JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2020, 71, 3, 373-382
www.jpp.krakow.pl | DOI: 10.26402/jpp.2020.3.08

S. WUPPER[1], A. FISCHER[1], K. LUERSEN[1], I.R. IPHARRAGUERRE[1], K. CHIKAMOTO[2], T. FURUNE[2], Y. ISHIDA[2], K. TERAO[2], G. RIMBACH[1]

EFFECTS OF DIETARY GAMMA-CYCLODEXTRIN ON VOLUNTARY ACTIVITY AND MUSCLE STRENGTH IN MICE

[1]Institute of Human Nutrition and Food Science, University of Kiel, Kiel, Germany;
[2]CycloChem Bio Co., Ltd., 7-4-5 Minatojima-minamimachi, Chuo-ku, Kobe, Japan

Gamma-cyclodextrin (γCD) is a cyclic oligosaccharide consisting of eight α-(1,4)-linked glucopyranose subunits, which is often used in the food and pharmaceutical industries. However, little is known regarding the metabolic activity of "empty" γCD *per se*. Therefore, in the present study young C57BL/6 male mice received a control diet (CON) or an experimental diet that was supplemented with 12.88% γCD exchanged against corn starch. After 6 weeks of treatment, the voluntary wheel running activity was monitored and the muscle strength of mice was measured by employing Kondziela's inverted screen test and forelimb grip strength assay. The γCD-treated mice covered a significantly larger distance per night (CON 8.6 km, γCD 12.4 km) and were significantly longer active (CON 340 min, γCD 437 min). Moreover, γCD-treated mice significantly performed better at the inverted screen test indicated by an enhanced Kondziela score (CON 3.10, γCD 4.63). These data suggest that dietary γCD leads to an increased endurance. We also found a slightly anti-glycemic effect of γCD during oral glucose tolerance test. However, our mice from the γCD group exhibited no difference in terms of GLUT2 protein level in ileum tissue nor increased muscle glycogen storage. Furthermore, γCD exhibited no DPP-4 inhibitory activity *in vitro*. By analysing candidate muscle genes and proteins related to endurance and muscle performance we did not observe any differences in terms of Sirt1, Pgc1α, Cpt1b, Mef2c, Myh1 and Myh2 gene expression levels as well as total oxidative phosphorylation (OXPHOS), mtTFA and GLUT4 protein expression levels in skeletal muscle in response to γCD. We could not fully establish the exact underlying molecular mechanisms of the fitness improvement by dietary γCD which warrants further investigations.

Key words: *gamma-cyclodextrin, exercise, skeletal muscle, muscle strength, wheel running, glucose transporter, sirtuin 1, total oxidative phosphorylation, sports nutrition*

INTRODUCTION

Cyclodextrins (CDs) are natural conversion compounds of starch, first discovered in 1891 by Villiers (1). CDs are cyclic oligosaccharides consisting of α-(1,4)-linked glucopyranose subunits. The most prominent members of the CD family are α- (six glucopyranose units), β- (seven glucopyranose units) and γCD (eight glucopyranose units, *Fig. 1*). CDs are characterized by a toroid molecular structure consisting of a hydrophilic outer wall and a less hydrophilic inner wall (1). Through this steric arrangement of glucose units, CDs are soluble in water and can function as important molecular complexation agents (2, 3). In fact, we have previously shown that various nutritional supplements including R-α lipoic acid (4, 5), tocotrienols (6) and Brazilian green propolis (7) exhibit an improved bioactivity in mice attributable to the encapsulation into γCD. γCD differs from αCD and βCD in that it is almost completely degraded by salivary and pancreatic α-amylase, similar to digestion of starch and linear dextrins (8-10). Furthermore, γCD shows the highest water solubility and bioavailability with the least toxicity among all parental CDs (1, 2, 11). A dietary dose of up to 20% γCD has been shown to be tolerated without toxic side effects in studies with Beagle dogs and Wistar rats (9, 12). In humans, a single dose of 8 g γCD in 100 g yogurt was tolerated equally well as the same amount of maltodextrin (13). Accordingly, γCD acts as a drug carrier and is often used in the food and pharmaceutical industries (2).

Adequate exercise training is a robust strategy to increase strength, endurance and neuroplasticity (14, 15). Furthermore, it is well established that diet composition can affect exercise capacity by altering the availability and utilization of the major energy source (16). Ingestion of carbohydrates prior to and during exercise can enhance endurance capacity. Expanding and maintaining glycogen stores is thereby an often discussed mechanism behind this performance improvement through carbohydrates (17, 18). Moreover, the beneficial effect may depend on the ingested carbohydrate type, which can differently influence factors like glycemic response, gastric emptying, fluid delivery, absorption rates and gastrointestinal comfort (18). These factors define the rate of carbohydrate availability, thereby determining the pre- or post-exercise glycogen stores (18). Thus, identifying natural conversion compounds of starch which increase exercise capacity and deciphering their underlying molecular mechanisms may lead to the development

of new strategies to further improve the beneficial effects of exercise by dietary means.

Voluntary activity in mice can be determined *via* recording wheel running behaviour. This wheel running behaviour of mice is often used to establish physical performance and endurance (19). Voluntary wheel running occurs within routine diurnal rhythmicity patterns in a non-stressed laboratory environment. Further advantages from voluntary wheel running are similarity to natural running behaviour of mice and the absence of direct interference from the researcher. Besides, it appears to satisfy playing and escaping (20, 21). The voluntary wheel running activity differs by sex, strain, age, design of the running wheel, diet and environment conditions (21). The alternative to voluntary wheel running is forced exercise. Forced exercise models, which use aversive stimuli to induce exercise, have the advantage to ensure reproducible distances and speed. However, one has to be aware that experimental parameters such as performance in daylight hours contrary to the murine diurnal pattern may be confounding factors in terms of an appropriate physiological response (19-21).

In our recently published mouse study, γCD-fed mice were originally considered as an experimental group receiving the vehicle control diet (22). However, phenotypic data obtained from voluntary wheel running and measuring of muscle strength revealed significant differences between control mice (not receiving the vehicle control) and mice supplemented with γCD. Mice from the γCD group showed increased voluntary activity in the running wheel and enhanced muscle strength in exercise assays indicating that γCD has beneficial effects on exercise capacity. To illuminate the underlying molecular mechanism, gene and protein expression levels of candidate genes of the energy metabolism and myogenesis were examined in skeletal muscle tissue.

MATERIALS AND METHODS

Mice and diet

In the present study, we employed the murine inbred strain C57BL/6NRj that has been shown to be appropriate for voluntary wheel running studies (21). Five–weeks-old male C57BL/6NRj mice were purchased from Janvier Labs (Saint Berthevin Cedex, France). Mice (initial body weight = 17.5–22.4 g) were housed in groups (5 animals per cage) in Makrolon cages with environmental enrichment under controlled climatic conditions (22 – 24ºC, 55% relative humidity, 12 h light/dark cycle) at the Institute of Human Nutrition and Food Science at the University of Kiel. The mice had free access to tap water and the experimental diets throughout the feeding study (22). Mice were fed a purified, semisynthetic, energy-dense, high-fat, high-fructose diet (Ssniff S0065-E230) based on casein, corn starch and pork lard as previously described (22). After a 2-week adaptation phase, the experiment began by dividing the mice randomly into groups with 10 mice each. Next to the control group (CON), there was the supplementation group that received the experimental diet supplemented with 12.88% γCD (CycloChem Bio Co., Ltd., Kobe, Japan) exchanged against corn starch.

Over the entire study period, the health conditions of mice were controlled daily. Mice were weighed weekly and fed daily. We lost two animals over the entire time period, one before the intervention started and one from the γCD group. After 6 weeks on the experimental diets, the mice were fasted for 5 hours prior to euthanization with carbon dioxide. Blood and tissue samples were collected as described previously (22).

Animal studies were performed according to German and international regulations of animal welfare. The experimental protocol was approved by the local authority on 31 August 2018 (Ministry of Energy, Agriculture, the Environment, Nature and Digitalization, Schleswig-Holstein, V 242-28307/2018).

Voluntary wheel running

A 11.8 cm diameter upright running wheel as part of the PhenoMaster system (TSE Systems GmbH, Bad Homburg, Germany) was placed in isolated cages to measure voluntary wheel running activity. Mice were placed separately in a cage with running wheel access for 12 hours overnight with free access to tap water and experimental diets. To record total distance, time and speed, the software TSE PhenoMaster V5.1.5 (2014-4301) was used.

Measuring the musce strength of mice

To determine the muscle strength of animals using all four limbs, the Kondziela's inverted screen test was performed (23). Each mouse was placed in the centre of the screen, which was built according to Deacon (23). The screen was smoothly inverted, with the head of the mouse declining first representing the starting point t_0. The screen was held steadily 20 – 30 cm above a soft surface covered with litter. The time point when the mouse fell off the screen was recorded to a maximum of 20 min. Depending on the hanging time, score points were awarded from 1 (< 5 min) to 5 (≥ 20 min).

Forelimb grip strength was measured by a weight lifting test employing seven weights, which constitutes the apparatus according to Deacon (23). Each weight consists of a ball of tangled fine gauge stainless steel wire, a scale collector (12 g) attached to a series of steel chain links (12 – 14 g). The mass of the weights were therefore: 12, 26, 40, 54, 66, 80 and 94 g. The number of links ranged from zero to six. Each mouse was held by the middle of the tail and lowered to grasp the first weight. As it grasped the scale collector, it was raised until the link was clear off the bench. A hold for 3 s was the threshold criterion. If the weight was dropped before 3 s, the mouse was given a second and third chance (23). A final total score was calculated as the product of the number of links in the heaviest chain held for the full 3 sec, multiplied by the time (s) it was held. If the heaviest weight was dropped before 3 s an appropriate intermediate value was calculated for the time of chain which could not be lifted for full 3 s. Thus, a mouse holding a 5-link weight for 3 s, but unable to lift a 6-link weight, is assigned a score of 15 (5*3). If it held the 6-link weight 3 times for 1 sec, it scores 16 (5*3+1).

Oral glucose tolerance test in mice

After 6 weeks on the experimental diets, mice from each experimental group were fasted for 5 – 6 hours, only receiving water *ad libitum*, prior to the oral glucose tolerance test (oGTT). For the oGTT, 2 g glucose/kg body weight was administered orally by gavage, and glucose levels were measured in blood taken from the tail tip (glucometer, Abbott Freestyle Lite). The blood glucose level was determined immediately prior glucose administration (at 0 min) as fasting blood glucose level and then after 15, 30 and 60 min of glucose administration.

Glycogen in muscle

The glycogen content of muscle was measured with a commercially available fluorometric assay (MAK016, Sigma-Aldrich Chemie GmbH, Darmstadt, Germany) according to the manufacturer's protocol.

RNA isolation and one-step quantitative reverse transcription real-time polymerase chain reaction

Total RNA was isolated from muscle tissue stored in RNAlater (Qiagen, Hilden, Germany) with peqGOLD TriFAst (VWR International, Darmstadt, Germany) following the manufacturer's instructions and as described previously (24). A one-step quantitative reverse transcription real-time polymerase chain reaction (one-step qRT-PCR) was carried out with a SensiFAST SYBR No-ROX One-step Kit (Bioline, Luckenwalde, Germany) and SYBR Green detection according to the manufacturer's protocol using a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia). Primers were designed with Primer 3 Input software (version 4.1.0) and purchased from Eurofins MWG (Ebersberg, Germany). Corresponding primer sequences and annealing temperatures are given in *Table 1*. RNA amplification for each sample was conducted in duplicate, and each run included a standard curve and a no-template control. Relative mRNA levels of target genes were normalized to Ap3d1 gene expression, and fold changes relative to the control group are given.

Western blot analysis

Protein expression was determined in whole cell lysates prepared from frozen muscle and ileum tissue, respectively. Protein concentrations were determined with a Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Bremen, Germany) according to the manufacturer's instructions.

For Western blotting, samples containing 30 µg of protein each for muscle tissue and 35 µg of protein each for ileum tissue were heated at 37ºC for 20 min with loading buffer, and a Western blot analysis was performed as previously described in detail (25). Target proteins were identified using the corresponding primary antibodies (glucose transporter 2, GLUT2: Novus Biologicals, Colorado, USA; glucose transporter 4, GLUT4: Sigma-Aldrich; transcription factor A, mitochondrial, mtTFA:

Table 1. Primer sequences and annealing temperatures for one-step quantitative reverse transcription real-time polymerase chain reaction.

Primer	Gene	Gene ID	Primer sequence	T_a [°C][1]
Ap3d1	Adaptor-related protein complex 3, delta 1 subunit	11776	F[2]: AGGAGCTGAAGCAGGACAAC	55
			R[3]: CGCTTGAATGTGAACTTGGA	
Cpt1b	Carnitine palmitoyltransferase 1b	12895	F: CTACCACAAAGGTCGCTTCT	57
			R: GGATTCTCTGGAACTGCATC	
Mef2c	Myocyte enhancer factor 2C	17260	F: CTGTCAGCACACTGGGAAAC	60
			R: CCTGTGTTACCTGCACTTGG	
Myh1	*Mus musculus* myosin, heavy polypeptide 1, skeletal muscle	17879	F: GCGAATCGAGGCTCAGAACAA	61
			R: GTAGTTCCGCCTTCGGTCTTG	
Myh2	*Mus musculus* myosin, heavy polypeptide 2, skeletal muscle	17882	F: AAGTGACTGTGAAAACAGAAGCA	55
			R: GCAGCCATTTGTAAGGGTTGAC	
Pgc1α	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	19017	F: TGCCCAGATCTTCCTGAACT	57
			R: TCTGTGAGAACCGCTAGCAA	
Sirt1	Sirtuin 1	93759	F: GTCTCCTGTGGGATTCCTGA	61
			R: ACACAGAGACGGCTGGAACT	

[1]T_a, annealing temperature; [2]F, forward; [3]R, reverse.

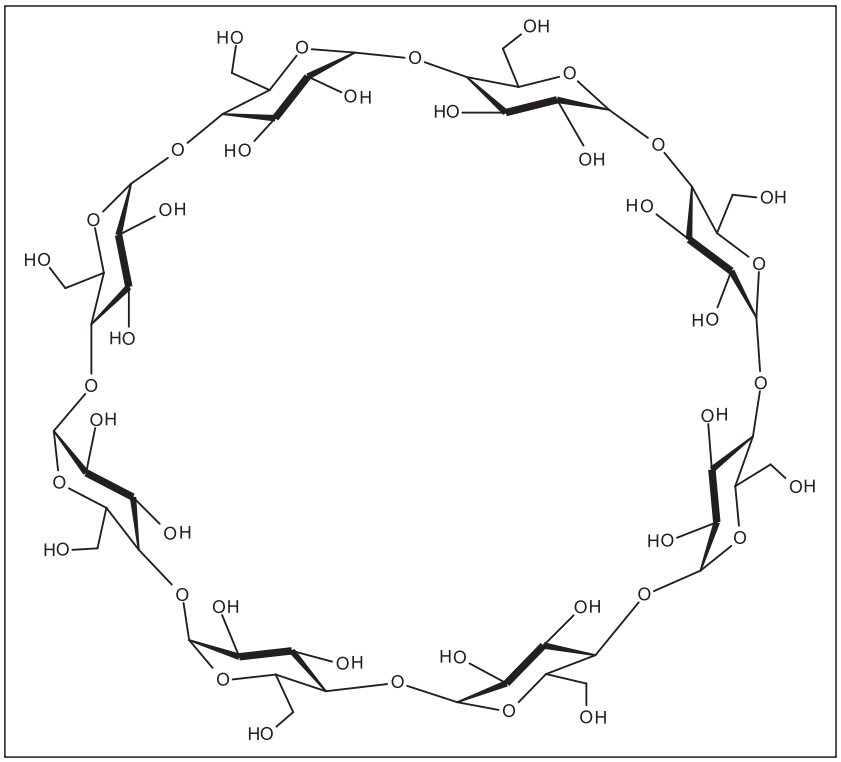


Fig. 1. Chemical structure of γ-cyclodextrin.

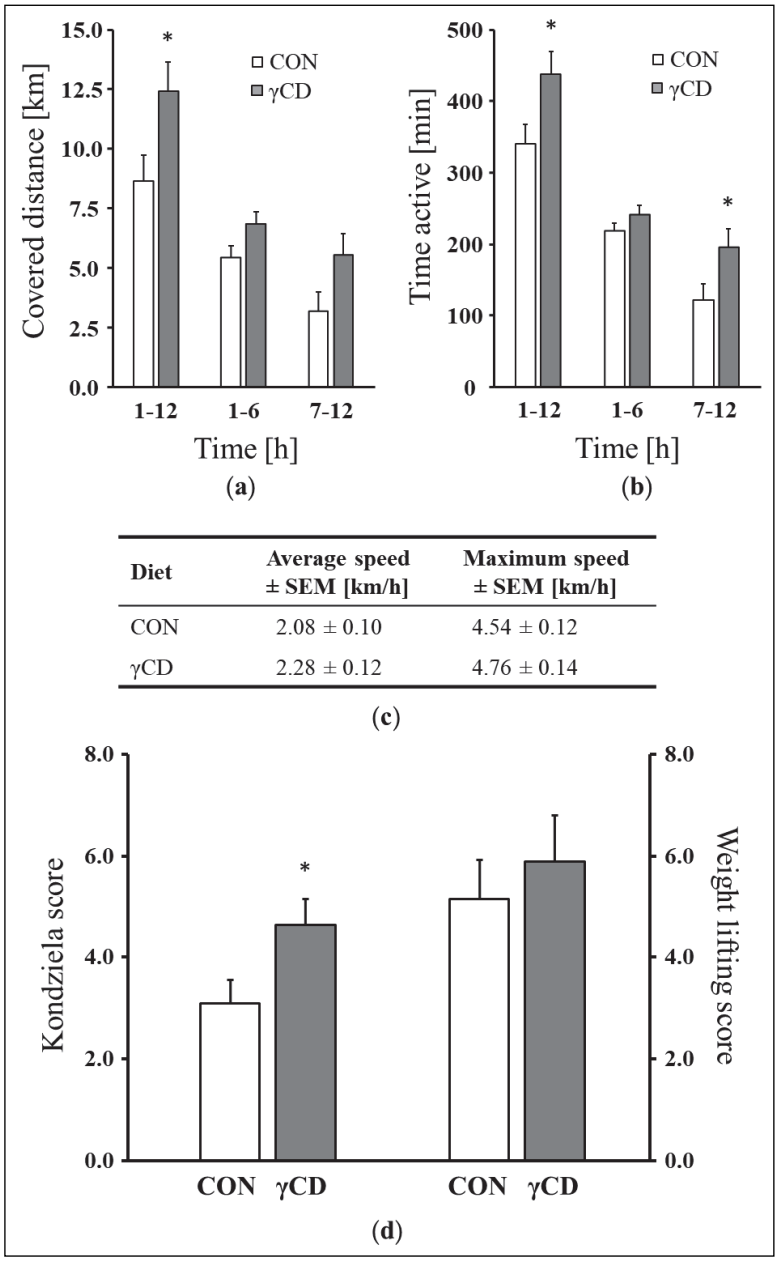
Santa Cruz Biotechnologies, Dallas, USA; OXPHOS rodent antibody cocktail: Abcam, Cambridge, United Kingdom) and secondary antibodies. Immunoreactive proteins were detected with enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific). Bands were visualized in a ChemiDoc XRS system (Bio-Rad, Munich, Germany). Target protein expression was related to the total protein load per lane.

Dipeptidyl peptidase-4 inhibition assay

Human dipeptidyl peptidase-4 (DPP-4) inhibition assay with γCD as potential inhibitor was performed *in-vitro* using a human inhibitor screening kit (MAK203, Sigma-Aldrich) according to the manufacturer's protocol. γCD was solved in assay buffer and analysed in concentrations of 1, 10, 100 µg/mL and 1 g/mL.

Statistical analysis

For the statistical analysis, the model of variance analysis was used. The voluntary wheel running performance was analysed using a mixed-effect model in which mice nested within cages was used as a random variable, whereas diet and run were considered as fixed variables. However, run was not significant and therefore not used for statistical analysis. The same mixed-model was used to analyse the muscle strength data, area under the curve (AUC) from oGTT, fasting blood glucose data and glycogen in muscle. The oGTT was analysed using a mixed-effect model with repeated measures in time. In the model, mice nested within cages was entered as a random variable and diet, run, time and the two-way interaction of diet and time were considered as fixed effects. However, run and the two-way interaction of diet and time were not significant and therefore excluded from the model. Least squares means were separated into significant effects using the Tukey adjustment. Differences among treatments were considered to be significant when $P < 0.05$. Statistical analyses were performed with SAS (release 9.2, SAS Institute). Data from qRT-PCR and Western blot were analysed for normality of distribution (Kolmogorov-Smirnov and Shapiro-Wilk test). In the case of normally distributed data, the *t*-test was applied. In



Diet	Average speed ± SEM [km/h]	Maximum speed ± SEM [km/h]
CON	2.08 ± 0.10	4.54 ± 0.12
γCD	2.28 ± 0.12	4.76 ± 0.14

(c)

Fig. 2. The mice supplemented with γ-cyclodextrin (γCD) showed a higher voluntary activity and increased muscle strength compared to the control mice. Mice were fed (*ad libitum*) for 6 weeks with a Western-type control diet (CON) and control diet supplemented with 12.88% γCD (γCD) exchanged against corn starch, respectively, before they were placed in a cage with running wheel access for 12 hours overnight.

a) γCD mice covered a significantly larger distance per night (1 – 12) compared to control mice in the running wheel. When looking at the first and the second half of the night separately, the γCD mice covered barely significantly larger distances ($P = 0.061$ and $P = 0.057$, respectively). Bars represent the least squares means (LSM) + standard error of the mean (SEM).

b) However, the mice supplemented with γCD were significantly longer active during the whole night and in the second part of the night compared to CON. Bars represent the LSM + SEM.

c) Average and maximum speed in running wheel were not significantly different between groups. Data are expressed as LSM ± SEM.

d) Kondziela's inverted screen test was performed and the maximum time of 20 minutes was the abort criterion. The score from the Kondziela's inverted screen test was significantly higher in γCD mice compared to CON mice. The score from the weight lifting test showed no significantly differences between the groups. Bars represent the LSM + SEM. All data were analysed with ANCOVA, $*P < 0.05$, n = 8 – 10 mice/diet.

the absence of normally distributed data, the Mann-Whitney U test was applied. The results were considered statistically significant at $P < 0.05$. IBM SPSS statistical software (version 25, IBM: Armonk, NY, USA) was used for the analyses.

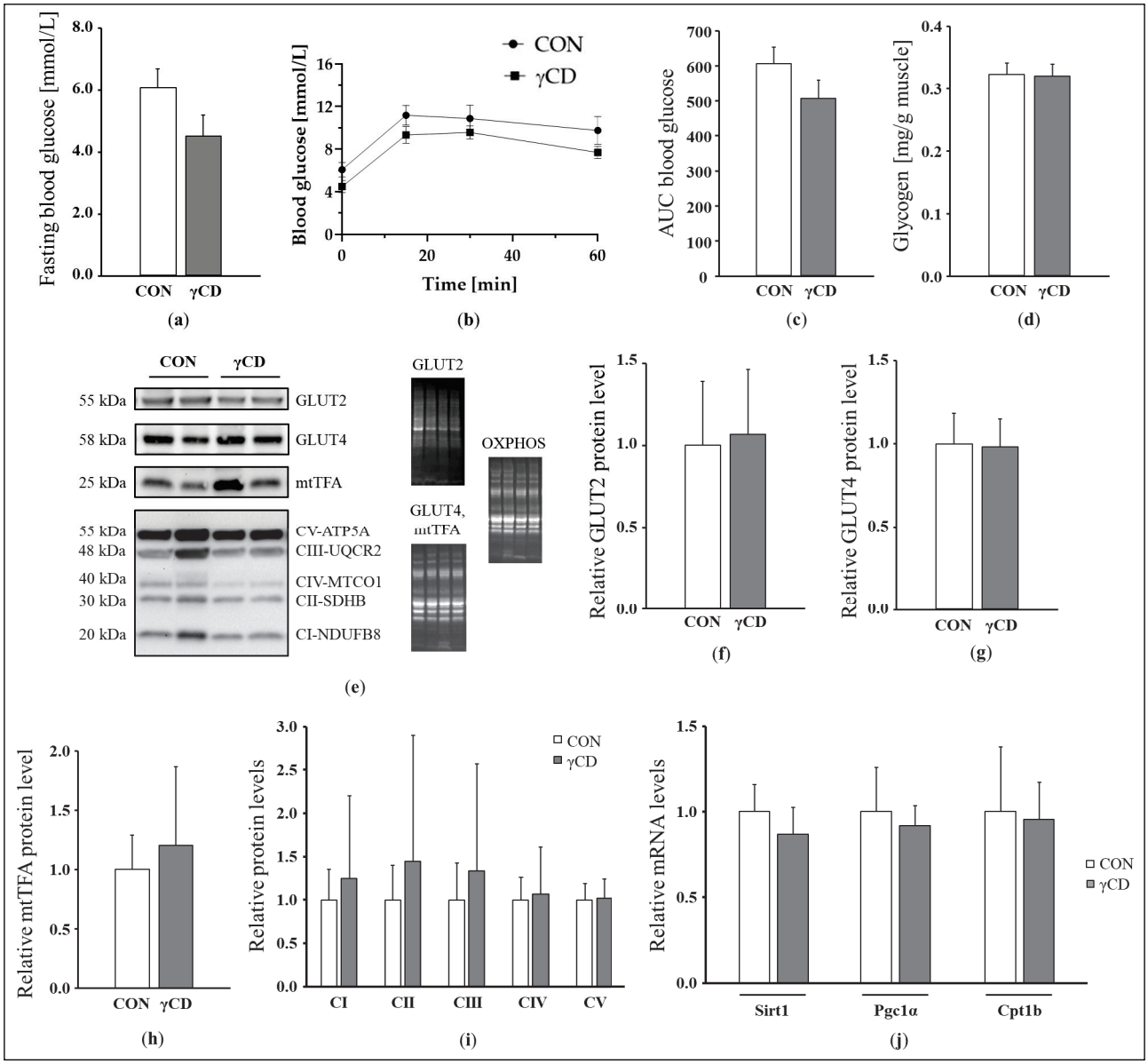


Fig. 3. Effect of γ-cyclodextrin on glucose metabolism and muscle parameters that are known to be related to muscle strength and endurance.

a) Fasting blood glucose levels from oral glucose tolerance test (oGTT) in mice fed (*ad libitum*) a Western-type diet supplemented with 0 (control, CON) or 12.88% γ-cyclodextrin (γCD) exchanged against corn starch over a 6-week experimental period. Bars represent the least squares means (LSM) + standard error of the mean (SEM).

b) oGTT (LSM ± SEM) and

c) area under the curve (AUC) of oGTT. Bars represent the LSM + SEM.

d) Glycogen content in muscle was not different between CON and γCD. Bars represent the LSM + SEM. Data were analysed with ANCOVA, n = 8 – 10 mice/diet.

Western blot (e) and densitometric analysis (f, g, h, i) of glucose transporter 2 (GLUT2) in ileum tissue and glucose transporter 4 (GLUT4), transcription factor A, mitochondrial (mtTFA) and mitochondrial respiratory complexes subunits in muscle tissue. Protein levels were determined by Western blotting, and a representative blot with the corresponding stain-free UV image is shown. Protein bands were analysed densitometrically. Values were related to total protein load per lane, and are expressed in relation to the CON group. Bars represent the mean + standard deviation (n = 8 – 10 mice/diet), analysed with t-test.

j) No effect of dietary γCD on mRNA levels of sirtuin 1 (Sirt1), peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (Pgc1α) and carnitine palmitoyltransferase 1b (Cpt1b) in skeletal muscle. Gene expression was analysed *via* a one-step quantitative reverse transcription real-time polymerase chain reaction (one-step qRT-PCR). All qRT-PCR data were normalized to Ap3d1 gene expression and are expressed in relation to the CON group. Data are given as the mean + standard deviation (n = 8 – 10 mice/diet) and were analysed with t-test.

RESULTS

Dietary γCD increased voluntary activity and muscle strength

The mice supplemented with γCD showed a higher voluntary activity in the running wheel compared to CON mice, especially in the second part of the night. The γCD mice covered significantly larger distances with an average distance of 12.4 ± 1.2 km (CON 8.6 ± 1.1 km) per night (*Fig. 2a*) and were significantly longer active during the whole night (CON 340 ± 28 min, γCD 437 ± 31 min; *Fig. 2b*). However, average and maximum speed were not significantly different between the groups (*Fig. 2c*). To get a better understanding of running characteristics, we separated the night period into two halves (first six hours versus last six hours). In the first half of the night, the running distance of the γCD supplemented mice was barely significantly different from controls (CON 5.4 ± 0.5 km, γCD 5.6 ± 0.9 km, P = 0.061; *Fig. 2a*), while the active time of both groups was quite similar (CON 218 ± 10 min, γCD 242 ± 12 min; *Fig. 2b*). In the second half of the night, γCD-treated mice were significantly longer active (CON 121 ± 23 min, γCD 196 ± 25 min; *Fig. 2b*). However, the covered distance was only barely significantly different (CON 3.2 ± 0.8 km, γCD 5.6 ± 0.9 km, P = 0.057; *Fig. 2a*).

To test the muscle strength of our mice, the Kondziela's inverted screen test as well as the weight lifting test were performed. From the mice supplemented with γCD, 5 out of 8 animals (62.5%) reached the abort criterion timepoint of 20 min, whereas in the CON group only 4 out of 10 animals reached the 20 min (40.0%). Therefore, γCD mice hang significantly longer at the inverted screen, shown as Kondziela score (CON 3.1 ± 0.5, γCD 4.6 ± 0.5; *Fig. 2d*). However, the weight lifting score representing forelimb grip strength showed no significant differences between the two groups (CON 5.1 ± 0.8, γCD 5.9 ± 0.9; *Fig. 2d*).

γCD supplementation did not significantly improve glucose metabolism

The effect of dietary γCD on glucose metabolism is shown in *Fig. 3*. Fasting blood glucose levels of γCD-treated mice were 26% lower than in control mice but did not reach statistical significance (P = 0.105; *Fig. 3a*). Furthermore, administration of dietary γCD slightly decreased glycemia over time during the oGTT and the corresponding AUC (*Fig. 3b* and *3c*). The peak blood glucose occurred at 15 – 30 min post glucose administration in both groups (*Fig. 3b*). To examine if γCD affects glucose transport in the intestine, GLUT2 protein levels in ileum tissue were determined. There was no significant difference between CON and γCD in terms of GLUT2 protein level (*Fig. 3e* and *3f*). The glycogen content in muscle of γCD mice was not significantly different between groups (*Fig. 3d*). GLUT4 protein level in muscle tissue from CON and γCD mice showed no significant difference (*Fig. 3e* and *3g*). The *in vitro* human DPP-4 inhibition assay exhibited no DPP-4 inhibitory activity of γCD at 1, 10, 100 µg/mL or 1 mg/mL, respectively (data not shown).

No effect of γCD on gene expression levels of regulators of mitochondrial biogenesis and muscle development in skeletal muscle

To examine if γCD affects the mitochondria of skeletal muscle cells, the steady-state mRNA levels of the genes encoding proteins centrally involved in mitochondrial biogenesis and function were determined *via* qRT-PCR. Dietary γCD had no effect on gene expression of sirtuin 1 (Sirt1), peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (Pgc1α) and carnitine palmitoyltransferase 1b (Cpt1b) in skeletal muscle (*Fig. 3j*).

Furthermore, myocyte enhancer factor 2C (Mef2c) was examined *via* qRT-PCR to determine skeletal muscle development and function in CON versus γCD mice. There was no significant difference between the groups in terms of Mef2c mRNA levels (CON 1.00 ± 0.18, γCD 0.93 ± 0.08, data not shown). To determine muscle fibre type composition, the mRNA levels of *Mus musculus* myosin, heavy polypeptide 1, skeletal muscle (Myh1) and *Mus musculus* myosin, heavy polypeptide 2, skeletal muscle (Myh2) were measured *via* qRT-PCR. γCD supplementation had no significant effect on muscle fibre type composition regarding Myh1 (CON 1.00 ± 0.20, γCD 1.16 ± 0.30, data not shown) and Myh2 (CON 1.00 ± 1.39, γCD 0.89 ± 0.80, data not shown).

No difference in mitochondrial total oxidative phosphorylation protein levels in muscle

The levels of mitochondrial- and nuclear-encoded protein subunits involved in OXPHOS were evaluated using muscle extracts (*Fig. 3e* and *3i*). The densitometric analysis (*Fig. 3i*) showed no significant differences in protein levels of mitochondrial respiratory complexes subunits between CON and γCD mice. While complexes IV (CIV) and CV exhibited almost similar protein levels between the two groups, CI, CII and CIII were slightly increased in γCD-treated mice. Even the protein level of mtTFA in muscle tissue showed no significant differences between the groups (*Fig. 3e* and *3h*).

DISCUSSION

Little is known regarding the metabolic activity of "empty" γCD *per se*. Therefore, in the present mouse study we supplemented our experimental diet with 12.88% γCD exchanged against corn starch. Accordingly, the cyclic oligosaccharide γCD was the main complex carbohydrate source for γCD-treated mice when compared to the remaining 3.62% corn starch. A similar γCD concentration has been previously used in a feeding study in dogs without toxic side effects (12).

We observed a significant improvement in nocturnal voluntary wheel running activity by 44% and an enhanced muscle strength in our γCD-treated mice. The mice from CON group ran on average 8.6 km overnight, which is similar to the running distances reported for untreated male C57BL/6 mice in previous studies (20, 21, 26). In addition, the activity pattern of our untreated mice is consistent with published data. Under normal conditions, mice ran most intensely the first several hours of the active dark cycle (19, 20, 26). Accordingly, our CON mice ran a significantly higher distance during the first half of the night comparing with the second half. The main observed difference between CON and γCD mice was in the second half of the night regarding covered distance and running time. γCD mice spent 61% more time running than CON mice during the last six hours of the dark cycle. A detailed analysis of mice running trends revealed that during their actual running activity there was no difference in average and maximum running speed between untreated and treated mice. Our data suggest that dietary γCD caused an increased endurance in our mice. Phelps *et al.* (27) described an exhaustion phenotype when they compared the running activity of wild-type C57BL/6 female mice and α-klotho-deficient mice. Here, the klotho-deficient mice ran significantly less than the wild-type control. Similar to our results for γCD supplemented animals, the mutant mice ran at the same speed as the wild-type mice. However, in contrast to

our γCD mice, α-klotho-deficient mice spent less time running than the control wild-type animals, indicating that the lack of α-klotho protein resulted in a more quickly exhaustion (27). Accordingly, the running distance in combination with running time reflects endurance capacity in an appropriate way.

It has been assumed that voluntary wheel running could serve also as a measure of muscle performance (19). Consistent with that, we observed a significant increase in all four limbs muscle strength in γCD mice *via* Kondziela's inverted screen test although the underlying molecular mechanisms remain to be further elucidated. So far, administration of γCD in mice led to a more active and stronger phenotype, probably through increasing endurance.

Previous studies have shown that γCD is almost fully digested and absorbed within the small intestine (10, 28), whereas absorption of intact γCD from gastrointestinal tract is extremely low (0.02%) (29). Moreover, according to the findings by Spears *et al.* (28), an impact of γCD on intestinal microbiota is rather unlikely. Hence, the gastrointestinal tract with its digesting enzymes is most probably the primary site of action of γCD. Although γCD is resistant towards degradation by β-amylase, an enzyme that hydrolyzes starch from the non-reducing end, it is a substrate of α-amylase, which hydrolyzes α-bonds from large polysaccharides like starch (9). Accordingly, γCD is almost completely degraded to malto-triose, maltose and glucose by amylases (8-10). The degradation of γCD by α-amylase starts with a ring-opening reaction which results in linear malto-octaose that can be further degraded by amylases (10). Owing to its cone-shaped structure and its hydrophobic core, the ring-opening reaction by α-amylase represents the slowest step of the degradation process of γCD. Accordingly, γCD is most probably slower digested than the easily digestible linear polysaccharide maltodextrin (10, 30). In this context, it is interesting that depending on its actual complex structure starch is classified as slowly or rapidly digestible starch. Compared to the rapidly digestible form, the amylase-mediated breakdown of slowly digestible starch is hindered, which affects the rate of glucose release and glucose absorption (31). The impact of meals with different glycemic indexes (GI) on exercise was examined by Kirwan *et al.* (32). A pre-exercise meal with a moderate GI significantly increased performance time in young men in contrast to a high GI meal prior to exercise or water as control. In addition, consuming the moderate GI meal maintained euglycemia during exercise over an extended period of time (32). In line with this assumption, it has been reported that γCD results in a lower postprandial glucose response when compared with maltodextrin (30) and provides glucose over a prolonged period of time during exercise. Asp *et al.* (30) performed a study with 32 healthy adult subjects who received 50 g of carbohydrate from γCD or maltodextrin *via* beverages. In this study, γCD reduced the postprandial glycemic and insulinemic response compared with the rapidly digested maltodextrin in humans without carbohydrate malabsorption. Additionally, inhibitory effects of CDs on the activity of amylase have been demonstrated (11, 33, 34). When amylose is used as substrate, α-amylase activity is competitively inhibited by γCD (34). This can additionally attenuate the postprandial glycemic and insulinemic response and may contribute to a steady and longer lasting supply of glucose through a slowed polysaccharide digestion rate in γCD supplemented animals.

Remarkably, Takii *et al.* (35) reported an enhancement of swimming endurance in mice by highly branched cyclic dextrin (HBCD). Furuyashiki *et al.* (36) compared the efficacy of HBCD with maltodextrin during endurance exercise on the rating of perceived exertion (RPE) in humans. Interestingly, the increase in RPE was significantly lower after ingesting HBCD than maltodextrin. It has to be noted though that HBCD differs structurally from γCD, but this likewise digested cyclic dextrins may affect endurance capacity in a similar way as γCD.

However, the improvement in voluntary wheel running activity and enhanced muscle strength by γCD could also be a result of behavioral change in mice. Two-hydroxypropyl-γCD showed positive effects in Niemann-Pick type C (NPC1) patient-derived fibroblasts through restoring cellular homeostasis (37). Besides, there is evidence that hydroxypropyl-β-CD treatment *via* subcutaneous injection improved learning and memory deficits in a transgenic mouse model of Alzheimer disease (38). An overview of βCDs and its derivatives in the treatment of neurodegenerative diseases is given by Coisne *et al.* (39). Unlike our experimental design, the injection or nasal delivery of CD partly avoid the gastrointestinal tract with its digesting enzymes, thus resulting in primary intact CD molecules in the circulatory system. The extent to which dietary cyclodextrins have an influence on brain function remains open.

We examined several muscle parameters that are known to be related to muscle strength and endurance in order to find out whether γCD supplementation affected muscle physiology. The glucose transporter GLUT4 mediates insulin-stimulated glucose transport in muscle tissue (40) and muscle glycogen plays an important role in athletic performance (41), since pre-exercise muscle glycogen content has been shown to determine muscle time to fatigue (42). However, there was no effect of dietary γCD on muscle GLUT4 protein and glycogen content in our mice.

Endurance capacity depends among other things on muscle fibre type proportions (43). So, we took a deeper look in skeletal muscle structure of our mice. Dietary γCD did not alter gene expression of Mef2c, a key regulator of muscle development. Even the muscle fibre type composition was not shifted in γCD-treated mice after 6 weeks on experimental diet and one night in the running wheel compared to CON mice.

Pgc1α is a transcription factor in muscle, adipose tissue and other tissues, controlling oxidative metabolism including muscle fibre type switching (44). Recently published studies with PGC1α transgenic mice have shown that increased skeletal muscle PGC1α expression decreased fatigability (45) and increased exercise ability (46, 47). Additionally, enhanced oxidative capacity and endurance were accompanied with enriched muscle expression of genes and proteins involved in mitochondrial biogenesis and function, including Sirt1, Pgc1α and mtTfa (48) as well as increased mitochondrial OXPHOS efficiency (49). Besides, it has been shown that endurance training increased the expression of oxidative and lipid metabolism markers like CPT1 (50) because mitochondrial fatty acid oxidation with its key enzyme CPT1 is the main muscle energy source during endurance exercise (51, 52). Moreover, Miklosz *et al.* (53) showed that a single exercise bout induced mRNA and protein expression of pro-lipolytic-related proteins in skeletal muscle of male rats, such as adipose triglyceride lipase and hormone sensitive lipase. However, we did not observe differences in terms of Sirt1, Pgc1α and Cpt1b gene expression levels as well as OXPHOS and mtTFA protein expression levels in skeletal muscle in response to dietary γCD and exercise.

A strength of this study was that we conducted a controlled feeding trial with comprehensive phenotyping. Furthermore, we determined many biomarkers related to skeletal muscle function and strength in our mice. In this context, it has to be noted that we partly measured potential targets of gCD only on the mRNA or protein levels, respectively. Besides, GLUT4 membrane translocation would have been interesting in a comparison between control and γCD mice. Another limitation of our study was that we examined the effects of only a single concentration of γCD over a relatively short experimental period of 6 weeks. Otherwise, it may be possible that the significant improvement in running wheel activity and muscle strength was not mainly

mediated *via* a direct transcriptional response or part of a structural and metabolic adaptation in the skeletal muscle but rather mediated *via* altered glucose availability. The underlying mechanisms by which cyclic dextrins improve endurance are still not fully understood and further studies are needed to elucidate how γCD can provide a blunted postprandial glucose response and affect endurance. In fact, a higher dietary γCD concentration may result in a more pronounced attenuation in postprandial glucose response and insulin secretion. In this context, a limitation of the present mouse feeding trial was that we conducted blood glucose measurements without having measured insulin and only at one time point. Corresponding analyses before, during and after exercise would have been interesting (32).

Overall, based on present and literature data it is suggested that γCD moderately improves endurance and muscle strength, which renders it as a putative carbohydrate source in sports nutrition. Endurance athletes may benefit from the blunted postprandial glucose response after γCD intake. Therefore, present findings in mice should be verified in further studies in humans including athletes. In particular, since it has been shown that regular physical activity improves nitric oxide-dependent endothelial function in healthy adults (54), it would be of interest to study if γCD could enhance the cardiovascular effects of aerobic training. Furthermore, endurance and muscle strength decline with age (55, 56). Thus, it is plausible to address the question whether γCD may partly counteract age-dependent decline in endurance and muscle strength in ageing rodent models as well as in the elderly.

In conclusion, our results suggest that administration of γCD moderately enhanced endurance capacity, indicated by increased voluntary activity and enhanced muscle strength in mice. Exact underlying molecular mechanisms still need to be elucidated.

List of abbreviations: AUC, area under the curve; BCA, bicinchoninic acid; C, complex; CD, cyclodextrin; CON, control; Cpt1b, carnitine palmitoyltransferase 1b; DPP-4, dipeptidyl peptidase-4; ECL, enhanced chemiluminescence; F, forward; GI, glycemic index; GLUT2, glucose transporter 2; GLUT4, glucose transporter 4; HBCD, highly branched cyclic dextrin; LSM, least squares means; Mef2c, myocyte enhancer factor 2C; mtTFA, transcription factor A, mitochondrial; Myh1, *Mus musculus* myosin, heavy polypeptide 1, skeletal muscle; Myh2, *Mus musculus* myosin, heavy polypeptide 2, skeletal muscle; oGTT, oral glucose tolerance test; one-step qRT-PCR, one-step quantitative reverse transcription real-time polymerase chain reaction; OXPHOS, total oxidative phosphorylation; Pgc1α, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; R, reverse; RPE, rating of perceived exertion; SEM, standard error of the mean; Sirt1, sirtuin 1; Ta, annealing temperature; γCD, γ-cyclodextrin.

Author contributions: Conceptualization: S.W., A.F., K.L., K.C., T.F., Y.I., K.T. and G.R.; methodology: S.W., A.F., K.L., I.I and Y.I.; data curation: S.W., A.F., K.L., I.I. and G.R.; writing - original draft preparation: S.W. and G.R.; writing - review, editing: S.W., A.F., K.L., I.I., K.C., T.F., Y.I., K.T. and G.R.; supervision, K.L., K.T. and G.R.; funding acquisition, G.R. All authors approved this final version of the work.

Funding: This study was partly financially supported by CycloChem Bio Co., Ltd.

Acknowledgements: We acknowledge the excellent technical assistance of Vivien Schmuck.

Conflict of interests: Keiji Terao is the president of CycloChem Bio Co., Ltd. Keita Chikamoto, Takahiro Furune and Yoshiyuki Ishida are employees of CycloChem Bio Co., Ltd.

REFERENCES

1. Del Valle EM. Cyclodextrins and their uses: a review. *Process Biochem* 2004; 39: 1033-1046.
2. Li Z, Wang M, Wang F, *et al.* Gamma-cyclodextrin: a review on enzymatic production and applications. *Appl Microbiol Biotechnol* 2007; 77: 245-255.
3. Singh M, Sharma R, Banerjee UC. Biotechnological applications of cyclodextrins. *Biotechnol Adv* 2002; 20: 341-359.
4. Nikolai S, Huebbe P, Metges CC, *et al.* R-α lipoic acid γ-cyclodextrin complex increases energy expenditure: a 4-month feeding study in mice. *Nutrition* 2014; 30: 228-233.
5. Ikuta N, Sugiyama H, Shimosegawa H, *et al.* Analysis of the enhanced stability of r(+)-alpha lipoic acid by the complex formation with cyclodextrins. *Int J Mol Sci* 2013; 14: 3639-3655.
6. Schloesser A, Esatbeyoglu T, Piegholdt S, *et al.* Dietary tocotrienol/γ-cyclodextrin complex increases mitochondrial membrane potential and ATP concentrations in the brains of aged mice. *Oxid Med Cell Longev* 2015; 2015: 789710. doi: 10.1155/2015/789710
7. Rimbach G, Fischer A, Schloesser A, *et al.* Anti-inflammatory properties of Brazilian green propolis encapsulated in a γ-cyclodextrin complex in mice fed a Western-type diet. *Int J Mol Sci* 2017; 18: 1140. doi: 10.3390/ijms18061141
8. Munro IC, Newberne PM, Young VR, Bar A. Safety assessment of gamma-cyclodextrin. *Regul Toxicol Pharmacol* 2004; 39 (Suppl. 1): S3-S13.
9. Saokham P, Loftsson T. γ-Cyclodextrin. *Int J Pharm* 2017; 516: 278-292.
10. Harangi J, Beke G, Harangi M, *et al.* The digestable parent cyclodextrin. *J Incl Phenom Macrocycl Chem* 2012; 73: 335-339.
11. Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. *J Pharm Sci* 1997; 86: 147-162.
12. Til HP, Bar A. Subchronic (13-week) oral toxicity study of gamma-cyclodextrin in dogs. *Regul Toxicol Pharmacol* 1998; 27: 159-165.
13. Koutsou GA, Storey DM, Bar A. Gastrointestinal tolerance of gamma-cyclodextrin in humans. *Food Addit Contam* 1999; 16: 313-317.
14. Argiles JM, Busquets S, Lopez-Soriano FJ, Costelli P, Penna F. Are there any benefits of exercise training in cancer cachexia? *J Cachexia Sarcopenia Muscle* 2012; 3: 73-76.
15. De Assis GG de, Gasanov EV, de Sousa MB, Kozacz A, Murawska-Cialowicz E. Brain derived neurotrophic factor, a link of aerobic metabolism to neuroplasticity. *J Physiol Pharmacol* 2018; 69: 351-358.
16. Burke LM, Hawley JA, Jeukendrup A, Morton JP, Stellingwerff T, Maughan RJ. Toward a common understanding of diet-exercise strategies to manipulate fuel availability for training and competition preparation in endurance sport. *Int J Sport Nutr Exerc Metab* 2018; 28: 451-463.
17. Jeukendrup AE. Carbohydrate intake during exercise and performance. *Nutrition* 2004; 20: 669-677.
18. Ormsbee MJ, Bach CW, Baur DA. Pre-exercise nutrition: the role of macronutrients, modified starches and supplements on metabolism and endurance performance. *Nutrients* 2014; 6: 1782-1808.
19. Goh J, Ladiges W. Voluntary wheel running in mice. *Curr Protoc Mouse Biol* 2015; 5: 283-290.
20. Bono JP de, Adlam D, Paterson DJ, Channon KM. Novel quantitative phenotypes of exercise training in mouse

models. *Am J Physiol Regul Integr Comp Physiol* 2006; 290: R926-R934.
21. Manzanares G, Brito-da-Silva G, Gandra PG. Voluntary wheel running: patterns and physiological effects in mice. *Braz J Med Biol Res* 2018; 52: e7830. doi: 10.1590/1414-431X20187830
22. Wupper S, Fischer A, Luersen K, *et al.* High dietary kuding tea extract supplementation induces hepatic xenobiotic-metabolizing enzymes - a 6-week feeding study in mice. *Nutrients* 2019; 12: 40. doi: 10.3390/nu12010040
23. Deacon RM. Measuring the strength of mice. *J Vis Exp* 2013; Jun 2 (76): 2610. doi: 10.3791/2610.
24. Pallauf K, Chin D, Gunther I, *et al.* Resveratrol, lunularin and dihydroresveratrol do not act as caloric restriction mimetics when administered intraperitoneally in mice. *Sci Rep* 2019; 9: 4445. doi: 10.1038/s41598-019-41050-2
25. Schloesser A, Campbell G, Gluer C-C, Rimbach G, Huebbe P. Restriction on an energy-dense diet improves markers of metabolic health and cellular aging in mice through decreasing hepatic mTOR activity. *Rejuvenation Res* 2015; 18: 30-39.
26. Landisch RM, Kosir AM, Nelson SA, Baltgalvis KA, Lowe DA. Adaptive and nonadaptive responses to voluntary wheel running by mdx mice. *Muscle Nerve* 2008; 38: 1290-1303.
27. Phelps M, Pettan-Brewer C, Ladiges W, Yablonka-Reuveni Z. Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice. *Biogerontology* 2013; 14: 729-739.
28. Spears JK, Karr-Lilienthal LK, Grieshop CM, Flickinger EA, Wolf BW, Fahey GC. Pullulans and gamma-cyclodextrin affect apparent digestibility and metabolism in healthy adult ileal cannulated dogs. *J Nutr* 2005; 135: 1946-1952.
29. Bie AT de, van Ommen B, Bar A. Disposition of 14C-gamma-cyclodextrin in germ-free and conventional rats. *Regul Toxicol Pharmacol* 1998; 27: 150-158.
30. Asp ML, Hertzler SR, Chow J, Wolf BW. Gamma-cyclodextrin lowers postprandial glycemia and insulinemia without carbohydrate malabsorption in healthy adults. *J Am Coll Nutr* 2006; 25: 49-55.
31. Englyst HN, Kingman SM, Cummings JH. Classification and measurement of nutritionally important starch fractions. *Eur J Clin Nutr* 1992; 46 (Suppl. 2): S33-S50.
32. Kirwan JP, Cyr-Campbell D, Campbell WW, Scheiber J, Evans WJ. Effects of moderate and high glycemic index meals on metabolism and exercise performance. *Metab Clin Exp* 2001; 50: 849-855.
33. Wang M, Jin Z, Liu L, *et al.* Inhibition of cyclodextrins on the activity of α-amylase. *J Incl Phenom Macrocycl Chem* 2018; 90: 351-356.
34. Koukiekolo R, Desseaux V, Moreau Y, Marchis-Mouren G, Santimone M. Mechanism of porcine pancreatic alpha-amylase. Inhibition of amylose and maltopentaose hydrolysis by alpha-, beta- and gamma-cyclodextrins. *Eur J Biochem* 2001; 268: 841-848.
35. Takii H, Ishihara K, Kometani T, Okada S, Fushiki T. Enhancement of swimming endurance in mice by highly branched cyclic dextrin. *Biosci Biotechnol Biochem* 1999; 63: 2045-2052.
36. Furuyashiki T, Tanimoto H, Yokoyama Y, Kitaura Y, Kuriki T, Shimomura Y. Effects of ingesting highly branched cyclic dextrin during endurance exercise on rating of perceived exertion and blood components associated with energy metabolism. *Biosci Biotechnol Biochem* 2014; 78: 2117-2119.
37. Singhal A, Krystofiak ES, Jerome WG, Song B. 2-Hydroxypropyl-gamma-cyclodextrin overcomes NPC1 deficiency by enhancing lysosome-ER association and autophagy. *Sci Rep* 2020; 10: 8663. doi:10.1038/s41598-020-65627-4
38. Yao J, Ho D, Calingasan NY, Lin MT, Beal MF. Neuroprotection by cyclodextrin in cell and mouse models of Alzheimer disease. *J Exp Med* 2012; 209: 2501-2513.
39. Coisne C, Tilloy S, Monflier E, Wils D, Fenart L, Gosselet F. Cyclodextrins as emerging therapeutic tools in the treatment of cholesterol-associated vascular and neurodegenerative diseases. *Molecules* 2016; 21: 1748. doi:10.3390/molecules21121748
40. Pessin JE, Saltiel AR. Signaling pathways in insulin action: Molecular targets of insulin resistance. *J Clin Invest* 2000; 106: 165-169.
41. Balsom PD, Gaitanos GC, Soderlund K, Ekblom B. High-intensity exercise and muscle glycogen availability in humans. *Acta Physiol Scand* 1999; 165: 337-345.
42. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 1967; 71: 140-150.
43. Potthoff MJ, Wu H, Arnold MA, *et al.* Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. *J Clin Invest* 2007; 117: 2459-2467.
44. Liang H, Ward WF. PGC-1α: a key regulator of energy metabolism. *Adv Physiol Educ* 2006; 30: 145-151.
45. Lin J, Wu H, Tarr PT, *et al.* Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 2002; 418: 797-801.
46. Calvo JA, Daniels TG, Wang X, *et al.* Muscle-specific expression of PPARgamma coactivator-1alpha improves exercise performance and increases peak oxygen uptake. *J Appl Physiol* 2008; 104: 1304-1312.
47. Tadaishi M, Miura S, Kai Y, Kano Y, Oishi Y, Ezaki O. Skeletal muscle-specific expression of PGC-1α-b, an exercise-responsive isoform, increases exercise capacity and peak oxygen uptake. *PLoS One* 2011; 6: e28290. doi:10.1371/journal.pone.0028290
48. Lagouge M, Argmann C, Gerhart-Hines Z, *et al.* Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 2006; 127: 1109-1022.
49. Fiorenza M, Lemminger AK, Marker M, *et al.* High-intensity exercise training enhances mitochondrial oxidative phosphorylation efficiency in a temperature-dependent manner in human skeletal muscle: implications for exercise performance. *FASEB J* 2019; 33: 8976-8989.
50. Baati N, Feillet-Coudray C, Fouret G, *et al.* New evidence of exercise training benefits in myostatin-deficient mice: effect on lipidomic abnormalities. *Biochem Biophys Res Commun* 2019; 516: 89-95.
51. Henique C, Mansouri A, Vavrova E, *et al.* Increasing mitochondrial muscle fatty acid oxidation induces skeletal muscle remodeling toward an oxidative phenotype. *FASEB J* 2015; 29: 2473-2483.
52. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* 1997; 244: 1-14. doi: 10.1111/j.1432-1033.1997.00001.x
53. Miklosz A, Baranowski M, Lukaszuk B, Zabielski P, Chabowski A, Gorski J. Effect of acute exercise on mRNA and protein expression of main components of the lipolytic complex in different skeletal muscle types in the rat. *J Physiol Pharmacol* 2019; 70: 425-433.
54. Glodzik J, Rewiuk K, Adamiak J, *et al.* Controlled aerobic training improves endothelial function and modifies vascular remodeling in healthy adults with high normal blood pressure. *J Physiol Pharmacol* 2018; 69: 699-707.
55. Keller K, Engelhardt M. Strength and muscle mass loss with aging process. Age and strength loss. *Muscles Ligaments Tendons J* 2013; 3: 346-350.

56. Donato AJ, Tench K, Glueck DH, Seals DR, Eskurza I, Tanaka H. Declines in physiological functional capacity with age: A longitudinal study in peak swimming performance. *J Appl Physiol* 2003; 94: 764-769.

Received: May 5, 2020
Accepted: June 30, 2020

Author's address: Dr. Svenja Wüpper, Institute of Human Nutrition and Food Science, University of Kiel, Hermann-Rodewald-Strasse 6, 24118 Kiel, Germany.
E-mail: wuepper@foodsci.uni-kiel.de