Cornu cervi pantotrichum supplementation improves physiological adaptions during intensive endurance training

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ABSTRACT. Cornu cervi pantotrichum (CCP), used in traditional Chinese medicine, is a well-known yang-invigorating agent with multifunctional bioactivities. We previously showed, through an acute exercise challenge, that short-term CCP supplementation improved physical activities and fatigue-associated biochemical indices. Questions about the long-term effects of CCP treatment on exercise performance and physical fatigue, as well as safety, with intensive exercise training need further research. ICR-strain mice were randomly assigned to three groups: (1) sedentary control and vehicle treatment (SC); (2) exercise training with vehicle treatment (ET); and (3) ET with CCP treatment at 4,108 mg/kg/day (ET+CCP). We assessed the physical performance, body compositions, and serum levels of lactate, ammonia, glucose and creatine kinase (CK) after an acute exercise challenge. The ET and ET+CCP groups had significantly increased grip strength and endurance swimming time, and decreased serum lactate and ammonia levels after the acute exercise challenge than the SC group. Moreover, serum ammonia and CK levels in the ET+CCP group were significantly decreased when compared to that of the ET only group. In regard to the body composition, the ET+CCP group inhibits the decrease in fat tissue, and related biochemical changes induced by the high intensity endurance training CCP supplementation combined with high-intensity endurance exercise could significantly improve the physiological adaptions related to fatigue or energy consumption and maintain the fat composition when compared to treatment with training only. Therefore, CCP may potentially improve the physiological adaptions in intensive exercise training.

KEY WORDS: ammonia, Chinese yang agents, creatine kinase, intensive training, physiological adaption
Appropriate and programmed exercise or training is beneficial for cardiovascular function and the circulatory system and improving exercise performance. It is also considered a preventive medicine, because it can reduce the incidence of chronic disease [25]. High-intensity or exhaustive workouts and consecutive competitions affect the body’s homeostasis, leading to pathological alternations. Physiological adaptations, such as oxidative, immune and metabolic systems, are consequences of regular exercise to maintain good performance under additional stress. However, these systems may be overwhelmed by long-term or high-intensity exercise in the body tissues [1, 9, 11]. Available nutritional supplements include whey protein [5], chicken essence [13], curcumin [14] and resveratrol [31] and have been proven to have beneficial effects, such as enhancement of exercise performance, mitigation of fatigue and increase in recovery efficiency after exercise [21]. Naturally occurring chemicals found in plant food, phytochemicals or crude extracts from natural products can be investigated to understand their potential benefits on exercise physiology and to obtain better insight into their different bioactivities for their potential use in the promotion of health.

In our previous study, we show that supplementation with CCP at the recommended doses (2,054 and 4,108 mg/kg/day) improves grip strength and exercise-relevant physiological biomarkers without exercise training [12]. We used an animal model involving intensive exercise training to further investigate whether CCP had additive effects on the promotion of exercise performance, physiological homeostasis or the amelioration of physical fatigue. We hypothesized that daily supplementation with CCP would have better beneficial effects on physiological adaptations during intensive exercise training intervention.

MATERIALS AND METHODS

Materials

The CCP used in this study was prepared by ethanol extraction (International Total Solution, Inc., Taipei City, Taiwan) with quality and safety certification. Briefly, the raw CCP materials were sliced and extracted three times with a material-ethanol ratio of 1:5. The extracts were filtered to remove debris and concentrated by vacuum drying. The final product obtained was the CCP powder, which was used in this study.

Animals and experiment design

Male 6-week-old ICR-strain mice, purchased from BioLASCO (Yi-Lan, Taiwan), with a specific pathogen-free condition were used in this study. All animals were given a standard laboratory diet (No. 5001; PMI Nutrition International, Brentwood, MO, U.S.A.), distilled water ad libitum, and maintained under a regular photoperiod (12-hr light/12-hr dark) at the conditioned temperature and humidity (24 ± 1°C; 60 ± 5%). The Institutional Animal Care and Use Committee (IACUC) of National Taiwan Sport University evaluated all animal experiments and approved this study as protocol number IACUC-10206.

The administrated doses for this animal experiment could be converted from a human-equivalent dose (HED) based on body surface area, provided by the U.S. Food and Drug Administration, by using different conversion coefficients according to different animal species; the conversion coefficient is 12.3 for mouse species. The administrated dose of CCP in the current study was 4,018 mg/kg of body weight (BW) for the animal experiments, which was equivalent to the recommended dose of approximately 20 g for a 60-kg human. The details of the formula are described in a previous study [5].

All experimental mice were provided with a 1-week acclimation period to adjust to their new environment and diet. Subsequently, they were randomly assigned to three groups (8 mice/group) for the training treatment with either the vehicle or CCP. The groups were as follows: (1) the sedentary control treatment (SC), (2) intensive aerobic exercise training (ET) or (3) the intensive aerobic exercise training with 4,108 mg/kg of CCP supplementation (ET+CCP). The sedentary and ET groups received the same volume of vehicle solution dose-equivalent for their individual body weight. The vehicle and CCP were administrated to each animal by oral gavage daily for consecutive 6 weeks.

ET protocol

In previous definitions of animal swimming endurance, the loading, less than 3% equivalent to the body weight, was defined as aerobic exercise. A frequency of three weekly sections was considered a moderate training protocol, and a five times a week was considered heavy training [24]. Animals in the ET and ET+CCP groups underwent an aerobic swimming training program adapted from our previous study [5]. The specifications of the container were 65 cm and 20 cm in high and diameter, respectively, filled with tap water to a depth of 40 cm, and maintained at 28 ± 1°C. They were trained for 30 min on the first day, 45 min on the second day and 60 min a day thereafter, for 5 days/week during the first week to acclimatize them to water. After the mice had adapted to the swimming exercise, they were subjected to a forced swimming task with a weight, equivalent to 1% to 2% of their body weight, attached to their tails for 5 weeks. The training period consisted of 5 weeks, 60 min per day and 5 sessions per week. The mice began the forced swimming training with a 1% body weight overload attached to their bodies in the first week, i.e., week 2. The training load was then increased to 2% of their body weight during the last 4 weeks (i.e., weeks 3–6). The training protocol is illustrated in Fig. 1. Body weight was measured weekly, and the load was calculated and increased accordingly.

Forelimb grip strength

The forelimb grip strength of mice undergoing vehicle or CCP treatments was measured by a low-force testing system (Model-RX-5, Aikoh Engineering, Nagoya, Japan). The amount of tensile force exerted by each mouse was measured using a force transducer equipped with a metal bar (2 mm in diameter and 7.5 cm in length). The detailed procedures are described in our previous reports [10]. The grip strength was recorded as the maximal force (g).
Swimming exercise performance test

The swim-to-exhaustion exercise test involved mice carrying constant loads corresponding to 5% BW and assessed endurance time, as previously described [5, 13, 14].

Determination of fatigue-associated serum biomarkers

The effects of ET and ET+CCP intervention on serum lactate, ammonia and glucose levels and creatine kinase (CK) activity were evaluated after exercise. The same intensity of challenge, a 15-min swimming test without weight-loading, was performed after 6 weeks from administration. Blood samples were immediately collected from the submandibular duct of the animals after the swimming exercise. The serum was prepared by centrifugation at 1,500 × g, 4°C for 10 min. Lactate, ammonia and glucose levels and CK activity were determined by using an auto-analyzer (Hitachi 7060, Hitachi, Tokyo, Japan). Other clinical biochemical variables were determined using an automatic analyzer (Hitachi 7080, Hitachi) at the end of experiment without fasting.

Histological staining of tissues

After sacrificing the mice, the important organs including the liver, muscles (gastrocnemius and soleus muscles of the lower legs), kidney, testes, epididymal fat pad (EPF), brown adipocyte tissue (BAT), heart and lung were dissected, weighed and snap-frozen in liquid nitrogen before they were stored at −80°C. Another set of liver, muscle, heart, kidney, lung and testes tissues was removed intact and fixed in 10% neutral-buffered formalin for at least 24 hr for histopathologic evaluation, as previously described [5]. Tissues were embedded in paraffin, cut into 4-μm thick slices for morphological and pathological evaluation, stained with hematoxylin and eosin (H&E), and examined using a light microscope equipped with a charge-coupled device (CCD) camera (BX-51, Olympus, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SEM. Statistical differences among groups were analyzed by a one-way analysis of variance (ANOVA) using SAS, v9.0 (SAS Inst., Cary, NC, U.S.A.). Tukey (HSD) post-hoc analysis was used for further comparisons within each testing group. A P value <0.05 was considered statistically significant.

RESULTS

Effect of CCP supplementation with training on forelimb grip strength

Grip strength differed significantly among the treatment groups (F[2,21]=15.684, P<0.05, η²=0.599). Grip strength was significantly higher in ET mice and ET+CCP mice, by 1.18- and 1.19-fold (all P<0.01), respectively, when compared to that of SC mice (Fig. 2).

Effect of CCP supplementation with training on the exhaustive swimming test

The exhaustive swimming test is a practical method for assessing exercise performance, as well as an indicator of fatigue during endurance exercises. The swimming time was significantly longer by 1.94- (P=0.0252) and 1.85-fold (P= 0.0415) with ET and ET+CCP treatment, respectively, when compared to the SC group (Fig. 3).
Effect of CCP supplementation with training on fatigue-associated biomarkers after exercise challenge

The status of muscle fatigue after exercise can be assessed by important biochemical biomarkers, including lactate, ammonia and glucose levels and CK activity [5, 10, 13, 14, 16, 21]. As shown in Fig. 4a, serum lactate levels significantly differed among the treatment groups ($F[2,21]=92.38$, $P<0.05$, $η^2=0.898$). It was reduced by 52% in ET mice and by 59.6% ($P<0.001$) in ET+CCP mice, respectively, when compared to the SC group. The serum lactate level in ET+CCP group was not significantly lower than the ET only group ($P=0.137$). Serum ammonia levels differed significantly among the treatment groups ($F[2,21]=25.21$, $P<0.05$, $η^2=0.706$). ET and ET+CCP treatments significantly decreased serum ammonia levels by 34.7% and 70% ($P=0.0236$ and $P<0.0001$), respectively, relative to SC (Fig. 4b). Remarkably, serum ammonia levels in the ET+CCP group were also significantly lower than those in the ET only group ($P=0.0210$).

During exercise, efficient utilization of glucose is an important index for maintaining performance by a mechanism independent of the insulin signaling pathway [20]. However, the glucose levels were not significantly different between the groups. The CK levels significantly differed between the treatment groups ($F[2,21]= 4.39$, $P<0.05$, $η^2=0.295$) (Fig. 4c). Although ET treatment led to a reduction in CK levels, compared to SC, this reduction was not statistically significant (Fig. 4d). ET+CCP treatment reduced CK levels by 67.5% ($P=0.0110$), in comparison to SC.

Effect of CCP supplementation with training on the general characteristics of the growth curve, diet and organ weights

The development and validation of physiological bioactivities in the naturally occurring compounds are important for the promotion of health, but the safety of these compounds is important to consider them edible. Therefore, pathological evaluations could provide insights into subacute toxicity based on OECD guideline 407 (repeated dose 28-day oral toxicity). We evaluated the general characteristics, such as behavior, the growth curve, food intake and organ weights of mice with gradually increasing CCP supplementation and exercise training. As shown in Fig. 5, body weight did not significantly differ initially between the SC, ET and ET+CCP treatment groups until after 6 weeks of intervention ($F[2,21]=4.25$, $P<0.05$, $η^2=0.288$). The body weight of the ET group was significantly lower than that of SC and ET+CCP groups ($P=0.0333$ and $P=0.0282$, respectively), but no significant difference was observed between the ET+CCP and SC groups ($P=0.9300$).

The ET and ET+CCP treatment groups did not differ in observations of their behavior, such as activities and socialization, when compared to that of the SC group. The food and water intake in ET-treated mice were significantly higher by 1.07-fold ($P=0.0001$) and lower by 11.67% ($P=0.0010$), respectively, when compared to that of the SC group. There was no significant difference between the SC and ET+CCP groups (Table 1). In regard to their body compositions, the weight of the liver differed significantly between the treatment groups ($F[2,21]=9.04$, $P=0.0010$, $η^2=0.463$). The liver weight of the ET group was significantly lower than that of the SC and ET+CCP groups ($P=0.0110$ and $P<0.0001$, respectively), but no significant differences were observed between ET+CCP and SC groups ($P=0.3400$). The weight of the epididymal fat pad (EFP) differed significantly between the treatment groups ($F[2,21]=5.27$, $P=0.0140$, $η^2=0.334$). The EFP weight in ET mice was decreased by 39% relative to SC mice ($P=0.0069$), but there was no difference between the ET+CCP mice and ET and SC mice. The weight of brown adipose tissue (BAT) significantly differed among the treatment groups ($F[2,21]=39.23$, $P=0.0001$, $η^2=0.789$). ET treatment raised the BAT weight by 25% when compared to the control (SC group; $P=0.0248$). Moreover, CCP supplementation with ET treatment increased the BAT weight by 40% when compared to ET treatment only ($P=0.0248$). The other tissues or organs, including muscle, heart, kidney and testes did not exhibit significant differences among the three groups.
Fig. 4. Effect of *Cornu cervi pantotrichum* supplementation with training on serum (a) lactate; (b) ammonia; (c) glucose; and (d) creatine kinase (CK) levels after an acute exercise challenge. Data are expressed as mean ± SEM for the 8 mice in each group. Different letters indicate a significant difference of *P*<0.05, assessed by a one-way ANOVA.

Fig. 5. Effect of *Cornu cervi pantotrichum* supplementation with training on growth. Data are expressed as mean ± SEM for the 8 mice in each group. Different letters indicate a significant difference of *P*<0.05, assessed by a one-way ANOVA.
Effect of CCP supplementation with training on clinical biochemistries

The physiological and biochemical parameters were also assessed in order to evaluate the effects of ET and ET+CCP intervention. As shown in Table 2, the creatinine levels were significantly different among the treatment groups \((F[2,21]=5.12, P=0.015, \eta^2=0.328)\). The creatinine levels in ET mice increased by 25.9% \((P=0.0025)\) relative to SC mice, but there were no differences between the ET+CCP mice and SC and ET mice. Remarkably, triacylglycerol (TG) levels were significantly different among the treatment groups \((F[2,21]=19.581, P<0.0001, \eta^2=0.651)\). The TG in ET mice was reduced by 52.7% relative to SC mice \((P<0.0001)\), but the ET+CCP treatment significantly increased the TG level by 42.7% relative to treatment with ET \((P=0.038)\). The other biochemical parameters, including aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), albumin, total protein (TP), blood urea nitrogen (BUN), uric acid (UA), total cholesterol (TC) and glucose levels were not significantly different between the three groups.

Effect of CCP supplementation on histological examination

Morphological observations showed that no histologic changes were observed in the arrangement of sinusoid and hepatic cords in the liver between the groups (Fig. 6). Furthermore, Zenker’s degeneration, hypertrophy and hyperplasia were not found in the cardiomyocytes or skeletal muscles. In addition, the structure of the renal tubules and glomeruli did not differ between the treatment groups. The convoluted seminiferous tubules also consisted of a normal range of spermatogenic cells, including spermatogonia and spermatocytes, in each mouse.

DISCUSSION

In our survey of the use of nutritional supplementations by athletes, we found that the proportion of TCM supplements used was relatively higher than any other nutrient supplements. Among those, CCP supplements were one of the preferred candidates, because of its medical efficacies. Exercise performance and physiological effects with evidence-based validations have been...
Fig. 6. Effect of *Cornu cervi pantotrichum* supplementation with training on the morphology of (a) liver; (b) skeletal muscle; (c) heart; (d) kidney; (e) lung; and (f) testis in mice. Specimens were photographed by a light microscope (H&E stain, magnification: ×200; Scale bar, 40 μm).
addressed by several studies. Previous studies show that a 2-weeks supplementation of CCP could improve the grip strength and ameliorate the physiological biomarkers that are related to exercise, including lactate and ammonia levels, CK activity and superoxide dismutase without training intervention [13, 15]. Therefore, there were several studies investigating the effects of CCP supplementation combined with training or exercise intervention. Evidence has shown that the performance (strength and endurance) could be significantly improved [15, 26] via the possible regulation of troponin mRNA expression in skeletal muscle [4]. However, a contradictory report shows that CCP supplementation combined with 10 weeks of training did not significantly improve rowing performance in rowers [27]. In the current study, we also found that CCP supplementation combined with training (ET+CCP group) did not significantly elevate performance when compared to the group that received ET treatment only (Figs. 2 and 3), which is consistent with the previous report. Therefore, our data suggest that CCP with the implementation of exercise training does not further improve exercise performance significantly. However, the CCP supplementation could still bring more benefits to the physiological adaptations of fatigue-related biomarkers, including ammonia levels and CK activity, in response to exercise training (Fig. 4).

With regard to the body composition, we found that the weight of several organs significantly differed among the treatment groups. The weights of brown adipocyte tissue (BAT) in mice with ET or ET+CCP treatments were significantly higher than that of the SC group. The ET+CCP group was even more significantly higher than the ET group (Table 1). The BAT metabolic activity could be a casual factor of obesity and type 2 diabetes. It is defined as an adaption to defend against cold and maintain body temperature via the uncoupling of protein 1, in contrast to the function of white adipocytes, which is to store energy [23]. Previous reports also show that training enhances the regulation of Ucp1/Ucp3 mRNA, which could result in higher energy efficiency [6]. In the current study, ET+CCP treatment may induce an increased activation when compared to treatment with ET and result in a marked elevation in BAT weight, which leads to better metabolic or energy efficiency. From the point of view of energy expenditure, it could coincide with the findings from other biochemical analysis as shown in Table 2. The TG released from tissues was significantly higher in the ET+CCP treated group than that of the ET treated group. This suggests that the muscle could efficiently utilize the lipid as an energy source for aerobic exercise, which is the main energy system used for long distance running.

The findings from studies on energy expenditure during a marathon implicate lipid as the preferred fuel, which ensures an optimal-time marathon [3]. The pace that corresponds to one’s aerobic threshold is generally the approximated marathon pace. Marathon athletes want to increase their capacity of aerobic threshold, because it will help them to run at a faster pace for a longer duration before they tip into anaerobic metabolism, which cannot be sustained for long. Therefore, the programmed training strategy and the energy support system are essential for better performance. During practice training for a marathon, the athletes received highly intensive training recipes to improve their aerobic and anaerobic thresholds during their preparation and for a specific period of time. Their extremely low fat percentage, commonly found in long-distance runners or endurance athletes, could be an important factor for the prediction of their marathon race time [28] and correlates to their performance [2]. However, excessive sports activity can be deleterious to the young female population, because of their lower BMI and menstrual irregularity [7]. In the current study, the results from the changes in the growth curve and body composition revealed that the long-term CCP supplementation could significantly ameliorate the loss of body weight induced by intensive aerobic training (Fig. 5) and maintain the fat tissues (Table 1). From a physiological viewpoint, the maintenance of weight and fat composition is relevant to hormone homeostasis and could be beneficial towards the provision of sufficient lipids for long-term aerobic exercise.

Safety is a concern when considering the use of specific extracts or herbs as plant-derived nutritional, medicinal or health-care products. Previous results from studies involving elk velvet antler consumption show no deleterious effects on growth, development and behavior and may have a hepatobeneficial effect [8]. Although the liver damage-related indices (AST and ALT) were decreased in mice receiving ET+CCP, compared to mice receiving ET, this change did not reach statistical significance. Histological data related to the pathological effects of CCP provided in the current study are indicative of the bioactivity doses. We found no changes in the arrangement of the sinusoid and hepatic cords with CCP treatment in mice and no reactive hypertrophy or hyperplasia in the cardiomyocytes and rhabdomyocytes of the gastrocnemius muscles. Our results also showed that CCP supplementation did not induce any significant alternations in the structure of renal tubules and glomeruli, or in the alveolar, bronchial and interstitial space.

In summary, CCP supplementation (ET+CCP group) could significantly improve the physiological fatigue biomarkers (ammonia and CK) even better than exercise training alone. It suggests that CCP has potential effects on enhancing recovery or physiological adaptations after intensive exercise training. CCP supplementation could also be beneficial for the improvement of energy utilization and the maintenance of body weight or composition under the influence of intensive aerobic intervention for optimized physiological conditions. Evidence obtained from the pathological and biochemical evaluations also provides information regarding the safety of CCP supplementation. Therefore, CCP could be an optional nutritional supplement for intensive aerobic exercise with profound physiological benefits.

CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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REFERENCES


