


The effect of strength-endurance training on serum and urine metabolic profiles of female adolescent volleyball athletes

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Received: September 24, 2020 • Accepted: March 13, 2021

Published online: June 25, 2021

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ABSTRACT

Aim: Limited investigations on metabolic responses to exercise training in female adolescent volleyball athletes exist. The aim of this study was to obtain serum and urine metabolite markers in female adolescent volleyball athletes within 2-week strength-endurance training using a metabolomics approach coupled with biochemical analysis, which would be potential biomarkers for evaluating the physiological state of athletes. *Methods:* Twelve female adolescent volleyball athletes were recruited for 2-week strength-endurance training. Differential serum and urine metabolic profiles between the pre- and post-training group were obtained on gas chromatography coupled to mass spectrometry (GC-MS) and data subsequently underwent orthogonal partial least-squares analysis (OPLS). *Results:* Strength-endurance training exerted a significant influence on the athletes' serum and urine metabolic profiles. The changed metabolites were

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primarily involved in energy metabolism, lipid metabolism and amino acids metabolism. Results support the hypothesis that female athletes displayed an increased propensity to oxidize lipids as the major energy source. Exposure to strength-endurance training also led to a significant increase in cortisol, but a decrease in testosterone, indicating disordered hormone adjustment. Exercise-induced oxidative stress occurred, as was evidenced by the decrease in reduced glutathione, and increases in blood malondialdehyde and oxidized glutathione. Since the muscle damage markers creatine kinase and lactate dehydrogenase did not show significant changes, the training might not cause cell membrane damage and the athletes did not cross the adaptive injury level. *Conclusion:* By measurement of endogenous metabolites, the metabolomics study has the potential to reveal the global physiological changes in response to exercise training.

KEYWORDS

female adolescent volleyball athletes, strength-endurance training, physiology, metabolomics

INTRODUCTION

Volleyball is an intermittent and explosive sport involving numerous repeated sprinting, jumping and diving movements [1, 2]. Well-planned training loads lead to favorable changes in the biochemical and physiological characteristics of the athletes. However, a persistent, high exercise volume and intensity could lead to declined performance, and subsequent accelerated fatigability [3]. Volleyball training loads applied during competitive phase are focused primarily on improving strength-endurance as well as technique and tactics of the game [4]. To assure an increase in athletes' performance, training loads must always be adjusted to an athlete's individual adaptive ability [5]. Currently, various biological, hormonal and immunological markers have been developed to evaluate the physical status in volleyball athletes during exercise training [4, 6]. However, these markers are usually preselected based on some expected hypothesis, they could not sensitively reflect all physiological differences and would be unable to explain the interaction between a variety of metabolites [7]. Therefore, a more comprehensive metabolic profiling has been considered in order to identify global physiological changes in response to training.

Metabolomics is a powerful analytical platform to determine endogenous metabolites and evaluate the global and dynamic metabolic response to external stimuli of living systems [8]. Physical exercise is one of the major modulators of metabolism that increases the rate of metabolic processes and modulates the levels of different metabolites [9]. Hence, by measurement of the endogenous compounds, metabolomics studies have the potential to reveal the global metabolic perturbation and to identify the biomarkers associated with exercise performance, fatigue or exercise-related disorders [10]. Recently, metabolomics has attracted increasing interest in the field of sports medicine [11–14]. In our previous research, metabolomics strategy was used to evaluate the alteration in biochemical composition and gender difference during exhaustive exercise in male and female rats [15]. The blood metabolic profiles were investigated by GC-MS and data subsequently underwent OPLS analysis. A significant gender-related difference in the metabolic profiles was found between male and female rats during exhaustive exercise and the metabolomics study showed great potential for monitoring the changes of physiological state.



Very few investigations on metabolic responses to exercise training in female adolescent volleyball athletes have been reported [16]. In the present study, we investigated the metabolic profiles of female adolescent volleyball athletes subjected to strength-endurance training by our established platform. We hypothesized that the metabolites that change in the serum and urine of the post-training group would be potential biomarkers for evaluating the physiological state of athletes, combined with the interpretation of the biochemical analysis.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Ethical Committee of Changzheng Hospital affiliated to The Second Military Medical University (Approval Notice No.: 2018SL001). All the participants and their parents were fully informed about the testing procedures and signed informed consents.

Reagents

N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), methoxyamine, heptadecanoic acid, and 4-chlorophenyl-alanine were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). Urease was purchased from Worthington (Worthington, USA). Methanol (HPLC Grade) and chloroform were obtained from Thermo Fisher (Thermo Fisher, USA). Pyridine was purchased from China National Pharmaceutical Group Corporation (China National Pharmaceutical Group Corporation, China). Pure water was produced by a Milli-Q purification system (Millipore, USA).

Participants

A total of twelve elite female adolescent volleyball athletes were recruited for the current study. The preliminary trial was performed one week prior to enrolment into the current study, and all participants passed a physical examination. Maximal oxygen consumption (VO_{2max}) test was conducted on a treadmill using Bruce [17]. In order to estimate the one-repetition maximal (1RM), participants were required to perform ten repetitions at 50% of 1RM estimated according to each participant's capacity. Then, a subsequent trail was performed for 1RM with heavier weights until the 1RM was determined within three attempts, with 3 min of rest between trials [18]. The physical characteristics of participants are presented in Table 1. The study, conducted during the in-season competition phase of the adolescent volleyball season, involved a 2-week integrity training cycle before a national competition. The training protocol consisted of two prime contents, i.e. the aerobic endurance exercise and the large-load exercise. The program of aerobic endurance exercise consisted of 3×2000 m variable speed running (500 m jog (about 65% VO_{2max}) + 100 m sprint (about 75% VO_{2max}) + 800 m jog (about 65% VO_{2max}) + 200 m sprint (about 70% VO_{2max}) + 400 m jog (about 65% VO_{2max})), the time was at least 35 minutes, and the interval was 10 minutes. The large-load exercise program included squatting slowly - getting up quickly with barbell, and weighted sit-ups. Participants performed five sets of 3 repetitions squatting



slowly - getting up quickly with barbell at an intensity corresponding to 65–70% of 1RM, with a 1-min rest period between sets. The weighted sit-ups had the same duration and rest period. The training schedule included 11 sessions each week for 30 hours. The total running distance for aerobic training was 72 km and the weight lifting training volume was $10,302 \pm 1,591$ kg. All training sessions were supervised by coaches and all of the participants completed the 2-week training program. The first sampling of venous blood and urine was carried out at 7:00 AM before the exercise training. The last sampling was carried out immediately after completion of the 2-week training session. Participants fasted overnight and resting venous blood and urine samples were collected [10]. Collected blood was incubated for 20 min at room temperature to allow clotting, and then centrifuged at 3,000 rpm for 10 min at 4 °C to obtain serum. All serum and urine samples were stored at –80 °C before use. Participants were instructed to maintain a normal diet for the duration of study without drugs, nutritional supplements and alcoholic drinks. The daily energy intake of the participants was $51 \pm 6\%$, $35 \pm 6\%$, and $14 \pm 4\%$ carbohydrate, fat, and protein, respectively.

Biochemical assay

Serum testosterone (T) and cortisol (CORT) were analyzed using ELISA assay kits strictly according to the manufacturer's instructions and measured by the microplate reader (Biotek, USA). Lipid peroxidation was evaluated by measuring serum MDA concentrations according to the thiobarbituric acid (TBA) method as commercially recommended. Plasma reduced glutathione (GSH) and oxidized glutathione (GSSG) were evaluated as markers of oxidative stress using commercial assay kits based on colorimetric method. All the commercial assay kits were bought from Nanjing Jiancheng Bioengineering Institute, China. Serum blood urea nitrogen (BUN), creatine kinase (CK) and lactate dehydrogenase (LDH) were measured using an automated biochemistry analyzer (Hitachi, Japan).

Metabolomics analysis

Urine samples were chemically derivatized following our previously published procedure with minor modification [19]. Each 200 μ L urine sample was added into a 1.5 mL centrifuge tube and centrifuged at 12,000 rpm (4 °C) for 10 min. 50 μ L of the supernatant was transferred to a new tube followed by the addition of urease (30 U) and incubation at 37 °C for 15 min. Then 10 μ L of 4-chlorophenyl-alanine (0.3 mg/mL in water) and 10 μ L of heptadecanoic acid (1 mg/mL in methanol) were added to each sample as internal standard solutions, and 170 μ L methanol was added to extract the metabolites from each vial. After vortex mixing for 30s, the mixtures were centrifuged at 12,000 rpm (4 °C) for 10 min. A 200 μ L aliquot of supernatant was transferred into a GC vial and dried under N₂ at 30 °C. Eighty μ L of methoxyamine (15 mg/mL) in pyridine was added to the residue and the methoximation reaction was carried out for 90 min in an air-shaker at 220 rpm (30 °C), followed by trimethylsilylation reaction for 60 min by the addition of 80 μ L BSTFA at 70 °C. Serum samples were chemically derivatized following our previously published procedure [15].

The GC-MS analysis method followed our previous research of Zhou et al. [15]. One μ L of the derivatized sample was injected onto a HP-5MS capillary column (30 m \times 250 μ m inner



diameter, 0.25 μm film thickness, Agilent J&W Scientific, USA) on an Agilent 7890A GC/5975C MSD (Agilent J&W Scientific, USA). The optimized GC-MS gradient temperature programming was selected as in our previous experiment [19]: the GC oven was started at 80 °C for 2 min, then the temperature was increased step-wise, starting at 10 °C/min to 140 °C, 4 °C/min to 210 °C, 10 °C/min to 240 °C, 25 °C/min to 290 °C and then maintained at 290 °C for 3 min. The temperature of injection port, transfer interface, and ion source was set to 270, 280, and 230 °C, respectively. Mass data were acquired in scan mode (m/z 50–660) at the rate of 20 spectra/s and the electron impact ionization was 70 eV. Helium was used as carrier gas through the column with a constant flow rate of 1.0 mL/min. The sample was injected in the splitless mode. The solvent delay time was set to 5.0 min.

Data analysis

All the GC-MS raw files were converted to CDF format, and subsequently processed by the XCMS toolbox (https://xcmsonline.scripps.edu/landing_page.php?pgcontent=toolbox) using XCMS's default settings with the following exceptions: `xcmsSet` (full width at half-maximum: `fwhm` = 5; S/N cutoff value: `snthresh` = 10, `max` = 15) to carry out baseline correction, peak deconvolution and alignment [20]. The resulting table (CSV file) was exported into Microsoft Excel (Microsoft Inc., USA), where normalization was performed. The resulting three-dimensional output data including peak index information (retention time– m/z pair), sample names (observations) and normalized peak area percentage (variables) were introduced to the SIMCA-P 11.0 Software (Umetrics, Umea, Sweden) for multivariate statistical analysis. Un-supervised principal component analysis (PCA) and supervised orthogonal partial least squares (OPLS) were employed to identify the general separation and cluster between pre- and post-training groups [21]. In the current study, the validation of OPLS models was performed with the default 7-fold cross-validation in SIMCA-P software package with one-seventh of the samples being excluded from the mathematical model in each round [19, 22]. R^2Y and Q^2Y values of the cross-validation were obtained to estimate the ability of prediction and reliability of the model [22]. In addition, permutation tests ($n = 999$) were also employed to evaluate the statistical significance of the OPLS calibration and CV-figures of merit [23]. Then the differential variables were examined and selected based on variable importance in the projection (VIP) value >1.0 from the 7-fold cross-validated OPLS model. In parallel, univariate statistical analysis, Wilcoxon-Mann-Whitney test (SPSS, Chicago, IL, USA), was performed on those variations to evaluate their significance with the P -value set at 0.05 [22, 23]. Mean ranks were obtained from the Wilcoxon-Mann-Whitney test and the corresponding fold change (FC) was calculated using those metabolites' mean ranks in the post-training group divided by the mean ranks in the pre-training group. The FC value showed how these selected metabolites varied between groups. FC with a value >1.0 indicates a relatively higher-level presence in the post-training group, whereas a value <1.0 means a relatively lower-level presence in the post-training group as compared to the pre-training group. Compound identification was performed by comparing the mass fragments with NIST 2011 standard mass spectral database (Agilent, USA) with a similarity of more than 70% [19]. The comprehensive metabolic pathway analysis was mapped by integration of altered metabolites by means of Metaboanalyst 3.0 (the most influential metabolic pathway had a pathway impact value >0.05 , $-\log(p) >2.0$) [24] and the Kyoto Encyclopedia of Genes and Genomes (KEGG).



The biochemical parameter data were analyzed by paired t test using SPSS 18.0 (SPSS Inc. Chicago, IL, USA) to evaluate the differences before and after training. The normality and homoscedasticity assumptions were checked, respectively, with the Shapiro-Wilk and the Levene tests before paired t test. The data were reported as mean \pm Standard Deviation (SD) and statistical significance was set at a $p < 0.05$.

RESULTS

Effects of exercise training on biochemical parameters

The effects of strength-endurance training on T, CORT, BUN, MDA, GSH, GSSG, CK and LDH were examined in female adolescent volleyball athletes. As shown in Fig. 1, exposure to the 2-week strength-endurance training led to increases in CORT, BUN, MDA and GSSG and to decreases in T and GSH levels in comparison with the pre-training group. Additionally, slight and non-significant increases in CK and LDH levels were found with exercise training.

Effects of exercise training on serum and urine metabolic profiles

The degrees of dispersion of serum and urine data points between pre- and post-training groups were clear and unambiguous in the PCA score plots (Fig. 2). The OPLS model, then, was employed to refine the clusters of the acquired data and to understand which variables contribute to class separating. Figure 3 A and C illustrate the OPLS score plots of the athletes' serum and urine samples from pre- and post-training groups, respectively. The calculated OPLS model parameters were $R^2Y = 86.5\%$ and $Q^2Y = 69.2\%$ of serum samples; $R^2Y = 97.5\%$ and $Q^2Y = 90.1\%$ of urine samples, indicating good ability of prediction and reliability of the model and also suggesting the significant impact of strength-endurance training on the female adolescent volleyball athletes' metabolome. All blue Q2-values shown on the left in permutation plots were lower than the original points on the right, suggesting that the original models were statistically significant (p -value <0.005) (Fig. 3B and D).

To further identify the endogenous variations in serum and urine between the pre-training and the post-training group, V-plots (Fig. 4A and B) were employed to display the

Table 1. Physical characteristics of participants at the start of the study

	Female athletes
Age (year)	13.7 \pm 1.2
Height (cm)	178.3 \pm 4.9
Body weight (kg)	62.1 \pm 8.4
Training experience (year)	4.1 \pm 1.6
VO _{2max} (mL•kg ⁻¹ •min ⁻¹)	43.3 \pm 6.2
IRM (squat, kg)	50.5 \pm 7.8

VO_{2max}: maximal oxygen consumption; IRM: one-repetition maximal.



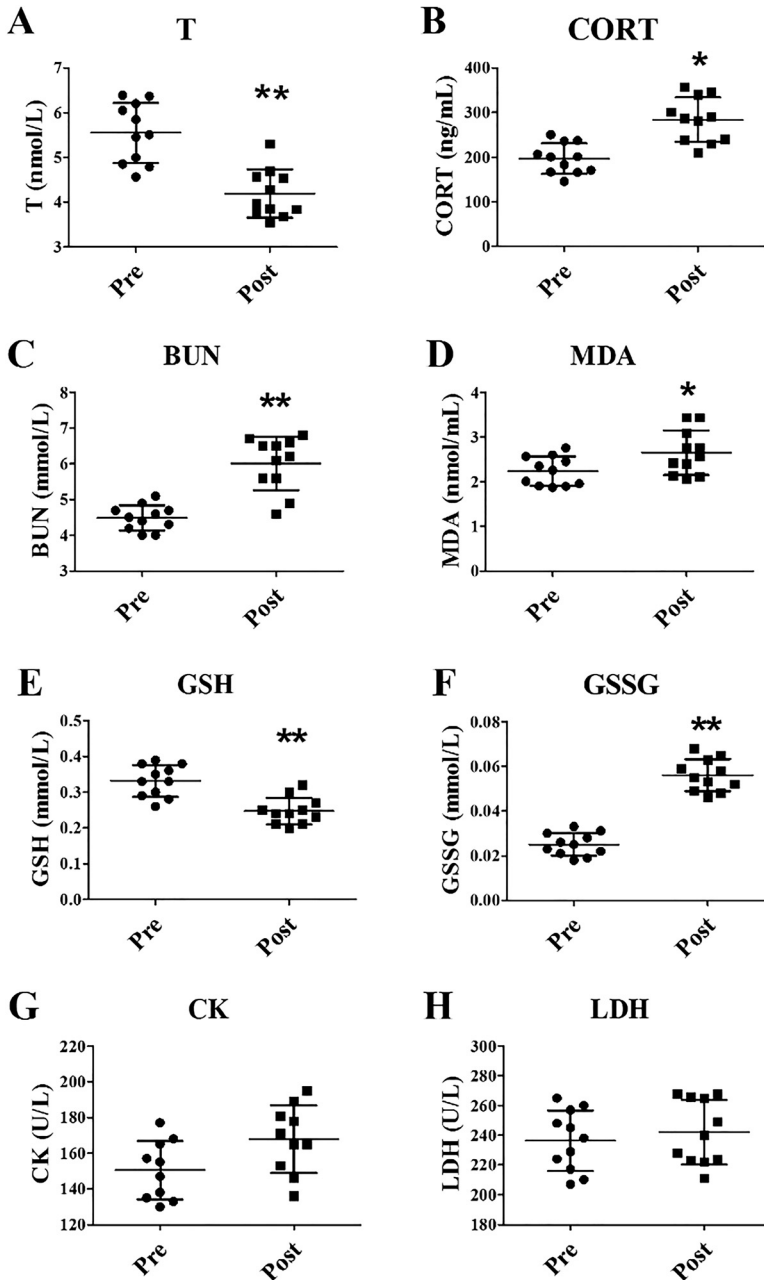


Fig. 1. Effect of exercise training on the levels of T (testosterone), CORT (cortisol), BUN (blood urea nitrogen), MDA (malondialdehyde), GSH (reduced glutathione), GSSG (oxidized glutathione), CK (creatin kinase) and LDH (lactate dehydrogenase) in female adolescent volleyball athletes ($n = 12$). The data were analyzed by paired t test and statistical significance was set at $p < 0.05$. Pre: pre-training group; Post: post-training group. *: $p < 0.05$, **: $p < 0.01$, compared with the pre-training group



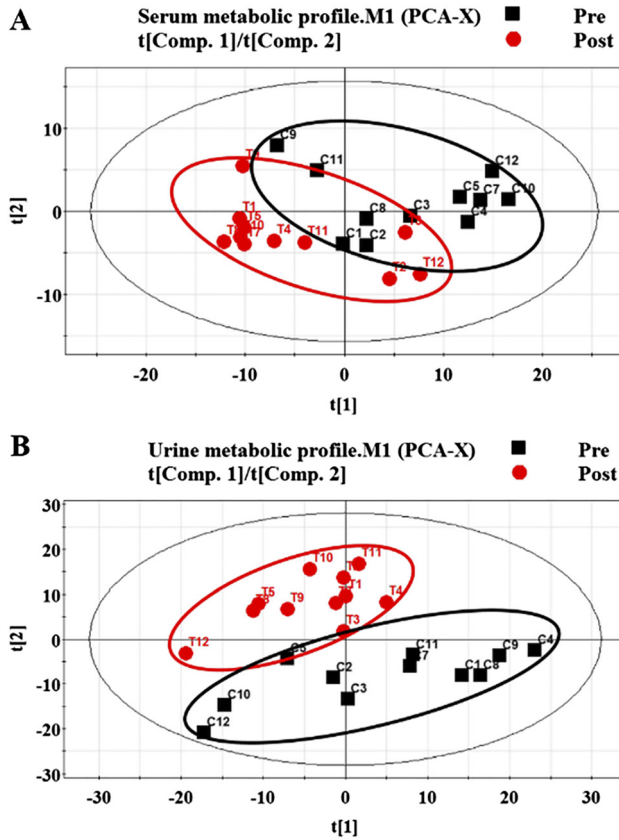


Fig. 2. **A, B:** Score plots of PCA analysis to compare the metabolomes of serum and urine from pre- and post-training groups ($n = 12$), respectively. The degree of dispersion of serum and urine data points between pre- and post-training groups was clear and unambiguous. Pre: pre-training group; Post: post-training group

contribution of detected metabolites in response to separation trends observed in score plots. The differentially expressed metabolites were selected based on the VIP values >1.0 and p values, calculated statistically with Wilcoxon-Mann-Whitney test being less than 0.05. A total of 17 and 14 metabolites in serum and urine samples were identified, respectively (Table 2). The altered metabolites included increased alanine, valine, glycerol, isoleucine, glycine, serine, threonine, 5-oxoproline, glutamic acid, ornithine, palmitic acid, linoleic acid, oleic acid, and octadecanoic acid and decreased threonic acid, aspartic acid, and citric acid in the serum; and increased glycine, threonine, threonic acid, phosphoric acid, myo-inositol and uric acid and decreased valine, leucine, 5-oxoproline, ornithine, aconitic acid, citric acid, fumaric acid, and histidine in the urine of the post-training group as compared with the pre-training group. The heatmap (Fig. 4C) was generated with those differential



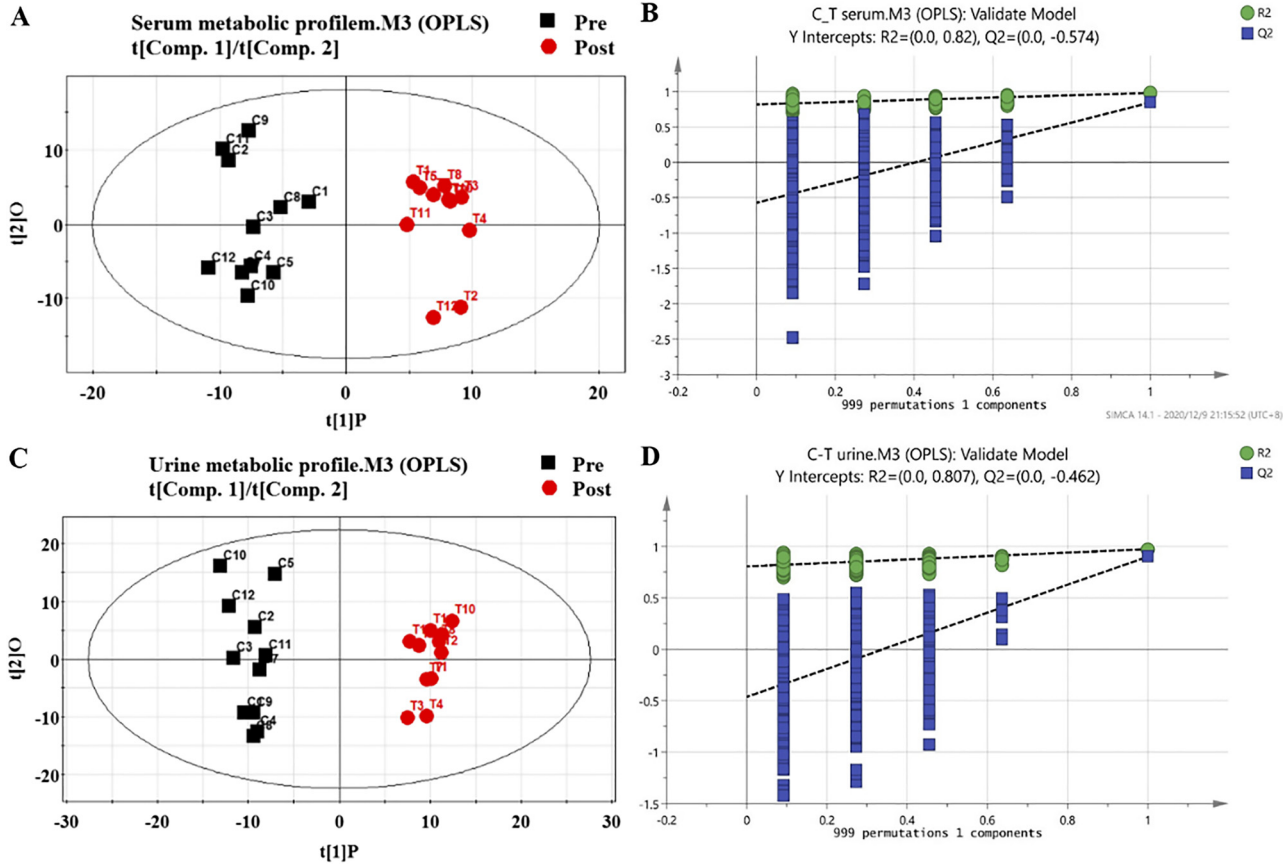


Fig. 3. A, C: Score plots of OPLS analysis to compare the metabolome of serum and urine from pre- and post-training groups ($n = 12$), respectively. The score plots showed distinct clustering of metabolites with training period. Metabolic patterns were grouped by different colored points. B, D: Permutation plots of the OPLS model of serum and urine samples between pre- and post-training groups, respectively. All blue Q2-values shown on the left in permutation plots were lower than the original points on the right, suggesting that the original models were statistically significant (p -value<0.005). Pre: pre-training group; Post: post-training group



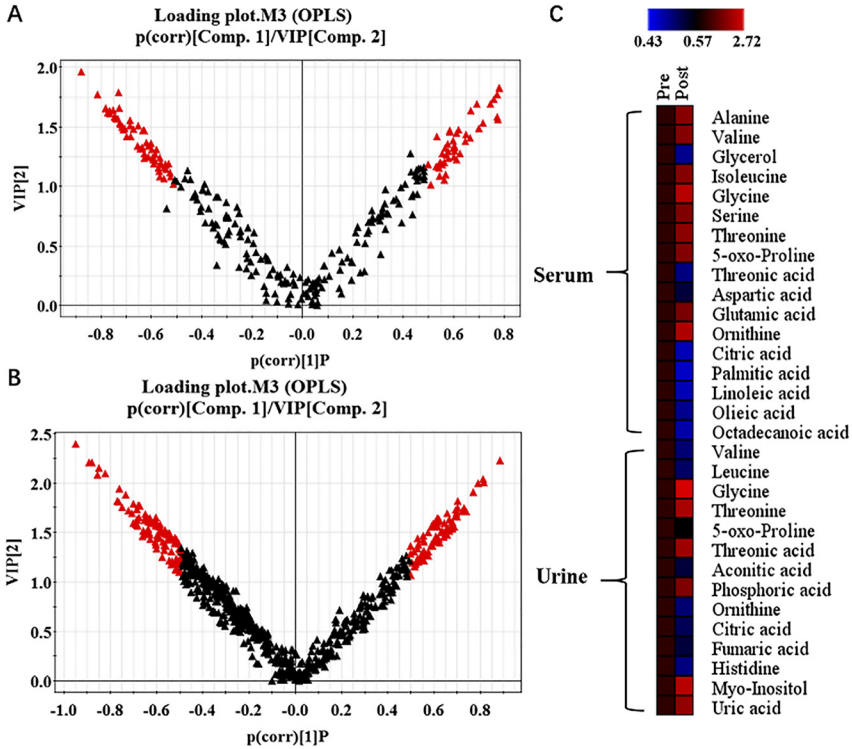


Fig. 4. **A, B**: The V-plots were created to display the contribution of detected metabolites in response to separation trends observed in score plots. The red triangle (\blacktriangle) indicates changed metabolites that differentiated the pre- and post-training groups. **C**: The heatmap showed changes in serum and urine metabolites compared to pre-training at post-training group. Shades of red and blue represent fold increase and fold decrease of a metabolite, respectively, in the post-training group relative to pre-training. Pre: pre-training group; Post: post-training group

serum and urine metabolites contributing to the separation of post-training from pre-training.

The metabolic pathway analyzed by MetaboAnalyst 3.0 based on the potential biomarkers revealed that ten metabolic pathways were defined as disturbed in the serum and urine profiles of athletes (Fig. 5), including glutathione metabolism, alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, arginine biosynthesis, glycine, serine and threonine metabolism, linoleic acid metabolism, arginine and proline metabolism, D-glutamine and D-glutamate metabolism, the citrate cycle and aminoacyl-tRNA biosynthesis.



Table 2. Altered serum and urine metabolites in female adolescent volleyball athletes after 2-week training (n = 12)

Metabolites ^a	Serum				Urine				
	RT (min) ^b	VIP ^c	FC ^d	p ^e	Metabolites ^a	RT (min) ^b	VIP ^c	FC ^d	p ^e
Alanine	6.10	1.38	1.94	0.003	Valine	7.70	1.25	0.49	0.004
Valine	7.68	1.32	1.91	0.007	Leucine	8.45	1.52	0.50	0.005
Glycerol	8.54	1.49	2.13	0.001	Glycine	9.05	1.31	2.72	<0.001
Isoleucine	8.82	1.42	1.91	0.002	Threonine	10.39	1.63	2.29	0.001
Glycine	9.01	1.83	2.41	<0.001	5-oxo-Proline	12.96	1.45	0.57	0.023
Serine	9.91	1.25	1.86	0.009	Threonic acid	14.02	1.46	2.16	0.002
Threonine	10.38	1.45	1.97	0.002	Aconitic acid	18.07	1.43	0.53	0.010
5-oxo-Proline	12.88	1.09	1.88	0.008	Phosphoric acid	18.65	1.46	1.84	0.014
Threonic acid	13.96	1.48	0.48	0.001	Ornithine	19.67	1.48	0.49	0.004
Aspartic acid	14.78	1.18	0.53	0.008	Citric acid	20.06	1.44	0.51	0.006
Glutamic acid	15.05	1.21	1.78	0.015	Fumaric acid	21.12	1.20	0.53	0.011
Ornithine	19.66	1.77	2.33	0.001	Histidine	22.13	1.52	0.48	0.003
Citric acid	19.91	1.51	0.44	0.001	Myo-Inositol	26.18	1.75	2.42	<0.001
Palmitic acid	24.51	1.66	2.33	<0.001	Uric acid	26.53	1.52	2.05	0.004
Linoleic acid	27.66	1.65	2.27	0.001					
Oleic acid	27.75	1.42	2.13	0.002					
Octadecanoic acid	28.13	1.57	2.22	<0.001					

^aMetabolites: Metabolites were identified with NIST 2011 standard mass spectral database with a similarity >70%. ^bRT: The retention time of each metabolite from GC-MS. ^cVIP: Variable importance in the project was obtained from OPLS model with a threshold of 1.0. ^dFC: Fold change was calculated using those metabolites' mean ranks in the post-training group divided by the mean ranks in the pre-training group. Mean ranks were calculated using the nonparametric Wilcoxon-Mann-Whitey test by SPSS 18.0. FC with a value >1.0 indicates a relatively higher-level presence in the post-training group as compared to the pre-training group, whereas a value <1.0 means a relatively lower-level presence in the post-training group as compared to the pre-training group. ^ep: Correlation coefficient from Wilcoxon-Mann-Whitney test and P < 0.05 means the change is significant.



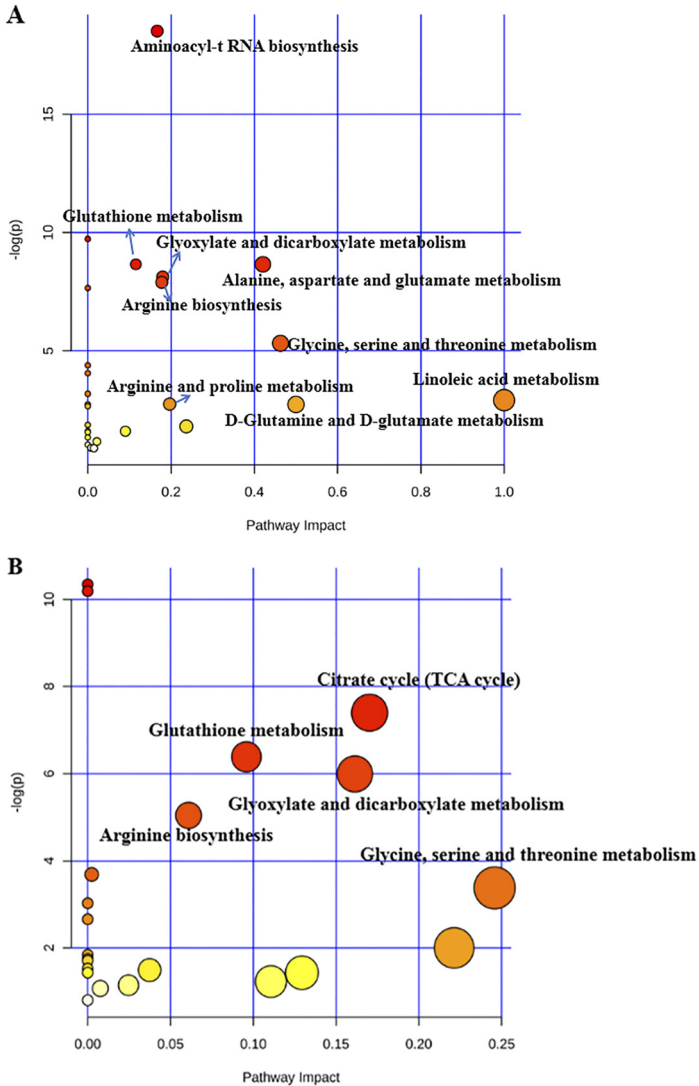


Fig. 5. Pathway and network analysis of differentially expressed metabolites in female athletes. Network pathway was identified by Metaboanalyst 3.0 software. Metabolism was inferred from changes in levels of intermediates during substance metabolism in serum (A) and urine (B). The most influential metabolic pathway had a pathway impact >0.05 and $-\log(p) >2.0$. Ten metabolic pathways were defined as disturbed in the serum and urine profiles of athletes, including glutathione metabolism, alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, arginine biosynthesis, glycine, serine and threonine metabolism, linoleic acid metabolism, arginine and proline metabolism, D-glutamine and D-glutamate metabolism, citrate cycle and aminoacyl-tRNA biosynthesis

DISCUSSION

The current study demonstrated that a 2-week strength-endurance training induced significant effects on biochemical parameters and metabolic responses in female adolescent volleyball athletes. Exposure to the strength-endurance training led to significant increases in CORT, BUN, MDA, and GSSG and to decreases in T and GSH levels in comparison with the pre-training group. There were also slight and non-significant increases in CK and LDH levels. Additionally, by means of high throughput metabolomics, the variations in serum and urine between the pre-training and post-training groups were well differentiated and mainly involved various metabolic pathways, indicating a metabolic disturbance induced by exercise training.

The effects of exercise training on biochemical parameters

It is well established that endogenous hormones are essential for physiological adaptation during exercise training, and the changes of testosterone (which was considered as anabolic hormone) and cortisol (which was considered as catabolic hormone) are important to assess endocrine homeostasis and evaluate athletic performance [25]. In the current study, serum CORT was significantly increased, whereas the level of serum T was significantly decreased during training, indicating that the 2-week strength-endurance training affected the balance of hormone adjustment in female adolescent volleyball athletes [26]. The basal levels of urea derived from nitrogen metabolism are reported to be amplified by exercise [27]. In this study, the BUN levels were significantly increased by exercise training, suggesting an increased breakdown of nitrogen-containing compounds. Similarly, Lemon and Mullin observed significantly increased serum urea excretion during bicycle ergometer exercise [28].

It has been known that exercise-induced membrane damage might be related to free radical mediated lipid peroxidation, and serum MDA is one of the byproducts in the lipid peroxidation process that has been used as a marker of oxidative stress [29]. MDA level was increased during exercise training, which implied that oxidative stress occurred in female adolescent volleyball athletes. In the current study, plasma GSH/GSSG was significantly reduced by exercise training, also reflecting exercise-induced oxidative stress. Considering the fact that GSH/GSSG in blood plasma represents a clinical measure of oxidative stress, reduction of GSH/GSSG indicates a shift in redox balance towards a more oxidizing environment [30]. The finding is in agreement with the previous study by Seifi-Skishahr et al. [31]. These authors found that plasma GSH/GSSG showed a sharp significant reduction immediately after one session of aerobic exercise in subjects with different physical training status, indicating the occurrence of exercise-induced oxidative stress. For the most part, increased levels of tissue enzymes in serum have been treated as indirect indicators of increased cell permeability caused by tissue membrane damage [32]. In the current study, post-training CK and LDH values had a trend of increase, but without statistical significance, suggesting that the 2-week strength-endurance training might not cause cell membrane damage and the athletes did not cross the adaptive injury level.

The effects of exercise training on metabolic profiles

In the OPLS scores plots, the closer the dots, the more similarity there was among the samples' metabolite compositions. However, there was significant difference in the serum and urine



metabolic profiles between pre- and post-training groups, showing that the athletes underwent an obvious metabolic perturbation after exercise. The metabolic pathway analyzed by MetaboAnalyst 3.0 and the correlation networks constructed by KEGG based on the potential biomarkers revealed the detailed impacts of strength-endurance training related alterations in female athletes. The strength-endurance training significantly decreased the level of the tricarboxylic acid cycle (TCA) intermediate citric acid in the serum and decreased aconitic acid, citric acid and fumaric acid in the urine. It is generally accepted that energy expenditure will be elevated with increasing exercise volume, and the TCA cycle will be activated accordingly to oxidize and produce more ATP for the body [9]. Consequently, the TCA intermediates were reduced by consumption. Generally, free fatty acids represent a major energy source supporting whole body energy flux [33]. Especially during moderately strenuous exercise (intensity: 55–75% of $\text{VO}_{2\text{max}}$), there is a progressive decline in the proportion of energy derived from muscle glycogen and a progressive increase in lipid oxidation [34]. In the current study, glycerol and a variety of fatty acids including oleic acid, linoleic acid, palmitic acid, and octadecanoic acid were increased in the serum after exercise, indicating enhanced lipid metabolism with exercise training [35].

Valine, leucine and iso-leucine, the branched-chain amino acids (BCAAs) participating in blood glucose regulation, were found significantly increased in the athletes' serum while decreased in their urine. It has been reported that skeletal muscle is a major site of BCAAs' utilization [36]. As a result of the reduced availability of muscular glycogen during training, there were higher blood levels of BCAAs on account of increased proteolysis in skeletal muscle [37]. The other amino acids alanine, serine, and threonine were also significantly increased in the serum after training. It is well known that, during exercise, there is a net breakdown of protein created by a decrease in the rate of protein synthesis [38]. The suppression of protein synthesis during exercise leaves amino acids available for catabolism. However, the exercise-induced increase in serum amino acids measured was similar to our previous research [15], which demonstrated that female athletes might rely more on lipolysis as an energy source, other than amino acid oxidation during training. The results of other studies also suggest that, for a given relative work intensity, fatty acids comprise a greater proportion of the energy source in women [35]. Additionally, because of the relative abundance of ovarian hormones in women, the regulation of metabolic pathways might be altered to favor lipid oxidation during exercise [39, 40].

As mentioned in the biochemical part, the levels of MDA and GSSG were significantly elevated and GSH was significantly reduced after exercise, suggesting the occurrence of oxidative stress in female adolescent volleyball athletes. Furthermore, elevated levels of metabolites associated with the glutathione metabolism pathway, such as glutamic acid, glycine, and 5-oxoproline, were detected in serum and urine. Glutathione is a major non-enzymatic endogenous antioxidant [41]. The increased serum glutamate levels through the increased cystine/glutamate antiporter activity and high cystine influx to the cell were responsible for increased glutathione synthesis [41]. Via increased cystine/glutamate antiporter activity, extracellular cystine is imported into the cell in exchange for intracellular glutamate. The increase in the influx of cysteine during increased glutathione synthesis results in increased export of glutamate to the extracellular compartment and to the circulation, which ultimately results in the increase of extracellular glutamate levels [42]. The levels of threonic acid and uric acid were also significantly changed, suggesting that strength-endurance training induced diverse responses to



oxidative stress in female adolescent volleyball athletes [43]. Additionally, it has been suggested that altered glycine and histidine levels in the current study could contribute to relieving oxidative stress from exercise [12]. Hence, in this study, the 2-week strength-endurance training induced oxidative stress in female adolescent volleyball athletes, as was evidenced by the decrease in blood GSH, increases in blood GSSG, MDA, and in serum glutamic acid, glycine, 5-oxoproline levels. Urate is an end product of purine metabolism and is suggested to function as an antioxidant [43]. The alteration of urate concentration also suggested that the body's antioxidant system was activated during the training. Intense exercise can cause the accumulation of reactive free radicals, which lead to an imbalance between the oxidation system and its anti-oxidation system, thus can result in tissue damage, and be followed by inflammatory response [44]. The muscle damage markers (CK and LDH) did not show any significant changes, suggesting that the 2-week strength-endurance training might not cause cell membrane damage and the athletes did not cross the adaptive injury level. Females seem to have more antioxidant protection, which might be explained by the higher concentration of estrogens in females [45].

Limitations

Some limitations of the present study should be considered. The potential effect of the menstrual cycle phases was not controlled. As some authors have observed, there was no difference in substrate utilization at different phases of the menstrual cycle during exercise [46]. Chung et al. [47] also examined if the menstrual cycle phase (follicular and luteal phase) influenced oxidative stress to exercise. They found there were minimal differences in oxidative stress when comparing the luteal and follicular phases of the menstrual cycle in young women, and independently of menstrual cycle phase, exercise resulted in a similar increase in oxidative stress. Further research may assess any changes in the stress response at different phases of the menstrual cycle. In addition, as we know, selected body fluids are important in the detection of oxidative stress. GSH is found in all extracellular biological fluids, and here we chose plasma as an extracellular fluid circulating between body cells and exchanging oxidative biomarkers. However, according to the study from Seifi-Skishahr et al. [31], the changes in erythrocyte GSH/GSSG are not parallel with plasma GSH/GSSG, which suggests that the plasma redox biomarker might not accurately reflect tissue redox status compared with erythrocyte biomarkers. One of limitations of the current study is lack of data on erythrocyte oxidative stress biomarkers that is suggested for future studies. Besides, based on Giustarini's research [48], artifactual oxidation to GSSG of 5–15% of the GSH found in a sample can occur during sample preparation and storage, with consequent marked overestimation of GSSG. This can be prevented by derivatizing GSH with the alkylating agent N-ethylmaleimide (NEM), which will be considered in further investigation.

CONCLUSIONS

Based on metabolomics investigation and biochemical analysis, a 2-week strength-endurance training affected the hormone balance of female adolescent volleyball athletes, which is essential for physiological adaption during training. Exercise-induced oxidative stress also occurred, as was evidenced by the decrease in blood GSH, increases in blood GSSG, MDA, and in serum glutamic acid, glycine, and 5-oxoproline levels. The alteration of urate concentration also



suggested that the body's antioxidant system was activated during the training. The muscle damage markers (CK and LDH) did not show any significant changes, indicating that the 2-week strength-endurance training might not cause cell membrane damage and the athletes did not cross the adaptive injury level. However, exercise is associated with oxidative stress, which can induce adverse effects on the physiological state, therefore the intensity of strength-endurance training might be adjusted in the future. In addition, strength-endurance training exerted a significant influence on the athletes' endogenous metabolites, which might be primarily involved in energy metabolism, lipid metabolism and amino acids metabolism, and female athletes showed an increased propensity to utilize lipid oxidation as the major energy source during training. By measurement of the endogenous metabolites, metabolomics studies have the potential to reveal global metabolic perturbations and to identify the biomarkers associated with exercise-related disorders.

Funding: This work was supported by the Shanghai Administration of Sports under Grant 16Z006.

Competing Interests: The authors declare that they have no conflicts of interest regarding the publication of this article.

ACKNOWLEDGMENTS

The authors appreciate the contribution of all the members participating in this study.

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