Master's Thesis of Sport Science

Identification of Overtraining Biomarkers by Proteomic Analysis of Central Nervous System-derived Extracellular Vesicles

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Abstract

Individuals undergo training to optimize their physical capacity and performance. However, it is crucial to incorporate recovery periods to prevent fatigue and central nervous system (CNS)-related symptoms, such as negative mood, behavioral changes, lack of motivation, and poor sleep quality. The mechanism for CNS-related symptom manifestation is unknown, which has hindered overtraining syndrome biomarker identification. In CNS diseases, CNS-derived extracellular vesicles (EVs) present disease- or fatigue-related content, thus, they may be crucial for investigating overtraining biomarkers. Therefore, this study investigated CNS-derived EV protein content under overtraining conditions to identify potential biomarkers. Eight-week-old C57BL/6J mice underwent an 8-week downhill treadmill overtraining protocol. Aerobic exercise capacity, high-intensity exercise tolerance, muscular strength, and body coordination were assessed using incremental loading, exhaustion, grip strength, and rotarod tests. Pro-inflammatory cytokines were measured in blood plasma, skeletal muscle, and the CNS to evaluate the systemic inflammatory response to overtraining. Nanoparticle tracking analysis confirmed EV size, and Western blotting validated protein markers. Isolated EVs underwent proteomic analyses. Mice exhibited a significant decrease in exhaustion velocity during aerobic exercise, high-intensity tolerance, and muscular strength tests.

Both skeletal muscle and brain tissues displayed significantly elevated levels of pro-inflammatory cytokines in the overtraining group, whereas blood pro-inflammatory cytokine levels were higher in the overtraining group. Proteomic analysis of CNS-derived EVs revealed a decrease in fat metabolism-related proteins. Valosine-containing proteins and catalase, upregulated in organs experiencing oxidative stress, showed increased levels. Therefore, CNS-derived EV protein content indicated CNS fatigue under overtraining conditions.

Keyword : Overtraining, Extracellular Vesicles (EV), Exosome, Central nervous system (CNS), CNS fatigue
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I. Introduction

1. Need for research

The primary objective of training for all sports participants is to enhance their physical capacity. High-intensity and voluminous training programs play pivotal roles in inducing overcompensation, ultimately leading to improved physical performance. However, to facilitate training adaptations, it is imperative to incorporate adequate recovery periods. Fatigue resulting from overloaded training sessions may induce acute performance decrement (Halson & Jeukendrup, 2004). An imbalance between the training and recovery cycle can manifest to functional overreaching (FOR), nonfunctional overreaching (NFOR), or overtraining syndrome (Meeusen et al., 2013). According to Meeussen, while FOR occurs a short-term performance decrement without serious psychological or physiological issue, it eventually leads to an improvement in performance after days of recovery. Trainees who do not undergo sufficient recovery at FOR may experience NFOR, which may take weeks to fully recover with involvment of psychological or physiological issue. Prolonged exposure to such training without any recovery period in NFOR state may ultimately lead to overtraining syndrome (Meeusen et al., 2013).

In addition to physical function decline, overtraining syndrome typically involves increased inflammation level, negative mood and behaviors, lack of motivation (H. KUIPERS, 1988), and poor sleep quality (Campbell et al., 2021). These symptoms may develop into persistent malfunction states, which are followed by long term slumps, depression, choronic mental disease, and early

retirement for athletes in fields. Therefore, prevention of overtraining syndrome through strategic training sessions is crucial. However, despite the identification of overtraining symptoms, the mechanisms underlying overtraining syndrome remain unclear.

ResearcResearchers have explored the mechanisms and the relationship between overtraining symptoms and central nervous system (CNS) fatigue. Symptoms such as depression, linked to imbalanced serotonin and dopamine levels in the CNS (Meeusen et al., 2007), and degraded sleep quality, associated with disrupted circadian rhythm-related genes (Zisapel, 2001), have been investigated. While the mechanisms of CNS-related diseases are well-established, there is currently no clear mechanism or reliable marker for overtraining syndrome or CNS fatigue (Anderson et al., 2021; Halson & Jeukendrup, 2004; Purvis et al., 2010).

This study focused on CNS-derived extracellular vesicle (EV) proteins as potential biomarkers for CNS fatigue in overtraining syndrome. EVs, secreted from cells and tissues, mediate intercellular communication and reflect the conditions of the originating tissue and cell through miRNAs, mRNA, and proteins (Tkach & Théry, 2016). In cases of parkinson's disease, increased α-synuclein in CNS-derived EVs serve as a biomarker, indicating its elevation within the brain and reflecting CNS conditions (Si et al., 2019). Given the absence of a clear mechanism or biomarker for overtraining syndrome, this study aimed to identify overtraining syndrome by investigating the protein content of CNS-derived EVs under overtraining conditions and identify potential biomarkers.

2. Purpose of Research

First, Investigate the protein contents of CNS-derived EVs under overtrained condition. Secondly, Identify biomarkers for overtraining by CNS-derived EVs.

3. Hypothesis

To clarify the purpose of this study, the following hypotheses were established. First, CNS-derived EV proteins will change under overtrained condition. Second, CNS-derived EV proteins has the role as a biomarker of overtraining by reflecting CNS fatigue.

II. Study Background

1. Overtraining syndrome

FOR and NFOR occurs after several training sessions with high intensity. It acutly decreases physical performance, but it can be recovered with rest sessions. Prolonged exposure to such training without any recovery period in NFOR state may ultimately lead to overtraining syndrome (Meeusen et al., 2013). In this reason, regulation of training intensity and monitoring fatigue is important to prevent overtraining syndrome. Beside decrement of physical performance, there is no clearfied marker to identify overtraining syndrome.

Overtraining syndrome typically involves in CNS related symptoms, such as negative mood and behaviors, lack of motivation (H. KUIPERS, 1988), and poor sleep quality (Campbell et al., 2021; Meeusen et al., 2013). In this reasons, researchers have been investigating CNS under overtraining to understand the mechanisms of overtraining and relation ship between CNS and overtraining. Researchers have observed that overtraining leads to an elevation of pro-inflammatory cytokines in the blood, skeletal muscle, and hypothalamus (da Rocha et al., 2019). Smith suggested that the accumulation of fatigue in skeletal muscle induces circulation of inflammatory cytokines through the blood, influencing the CNS. The impacted CNS may trigger mood-related behavioral disorders (Smith, 2000). Despie of the theory of Smith, clearfing follow-up study have not been done yet. In this reason, mechanisms of overtraining and relationship between CNS are still uncleared.

2. Exercise and EV

Extracellular vesicle (EV) are secreted from cell and tissue, mediates intercellular communication and represent condition of originated tissue and cell by their contents such as miRNA and proteins with in their lypid bylayer (Tkach & Théry, 2016).

It is well known that exercise stimulates the release of molecules that supports the situations. Reaserchers found EVs are circulating during various situation such as inflammatory situations, disease, and exercise with various contents that represents the situation During acute exercise, EVs are including glycolysis related protein mostly in there vesicle, sending signal through whole body as response of exercise stimulation (Whitham et al., 2018). Beside EV proteins, it is researched that EV from acute exercise induced fatigue has shown PI3K-AKT, mitogen-activated protein kinase, and insulin pathway related miRNAs at the urinary EVs (Park & Moon, 2022). Beside acute exercise, T cell dysfunction signals are included at tumer derived EVs (Seo et al., 2018).

3. CNS derived EV

EVs are secrets from almost all kind of cells and organs. CNS-derived EVs are characterizable by L1 cell adhesion molecule (L1CAM), known as CNS-derived EVs membrane protein marker. CNS-derived EVs are isolates by immunoprecipitation based two-step EV isolation method (Mustapic et al., 2017).

As mentioned prevously, CNS-derived EVs are useful method for investigating CNS or brain related disease, such as Parkinson's disease (Shi et al., 2014), alzhimer (Pulliam et al., 2019), and amyotrophic lateral sclerosis (Basso et al., 2013). In detail, An increase in α-synuclein in CNS-derived EVs serves as a potential biomarker for Parkinson's disease, as it is associated with its elevation within the brain. It shows that CNS-derived EVs are indicating CNS condition (Shi et al., 2014).

III. Material and Method

1. Animal experiment

For For the animal experiments, C57BL/6J mice (8 weeks old) were categorized into sedentary (SED, n = 7), exercise control (EX, n = 8), and overtraining (OT, n = 9) groups. The mice underwent an 8-week downhill treadmill protocol known to induce overtraining syndrome (Pereira et al., 2012). Forty-eight hours before the commencement of the 8-week exercise protocol, exhaustion velocity (EhV) was measured through the incremental loading test (ILT).

The 8-week exercise program initiated at an intensity of 60% of EhV for 15 min in the first week and increased by 15 min each week until reaching 60 min in the fourth week. After 4 weeks of exercise, ILT was conducted to determine the new EhV. The EX group maintained an intensity of 60% EhV and exercised for 60 min from the fifth to the eighth week. In the sixth week, the OT group exercised at an intensity of 70% of EhV for 60 min with a –14% inclination. The intensity and duration increased by 75% after 75 min. In the eighth week, the OT group performed two sessions of 75 min of exercise at 75% EhV with a 4 h rest period.

ILT, exhaustion test, grip strength test, and rotarod test were conducted 48 h before the commencement of the 8-week exercise, 48 h after the last exercise session in the fourth week, and 48 h after the last exercise session in the eighth week. To mitigate acute exercise effects, the mice were euthanized 48 h after the last exercise capacity test session.

The ILT involved mice running at an intensity of 6 m/min at 0%, with increments of 1 m/min every minute until exhaustion, defined by the mice stopping at the end of the treadmill five times in 1 min. Physical prodding was used to encourage mice. The EhV determined the intensity of the OT protocols.

The grip strength test, performed 4 h after ILT, assessed muscle strength. Mice were gently held by their tails and allowed to grasp the horizontally positioned metal grid of a Grip Strength Device (Bioseb). Each mouse underwent three trials with a 3 min rest between trials.

Twenty-four hours after ILT, mice ran at 36 m/min until exhaustion with physical stimulation. Exhaustion was determined when the mice stopped at the end of the treadmill five times in 1 min, and the failure time was recorded.

Motor coordination and balance were evaluated using the rotarod test (Jeong-Do Bio & Planet Co., Ltd.). Mice were individually placed on the rotarod at an initial intensity of 10 rpm, reaching a final intensity of 40 rpm after 300 s. Mice underwent a 5 min warm-up at 10 rpm and two main trials with a 5 min rest between trials in the cage. The latency to fall and the number of falls were recorded.

2. Blood Plasma EV Collection

For animal experiments, blood was collected via cardiac puncture using a 1 mL syringe (Sterile Hypodermic Syringe, Korea Vaccine Co., Ltd.) and transferred to an anticoagulant-treated 3 mL tube (13 × 75 mm 3.0 mL BD Vacutainer® plastic P700 plasma tube. Lavender BD Hemogard™ closure, SKU: 366473, BD).

The whole blood was centrifuged for 10 min at 1,000xG, 4°C and the supernatant underwent an additional centrifugation for 10 min at 2,000 × g, 4°C. Isolated plasma was aliquoted into 300 μL portions for EV isolation and 10 μL aliquots. Plasma was mixed with an equal amount of distilled water (DW) and centrifuged for 10 min at 4,500 × g, 4°C. The supernatant was mixed with Exoquick exosome precipitation kit following the manufacturer's protocol (63 μL of Exoquick solution per 250 μL of biofluid sample). After 1 h of incubation, samples were centrifuged for 30 min at 1,500 × g at 4°C. The pellet was resuspended in phosphate-buffered saline (PBS) over 3 h on a rotating mixer (KBT, Korea) after gentle mixing by pipet.

3. CNS-derived EV isolation

To isolate EVs from the CNS, the L1 cell adhesion molecule (L1CAM), a membrane protein of CNS-derived EVs, was chosen for immunoprecipitation. An IgG isotype control (Mouse IgG2a kappa Isotype Control Biotin) served as a negative control. Fifty microliters of 3% bovine serum albumin (BSA) containing 4 μg of L1CAM antibody (CD171 Monoclonal Antibody (eBio5G3 (5G3)), Biotin, eBioscience™) was added to 500 μL of EV + PBS and incubated for 1 h at 4°C with gentle rotation on a mixer.

After 1 h of incubation, 25 μL of 3% BSA containing 15 μL of Streptavidin beads (Thermo Scientific™ Pierce™ Streptavidin Plus UltraLink™ Resin) was added and incubated for 30 min at 4°C with continuous mixing. Samples were then centrifuged for 10 min at 200 × g, 4°C, and the supernatant was discarded. The remaining pellet was resuspended in 200 μL of 0.1 M Glycine-HCl pH 2.2 (0.1 M Glycine-HCl buffer, Tech&Innovation) with 10 s of vigorous vortexing, followed by gentle rotation for 1 min, and centrifuged for 10 min at 4,500 × g, 4°C to detach L1CAM + EV from the antibody-beads complex.

One hundred microliters of 1 M Tris-HCl (Invitrogen™ UltraPure™ 1M Tris-HCl, pH 8.0) and DW was added to the supernatants in a fresh 1.5 mL tube to normalize the pH at 7. Then, 10% of the sample was aliquoted and diluted to 1:200 in PBS for nanoparticle tracking analysis (NTA). A total of 200 μL of M-PER with protease inhibitor and phosphorylation inhibitor was added to the remaining 90% of the sample to lyse and extract proteins from L1CAM + EV. The final suspensions were stored at –80°C for the next step

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4. NTA

The mean diameter (nm) and concentration (particles/ml) of EV were determined using a Nanosight LM10 system with a 642 nm laser module and NTA 3.1 nanoparticle tracking software (Malvern Instruments, Malvern, UK)

5. Western blot

Samples were loaded onto a 4–12% NuPAGE Bis-Tris Mini gel (Invitrogen) and ran at 200 V for 30 min. The iBlot™ 2 Gel Transfer Device (Invitrogen) was used for transfer following the manufacturer's protocol.

Primary antibodies including L1CAM (CD171 Monoclonal Antibody (eBio5G3 (5G3)), Biotin, eBioscience™), CD81 (CD81 Antibody (B-11), Santa Cruz), NeuN (NeuN (D4G4O) XP® Rabbit mAb, Cell Signaling), CD9 (Anti-CD9 Antibody, Mouse Monoclonal, Sino Biological Inc.), IL-1β (IL-1β (3A6) Mouse mAb, Cell Signaling), IL-6 (IL-6 Monoclonal antibody, Bioteintech), TNF-α (TNF Alpha Polyclonal antibody, Cell Signaling), VCP (VCP (7F3) Rabbit mAb, Cell Signaling), catalase (CAT; Catalase Antibody (H-9), Santa Curz), and GAPDH (GAPDH (14C10) Rabbit mAb, Cell Signaling) were used, with Streptavidin-HRP (Invitrogen™), anti-mouse (m-IgGκ BP-HRP, Santa Cruz), and anti-rabbit (Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling) serving as secondary antibodies

6. Proteomic analysis

In the FASP digestion section, protein samples were quantified using the BCA assay. The digestion process involved protein reduction with 500 mM TCEP, alkylation with 500 mM IAA, urea treatment, and trypsin digestion in two rounds. After each step, components were separated via centrifugation, and the final product was dried under vacuum.

Following FASP digestion, desalting was performed using C18 Micro Spin-Columns. This involved sequential centrifugation with different solvents to remove salts. The samples were dissolved in solvent A, loaded onto the column, and centrifuged to separate the flow-through. Solvent A was repeatedly added and removed, and finally, Solvent B was used to elute the peptides. Desalted samples were then dried for storage.

For LC-MS/MS analysis, specific column types and mobile phases were utilized. Chromatographic conditions included a gradient of Solvent B over time. The mass range for analysis was set between 400 and 2,000 m/z. Data analysis was conducted on LC-MS/MS data from each sample using Proteome Discoverer. The Mus musculus database from UniProt was used, considering modifications such as acetyl, oxidation, carbamyl, and carbamidomethyl groups during the analysis.

7. Cytokine array

Samples were analyzed using a semi-quantitative Mouse Cytokine Array GS1 (RayBiotech, Inc.), detecting 20 proteins in one experiment. Following the manufacturer's instructions, the microarray-based antibody array was blocked, treated with samples, and incubated with a secondary biotinylated antibody mixture. After washing, Cy3-conjugated streptavidin was used for fluorescent detection. Signals were detected using a laser scanner (GenePix 4100A, Molecular Devices) and analyzed with GenePro7.0 software (Molecular Devices).

8. Statistical analysis

Statistical analyses were performed using GraphPad Prism v.9 software (GraphPad Software Inc.). Mean ± standard deviation (SD) is presented in all diagrams and data. Student's t-test was conducted for the EX and OT groups. One-way analysis of variance (ANOVA) identified statistically significant differences among SED, EX, and OT. A two-way ANOVA test was performed to detect changes in physical performance throughout the weeks between the SED, EX, and OT groups. A p-value < 0.05 was accepted as statistically significant.

IV. Results

1. 8-weeks of overtraining occurred physical performance decrement

In Figure 1A and B, it can be seen that SED mice gained more body weight than EX and OT over the 8 weeks, but no statistical significance was found in any of the mice groups.

A performance parameter test was conducted before training (W0), after the fourth week, and after W8. ILT was utilized to investigate maximal velocity during aerobic training. Figure 1C illustrates an increase in exhaustive velocity from W0 to W4 for both EX ($p < 0.05$) and OT ($p < 0.01$) with statistical significance. After W8, EX showed an increased EhV from W4 to W8, but the difference was not statistically significant, whereas W0 to W8 showed higher significance ($p < 0.001$) than W0 to W4. OT significantly decreased EhV from W4 to W8 ($p < 0.001$), with a lower average than that at W0.

Exhaustion tests were conducted to investigate high-intensity exercise tolerance. EX significantly increased the time to exhaustion from W0 to W8 ($p < 0.01$), while OT significantly decreased the time to exhaustion from W4 to W8 ($p < 0.05$), although it showed a tendency to increase from W0 to W4. SED had no tendency.

Grip strength tests were performed to investigate skeletal muscle strength. To eliminate body weight-related variance, grip strength results were normalized to the body weight of the corresponding week. SED and EX showed no differences between W0, W4, and W8, whereas OT showed

significantly decreased results from W0 to W8 ($p < 0.001$).

Rotarod tests were conducted to investigate body balance and coordination. The EX and OT groups had an increased latency to fall from W0 to W4 ($p < 0.05$, $p = 0.0643$), whereas both had no tendency from W4 to W8. The error counts tended to decrease from W0 to W4 for SED, EX, and OT. Only OT increased from W4 to W8, but the difference was not significant.

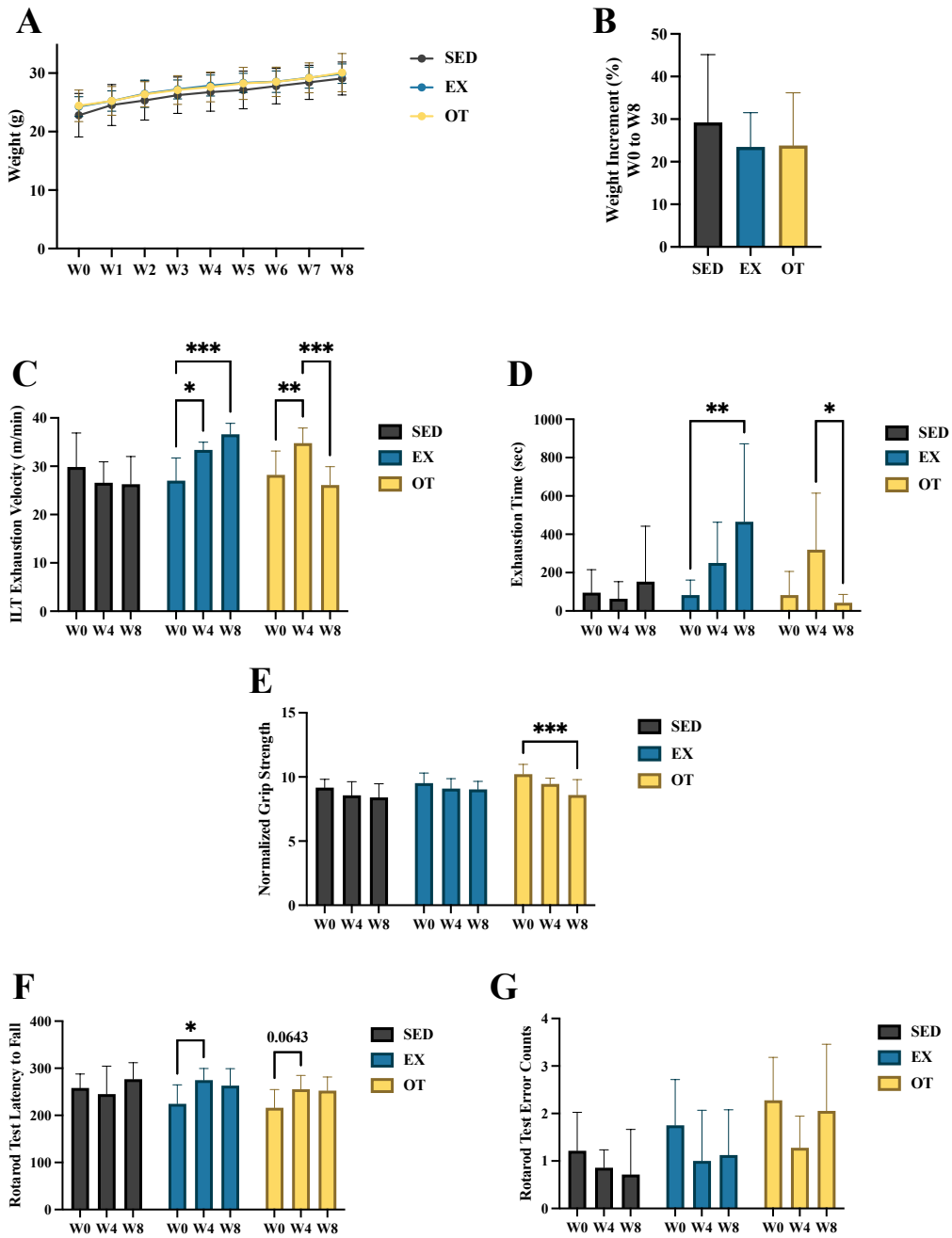

Figure 1. (A) Body weight from W0 to W8. (B) Body weight increment through W0 to W8. (C) Exhausted velocity at the incremental loading test from every W0, W4, W8. (D) Exhaustion time at exhaustion test from every W0, W4, W8, within velocity of 36 m/min. (E) Grip strength result normalized by corresponding week. (F) Result of Latency to fall at 300sec rotarod test. (G) Error counts at 300sec rotarod test. Data from (A)-(G) (SED = 7, EX = 8, OT = 9) are presenting as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with two-way ANOVA for (A), (C)-(G), One-way ANOVA for (B)

2. Overtraining induced pro-inflammatory cytokines in blood, quadriceps, and hypothalamus

Studies by Rocha et al., 2019, indicated that overtraining increases pro-inflammatory cytokine levels in blood, skeletal muscles, and hypothalamus. Samples were collected from euthanized mice in each group for cytokine array and Western blot analyses to validate overtraining.

IL-1β and IL-2, known as pro-inflammatory cytokines, were higher in OT than EX in blood plasma (Fig. 2A,B; $p = 0.113$, $p = 0.0859$). IL-1β and TNFα levels tended to be higher in OT than EX in the gastrocnemius (Fig. 2C,D; $p = 0.0869$, $p = 0.1549$). Lastly, the IL1-β level was significantly higher in OT at the hypothalamus (Fig. 2E; $p = 0.0113$).

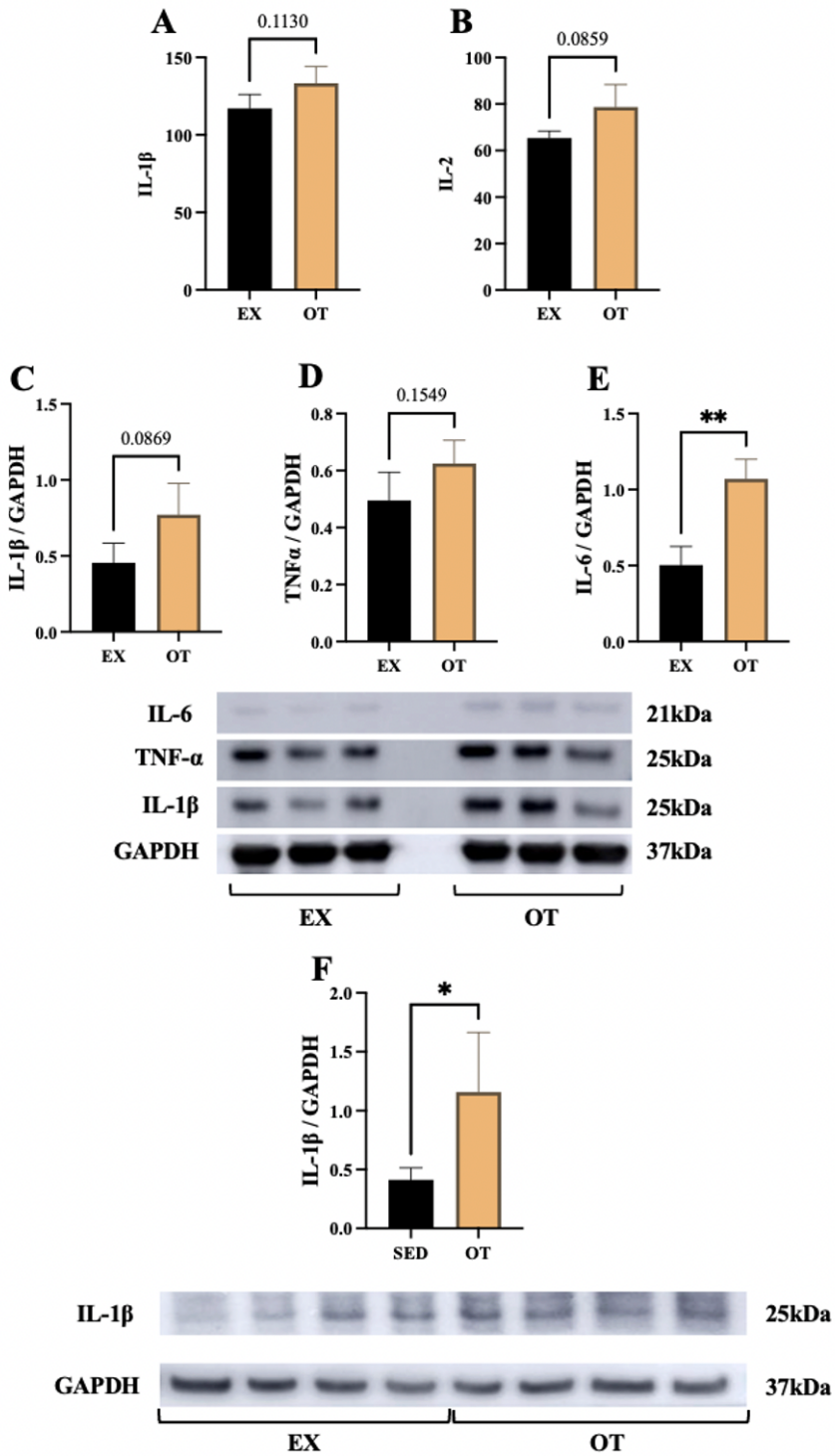

Figure 2. (A) Blood plasma level of IL-1β. (B) Blood plasma level of IL-2. (C) Western blot result of IL-1β at gastrocnemius. (D) TNFα level at gastrocnemius (E) Western blot result of IL-1β at hypothalamus. Data from (A)-(E) (n = 3), (F) (n = 4) are presenting as mean ± SD. *$p < 0.05$, **$p < 0.01$ with unpaired t-test for (A)-(F).

3. Isolated EVs were validated through its size and CNS-derived EVs protein markers

To validate CNS-derived EVs, NTA measured the number and size of particles, and Western blot analysis measured the levels of EV protein markers (CD81 and CD9) and CNS-derived EV markers (L1CAM and NeuN).

EVs typically range in size from 50 to 500 nm, with exosomes, a subset of small EVs, falling in the range of 50 to 200 nm. The average diameter of the particles was approximately 211 nm. In the Western blot image (Fig. 3C), immunoprecipitated EV with L1CAM (L1CAM + EV) exhibited higher levels of L1CAM and NeuN than total EVs. The EV markers CD81 and CD9 showed equivalent signal intensities between L1CAM + EV and total EVs, while the IgG control showed no signal

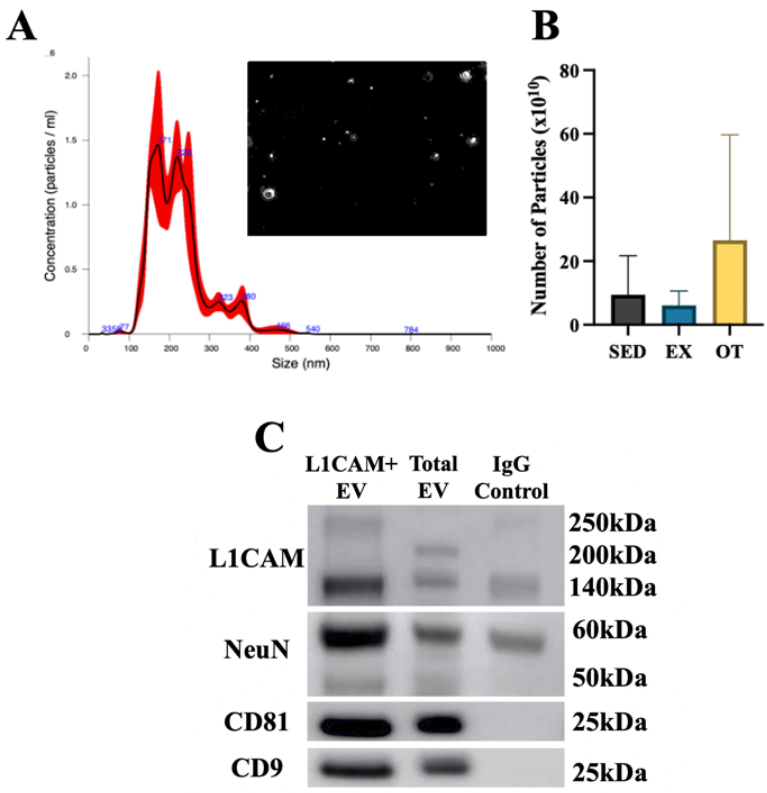

Figure 3. (A) Particle concentration and particle size. (B) Number of particles per 1ml of blood plasma. (C) Validating CNS-derived EV through western blot result of CNS-derived EV by compare with Total EV and IgG isotype control of IP. EV markers CD81 and CD9, CNS derived EV marker L1CAM and NeuN are validated by WB. Data from (A), (B) (n = SED = 5, EX = 3, OT = 4) are presenting as mean ± SD. Statistical analysis has done with One-way ANOVA.

4. Effect of overtraining on CNS-derived EV protein components

Proteomic Proteomic analysis was conducted to explore the impact of overtraining on EV protein components. In comparison to EX, OT had 290 downregulated proteins and 268 upregulated proteins, while EX had 313 upregulated proteins and 245 downregulated proteins compared to SED. After filtration, OT exhibited 14 upregulated proteins and two downregulated proteins compared to EX, and 12 downregulated proteins and two upregulated proteins compared to SED. EX had 41 upregulated proteins and one downregulated protein compared to SED.

Gene Ontology analysis using DAVID Bioinformatics Resources parameters revealed insights into the roles of identified CNS-derived EV proteins. In OT compared to SED, 12 upregulated proteins were associated with fatty acid metabolic processes, lipid metabolic processes, long-chain fatty acid transport, NADH metabolic processes, fatty acid beta-oxidation, and aerobic respiration.

In OT compared to EX, 14 downregulated proteins were linked to lipid metabolic processes, fatty acid beta-oxidation, ketone body catabolic processes, fatty acid beta-oxidation using acyl-CoA dehydrogenase, tricarboxylic acid cycle, response to starvation, regulation of cytosolic calcium ion concentration, adipose tissue development, response to activity, response to hormones, and MAPK cascade.

Among the 41 proteins upregulated during exercise, 12 were involved in lipid metabolic processes, 10 in fatty acid metabolic processes, eight in tricarboxylic acid cycles, and seven in fatty acid beta-oxidation and

carbohydrate metabolic processes. Four proteins were associated with acetyl-CoA metabolic processes, pyruvate metabolic processes, NADH metabolic processes, gluconeogenesis, and response to xenobiotic stimuli.

Notably, both valosin-containing protein (VCP) and CAT were significantly upregulated in the OT group compared to the EX and SED groups. These proteins were associated with amyotrophic lateral sclerosis and pathways of neurodegeneration-related multiple diseases according to KEGG pathway analysis using DAVID Bioinformatics Resources

Lastly, both valosin containing protein (VCP) and catalase (CAT) were significantly up-regulated in the OT groups compare to EX and SED groups (Table. 1, 2). These proteins were associated with Amyotrophic lateral sclerosis and Pathways of neurodegeneration – multiple diseases according to KEGG pathways analysis through DAVID Bioinformatics Resources parameters.

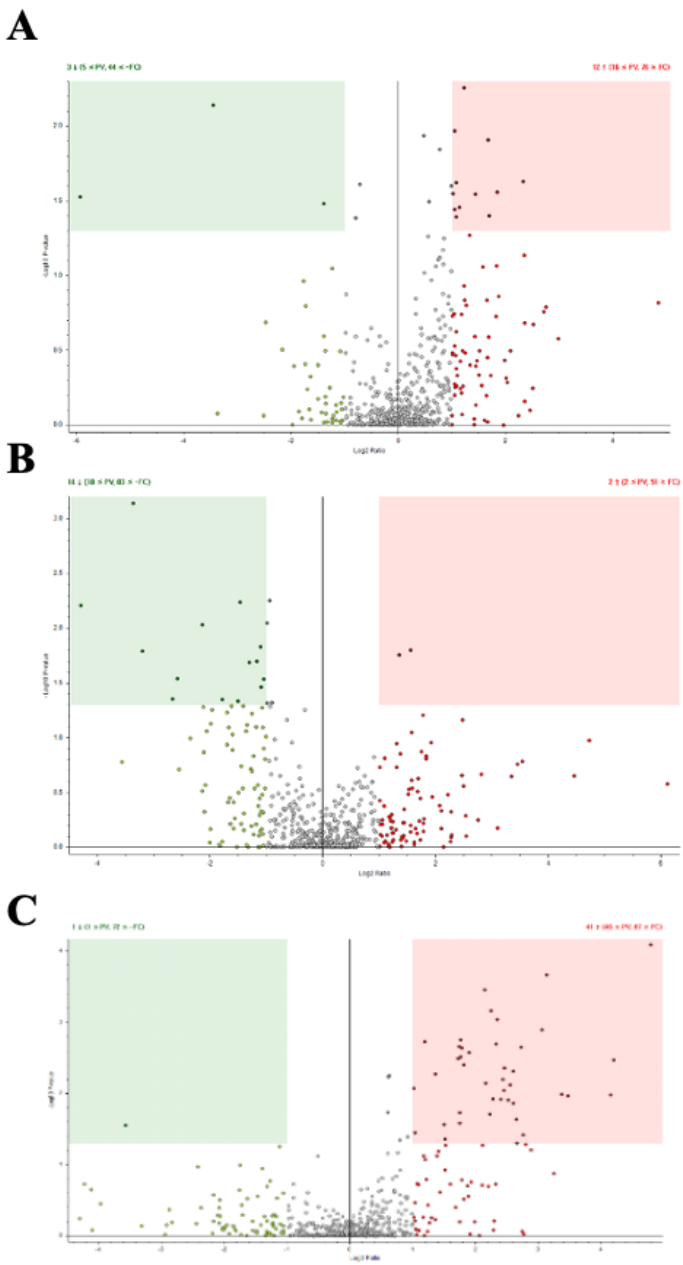

Figure 4. (A) Volcano plot of CNS-derived EV of EX/SED. (B) Volcano plot of CNS-derived EV protein of OT/EX. (C) Volcano plot of CNS-derived protein EV of OT/SED. Data has filtered by *p*-value < 0.05, Ratio ≥ |2|.

Table 1. Differently expressed proteins in OT/SED

Accession no.	Protein name	Gene symbols	Abundance ratio: (OT) / (SED)	*p*-value: (OT) / (SED)
Q9WVA4	Transgelin 2	Tagln2	2.35	0.0056
P06330		-	0.09	0.0072
Q924X2	Carnitine palmitoyltransferase 1b	Cpt1b	2.08	0.0108
P16015	Carbonic anhydrase 3	Car3	3.19	0.0124
Q8K426	Resistin-like gamma	Retnlg	5.03	0.0236
P03921	NADH dehydrogenase subunit 5	ND5	2.12	0.0238
P04117	Fatty acid binding protein 4, adipocyte	Fabp4	3.61	0.0276
Q8QZS1	3-Hydroxyisobutyryl-coenzyme A hydrolase	Hibch	2.04	0.0283
Q8QZT1	Acetyl-coenzyme A acetyltransferase 1	Acat1	2.71	0.0285
P01872	Immunoglobulin heavy constant mu	Ighm	0.02	0.0297
A0A075B696		-	0.38	0.0332
P24270	Catalase	Cat	2.20	0.0351
A0A0G2JGY4		-	2.08	0.0361
Q01853	Valosin-containing protein	Vcp	3.24	0.0400
P19096	Fatty acid synthase	Fasn	2.12	0.0406

Table 2. Differently expressed proteins in OT/EX

Accession no.	Protein name	Gene symbols	Abundance ratio: (OT) / (EX)	*p*-value: (OT) / (EX)
Q8QZT1	Acetyl-coenzyme A acetyltransferase 1	Acat1	0.10	0.0007
P16015	Cysteinyl-tRNA synthetase 1	Car3	0.36	0.0058
Q9Z2I9	Succinate-coenzyme A ligase, ADP-forming, beta subunit	Sucla2	0.05	0.0062
Q8BWT1	Acetyl-coenzyme A acyltransferase 2	Acaa2	0.23	0.0094
Q924X2	Carnitine palmitoyltransferase 1b	Cpt1b	0.47	0.0149
Q01853	Valosin-containing protein	Vcp	2.96	0.0159
P62259	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	Ywhae	0.11	0.0162
P24270	Catalases	Cat	2.57	0.0176
P03921	NADH dehydrogenase subunit 5	ND5	0.45	0.0201
A0A0G2JGY4	-	-	0.41	0.0205
D3Z148	-	-	0.17	0.0290
Q99LC5	Electron transferring flavoprotein, alpha polypeptide	Etfa	0.49	0.0291
P50544	Acyl-coenzyme A dehydrogenase, very long chain	Acadvl	0.47	0.0345
Q9D0K2	3-Oxoacid coenzyme A transferase 1	Oxct1	0.16	0.0443
Q9CZ13	Ubiquinol-cytochrome C reductase core protein 1	Uqcrc1	0.29	0.0448
G3UZJ4	-	-	0.36	0.0465

Table 3. Differently expressed proteins in EX/SED

Accession no.	Protein name	Gene symbols	Abundance ratio: (EX) / (SED)	*p*-value: (EX) / (SED)
Q8QZT1	Acetyl-coenzyme A acetyltransferase 1	Acat1	27.79	0.0001
P16015	Cysteinyl-tRNA synthetase 1	Ca3	8.79	0.0002
Q924X2	Carnitine palmitoyltransferase 1b, muscle	Cpt1b	4.45	0.0004
P03921	-	Mtnd5	4.77	0.0007
A0A0G2JGY4	Malate dehydrogenase 2, NAD (mitochondrial)	Mdh2	5.11	0.0009
Q8BWT1	Acetyl-coenzyme A acyltransferase 2	Acaa2	8.37	0.0013
Q9WVA4	Transgelin 2	Tagln2	3.41	0.0018
P63101	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Ywhaz	2.30	0.0019
P19096	Fatty acid synthase	Fasn	5.04	0.0020
Q99JY0	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta	Hadhb	3.36	0.0022
P13707	Glucose-6-phosphate dehydrogenase 2	Gpd1	6.66	0.0022
P50544	Acyl-coenzyme A dehydrogenase, very long chain	Acadvl	3.46	0.0023
P12787	Cytochrome C oxidase subunit 5A	Cox5a	3.75	0.0027
Q8VCT4	Carboxylesterase 1D	Ces1d	3.42	0.0031
Q99LC5	Electron transferring flavoprotein, alpha polypeptide	Etfa	3.33	0.0033
P62259	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	Ywhae	18.43	0.0034
Q61425	Hydroxyacyl-coenzyme A dehydrogenase	Hadh	3.53	0.0040
P04117	Fatty acid binding protein 4, adipocyte	Fabp4	5.53	0.0044
Q9CZ13	Ubiquinol-cytochrome C reductase core protein 1	Uqcrc1	6.10	0.0049

(Continued next page)

Table 3. Continued

Accession no.	Protein name	Gene symbols	Abundance ratio: (EX) / (SED)	*p*-value: (EX) / (SED)
Q8QZS1	3-Hydroxyisobutyryl-coenzyme A hydrolase	Hibch	2.58	0.0054
P12242	Uncoupling protein 1 (mitochondrial, proton carrier)	Ucp1	5.44	0.0064
A0A1L1STE6	Isocitrate dehydrogenase 3 (NAD+) alpha	Idh3a	4.49	0.0072
Q3V117	ATP citrate lyase	Acly	5.90	0.0077
P17182	Enolase 1B, retrotransposed	Eno1	2.03	0.0085
G3UZJ4	Prostaglandin D receptor	Prdx5	5.49	0.0092
Q99KI0	Aconitase 2, mitochondrial	Aco2	10.40	0.0105
Q9D0K2	3-Oxoacid coenzyme A transferase 1	Oxct1	17.86	0.0106
Q8BMF4	Dihydrolipoamide S-acetyltransferase	Dlat	11.17	0.0109
Q64521	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	Gpd2	4.87	0.0121
Q8BMS1	Hydroxyacyl-coenzyme A dehydrogenase trifunctional multienzyme complex subunit alpha	Hadha	5.32	0.0122
Q9CZU6	Citrate synthase	Cs	5.75	0.0124
Q9DCD0	Peroxiredoxin 6	Pgd	6.09	0.0137
A0A1B0GQX5	Lactate dehydrogenase A	Ldha	3.37	0.0188
H3BKG7	Fumarate hydratase 1	Fh1	4.71	0.0194
Q9Z2I9	Succinate-coenzyme A ligase, ADP-forming, beta subunit	Sucla2	6.31	0.0233
P52480	Pyruvate kinase, muscle	Pkm	3.38	0.0264
A0A0R4J083	Acyl-coenzyme A dehydrogenase, long-chain	Acadl	2.84	0.0276
Q64727	Vinculin	Vcl	2.06	0.0357
D3Z148	Caveolin 1, caveolae protein	Cav1	6.79	0.0386
Q99LC3	NADH:ubiquinone oxidoreductase subunit A10	Ndufa10	2.87	0.0441
A0A6I8MX27	Lactate dehydrogenase B	Ldhb	6.34	0.0499

Table 4. CNS-derived EV proteins up-regulated specifically in OT

Protein	Abundance ratio: (OT) / (EX)	Abundance ratio: (OT) / (SED)	Abundance ratio: (EX) / (SED)	*p*-value: (OT) / (EX)	*p*-value: (OT) / (SED)	*p*-value: (EX) / (SED)
Valosin containing protein	**2.955**	**3.239**	1.096	**0.016**	**0.04**	0.72
Catalase	**2.573**	**2.202**	0.856	**0.018**	**0.035**	0.828

V. Discussion

This In this study, we delved into the protein content of CNS-derived EVs in an overtrained mouse model, identifying VCP and CAT as specific to overtraining. These proteins are indicative of stress and disease conditions in the CNS (Michiels et al., 1994).

Existing research has consistently highlighted the overtraining syndrome's impact on performance decline and CNS fatigue (Armstrong et al., 2022; Kim et al., 2017). Despite recognizing the importance of preventing overtraining syndrome, accurate markers for prevention and understanding its underlying mechanisms remain elusive (Anderson et al., 2021).

EVs have emerged as pivotal components in investigating unknown biomarkers and disease mechanisms (Si et al., 2019; Tkach & Théry, 2016). Circulating EVs carry proteins and miRNAs associated with specific diseases, making them valuable in pathology research (Yuana et al., 2013). In the realm of exercise, EVs have been studied for their role in metabolism-related proteins or miRNAs during physical activity (Nederveen et al., 2021; Whitham et al., 2018). Consequently, we chose to discern overtraining and CNS fatigue through the protein content of EVs, particularly CNS-derived EVs, acknowledging the crucial relationship between the CNS and overtraining.

Building on the work of Pereira et al., who investigated overtraining using an 8-week downhill treadmill exercise mouse model (Pereira et al., 2012), our study observed a significant decrease in physical performance. Concurrently, pro-inflammatory cytokine IL-6 levels significantly increased in skeletal muscle, with those of TNF-α and IL-1β showing a tendency to increase. Pro-

inflammatory cytokines also exhibited an upward trend in levels in blood plasma. Notably, the IL-1β level significantly increased in the hypothalamus, indicative of CNS fatigue in overtraining, aligning with previous research.

While various aspects of overtraining have been explored in past studies, none have employed EVs to investigate the overtraining syndrome. Given the symptomatology's connection to the CNS, we utilized CNS-derived EVs to identify overtraining and potential biomarkers. Isolating CNS-derived EVs followed the two-step EV isolation method outlined by Mustapic (Mustapic et al., 2017). Similar to the Mustapic protocol, our validation of EV protein markers CD81 and CD9 as well as CNS-related markers through Western blot analysis confirmed their higher signals compared to the total exosome lysate.

Proteomic analysis revealed upregulated fat metabolism-related proteins in the EX group compared to the SED group. This finding indicates that EV contents are intricately linked to the energy demands of chronic exercise, supporting earlier research demonstrating the secretion and circulation of energy metabolism-related molecules via small vesicles (Garcia et al., 2016; Whitham et al., 2018).

In addition to the comparison between the EX and SED groups, a notable downregulation of proteins was observed in the OT group compared to the EX group. Such aberrant decreases in fat metabolism have been reported to be associated with neuromuscular junction denervation, mitochondrial dysfunction, excitotoxicity, impaired neuronal transport, cytoskeletal defects, inflammation, and reduced neurotransmitter release (Tracey et al., 2018).

Proteomic analysis also unveiled a significant upregulation of both VCP and CAT in the OT group compared to both the EX and SED groups (refer to Table

1 and 2). VCP is implicated in apoptosis-related pathways through endoplasmic reticulum stress during protein processing and is associated with various neuronal diseases, including Parkinson's disease, Alzheimer’s disease, and amyotrophic lateral sclerosis (Kakizuka, 2008). CAT, an antioxidant enzyme ubiquitous in aerobic organisms, breaks down hydrogen peroxide into water and oxygen, with observed upregulation in organs experiencing oxidative stress (Michiels et al., 1994).

Notably, prior studies have not explored fat metabolism in the brain or CNS. Additionally, the roles of VCP and CAT in the context of overtraining remain unexplored. Given their critical associations with neurodegenerative diseases, these proteins could introduce novel elements in the field of overtraining research, potentially serving as biomarkers for overtraining and CNS fatigue, pending further investigation.

VI. Conclusion

In conclusion, mice subjected to overtraining for 8 weeks exhibited symptoms indicative of overtraining, including elevated IL-1β levels in the hypothalamus. CNS-derived EVs were isolated using a two-step EV isolation method from blood plasma. These EVs from OT mice demonstrated heightened levels of CNS fatigue-related proteins, namely, VCP and CAT. These proteins exhibited significant associations with CNS-related diseases and pathways linked to cellular stress. While VCP and CAT showed upregulation due to overtraining, downregulated proteins were identified using DAVID Bioinformatics Resources, suggesting aberrant regulation of fat metabolism possibly contributing to fatigue in the brain and CNS.

Therefore, we propose that CNS-derived EVs may represent CNS fatigue induced by overtraining. Furthermore, VCP and CAT emerge as potential biomarkers of CNS fatigue in overtraining syndrome.

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