Appl Biochem Biotechnol (2016) 179:819–829
DOI 10.1007/s12010-016-2033-8

Whole Body Vibration Improves Insulin Resistance in db/db Mice: Amelioration of Lipid Accumulation and Oxidative Stress

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Received: 17 December 2015 / Accepted: 25 February 2016 /
Published online: 5 March 2016

Abstract Insulin resistance (IR) is the hallmark of type 2 diabetes mellitus (T2DM), which is one of the most important chronic noncommunicable diseases. Effective and feasible strategies to treat IR are still urgently needed. Previous research studies reported that whole body vibration (WBV) was beneficial for IR in clinical; however, its underlying mechanisms remains unknown. In the present study, db/db mice were treated with WBV administration 60 min/day for 12 weeks and the impaired insulin sensitivity was improved. Besides, liver steatosis was also ameliorated. Further explorations revealed that WBV could reduce the expression of SREBP1c and increase the expression of GSH-Px and consequently suppress oxidative stress. In conclusion, WBV attenuates oxidative stress to ameliorate liver steatosis and thus improves insulin resistance in db/db mice. Therefore, WBV administration is a promising treatment for individuals who suffered from central obesity and IR.

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Keywords Insulin resistance · Liver steatosis · Oxidative stress · Glutathione peroxidases · Whole body vibration

Introduction

Recent large-scale epidemiology surveys showed that the morbidity of type 2 diabetes mellitus (T2DM) has increased dramatically in the last years [1]. Being a major chronic noncommunicable disease that threatens human health, T2DM is characterized by insulin resistance (IR) and hyperglycemia [2–4]. Insulin resistance plays a fundamental role in the development of T2DM complications [5]. Thus, ameliorating insulin resistance is believed to be one of the most important strategies in T2DM treatment.

Although the mechanism of insulin resistance is still not completely understood, oxidative stress is considered to play a causal role [6–9]. Oxidative stress is caused by excessive generation of reactive oxygen species (ROS) which is mainly produced during oxidative phosphorylation in mitochondria. The main ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^·$). They play an important role in aging and diseases initiation through quickly reacting with various intracellular macromolecules such as DNA, proteins, and polyunsaturated fatty acids. Serving as a hub of nutrient metabolism, liver is abundant of mitochondria and thus vulnerable to ROS attack. Normally, blood glucose metabolism is regulated by a variety of hormones to keep a dynamic equilibrium. Liver functions as the major target of those hormones that play important roles in glycogenolysis and gluconeogenesis. Among the various hormones, insulin is the only hormone lowering blood glucose. Therefore, the regulation of blood glucose would be out of control if the liver fails to respond to insulin. There are two different types of obesity characterized by excessive accumulation of subcutaneous adipose tissue or visceral adipose tissue, respectively. Recent studies have shown that distribution of fat also contributes to the development of obesity and obesity-associated diseases [10, 11]. For example, central obesity patients, who have fat accumulation in visceral adipose tissues, particularly in liver are prone to developing complications such as metabolism syndrome.

Whole body vibration (WBV) is a newly developed training method for improving strength and power of muscle, mass and mineral destiny of bone, flexibility, postural control, granulation tissue formation, angiogenesis and wound healing, etc. [12–14]. Vibrations could be generally divided into synchronous vibration and side alternating vibration. The synchronous vibration could also be described as sinusoidal vibration, and it is the type that currently available WBV instruments provide [15]. During the vibration exercise, vibrations are generated by vibratory platform and transferred to the subject who contacted with the platform directly to bring various benefits [16]. There are several critical parameters such as frequency (Hz), accelerated speed (g), daily dose (time of a session), and repetitions (number of sessions). As a safe and simple modality of exercise, WBV could also be beneficial to metabolic syndrome [16]. In sedentary obese individuals, hypocaloric diet combined with WBV could improve insulin sensitivity more effectively compared with hypocaloric diet alone [17]. Aerobic exercise is recommended as an effective treatment for T2DM patients, and Behboudi et al. have proved that WBV possessed the same effect with aerobic exercise in decreasing fasting blood glucose [18]. However, the underlying mechanisms are not clear.

The purpose of our current study is to investigate whether the beneficial effect of WBV on metabolism is related to the improvement of lipid metabolism disorders in T2DM model and to

further explore the possible mechanisms. The results indicated that WBV could improve insulin resistance, alleviate liver steatosis, and ameliorate oxidative stress in db/db mice.

Methods and Materials

Materials and Reagents

Pierce BCA Protein Assay Kit was purchased from Thermo Scientific. SREBP1c antibody was purchased from Santa Cruz Biotechnology. CAT antibody was purchased from Proteintech Group. GR antibody was purchased from Bioss Biotechonogy. β-Actin and GSH-PX antibodies were purchased from Bioworld Technology. SOD1 and SOD2 antibodies were purchased from Abcam Biotechonogy. Mouse insulin ELISA kit was purchased from CUSABIO BIOTECH. The kits used to test the concentration of hepatic TG, MDA, and GSH/GSSG, and the activity of hepatic CAT, SOD, and GSH-PX were purchased from Nanjing Jiancheng Bioengineering Institute. The glucose analyzer and glucose test strips were purchased from Roche. Other chemicals used in this study were purchased from Sigma. The vibration platform and accelerometer were produced in Shanghai Huixia Instruments and Equipments Limited.

Animals and Experimental Procedures

Animal experiments were approved by Fourth Military Medical University Ethical committee. Eight 8-week old female C57BL/KsJ mice (18.07 ± 1.54 g) and 16 8-week old female C57L/6Jdb/db mice (29.73 ± 2.27 g) were purchased from Model Animal Research Center of Nanjing University. The db/db mice were originally obtained by Jackson Laboratory and bred in Nanjing University. They were accommodated under a standard environmental condition with light control (12-h light/12-h dark cycle) and temperature regulation (22 ± 1 °C). The mice consumed standard chow and water freely.

The female C57BL/KsJ mice were control group ($n = 8$), and the 16 female C57L/6Jdb/db mice were randomly divided into two groups including db/db group ($n = 8$) and WBV group ($n = 8$). The mice in WBV group were put in an empty cage which was directly and tightly attached to the top surface of vibrating platform, and vibration was applied with 45 Hz of frequency and accelerated speed of 0.5 g for 60 min/day for 12 weeks. The mechanical signals transmitted to animals were determined using an accelerometer which was attached closely to the bottom of the cage. Mice in db/db group were also placed in a same empty cage as which the mice in WBV group were in for the same amount of time. Effects of exposing to new environment could be excluded. Before execution, fasting blood glucose was analyzed. At the end, the mice were anesthetized and hepatic samples and serum were harvested for the assays. Hepatic frozen sections for lipid accumulation analysis were sliced immediately. Livers used for western blotting, analysis of antioxidation enzymes activity, determination of malondialdehyde (MDA), reduced glutathione (GSH) and oxidized GSH (GSSG), and triglyceride (TG) content determination were stored at −80 °C until use. The insulin level of the serum was detected with an ELISA kit following the instruction. The homeostasis model assessment (HOMA) for evaluating insulin resistance was determined by performing the following calculation: homeostasis model

assessment for insulin resistance = fasting blood glucose (mmol/L) × fasting plasma insulin (mIU/L)/22.5.

Oil Red O Staining

To evaluate the cellular neutral lipid accumulation, frozen liver samples were sliced at 10 μm and stained with Oil Red O solution (stock solution 5 mg/ml in isopropanol; working solution 6 ml Oil Red O stock solution and 4 ml distilled water) for 10 min at 20 °C. After staining, sections were washed with 0.01-M phosphate buffer solution and then observed with an Olympus light microscope.

Hepatic Triglyceride Determination

For determination of the hepatic triglyceride, a commercial available kit was used. Briefly, the liver samples were homogenized with absolute ethyl alcohol at the rate of 100 mg: 0.9 ml in ice bath, and then the homogenate was centrifuged at 570 g for 10 min. The supernatant was collected, and the content of triglyceride was analyzed following the manufacturer's instruction.

Activity of Antioxidation Enzymes as well as MDA and GSH Content Measurement

Commercial kits were used to measure the activity of antioxidation enzymes as well as MDA and GSH/GSSG in livers. Briefly, for testing MDA and activities of antioxidant enzymes, the liver samples were homogenized in ice bath with 0.9 % saline at the rate of 100 mg: 0.9 ml. Then, the homogenate was centrifuged at 570 g for 10 min. The supernatant was collected and measured following the instruction. For testing GSH/GSSG, the liver samples were homogenized in ice bath with agent given in the kit at the rate of 200 mg: 0.8 ml. Then, the homogenate was centrifuged at 1120 g for 10 min. The supernatant was collected and measured following the instruction.

Western Blot

Protein expression was detected and measured by western blotting assays. The liver samples were homogenized with RIPA containing phosphate and protease inhibitors for 30 min on ice and then centrifuged at 20,000*g*. The supernatant was collected and protein concentration was measured with the Pierce BCA Protein Assay Kit. Proteins were denatured by boiling at 95 °C for 5 min with loading buffer, and then 10 % SDS-PAGE was used to separate the denatured proteins. After that, proteins were transferred to polyvinylidene fluoride membranes. Five percent skimmed milk diluted with TBST buffer was used to block the nonspecific binding site for 60 min at room temperature. Primary antibodies were then applied and incubated at 4 °C overnight. After incubation, the membranes were washed three times for 10 min each with TBST buffer and then incubated with appropriate HRP-conjugated secondary antibody (1:3000 diluted in TBST) at 37 °C for 60 min. The protein bands were visualized with chemiluminescent reagents following the manufacturer's guidelines and quantified using Quantity One Software. β-Actin was used as reference protein.

Statistical Analysis

Data were expressed as means ± SD, and one-way ANOVA with LSD *t* test were applied to analyze the difference between two groups. Statistical analysis was performed with Graph Pad Prism 5, and $p < 0.05$ was considered statistically significant.

Results

WBV Administration Improved Insulin Resistance in db/db Mice

As is shown in Fig. 1a, b, after the WBV administration, fasting blood glucose trended lower and fasting blood insulin significantly decreased. Then, the HOMA-IR was measured and the results indicated that WBV could lower the HOMA-IR by about 25 %, shown in Fig. 1c.

WBV Administration Reduced Hepatic Lipid Accumulation Through Inhibiting SREBP-1c Expression in db/db Mice

Figure 2a shows that the body weights were significantly higher in db/db mice and WBV administration could not relieve the whole body weight gain. However, the liver weight was significantly decreased in WBV group (Fig. 2b). Liver lipid accumulation was examined by Oil Red O staining, and the results indicated that both the number and size of lipid droplets were remarkably decreased in WBV-treated group (Fig. 2c). Besides, WBV administration could reduce liver TG content from 7.16 ± 0.38 mmol/gprot to 4.93 ± 0.92 mmol/gprot (Fig. 2d). Figure 2e shows that the expression of SREBP-1c expression was also attenuated by around 35 % after given WBV, suggesting that WBV could actually diminish hepatic lipid content in db/db mice.

WBV protected the db/db mice from oxidative stress injury

To examine whether WBV could decrease liver oxidative stress in db/db mice, we further tested liver MDA level, both GSH and GSSG level, and antioxidant enzymes' expression and activity. As is shown in Fig. 3a–c, MDA and GSSG level was significantly decreased, while the reduced GSH level was visibly increased in WBV group, comparing with db/db mice. Then, we tested the activity of several antioxidant

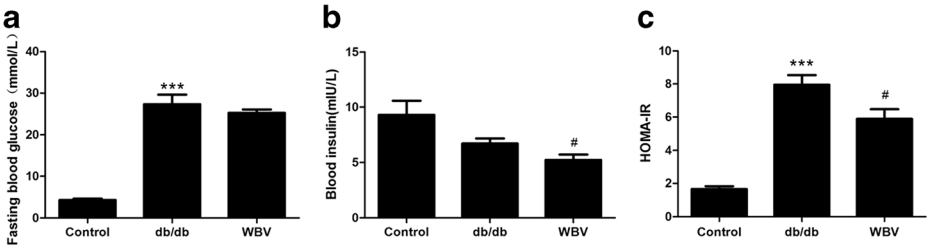

Fig. 1 Effect of WBV on insulin resistance in mice. **a** Level of fasting blood glucose. **b** Level of fasting blood insulin. **c** Data of HOMA-IR. ***$p < 0.001$, compared with control group; #$p < 0.05$, compared with db/db group

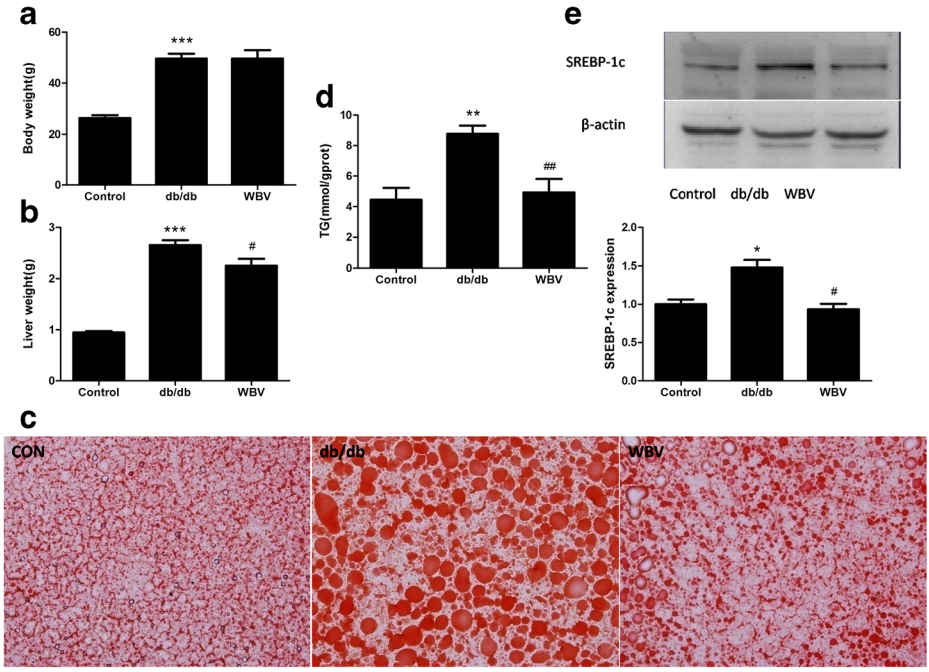

Fig. 2 The hepatic lipid accumulation and SREBP-1c expression of mice. **a** Weight of body. **b** Weight of liver. **c** Hepatic lipid accumulation was evaluated with frozen sections stained by Oil Red O (×40). **d** Hepatic TG content was determined with a commercial available kit. **e** Hepatic SREBP-1c expression was measured by Western Blot. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, compared with control; ##$p < 0.01$, #$p < 0.05$, compared with db/db mice

enzymes including CAT, SOD, and GSH-Px (Fig. 3d–f). The results indicated that WBV would not affect any of the enzymes' activity/unit mentioned above. In Fig. 3g, expressions of CAT, SOD2, and SOD1 were not altered by WBV. Expression of glutathione reductase trended higher, but the difference was not statistically significant. Only GSH-Px overexpressed obviously after WBV.

Discussion

The prevalence of T2DM and insulin resistance urgently needs preventive measures and effective treatments. WBV could not only ease the complications in T2DM patients such as painful peripheral neuropathy [19], osteoporosis, and chronic wounds [12] but also reduce glycosylated hemoglobin (HbA1c), cholesterol, and triglycerides [14]. As for the beneficial multi-effects of WBV, we aimed to examine the potential effect of it on insulin resistance and further explore the possible mechanisms in the current study.

After 12-week administration, WBV treatment could significantly decrease the fasting blood insulin level and index of HOMA-IR in db/db mice suggesting that WBV could effectively improve insulin resistance. To explore the mechanism of this therapeutic effect of WBV, we firstly paid attention to the change of liver, the most important organ taking part in body metabolism. The weight of the livers in WBV group decreased compared with db/db group through observably scavenging lipid in hepatocytes, which was proved by frozen

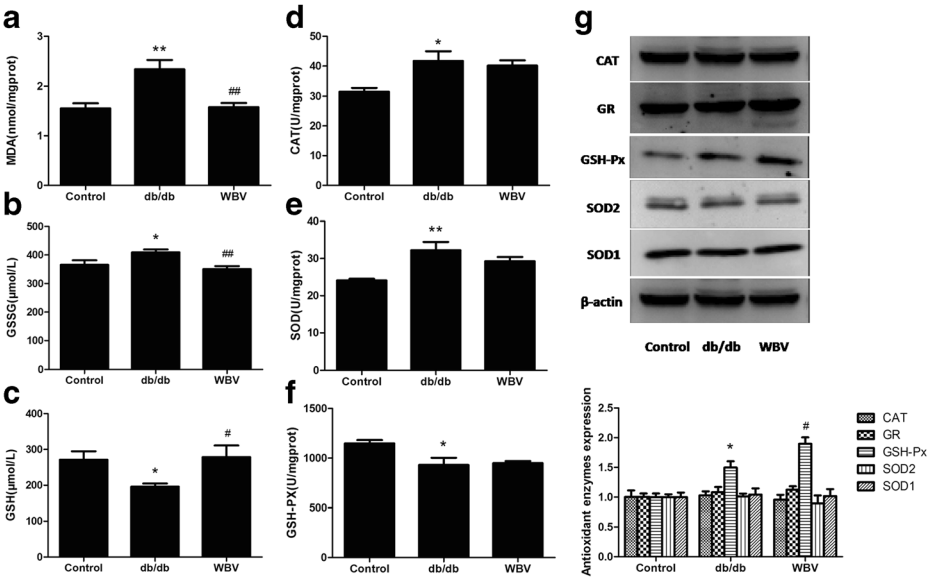

Fig. 3 Effects of WBV on db/db mice's redox homeostasis. **a** Hepatic MDA content. **b** Hepatic GSSG content. **c** Hepatic reduced GSH content. **d** Activity/unit of CAT. **e** Activity/unit of SOD. **f** Activity/unit of GSH-Px. **g** Expressions of CAT, GR, GSH-Px, SOD2, and SOD1 were determined by Western Blot. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with control; $\#\#p < 0.01$, $\#p < 0.05$, compared with db/db mice

sections and TG content determination. Belavy et al. reported that strict inactivity (bed rest) preferentially led to deposition of visceral adipose tissue and decrease of insulin sensitivity, but performing resistance exercise plus WBV restrained this [20]. WBV training for 12-week increased leg blood flow and reduced adiposity in patients with T2DM effectively [21]. An increased risk of insulin resistance and metabolism syndrome was closely associated with increased visceral adipose tissue mass. Therefore, diminishing the hepatic deposition would ameliorate those complications. Sun et al. reported that Rosiglitazone as an agonist of peroxisome proliferatior-activated receptor-α could reverse the alcohol-induced adipose tissue dysfunction, improve lipid homeostasis, thereby reduce fatty acid overflux to liver, and protect against liver injury [22]. Tan et al. found that SK0506, a mixture of 3 Chinese herbal extracts, could act on hyperlipidemia and visceral obesity and thus improve impaired insulin sensitivity in high-fat-fed mice [23]. In addition, timed-daily ingestion of whey protein with multimode exercise training program including resistance exercise, intervals, stretching/yoga/Plates, and endurance exercise (PRISE) could reduce visceral fat tissue mass of overweight/obese people, decrease their fasting blood glucose, and improve insulin resistance in a 16-week research [24]. Presumably, WBV improved insulin resistance through eliminating hepatic lipid accumulation in db/db mice.

Sterol regulatory element binding proteins (SREBPs) are transcription factors that regulate the expression of genes control synthesis of fatty acid and cholesterol. The identified SREBPs include three different subtypes: SREBP1a, SREBP1c, and SREBP2. As a transcription factor that enhances lipogenesis and adipogenesis, SREBP1c is mainly expressed in liver and white adipose tissue and is activated by nutritional status changing [25]. Since WBV could significantly reduce the lipid accumulation in db/db mice liver, we detected the expression of SREBP1c and found that WBV administration attenuated the expression of this protein by

about 35 %. Lu et al. reported that curcumin reversed increasement of SREBP1c expression induced by alcohol exposure and thus alleviated aberrant accumulation of lipid in hepatocytes [26]. Crude triterpenoid saponins from ilex latifolia (Da Ye Dong Qing) has protective effects on nonalcoholic fatty liver disease by attenuating lipid accumulation via inhibiting SREBP1c expression [27]. Therefore, we could infer that WBV administration could reduce visceral adipose tissue particularly in liver to improve insulin resistance through declining the expression of SREBP1c in db/db mice.

Some studies suggested that ROS played the causal role in hepatic steatosis formation, while some others suggested that aberrant accumulation of lipid in liver induced overproduction of ROS [28, 29]. As a key driving force exacerbates the progression from hepatic steatosis to nonalcoholic fatty liver disease, ROS directly depletes antioxidant molecules and inactivates antioxidant enzymes [30]. It has been reported that H_2O_2 could activate transcription of SREBP1c and accelerate lipid accumulation in HepG2 cells [31]. Kim et al. found that epigallocatechin gallate (ECCG) could suppress fat accumulation and adipocyte differentiation by inactivating FoxO1 and SREBP1c through its antioxidant effect in 3T3-L1 cells [25]. Therefore, we tested whether WBV-induced reduction of lipid accumulation was attributed to the decrease of oxidative stress. Compared with db/db mice without WBV administration, the content of MDA, an indicator of oxidative stress, was reduced by about 30 %. While oxidized GSH (GSSG) level was reduced in WBV mice compared with db/db mice without WBV, reduced GSH level was increased. These data showed that redox homeostasis was improved after WBV treatment. To further figure out the mechanisms that how the WBV administration improved the oxidative stress, we analyzed the activity and expression of a series of enzymes that played antioxidant function. Both of activity/unit and expression of catalase (CAT) and superoxidase (SOD) were not changed. Expression of GR showed increasing trend, but the difference was not statistically significant. While the activity/unit of GSH-Px was not improved by WBV administration, the expression of this enzyme was increased by about 25 %. As a tripeptide, glutathione (GSH) is composed of glutamate, cysteine, and glycine and ubiquitous in cell [32]. For its abundance, GSH serves as a defender against oxidative damage, free radical damage, and other types of toxicity in cells [33]. The GSH-Px were a series of important selenoperoxidases widely distributing in the body that could metabolize toxic peroxides such as hydrogen peroxide and lipid

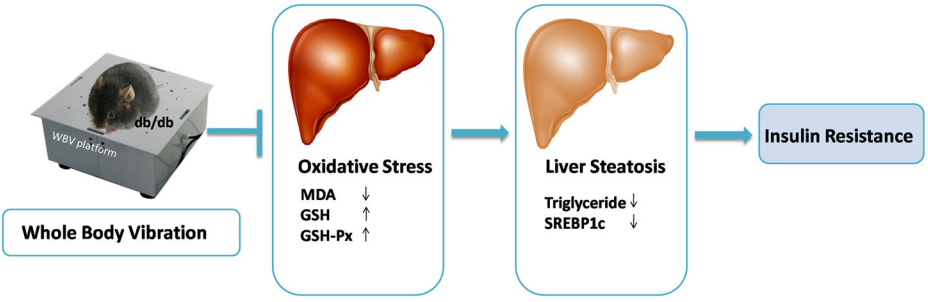

Fig. 4 Working diagram of how WBV improving insulin resistance. WBV inhibits oxidative stress through increasing GSH content and enhancing expression of GSH-Px, and then SREBP1c expression is attenuated. As liver steatosis is ameliorated, hepatic insulin resistance is finally improved

hydroperoxides into nontoxic hydroxyl compounds utilizing GSH as a co-substrate into oxidized glutathione (GSSG) to protect structure and function of cell against interference and damage by peroxides [34–37]. And then, GSSG is catalysed by GR and converts into reduced GSH [37]. Recently, a study including 879 cases of newly diagnosed T2DM and 1295 healthy controls was conducted by Wang et al. in a Chinese population. The result of this study indicated that the GSH-Px activity was decreased significantly, while the plasma MDA concentration was significantly increased in insulin resistance individuals compared with controls [38]. Deficiency of GSH-Px also could induce endothelial dysfunction, structural abnormally changes in vasculature and myocardium, and even heart failure [39, 40]. As newly reported, GSH-Px and paraoxonase (PON) alleviated insulin resistance through defending the oxidative injury by supplementation of fructose in Sprague-Dawley rats liver, and this protective function was intensified by ingesting melatonin through strengthening the activities of those two kinds of enzymes [41]. As a summary, WBV administration could increase the expression of GSH-Px to enhance the enzyme's total antioxidant ability unless increasing its activity/unit and then ameliorate oxidative stress in livers of db/db mice.

Guo et al. reviewed and summarized the beneficial mechanisms of aerobic exercise on hepatic lipid metabolism in nonalcoholic fatty liver disease (NALFD) [42]. Then, we made a comparison between the aerobic exercises with WBV administration. Firstly, the activity of β-oxidation in muscle of BALB/c mice was significantly intensified by swimming [43], as well as the liver fat content of individuals who underwent 3-month energy restriction and physical activity that were sharply lowered through reducing hepatic lipogenesis [44]. In addition, Cintra et al. found that exercise training played an important role in ameliorating fatty liver disease through significantly attenuating expression of SREBP1c [45]. Secondly, the physical training could protect the mice liver against oxidative injury through upregulating antioxidant enzymes such as SOD and GSH-Px [46]. Marosi et al. had the similar point of view that long-term exercise training had protective effects on withstanding oxidative damage by enhancing expression levels of antioxidant enzymes in aging rats [47]. Moreover, it was mentioned that WBV had the similar effect on glycaemia control compared with aerobic exercise for type 2 diabetes patients who are not interesting in or fit for doing exercise [18].

Figure 4 summarizes how WBV attenuates oxidative stress to ameliorate liver steatosis and then improves insulin resistance in db/db mice. WBV increases GSH content and promotes GSH-Px expression. Therefore, decrease of MDA level suggesting oxidative stress is alleviated, and the attenuated SREBP1c expression might attribute to this. As expression of the transcription factor that regulates genes related with lipogenesis is inhibited, fat accumulation in liver is also ameliorated. Finally, the insulin sensitivity of liver is improved. In conclusion, our research indicated that WBV administration attenuated hepatic oxidative stress level through increasing GSH content and the expression of GSH-Px. With the reduction of oxidative stress, the expression of SREBP1c was consequently decreased and hepatic lipogenesis suppressed with liver injury ameliorated. Ultimately, insulin resistance was improved by WBV administration in db/db mice. Although the exact mechanism of how the WBV administration increases GSH and expression of GSH-Px are yet to know, this beneficial nonpharmacological treatment could be a promising recommendation for individuals with insulin resistance and liver steatosis.

Acknowledgments This work was supported by Program for Chankiang Scholars, Innovative Research Team in University (PCSIRT) and National Nature Scientific Foundation (NSFC-81473010 and NSFC-3140040159). This work was also supported by the Natural Science Foundation of Shaanxi Province (2014JQ4135).

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