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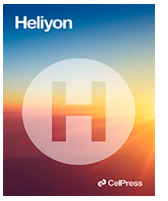

Research article

Boosting physical performance in SD rats through brain-targeted delivery of caffeine-loaded transferrin liposomes

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ABSTRACT

This study aimed to explore the impact of caffeine (CAF) encapsulated in transferrin-modified, sterically-stabilized liposomes (Tf-SSL) on the physical performance of rats, specifically forelimb grip strength, running, and swimming. The brain-targeted drug delivery system, Tf-SSL, was used for the administration of caffeine. 168 male Sprague-Dawley (SD) rats were randomly assigned to different groups, including swimming, running, running wheel, and strength groups. Each group was further subdivided into high, medium, and low dose free caffeine (HCAF, MCAF, LCAF) and Tf-SSL CAF groups, along with a control group (CON). The strength, swimming, and running groups underwent training for four weeks, three times per week. The running wheel group was placed in rearing cages for a one-week adaptation period. After the final training session, the resistance, swimming, running, and running wheel exercise capacities of the rats were tested. The rats were administered treatment via tail vein injection, while the blank CON group received 0.9 % saline solution without treatment throughout the entire process. The results demonstrated a Tf-SSL CAF group encapsulation rate of 70.58 ± 5.14 %. Increasing the concentration of supplemented caffeine led to enhanced forelimb grip strength in rats, with significant differences observed in HCAF alone group, medium-dose Tf-SSL CAF (MTf-SSL CAF), and high-dose Tf-SSL CAF (HTf-SSL CAF) groups compared to the CON group. In the running and swimming experiments, higher caffeine supplementation concentrations correlated with increased running and swimming time to exhaustion, and the MTf-SSL CAF group showed longer running and swimming time compared to the HCAF alone group. The results of rat striatal dopamine levels indicated that increased caffeine supplementation concentrations led to higher dopamine secretion, with significantly different striatal concentrations in the HCAF group, MTf-SSL CAF group, and HTf-SSL CAF group compared to the CON group. The running wheel experiment revealed that rats in the medium- and high-dose Tf-SSL CAF groups exhibited greater

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6-h running distances than the HCAF group and CON group. In conclusion, caffeine supplementation improved the physical performance of rats, with the high concentration CAF group outperforming the low and medium concentration groups. Furthermore, Tf-SSL CAF demonstrated superior physical enhancement compared to caffeine supplementation alone.

1. Introduction

The caffeine (CAF) is a widely consumed substance globally, with a prevalence in the diet of American adults, where 89 % of individuals reported consuming CAF at an average daily intake of 211 ± 3 mg/d, as evidenced by a survey [1]. Numerous studies have consistently demonstrated the beneficial effects of CAF on various aspects of physical performance, including muscular endurance, muscle strength, anaerobic capacity, aerobic endurance, and anti-fatigue capacity [2–5]. As a result, CAF has gained popularity not only among the general population but also as a performance enhancer for athletes. In fact, a study found that three-quarters of British athletes have utilized CAF prior to competing [6]. Furthermore, the International Society of Sports Nutrition has stated that CAF doses of 3 mg/kg body weight taken before exercise can positively impact athletic performance [7]. However, it is important to note that caution should be exercised regarding CAF dosages, as higher doses above 9 mg/kg may not yield additional performance benefits and can lead to various side effects associated with excessive CAF intake, such as tachycardia, headache, and anxiety [4,8,9]. Currently, liposomes play a crucial role in medical and pharmacological research, recognized as one of the most effective carriers for delivering various drugs to specific cells and tissues [10]. Comprising mainly of phospholipids extracted from soybeans or egg yolks, liposomes are artificial vesicles with one or multiple layers, capable of encapsulating both lipophilic and hydrophilic compounds [10,11]. The affinity of bioactive compounds for water or lipid membranes determines whether they are encapsulated in the lipophilic or hydrophilic regions of the liposomes. The preparation method significantly influences the nature, shape, size, stability, and drug delivery efficiency of liposomes [10].

In the medical field, liposome formulations have been approved for intravenous, intramuscular and oral administration in anticancer, antifungal and anti-inflammatory therapies [12–14]. Despite liposomes being widely utilized in critical areas such as pharmacology, oncology, and materials science, benefiting from advantages like enhanced efficacy, high biocompatibility, low immunogenicity, drug protection, extended drug half-life, and low toxicity. However, there are still very few research reports on improving the physical performance and ability of rats through the novel delivery method of liposome-encapsulated caffeine.

Currently, the majority of caffeine drug delivery methods revolve around conventional approaches, including capsules, coffee, sports drinks, chewing gum, chocolate bars, gels, mouthwashes, and aerosols. These methods rely on absorption mainly from the intestine into the bloodstream, where the caffeine is subsequently distributed throughout the body via the circulatory system or absorbed by the oral and nasal mucosa, ultimately activating specific sensors connected to the brain [15,16]. However, conventional drug delivery methods suffer from reduced drug utilization efficiency and imprecise targeting of specific cells, which can lead to a significant distribution of caffeine to non-target tissues, including the aorta, potentially causing toxicity [17].

To overcome these limitations, this study adopts liposomes as an innovative encapsulation system for caffeine, offering a novel drug delivery method. By employing liposomes, the study achieves efficient encapsulation and controlled release of caffeine, resulting in enhanced bioavailability and stability. Liposomes act as protective carriers, shielding caffeine molecules from rapid elimination, thereby prolonging the drug's circulation time in the body. Moreover, liposomes enable the gradual release of caffeine within the targeted organ or tissue, avoiding the rapid and intense peak responses associated with direct intravenous injections. This controlled release mechanism mitigates the risk of adverse reactions due to caffeine overdose and obviates the need for frequent dosing.

Moreover, caffeine liposomes offer improved drug distribution and enhanced selectivity for target tissues. When caffeine is administered orally, the blood-brain barrier plays a crucial role in preventing metabolic waste and toxic substances from entering the central nervous system through blood circulation. Therefore, crossing the blood-brain barrier is a challenge. Previous studies have demonstrated that by binding therapeutic drugs to specific proteins, such as transferrin (Tf), they can effectively cross the blood-brain barrier. The transferrin receptor (TfR) is highly expressed on the blood-brain barrier and has the ability to recognize transferrin carrying iron, thereby facilitating intracerebral transport of iron [18]. This property can be exploited to transport drugs into the brain, and the use of transferrin or TfR as a target for drug delivery to the brain has been validated in animal models. Transferrin's targeting capability allows it to circulate without entering ordinary non-targeted cells, exhibiting high precision in drug delivery.

Various Nano preparations, such as liposomes and nanoparticles, have been explored to overcome the challenges of crossing the blood-brain barrier. Liposomes and nanoparticles can encapsulate both lipid-soluble and water-soluble drugs, enabling them to reach the surface of cerebral vessels and successfully cross the blood-brain barrier. Depending on factors such as size, surface charge, and attached ligands, liposomes can preferentially accumulate in specific tissues or organs, enabling targeted drug delivery. This approach enhances the efficacy of caffeine while reducing its toxicity. Through a brain-targeted delivery system, specifically transferrin-modified liposomes encapsulating caffeine, it is possible to achieve better effects in enhancing the physical performance of rats. Additionally, a medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine (MTf-SSL CAF) is expected to show a greater enhancement in physical performance compared to the free caffeine group, especially compared to the high-dose caffeine group (HCAF).By using liposomes, caffeine can be released in a more stable and controlled manner, leading to improved bioavailability and tissue distribution, as well as reduced risks of adverse reactions associated with rapid and intense peak responses. Although the preparation process for intravenous administration of caffeine liposomes may be more complex and entail higher production costs, their excellent targeting effect allows for reduced dosage and improved safety. Consequently, exercise enhancement can

be achieved without the need for impractical high doses of caffeine, thereby minimizing potential caffeine toxicity and associated side effects while effectively enhancing exercise capacity. Therefore, the objective of this study is to investigate the efficacy of Tf-modified liposomes in efficiently loading CAF and specifically targeting it to the brain. The primary aim is to enhance the physical performance of rats, particularly in terms of forelimb grip strength, running, and swimming, while ensuring high efficiency, low toxicity, and safety.

2. Materials and methods

2.1. Experimental animals

The present experiment employed a randomized controlled double-blind trial. A total of 168 male SD rats, aged 8–9 weeks and weighing 200 ± 10 g, were obtained from Beijing Viton Lever Laboratory Animal Technology Co. The rats were housed in cages with two animals each, and the experimental environment was maintained at a controlled room temperature of 25 °C (77.0 °F), relative air humidity of 50–60 %, and a 12-h light/dark cycle. All rats were fed a normal standard diet. All animal handling procedures strictly adhered to the Regulations for the Management of Laboratory Animals and received ethical approval from the Sports Science Experiment Ethics Committee of Beijing Sport University with the registration number of (2023183A). The rats were randomly divided into four main groups: swimming, running, running wheel, and strength groups. Groups were assigned randomly using computer-generated random numbers. Each group was subsequently subdivided into seven subgroups, which included the LCAF group, MCAF group, HCAF group, LTf-SSL CAF group, MTf-SSL CAF group, HTf-SSL CAF group, and CON group. The LCAF group, MCAF group, and HCAF group were injected with low, medium, and high doses of caffeine, respectively. The LTf-SSL CAF group, MTf-SSL CAF group, and HTf-SSL CAF group were injected with low, medium, and high doses transferrin-modified, sterically-stabilized liposome-encapsulated caffein respectively. The CON group was injected with caffeine-free saline solution. Each subgroup consisted of six animals. The CAF concentrations for the high, medium, and low dose groups were set at 1, 2, and 4 mg/kg, respectively. All rats underwent resistance, swimming, running, and wheel running ability tests 24 h after the final training session. Caffeine and saline solution were used in the experiment, with saline serving as the solvent for dissolving caffeine. Treatment was administered via tail vein injection in rats, while the CON group received an equivalent amount of 0.9 % treatment-free saline throughout the process. The reagents required for this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of transferrin liposomes

Liposomes were prepared by lipid hydration method, suitably modified from the study of Pardakhty et al. [19]. Briefly, films containing soy phosphatidylcholine (SPC, 99 % purity)/cholesterol/1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[poly (ethylene glycol)]-maleimide (DSPE-PEG(3400)-MAL)/1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol) 2000] (DSPE-PEG-2000) films were hydrated with phosphate-buffered saline (PBS, pH 7.4) for 20 min at 25 °C (77.0 °F) under sonication to form SSL with an active maleimide functional group (SSL-MAL).

Tf-SSL was formed by incubating sulfotransferrin with SSL-MAL (Tf:DSPE-PEG(3400)-MAL = 1:4, mol/mol) for 12 h at 37 °C (98.6 °F), where 3400 denotes the molecular weight of polyethylene glycol attached to Tf. Tf-SSL was separated from free Tf using a SepharoseCL-4B chromatographic column, and the coupling efficiency of Tf to SSL was determined to be 75 ± 5.6 % using a BCA (Bicinchoninic Acid) protein quantification kit. In the construction of active targeting liposomes, the ligand portion is usually coupled to the liposome surface via polyethylene glycol as a linker.

2.3. Vesicle size measurement

The size of the vesicles was measured by static laser diffraction at 72 h after preparation with a Malvern particle analyzer (Malvern, MasterSizerX-100, UK) using a 100 mm focal length lens with a low power He–Ne laser as the light source. The basic size distribution obtained by this technique is volume-based and the size. The distribution is expressed in terms of the volume of the equivalent sphere (dv). The size distribution parameters and the derived diameter are calculated from the basic size distribution using the Size-x software.

2.4. Resistance and aerobic training and testing program for rats

2.4.1. Forelimb grip training and testing program for rats

The rats were trained on a custom-made vertical ladder (height × width: 90 cm × 15 cm) for a total of 4 weeks by tail-weighted ladder climbing with increasing load, i.e., the rats were placed at the bottom of the ladder and trained with tail-weighted ladder climbing for 3 groups/d, 4 times/group, with 60 s interval between groups. 40 % of body weight was used as the starting load, and the load was increased by 10 % of body weight every 2 days, with a final load of 120 % of body weight.

The grip force of the forepaw was measured with a grip force meter (Shenzhen Ryder Biotechnology Co., Ltd., China). Grip force was used to determine the maximum peak force generated by the rat pulling out a metal bar. The machine was mounted on a stable table while the rat was allowed to grasp the metal bar with its front paws while its tail was pulled backwards in the horizontal plane. The peak tension, i.e. the force applied to the metal bar before it lost its grip, was recorded in grams. The rat was pulled three times and the highest peak tension was recorded by the device. The body weight of each rat was measured and the results were expressed as grip force (G) per body weight (100 g). The CON group rats also underwent the same training and testing regimen.

2.4.2. Rat running training and testing protocol

Rats were trained on a treadmill with an inclination of 0° for 4 weeks, 3 times a week, with a gradual increase in the duration and intensity of the exercise training. Week 1: 12 m/min for 30 min, week 2: 15 m/min for 30 min, week 3: 18 m/min for 35 min, week 4: 20 m/min for 40 min. Before each workout, a 5-min warm-up (8 m/min) was performed. In order to reduce the stress conditions that adversely affect the results, electric shocks were not used in this study, but a sponge was placed at the proximal end of the treadmill and the rats were gently tapped on the tail.

Rats were exercised at 20 m/min on a running platform until exhaustion to reflect their endurance. The time response of running at the time of exhaustion was recorded. The criterion of exhaustion was that the rats were unable to maintain an appropriate speed despite continuous hand stimulation for 1 min, at which point the rats were removed from the treadmill and their running time was recorded [20]. The CON group rats also underwent the same training and testing regimen.

2.4.3. Rat swimming training and testing protocol

The rats underwent a continuous swimming training regimen for 4 weeks. The training sessions were conducted 3 times per week, with the water temperature in the tanks maintained at approximately 30 °C (86.0 °F) [21,22].Swimming interventions were performed in transparent glass tanks (L × W × D 150 × 60 × 50 cm), and the animals were placed in an aquatic environment for a familiarization period of 3 days. Adaptive swimming was performed for 15 min on the first day of training, followed by a gradient of 5 min on each of the following days until the full 30 min of swimming, and then 30 min/day of swimming exercise until the end of the experiment.

The rats were placed in a tank with a water depth of 50 cm and a water temperature of 30 °C (86.0 °F) for the exhaustion swim test, during which a load equivalent to approximately 5 % of the animal's body weight was increased, and the load was adjusted with a lead weight tied to the animal's tail. The criterion for determining exhaustion was that the rat did not rise to the surface after 8 s of submersion, and the time of exhaustion was recorded [23]. The CON group rats also underwent the same training and testing regimen.

2.4.4. Rat running wheel test

Prior to the start of the experiment, rats were assigned to rearing cages with running wheels, 34 cm in diameter and 10 cm wide, for a week of acclimatization exercise, during which the animals had free access to food and water throughout the experiment. All exercising animals were allowed to run freely at their own speed. At the end of one week of acclimatization, different concentrations of caffeine or Tf-SSL CAF were injected into the rats, which were subsequently placed in a feeding cage to record the distance the rats ran over a 6-h period. Each running wheel was connected to a computer, and the distance and time of the rat's exercise could be recorded through the software. The main record was the distance of the rat's 6 h running wheel movement, and the CON group rats also underwent the same training and testing regimen.

2.5. ELISA analysis of striatal dopamine

The rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg per kilogram of body weight; the striatum was isolated from the brain. The entire procedure was performed using a rat dopamine ELISA kit (Wuhan Cusabio Biotechnology Co., Ltd., China) strictly according to the manufacturer's instructions. Briefly, 30 mg of striatum was rinsed in PBS and stored overnight at −20 °C (−4.0 °F), then homogenized in 300 μl of PBS. Two freeze-thaw cycles were performed to break the cell membrane. The homogenate was centrifuged at 20,000 g for 10 min at 4 °C (39.2 °F), and the supernatant was removed for immediate detection. The assigned antigen standard and sample are added to each well of a 96-well plate that is pre-coated with primary antibody. After the addition of biotin coupling agent and enzyme coupling agent to each well, the plate was incubated for 60 min at 37 °C (98.6 °F). The plate was then washed five times with distilled water. Within 30 min of the color development reaction, absorbance was measured at 450 nm using a microplate reader.

2.6. Caffeine encapsulation efficiency

The encapsulation efficiency (EE) of caffeine liposomes was measured using the dialysis bag method. One milliliter of caffeine liposomes was dropped into a cellulose acetate dialysis bag immersed in 150 ml of water and stirred to 400 rpm. A sample is taken from the receiver solution and a new solution is added to maintain a constant solution concentration. Caffeine was measured spectrophotometrically at 273 nm using a UV-spectrophotometer (UV-spectrophotometer DU720, Beckman Coulter, California, USA). The drug encapsulation rate (EE%) was calculated as follows: EE% = (Total drug) - (diffused drug)/(Total drug) * 100 %.

2.7. Statistics

SPSS 26.0 statistical analysis software was used to statistically process the experimental data, and the results of each test were expressed as mean ± standard deviation (mean ± SD) using analysis of variance (ANOVA) statistical method, and $P < 0.05$ was considered statistically significant. The significance level was set at $P < 0.05$, and the very significant level was set at $P < 0.01$. The bar graph depicting the results of the rats' physical performance was created using GraphPad Prism 9 software.

3. Results

3.1. Caffeine encapsulation efficiency and in vivo live-imaging of Tf-SSL

The average volume diameter of caffeine vesicles after 72 h of preparation was 117.52 ± 8.36 nm, and the caffeine encapsulation efficiency was 70.58 ± 5.14 %. Caffeine was successfully encapsulated within the lipid vesicles with high efficiency, consistent with literature protocols [18]. To evaluate the distribution of transferrin-modified and sterically-stabilized liposome-encapsulated caffeine in tissues, 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine Iodide (Dir)-labeled Tf-SSL was prepared and visualized. As shown in Fig. 1, the results showed that both Tf-SSL could be distributed in liver and spleen, and Tf-SSL(Dir) entered into brain distribution after drug administration and had strong brain targeting effect.

3.2. Motor ability of rats

3.2.1. Results of forelimb grip force measurement in rats

In this study, the effect of different concentrations of caffeine on the muscle strength of different groups of rats was determined using the rat forelimb grip assay. As shown in Fig. 2: The muscle strength of control rats was 211.33 ± 10.95 g/100 g body weight, compared with the CON group, the forelimb strength of rats supplemented with HCAF significantly increased to 231.50 ± 10.21 g/100 g body weight with a significant difference $p < 0.05$. The forelimb grip strength of rats in the MTf-SSL CAF was 232.33 ± 11.98 g/100 g body weight higher than that in the MCAF and HCAF groups, and was significantly different from the CON group $p < 0.05$. The forelimb grip strength of rats in the HTf-SSL CAF group was higher than that of the HCAF group and the MTf-SSL CAF group, measuring 238.17 ± 10.40 g/100 g body weight, and showed a highly significant difference compared to the CON group with $p < 0.01$.

3.2.2. Rat treadmill assay results

In this study, the effect of caffeine on muscle endurance in different groups of rats was evaluated by recording the running time of the rats from the running platform exercise to the time of exhaustion. As shown in Fig. 3, the running distance of rats gradually increased with the increase of caffeine supplementation concentration, and the running to exhaustion duration of rats in the CON group was 36.36 ± 3.48 min, in which the running duration of rats in the MTf-SSL CAF group was 45.61 ± 3.75 min higher than that in the MCAF and HCAF groups, and the HTf-SSL CAF group demonstrated a running duration of 47.98 ± 3.06 min, significantly surpassing the MTf-SSL CAF group and exhibiting a statistically significant difference compared to the CON group ($p < 0.01$).

3.2.3. Results of swimming measurement in rats

The present study also reflected the aerobic exercise capacity of rats by recording the duration of swimming to exhaustion. As shown in Fig. 4: The duration of swimming to exhaustion in CON rats was 30.14 ± 5.09 min, compared with the CON group the

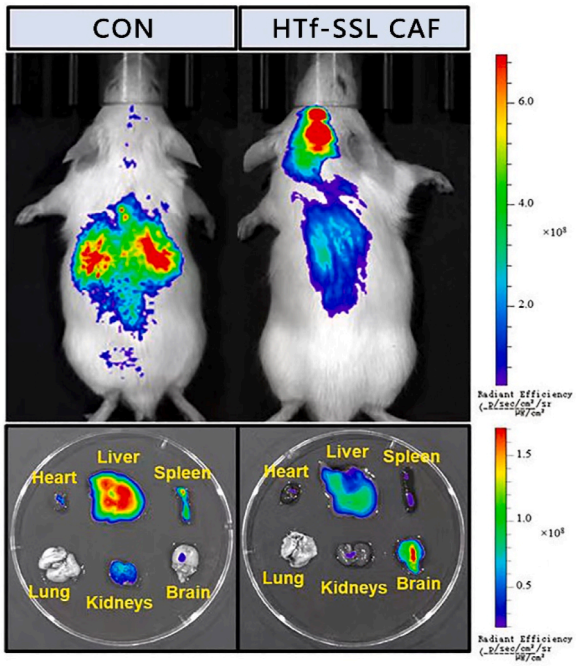

Fig. 1. *Invivo*live- imaging of liposomal drug-loaded Dir. CON: Control group; HTf-SSL CAF: High dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine.

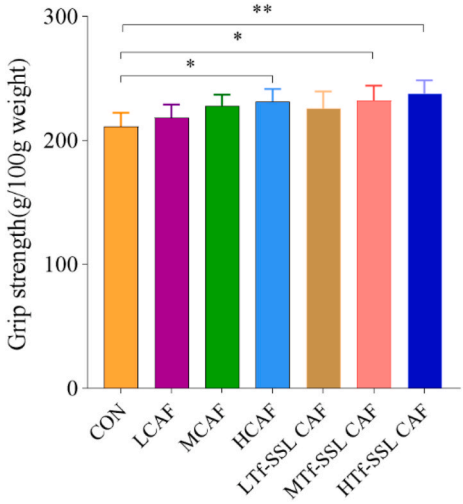

Fig. 2. Results of forelimb grip strength measurement in different groups of rats. CON: Control group; LCAF: Low dose caffeine; MCAF: Medium dose caffeine; HCAF: High dose caffeine; LTf-SSL CAF: Low dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; MTf-SSL CAF: Medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; HTf-SSL CAF: High dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; *p < 0.05, **p < 0.01 compared to the control group.

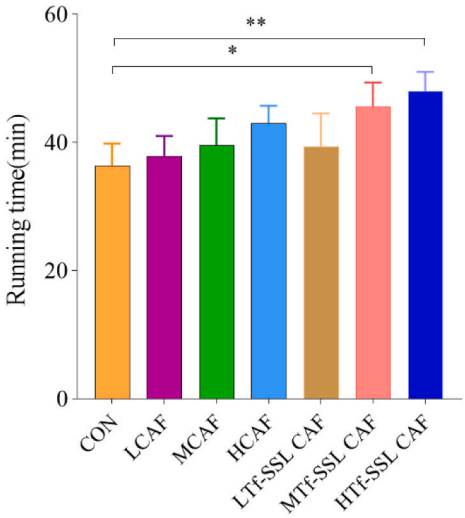

Fig. 3. Treadmill test results of rats in different groups. CON: Control group; LCAF: Low dose caffeine; MCAF: Medium dose caffeine; HCAF: High dose caffeine; LTf-SSL CAF: Low dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; MTf-SSL CAF: Medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; HTf-SSL CAF: High dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; *p < 0.05, **p < 0.01 compared to the control group.

duration of swimming in rats in the MTf-SSL CAF group was 43.02 ± 4.62 min higher than that in the MCAF and HCAF groups. The swimming time of rats in the HTf-SSL CAF group was 45.06 ± 5.08 min, which was higher than that of the MTf-SSL CAF group and significantly different from the CON group ($p < 0.01$).

3.2.4. Results of rat running wheel assay

In this study, the distance of 6 h voluntary running in the running wheel of rats was recorded. As shown in Fig. 5: the 6 h running distance of rats in the CON group was 844.83 ± 40.15 m. The running distance of rats in the MTf-SSL CAF group was 1071.00 ± 47.22 m compared to the CON group, which was higher than the MCAF and HCAF groups and was statistically different from the CON group $p < 0.01$. The running distance of rats in the HTf-SSL CAF group was 1146.10 ± 35.86 m, which was higher than that of the MTf-SSL CAF group and showed a highly significant difference compared to the CON group ($p < 0.01$).

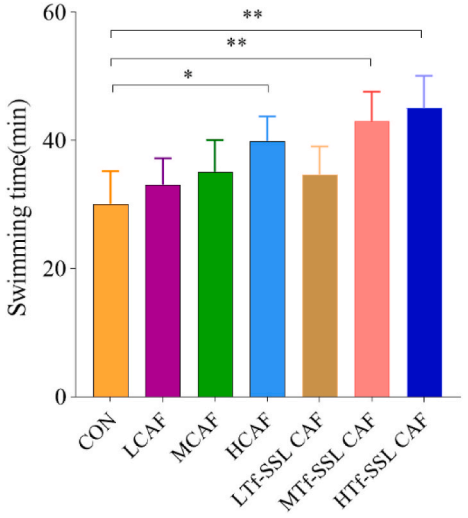

Fig. 4. Results of swimming measurement in different groups of rats. CON: Control group; LCAF: Low dose caffeine; MCAF: Medium dose caffeine; HCAF: High dose caffeine; LTf-SSL CAF: Low dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; MTf-SSL CAF: Medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; HTf-SSL CAF: High dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; *p < 0.05, **p < 0.01 compared to the control group.

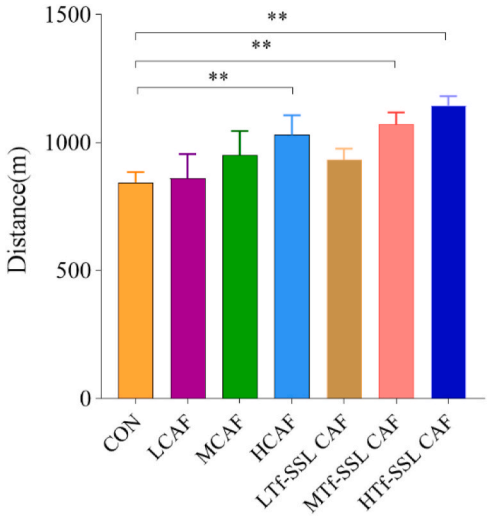

Fig. 5. Results of running wheel measurement in different groups of rats. CON: Control group; LCAF: Low dose caffeine; MCAF: Medium dose caffeine; HCAF: High dose caffeine; LTf-SSL CAF: Low dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; MTf-SSL CAF: Medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; HTf-SSL CAF: High dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; **p < 0.01 compared to the control group.

3.2.5. Dopamine secretion level in rat brain striatum

Dopamine is an important neurotransmitter associated with fatigue, and increased dopamine secretion facilitates improved physical exercise performance. As shown in Fig. 6, the level of striatal dopamine secretion in CON rats was 116.08 ± 4.53 ng/ml, compared to the CON group with a significant increase in striatal dopamine reaching 129.38 ± 6.57 ng/ml in the HCAF group, and there was a significant difference $p < 0.01$. The striatal dopamine secretion of rats in the MTf-SSL CAF group was 132.40 ± 4.12 ng/ml higher than that in the MCAF and HCAF groups and was significantly different from the CON group $p < 0.01$. The amount of striatal dopamine secretion was higher in the HTf-SSL CAF group than in the MTf-SSL CAF group 139.62 ± 3.43 ng/ml in rats and was very significantly different from the CON group $p < 0.01$.

4. Discussion

The main findings of this study were that supplementation with medium to high doses of caffeine had the effect of enhancing

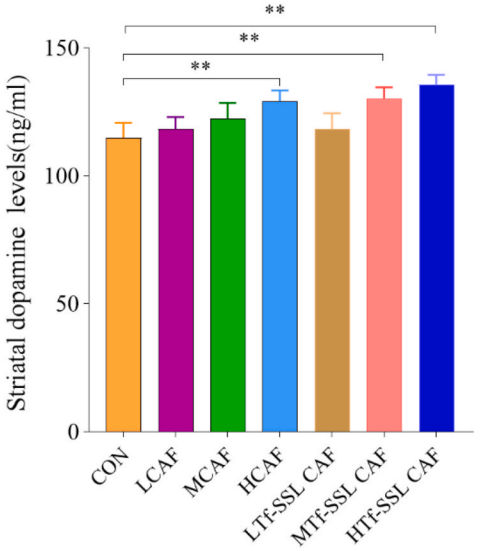

Fig. 6. Results of striatal dopamine assay in different groups of rats. CON: Control group; LCAF: Low dose caffeine; MCAF: Medium dose caffeine; HCAF: High dose caffeine; LTf-SSL CAF: Low dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; MTf-SSL CAF: Medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; HTf-SSL CAF: High dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine; **$p < 0.01$ compared to the control group.

physical performance (swimming, running and forelimb grip) in rats, and supplementation with low doses of caffeine did not cause the effect of enhancing physical performance in rats. In particular, the MTf-SSL group consistently exhibited higher performance values compared to the MCAF and HCAF groups, indicating that medium dose transferrin-modified, sterically-stabilized liposome-encapsulated caffeine may be more beneficial for enhancing the physical performance of rats.

Numerous experiments have also shown that caffeine intake has the effect of improving physical performance [2–5]. This was also confirmed in the present study, where the physical performance of the rats was gradually increased with increasing doses of caffeine. Caffeine is reported to be distributed throughout the body within 5 min of ingestion/injection and begins to produce peak effects 15 min later and lasts for approximately 1 h (typically 99 % of the ingested dose is absorbed within 45 min) [24,25]. It has been shown that caffeine improves muscular endurance mainly by direct stimulation of the central nervous system, leading to delayed fatigue, increased alertness and attention of the body [25]. In the present study, a significant increase in the duration of swimming to exhaustion was observed in the MCAF group, the HCAF group, the MTf-SSL CAF group and the HTf-SSL CAF group, which may be related to the antagonistic effect of caffeine on adenosine receptors. Adenosine has four G protein-coupled receptors, A1, A2a, A2b and A3, mainly distributed in different regions of the brain, each with a unique tissue distribution and pharmacological profile that is upregulated with increasing caffeine intake. Adenosine binding to A1 and A2a G protein-coupled receptors inhibits the release of various neurotransmitters (e.g., dopamine, catecholamines, glutamate, 5-hydroxytryptamine, etc.). Caffeine is structurally similar to adenosine; therefore, when ingested, it blocks the binding of adenosine to A1 and A2a receptors and promotes the release of these neurotransmitters [26], and the use of caffeine increases the release of dopamine in the striatum [27]. Therefore, the present study measured the level of dopamine in the striatum of rats, and its results also showed that with the gradual increase in the concentration of caffeine intake, the level of dopamine secretion in the striatum of rats was gradually increased and the locomotor capacity of rats was enhanced compared to the CON group. Among them, according to the results of this study, low doses of caffeine did not cause significant changes in dopamine, but high doses increased the release of dopamine in the brain. During prolonged wakefulness, high doses of caffeine produced more significant and beneficial effects on alertness than low and medium doses of caffeine, similar to the findings of Kalmar et al. [28]. The liposomal caffeine group was superior to the caffeine supplementation alone group, especially the MTf-SSL CAF group exhibited better exercise effects. The 6-h running distance of rats in the MTf-SSL CAF group was higher than that in the HCAF group, which reduced the intake of caffeine and improved the bioavailability and side effects of the drug. It also enabled the sustained and long-lasting release of caffeine and enhanced drug stability. Liposomes are one of the carriers for the introduction of various drugs into target cells, and their properties such as high efficiency, high biocompatibility, and low immunogenicity have been widely used in the medical field, which also seems to be confirmed in the present study. In addition, it has been shown, that caffeine also increases calcium release from the sarcoplasmic reticulum and motor unit recruitment, which may lead to more powerful muscle contractions [29], and this may be one of the reasons for caffeine's muscle strength enhancing properties.

The development of liposomal delivery systems for caffeine administration represents significant improvements over traditional methods. Liposomes can protect caffeine molecules from rapid degradation, ensure a stable supply of caffeine, and reduce the risk of adverse effects associated with peak concentrations, avoiding the peaks and troughs seen with conventional caffeine sources. This delivery precision may help athletes maintain optimal performance levels during competitions and training in the future. Currently, liposome-encapsulated drugs are already used in clinical and therapeutic fields [30,31]. For patients needing cognitive enhancement

or suffering from fatigue-related conditions, liposomal caffeine can provide a more stable and effective treatment option. Extensive research is still needed to explore the optimization of liposome formulations to improve drug loading capacity, stability, and targeting efficiency. Additionally, combining caffeine with other bioactive compounds in liposomal formulations may produce synergistic effects, although this requires further extensive research to validate. There is still a long way to go in the future.

5. Conclusion

In conclusion, caffeine supplementation had the effect of improving physical performance (including upper limb muscle strength and aerobic capacity) in rats, and the physical performance was enhanced with increasing doses of caffeine supplementation. Caffeine encapsulated in Tf-SSL superior effectiveness compared to standalone caffeine supplementation in enhancing strength and aerobic exercise capacity in rats. Therefore, caffeine-loaded liposomes have the potential to serve as a more effective caffeine supplement for improving athletic performance, requiring lower caffeine doses and consequently reducing caffeine toxicity.

Ethics declarations

This study adhered to the principles of the Helsinki Declaration and received approval from the Sports Science Experiment Ethics Committee of Beijing Sport University (Ethics Committee No.2023183A). The studies were conducted in accordance with the local legislation and institutional requirements.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Consent for publication

Not Applicable.

CRediT authorship contribution statement

Hezhang Yun: Writing – original draft, Software, Formal analysis, Data curation. **Wenbo Su:** Writing – original draft, Software, Project administration. **Ting You:** Methodology, Investigation, Data curation. **Jing Wang:** Writing – original draft, Resources, Methodology, Data curation. **Yuxuan Ying:** Formal analysis, Data curation. **Can Wang:** Validation, Software. **Yuyi Ren:** Methodology, Formal analysis. **Bin Lu:** Formal analysis, Data curation. **Yi Li:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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