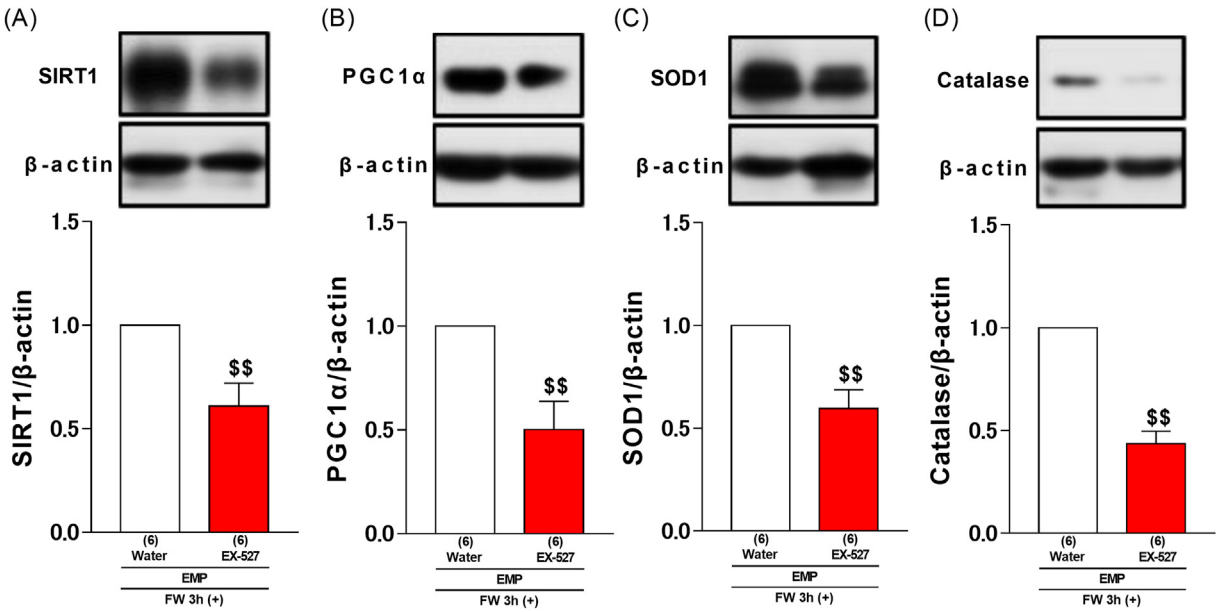

Fig. 5. Effect of EX-527 on EMP-induced enhanced levels of SIRT1, PGC1α, SOD1, and catalase expression in the soleus muscle. Quantification of values for SIRT1 (A), PGC1α (B), SOD1 (C), and catalase (D) normalized with β-actin levels in the soleus muscle. Numbers in square brackets indicate the number of animals in each group. Bars represent mean ± standard error mean. $$: $p < 0.01$ vs. EMP with the forced walking group.

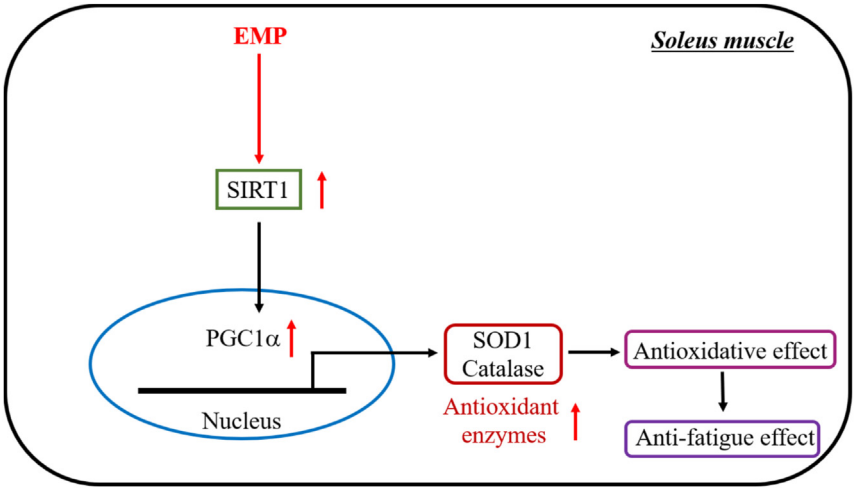

Fig. 6. Schematic representation of anti-fatigue effects of EMP.

In the present study, we determined the effects of EMP on forced walking-induced fatigue-like behavior. However, it remains unclear what components in EMPs are responsible for their anti-fatigue effects in mice. There were no significant differences in SIRT1 and catalase levels in the soleus muscle between the control and forced walking groups. This result indicates that SIRT1 and catalase in the soleus muscle are not affected by the fatigue caused by forced walking for 3 h. However, other researchers have reported that moderate exercise (for 2–6 weeks on a treadmill) activated SIRT1 and induced increased catalase expression.[32] As the organism responds differently to acute exercise and repeated chronic exercise regimens, this difference can be explained. The increase in SIRT1 and catalase levels in the soleus muscle following EMP administration indicates that they may be involved in the anti-fatigue effects of EMP. These questions need to be addressed in future studies.

In conclusion, the present study demonstrated that EMP improved physical endurance concomitantly reducing oxidative stress. Thus, it could be used as a pharmacologic tool for recovery from fatigue.

Credit authorship contribution statement

Osamu Nakagawasai: Conceptualization, Methodology, Writing - original draft, Project administration, Funding acquisition. Kohei Takahashi: Formal analysis, Writing - original draft, Visualization, Funding acquisition. Wakana Sakuma: Formal analysis, Investigation, Data Curation. Wataru Nemoto: Investigation, Data Curation. Ruka Kobayashi: Investigation. Tomohiro Hoshi: Investigation. Satoshi Matsumoto: Supervision, Resources. Takeshi Tadano: Supervision. Koichi Tan-No: Supervision.

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Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declaration of competing interest

Satoshi Matsumoto is employee of LS Corporation Co. Ltd.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2023.03.001.

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