Endurance exercise ameliorates low birthweight developed catch-up growth related metabolic dysfunctions in a mouse model

Liping Ju1)*, Wenxin Tong1)*, Miaoyan Qiu1)*, Weili Shen2), Jichao Sun3), Ying Chen1), Zhen Li4), Weiqing Wang1) and Jingyan Tian1)

1) Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases, Department of Endocrinology and Metabolism, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
2) Shanghai Institute of Hypertension, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
3) Laboratory of Endocrine and Metabolic Diseases, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and Shanghai Jiao Tong University School of Medicine, Shanghai, China
4) Department of General Surgery, Yangpu Hospital, Tongji University School of Medicine, Shanghai, China

Abstract. Low birthweight is known to predict high risk of metabolic diseases in adulthood, while regular endurance exercises are believed sufficient to improve metabolic dysfunction. In this study, we established a mouse model to determine whether long-term exercise training could ameliorate catch-up growth, and we explored the possible underlying mechanisms. By restricting maternal food intake during the last week of gestation, we successfully produced low birthweight pups. Further, normal birthweight mice and low birthweight mice were randomly distributed into one of three groups receiving either a normal fat diet, high fat diet, or high fat diet with exercise training. The growth/metabolism, mitochondrial content and functions were assessed at 6 months of age. Through group comparisons and correlation analyses, the 4th week was demonstrated to be the period of crucial growth and chosen to be the precise point of intervention, as the growth rate at this point is significantly correlated with body weight, intraperitoneal glucose tolerance test (IPGTT), Lee’s index and fat mass in adulthood. In addition, regular endurance exercises when started from 4 weeks remarkably ameliorated low birthweight outcomes and induced catch-up growth and glucose intolerance in the 25th week. Furthermore, real-time PCR and western blot results indicated that the effect of long-term exercise on mitochondrial functions alleviated catch-up related metabolic dysfunction. To conclude, long-term exercise training from the 4th week is sufficient to ameliorate catch-up growth and related metabolic disturbances in adulthood by promoting mitochondrial functions in skeletal muscle.

Keywords: Endurance exercise, Catch-up growth, Metabolic dysfunctions, Mitochondrial function

FETAL origins of adult diseases indicate that metabolic disorders in adulthood may be caused by abnormal nutrition and metabolism in earlier stages of development. For example, type 2 diabetes and obesity are associated with fetal growth restriction, which sets the individual on the path to later disease [1-3]. Numerous clinical observations have demonstrated that catch-up growth in early life significantly increases the incidence of metabolic disorders in adults with low birthweight [4-8]. However, environmental changes can reset the catch-up growth during a critical period of life when tissues maintain some plasticity and exhibit a higher proliferating and differentiating phase [9].

Previous research studies have indicated that regular exercise could alleviate many chronic diseases. Remarkably, exercise training is demonstrated to reduce adipose tissue mass and improve insulin sensitivity in obesity, metabolic syndrome, type 2 diabetes patients [10, 11]. Furthermore, aerobic exercise training is proved effectively to improve endurance exercise capacity in patients with mitochondrial myopa-
As skeletal muscle is the major targets of regular endurance exercise, some investigations indicated that mitochondria may be the key of long-term exercises ameliorate diseases [13]. Mitochondria, which form a reticular network within mammalian skeletal muscle, is the primary controllers of cellular metabolism [14]. Since diminished skeletal muscle mitochondrial mass and function are related to catch-up growth [15, 16], and regular exercises are proved to improve mitochondrial functions [17, 18], we hypothesis that long-term exercises may be an effective intervention to limit catch-up growth and ameliorate catch-up growth-related metabolic dysfunctions in adulthood.

In current study, we establish a mouse model to investigate the crucial phages of growth and the prospective time point for intervention. Further, we chose endurance exercises as our intervention and aimed to testify whether long-term exercise training could ameliorate catch-up growth and explored the possible underlying mechanisms.

**Materials and Methods**

**Mouse model**

The model consisted of 26 six-to-eight-week-old virgin female C57BL/6J mice and 13 eight-to-ten-week-old C57BL/6J males, which were bought from SLAC Laboratory (SLAC Laboratory Animal, Shanghai, China). Females and males were distributed as 2:1 into 13 cages and were caged in a temperature-controlled room with a 12:12-h light-dark cycle. Pregnancy was dated with vaginal plugs (day 1). Females were subsequently housed individually. Females were randomly assigned to a control group (C) or an under-nutrition group (U) on pregnancy day 11. Water and rodent chow (normal fat diet, NFD; 10% kcal for fat; Shilin Bio, Shanghai, China) were provided ad libitum to group C. Rodent chow for a day and rodent chow withdraw for a day and a half take turns alternately in group U from pregnancy day 11. A total of 2.5 g [19] of extra food was supplied to U on the evening of pregnancy day 19, just before the initiation of parturition. Male offspring of group C were assigned to the NBW group after delivery. Birth weights in group U which 2 standard deviations below the mean weight in NBW group were assigned to the low birthweight (LBW) group. All pregnancy females were permitted to procreate only once. Finally, 18 males from 7 litters in NBW groups and 16 males from 18 litters in LBW groups were further randomly distributed into three additional experimental groups after weaning: (1) normal fat diet (NFD); (2) high fat diet (HFD, 60% kcal for fat, Research Diets, D12492) (HFD); and (3) high fat diet with exercise training (Ex). We designated these six groups as NBW-NFD (n=6), NBW-HFD (n=6), NBW-HFD-Ex (n=6), LBW-NFD (n=4), LBW-HFD (n=6), and LBW-HFD-Ex (n=6), respectively.

The Institutional Animal Care and Use Committee of Shanghai Research Institute of Sports Science approved all procedures (IACUC #10-09011). All experiments involving animals were conducted in conformance with the principles of laboratory animal care (NIH Publication, 8th edition, 2011).

**Training protocol**

Ex groups were exercise-trained on a motor-driven rodent treadmill (Hangzhou, China) at room temperature. The intensity progressively increased after acclimation for 3 days for 4-6 weeks. An intensity of 13 m/minute, 12% slope, and 60 min/day was used for 7-25 weeks. Animals were placed on the treadmill every day at the same time (15:30–17:30) under the supervision of the same person.

**Energy intake**

The adolescence phase of C57BL/6J occurs at 4-9 weeks of age [20]. Food intake was estimated as the difference between the offered and remnant amount based on twice weekly measurements in the 4th, 9th and 24th week, respectively. Total energy intake was determined from the energy content in the diet and was calculated on a per-day basis.

**Body composition and growth rate**

Fat mass and lean mass were determined using an Animal Body Composition Analyzer (EchoMRI-100, Houston, TX, USA).

The Lee index [21], an index of obesity, was calculated as:

\[ \text{GR} = \frac{(W_n - W_{n-1})}{W_{n-1}} \]

where \( W \) is body weight and \( n \) is the age in weeks [22].
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**IPGTT and ITT**

Glucose tolerance was determined using an intraperitoneal (ip) glucose tolerance test (IPGTT) after overnight fasting (12–14 h) by removal to a clean cage without food in week 24. Mice were weighed before receiving an injection of glucose (Sinepharm, Shanghai, China) at a dose of 2 g/kg body weight. Blood was collected from the tail vein immediately before injection (0 min) and at 15, 30, 60, and 120 min after injection. Blood glucose concentration was measured using the glucose analyser Ascensia Elite (Bayer HealthCare AG, Leverkusen, Germany). Glucose tolerance is expressed as the area under the curve (AUC) of glucose concentrations between 0–120 min.

Intraperitoneal insulin tolerance testing (ITT) was performed in week 25. Mice were fasted for 6 h by removal to a clean cage without food and weighed before insulin injections (Humulin R, Lilly, USA) at a dose of 0.75 U/kg body weight. The measurement method was the same as for IPGTT.

**Tissue extraction and animal sacrifice**

Mice in exercise groups were stayed 36h [23, 24] after the last bout of exercise training. All of the mice were overnight fasted and sacrificed with an intraperitoneal injection of 10% chloral hydrate (100 μl/20 g body weight). The gastrocnemius muscles (GM) of the left posterior limb, subcutaneous adipose tissue (SAT), epididymis adipose tissue (EAT) and brown adipose tissue (BAT) were rapidly extracted, weighted, frozen in liquid nitrogen and stored at −80°C.

**MDA (malondialdehyde) assay**

MDA levels in gastrocnemius muscles were measured using a Lipid Peroxidation MDA Assay Kit (Beyotime, Shanghai, China) while an ATP Assay Kit (Beyotime, Shanghai, China) was used to assess the ATP levels of gastrocnemius muscles. All the measurements followed the manufacturer’s instructions. Protein concentrations in samples were determined using a BCA assay kit (Thermo Scientific, USA). MDA equivalents are expressed as nmol/mg tissue protein and normalized to NBW levels.

**Western blot**

We carefully weighed 30 mg gastrocnemius muscles from each mouse, then 500 μl RIPA was added to lyse the tissue. After mixed the tissue homogenate at 50HZ for 30 seconds three times the superna-
Mitochondrial D-loop
Forward: AATCTACCATCCTCCGT
Reverse: GACTAATGATTCTTCACCGT

18S rRNA
Forward: CATTCGAACGTCTGCCCTATC
Reverse: CCTGCTGCCTTCCTTGGA

Statistical analysis
The results are expressed as the mean±SEM. Statistical analyses for Body weight, Growth rate, IPGTT and ITT were performed using a repeated measure ANOVA. Group comparisons of other data were performed using multivariable ANOVA, with exercise as a covariate and followed by post hoc least significant difference (LSD) tests. Bivariate correlations for the dependent variables Birth weight, GR1-3 and GR1 were assessed with a Pearson correlation coefficients, and partial correlation analysis for the dependent variables GR1-7, GR4-7 and GR4 were assessed with exercise as a control variable. Multiple linear regression analysis was performed with exercise as an independent variable. All data were analyzed using SPSS for Windows Version 17.0. *p* values <0.05 were considered significant.

Results

Mouse model of catch-up growth
By restricting maternal food intake during the last week of gestation, we successfully resulted in low birthweight mice, with the average weight of 1.15±0.02 g, meeting a criterion of 2 standard deviations below the normal birthweight mice (1.29±0.02 g) (Fig. 1a), without affecting the normal pregnancy duration (7.5±1.4 vs 7.4±1.3 mice/litter, *p*>0.05), litter of mother mice (20.71±0.49 vs 20.43±0.50 days, *p*>0.05) and maternal body weight before delivery (28.57±0.57 vs 27.79±1.46 g, *p*<0.05).

We detected that the catch-up growth may start from puberty by tracking the body weights of different growth phages in each group, as the body weight differences were firstly observed from 7 weeks of age in the NFD groups (NBW: 20.39±0.42 g, LBW: 22.96±0.43 g, *p*<0.05) and from 5 weeks of age in the HFD groups (NBW-HFD: 18.54±0.31 g, LBW-HFD: 20.10±0.31 g, *p*<0.05) (Fig. 1b). Furthermore, the LBW developed catch-up growth related obesity trend while HFD exaggerate this phenomenon, supported by remarkable higher body weight and scores of Lee’s index at 25 weeks (Fig. 1c-d).

Correspondently, glucose measurements illustrated that LBW developed remarkable glucose intolerance and insulin insensitivity by the age of 6 months, while HFD exaggerate the trend, indicating that catch-up growth did result in metabolic disturbance in adulthood (Fig. 1e-f).

Crucial phages of catch-up growth
To further identify the crucial phages of catch-up growth, growth rate of each groups from 0-25 weeks were recorded. As shown by Fig. 1g, NBW and LBW shared the similar growth patterns of two spurs (0-3 weeks, and 4-7 weeks) and a nearly constant growth rates from the 11th to 25th week, with LBW induced a significantly higher growth rate in two spurs (*p*<0.05, compared with NBW). To noted, high fat diet after weaning significantly exaggerate the growth rates in the 4th week (*p*<0.05, compared with NBW-NFD; *p*<0.05, compared with LBW-NFD).

Body compositions of relative fat mass (RFM), related lean mass (RLM), subcutaneous adipose tissue (SAT), Epididymis adipose tissue (EAT), Brown adipose tissue (BAT), Gastrocnemius muscles (GM) were tested after sacrificed to further assess body weight differences (Table 1). Remarkably, LBW developed significant increase in RFM and decrease in RLM, while HFD exaggerate the trend. Moreover, we further identify that it is the SAT and EAT rather than BAT and GM contributed to the differences in body weight.

Furthermore, correlation analyses across all experimental groups were performed to estimate the contributions of each postnatal growth phages to the development of obesity and glucose intolerance. Table 2 demonstrates that the growth rate during the first 7 weeks (GR 1 – 7) were correlated with body weight, SAT, EAT, Lee’s index and glucose homeostasis at 6 months. Moreover, we further divided the GR 1-7 into the Growth rate during the first 3 weeks (GR 1 – 3), the 4th to 7th week of life (GR 4 – 7), the growth rate in the first week (GR 1) and the 4th week (GR 4) according to the growth spurts, and surprisingly found that only the GR 4 was significantly correlated with Lee’s index.

To conclude, we indicated that 0-7 weeks is the crucial growth phage, while the catch-up growth may be started from the 4th week. Therefore, we chose 4 week as the intervention time point, and subjected half the LBW-HFD and NBW-HFD groups to endurance exercise training, to investigate whether endurance exercise
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Fig. 1 Establishment of the catch-up growth mouse model.
(a) Birth weight of LBW and NBW groups.  (b) Continuous monitor of Body weight (0-25 w).  (c) Body weight of NBW, NBW-HFD, LBW and LBW-HFD in adulthood (25 w).  (d) Lee’s index of NBW, NBW-HFD, LBW and LBW-HFD in adulthood (25 w).  (e) Glucose levels during an intraperitoneal glucose tolerance test (2 g glucose/kg body weight) and Glucose AUC during the glucose tolerance test.  (f) Insulin tolerance test (0.75 U insulin/kg body weight) and Glucose AUC during the insulin tolerance test, glucose levels were represented as percentage of initial glucose.  (g) Growth rate (0-25 w). Data represent mean±SEM.  * p<0.05.
Long-term exercise is believed helpful to alleviate the majority metabolic diseases [17]. Hence, in this study, we aimed to testify whether endurance exercise is sufficient to ameliorate catch-up growth and the metabolic disturbance in adulthood.

As expected, exercise training effectively limited catch-up growth and high fat diet-related obesity, supported by the significantly decreased body weight and fat mass% in adulthood (Fig. 2a-b). In addition, exercise training effectively improved glucose tolerance and insulin sensitivity in both LBW-HFD-Ex and NBW-HFD-Ex in the 25th week (Fig. 2c-d).

**Endurance exercise improves mitochondrial functions in gastrocnemius muscle**

As regular endurance exercise is demonstrated to exert positive effects on mitochondrial biogenesis and function, therefore, we aimed to investigate whether regular exercises ameliorate catch-up growth by promoting mitochondria functions.

ATP assessment indicated that LBW mice developed serious ATP generation attenuation while HFD inducement exaggerate the trend, however, regular exercises could effectively up-regulated ATP generation, indicating the improvement of mitochondrial functions (Fig. 3a). In addition, mitochondrial DNA copy number, acted as a marker for mitochondrial content, was notable reduced in LBW mice and even dropped when treated with HFD, while is significantly remedied by long-term exercise treatment (Fig. 3b). Consistently, regulators of mtDNA transcription and replication, such as peroxidase body growth-activated receptor gamma auxiliary promoters (PGC-1α), the transcription factor nuclear respiratory factor 1 (Nrf1) and mitochondrial transcription factor A (Tfam) were down-regulated in LBW-HFD and NBW-HFD mice, which were also reversed by endurance physical exercise (Fig. 3c-e), indicating that long-term exercise pro-
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Promoted mitochondria biogenesis and increased the mitochondria content.

**Endurance exercise enhances mitochondrial oxidative balance in gastrocnemius muscle**

Furthermore, a MDA analysis, which represented the peroxidation levels of the membrane lipid, was conducted to indicate the oxidative conditions in each group. As expected, MDA levels in LBW were significantly up-regulated and even exaggerate by HFD inducement, while endurance exercise remarkably reduced the high MDA levels (Fig. 4a).

In addition, western blotting was conducted to analyze the protein levels of the antioxidant system, such as Nrf2 (a redox-sensitive, basic leucine zipper protein that regulates the transcription of several antioxidant genes) and antioxidant enzymes (catalase and MnSOD). Correspondingly, the remarkable reduced levels of Nrf2 and catalase in LBW-HFD group were reversed by regular endurance exercise administration (Fig. 4b-d), demonstrating that long-term exercise is sufficient to enhanced the anti-oxidative process. Interestingly, LBW seemed to have little effect on the expression of MnSOD while HFD remarkable reduced the MnSOD levels, however, regular exercises in NBW demonstrated more efficient in up-regulating MnSOD expressing (Fig. 4e).

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**Fig. 2** Endurance exercises ameliorate catch-up growth related obesity and metabolic dysfunctions.

(a) Body weight in adulthood (25 w). (b) Related percentage of fat mass and lean mass in the 25th week. (c) Glucose levels during an intraperitoneal glucose tolerance test (2 g glucose/kg body weight) and Glucose AUC during the glucose tolerance test. (d) Insulin tolerance test (0.75 U insulin/kg body weight) and Glucose AUC during the insulin tolerance test, glucose levels were represented as percentage of initial glucose. Data represent mean±SEM. * p<0.05.
Fig. 3  Endurance exercises promoted mitochondrial function in gastrocnemius muscle.
(a) ATP levels.  (b) mtDNA levels.  Q-PCR analysis of the mitochondrial biogenesis-related genes: (c) PGC-1α, (d) Nrf1 and (e) Tfam. Data represent mean±SEM, *p<0.05.

Fig. 4  Endurance exercise enhances mitochondrial oxidative balance in gastrocnemius muscle.
(a) MDA levels.  (b-e) Western blot images and quantitative band density analyses of endogenous antioxidants: Nrf2, Catalase, and MnSOD. Data represent mean±SEM, *p<0.05.
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Discussion

Low birthweight is proved to be relative with metabolic diseases in adulthood [4-8], as the detrimental effects of low birthweight are amplified by rapid postnatal catch-up growth, which is normally refers to the increasing growth rate after removed the pathological factors that inhibit growth [25]. Fortunately, environmental changes can reset the catch-up growth during a critical period of life [9]. Therefore, we aimed to indicate the crucial phases of growth and the prospective time point for interventions. In our study, two distinct growth spurts in rodents were observed, which was in accordance with a previous ICR mouse model study [22]. However, while the ICR mouse model study indicated that the growth rate in the first week (GR1) had the strongest positive correlation with body weight, fat mass, insulin sensitivity and glucose tolerance at 6 months of age, GR4 in our study was demonstrated to be the most critical growth phase, as GR4 is the only phase correlated with Lee’s index, IPGTT, body weight and fat mass.

Previous investigations established the low birthweight rat model by restricting the calorie intake during the suckling period [6, 22, 26]. However, inadequate nutrition during early postnatal life is occasionally demonstrated to adversely affect central nervous system development, cognitive function and the risk of infection [27, 28], not to mention that there is no criteria of the exact nutritional requirement of VLBW (very low birthweight) infants to achieve a postnatal growth [29]. To noted, in our study, we successfully established the low birthweight mouse model by restricting maternal food intake during the last week of gestation, without affecting the normal pregnancy duration and litter of mother mice, which provide a preferable way to establish the catch-up models.

As similar exercise training in a mouse model of catch-up growth has not been reported previously, we designed an exercise training protocol according to a rat model: 40 min/day at 12 m/min, 12% grade, 5 times/week (this intensity corresponds to 65-70% of maximal oxygen uptake) [30]. However, 8 weeks of regular exercise training failed to reduce body weight in our pre-investigations. Therefore, we further investigated the different grades of exercise intensity [31]: 12% grade, 5 times/week (a) 40 min/day at 15 m/min, (b) 60 min/day at 13 m/min, and (c) 60 min/day at 15 m/min. Finally, grade (b) was selected as our training procedure, for the reason that the exercise intensity changed body weight effectively without causing exhaustion.

As known, regulation of exercise training induced not only mitochondria biogenesis, but also fiber type transformation and angiogenesis in skeletal muscle [32, 33]. Previous studies reported that various types of aerobic training protocols may increase the percentage of type I fibers, such as endurance cycle training [34] and long distance running [35]. In addition, a greater proportion of type I fibers would favor resistance to body fat accumulation [36]. Since there was not related fiber type study in our research, future study may be focused on fiber type transformation to evaluate the role of fiber type composition in metabolism improvements.

Mitochondrial oxidative balance is the fundamental of standard mitochondrial functions, which is demonstrated to be accompanied by catch-up growth in heart [37, 38] and adipocyte [39]. Moreover, Resveratrol, a kind of antioxidant, has been indicated to ameliorate the catch-up growth related oxidative stress in skeletal muscle [40]. As known, regular exercise training is one of the traditional methods to enhance mitochondrial functions by increasing the antioxidants in skeletal muscle [41], hence, in this study, we analyzed whether long-term exercise could promoted mitochondrial functions in gastrocnemius muscle. As expected, endurance exercise significantly reduced the MDA levels and remarkable increase of protein levels of antioxidative enzymes MnSOD, Catalase and Nrf2 in skeletal muscle, indicating the improvement of antioxidative ability and the amelioration of oxidative stress.

However, there are still several limitations in this study. High-fat diet, the most common factor relative with the metabolic problems in current society, was chosen to be the amplification factor to exaggerate the catch-up growth trend. However, whether the observed worse metabolic dysfunctions in low birthweight groups were due to the exaggerated catch-up growth or the HFD-evoked metabolic disturbances still needs further investigations, while the NBW-Ex and LBW-Ex groups may be the leading choice in the further study to testify whether the low birthweight is an independent risk factor of metabolic diseases in adulthood. Furthermore, exercise induced losing weight may partly contribute to the decreased relative fat mass%, which may partly owe to the change of different adipokine levels [42], hence, the adipokine secretion profile may be the further focus of study.
In summary, our study in mice demonstrated that long-term exercise training beginning at the 4th week of age prevented rapid postnatal catch-up growth of low birthweight infants and exhibited long-term beneficial effects via increasing mitochondrial content and functions. Pharmaceutical therapy may still require in whom exercise is not possible due to extreme muscle wasting and dysfunction. Future studies should focus on mitochondria-targeted therapy, which may exhibit therapeutic potential for the prevention of catch-up growth related metabolic disorders.

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Disclosure

The authors declare that there is no conflict of interest concerning this article.

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