Original Article

Effects of Balanced Supplementation with Multiple Trace Elements on Oxidative Stress, Inflammation, and Immune Function in High-Fat Diet-Induced Rats

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Abstract

Background: A high-fat diet (HFD) significantly contributes to the development of chronic diseases, which have become a major public health concern. These diseases affect individuals' health and quality of life and hinder socioeconomic progress. Trace elements (TEs) are crucial in various physiological and biochemical processes. Although the effects of single or a few TEs have been extensively reported, considering the complex interactions between different TEs, the effects of balanced supplementation of multiple TEs still need further investigation. **Methods:** In this research, 11 TEs (B, V, Cr, Mn, Fe, Co, Cu, Zn, Se, Sr, and Mo) were selected and prepared as a mixed solution in specific proportions to supplement rats. The study investigated the effects of balanced supplementation of multiple TEs on inflammation, oxidative stress, and immune function in rats fed an HFD. **Results:** The results demonstrated that supplementing multiple TEs had several positive effects, including increased lipid metabolism in HFD rats, improved dyslipidemia, reduced weight and obesity incidence, enhanced antioxidant enzyme activity, and improved anti-inflammatory capacity. **Conclusions:** Therefore, the balanced supplementation of various TEs is expected to become an effective method to prevent and control the harmful effects of HFD

Keywords: *Anti-inflammatory, antioxidant, chronic disease, high-fat diet, immunomodulation, multiple trace elements*

Introduction

The prevalence of high-fat diets (HFDs) in modern society has led to a significant increase in health issues, including obesity, diabetes, and cardiovascular diseases. HFDs contribute to an energy imbalance and metabolic disruptions, which result in the accumulation of body fat and the development of chronic diseases.[1] The excessive intake of fats can elevate cholesterol levels, induce insulin resistance, and increase inflammation, further exacerbating these health problems. Addressing the adverse effects of HFDs through dietary modifications and public health interventions is crucial to improving overall health and reducing the burden of diet-related diseases.[2,3]

Trace elements (TEs) refer to elements in the human body with a content ranging from 0.01% to 0.005% of body weight. TEs are considered important micronutrients for maintaining human balance and regulating the activity and function of various metabolic enzymes. They play a crucial role in many physiological and biochemical processes, such as metabolic processes, energy metabolism, DNA synthesis, etc.[4,5] TEs are crucial for the development of chronic diseases, as chronic diseases are mostly caused by metabolic disorders in the body.[6] According to the 2020 World Health Organization report, more than 2 billion people worldwide are affected by micronutrient deficiencies, and the intake of micronutrients varies among countries and regions.[7] Studies have shown that the appropriate supplement of TEs has a positive effect on the prevention and treatment of diabetes. It can also regulate the lipid metabolism of experimental animals fed a high-fat diet (HFD).[8] However, most of the current studies only focus on one or several TEs, and there are few studies on simultaneous supplementation of multiple micronutrients, which makes it difficult to obtain the effect of balanced supplementation of multiple TEs on HFD.[9-11] In reality, because of the synergistic or antagonistic interactions between different elements, the balanced supplementation of multiple TEs is very important.

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In this study, we investigated the effects of a solution containing 11 TEs (B, V, Cr, Mn, Fe, Co, Cu, Zn, Se, Sr, and Mo) mixed in specific proportions to supplement the diet of rats fed a HFD. Our findings indicate that multi-TEs supplementation significantly improves lipid metabolism, corrects lipid abnormalities, reduces body weight and the incidence of obesity, enhances antioxidant enzyme activities, and improves anti-inflammatory capacity. These results underscore the importance of a balanced multi-element approach in counteracting the negative impacts of HFDs and highlight its potential for broader dietary interventions.

Materials and Methods

Animals and treatment

A total of 160 male Wistar rats (4 weeks old, weighing 100-120 g) were obtained from Beijing SPF Biotechnology Co., Ltd. The rats were housed in the Laboratory Animal Center under controlled conditions: a temperature of (22 ± 2) °C, a humidity of (60 ± 10) %, and a 12-hour light/dark cycle. After 1 week of acclimatization, the rats were randomly divided into four groups (40 rats/group): (1) a control group fed a TE-free maintenance diet with saline gavage; (2) a TE supplementation group (TE) fed a TE-free diet with TEs gavage; (3) a HFD group fed a HFD with saline gavage; and (4) a HFD supplemented with TEs group (HFD+TE) fed a HFD with TE gavage. The gavage volume was set at 2 mL/kg body weight, administered daily, and the experimental drinking water was deionized water without TEs. All feed for the experimental animals was provided by Beijing Keaoxieli Feed Co., Ltd (Beijing, China). The feeds included: (1) a TE-free maintenance feed (serial number: GB14921.1-2001) with main nutritional ingredients comprising ≤10% water, ≥18% crude protein, ≥4% crude fat, ≤5% crude fiber, 1.0-1.8% calcium, and 0.6-1.2% phosphorus; and (2) a high-fat, TE-free feed, which contained the aforementioned TE-free maintenance feed (78.9%), lard (10%), egg yolk powder (10%), cholesterol (1%), and sodium cholate (0.1%). There are no differences in the essential nutrients among different feeds. Conducted potential trace element source testing on the housing and all other environments of mice before the experiment, including bedding, water, feed troughs, etc.

The elemental solution given to rats by gavage contained H_3BO_3 (1.76 mg/mL), VO (SO_4) (13.5 μg/mL), $CrCl_3$ (4.1 μg/mL), $MnCl_2$ (0.68 mg/mL), $FeCl_2$ (1.62 mg/mL), $CoCl_2$ (8.1 μg/mL), $SrCl_2$ (0.54 μg/mL), $CuCl_2$ (108 μg/mL), $ZnCl_2$ (1.69 mg/mL), Na_2SeO_3 (8.1 μg/mL), and Na_2MoO_4 (13.5 μg/mL). All chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The rats were weighed weekly, and their diet and growth activity were observed and recorded daily.

Sample collection

The rats were divided into four batches at the end of the 4th, 8th, 12th, and 20th week of the experiment. Ten rats were randomly selected from each batch, subjected to a 12-hour fasting period, weighed, and then anesthetized by in-traperitoneal injection of 1% sodium pentobarbital. Blood was collected from the abdominal aorta, and serum was separated. Subsequently, the spleens of rats were taken on ice, partially ground, and analyzed by flow cytometry. The remaining spleen samples were frozen in liquid nitrogen and stored at -80°C for further testing.

Biochemical analysis

Determination of various serum indicators

The level of serum triglycerides (TG) was determined using the GPO-PAP enzyme method by commercial kits, while the level of serum total cholesterol (TC) was determined using the COD-PAP enzyme method by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). 2.5 μL of rat serum was added into the enzyme labeling plate, and other reagents were added according to the instructions of the kit and mixed well. The samples were incubated at 37°C for 10 minutes, and the absorbance at 510 nm was measured using an ELISA reader. Glutathione (GSH) was assayed by measuring the compound formed by its reaction with $C_{14}H_8N_2O_8S_2$ at 405 nm. Malondialdehyde (MDA) was determined by measuring the combination with $C_4H_4N_2O_2S$ at 532 nm. Total antioxidant capacity (T-AOC) was measured by spectrophotometry at 520 nm using a reagent kit. Glutathione peroxidase (GSH-Px) activity was measured at 412 nm by zymography according to the kit instructions. Serum sample GR was determined by UV colorimetric method using a kit. Serum superoxide dismutase (SOD) activity was determined by the hydroxylamine method using a kit. Catalase (CAT), xanthine oxidase (XOD) and myeloperoxidase (MPO) were determined using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) in accordance with the manufacturer's instructions.

The levels of serum interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) were estimated with an ELISA (Tecan Infinite 200pro, Switzerland) instrument using kits specific to rats (Shanghai Jianglai Industrial Limited ByShare Ltd., Shanghai, China); these kits were used in accordance with the manufacturer's protocol.

Determination of various indicators of spleen tissue fluid

The levels of GSH, MDA, GSH-Px, SOD, and CAT were measured in the spleen of rats according to the instructions of the kit.

Evaluation of immune function

1 mL of red blood cell lysis buffer (Solarbio, Beijing,

China) was added to 200 μL of blood cell suspension, incubated at 37°C for 10 minutes, centrifuged at 25 g for 7 minutes and resuspended using PBS. This lysis procedure was repeated lysis until the erythrocytes had been completely lysed. A cell concentration of 1×10^6 cells/mL was obtained.

For spleen cells, when the rats were executed, ground the removed spleen, rinsed with PBS buffer and collected the grinding solution. Filtered with a 200 mesh sieve and collected about 5 mL of spleen cell suspension into a centrifuge tube. Centrifuged at 25 g for 7 minutes, discarded the supernatant, and added 1 mL of PBS buffer for resuspension. After discarding the supernatant, added 1 mL of red blood cell lysis solution and mixed well. Incubated at 37°C for 10 minutes, centrifuged at 25 g for 7 minutes, and resuspended. Repeated the above lysis process until the erythrocytes had been completely lysed. After completed lysis, adjusted the concentration of the cell suspension to about 1×10^6 cells/mL.

Then, FITC-CD45 (1:250 dilution; AB_314005; BioLegend, San Diego, CA, USA), PE/Cyanine7-CD8a (1:100 dilution; AB_2814100; BioLegend), APC-CD4 (1:100 dilution; AB_313935; BioLegend) and PerCP/Cyanine5.5-CD3 (1:100 dilution; AB_2860754; BioLegend) antibodies were added, followed by incubation at room temperature (protected from light) for 15 minutes. Then, we performed centrifugation at 25 g for 10 minutes and discarded the supernatant. This was followed by a PBS wash and resuspension in 500 μL of PBS. Data were acquired using a CytoFLEX Flow Cytometer and processed using FlowJo software (FlowJo LLC, Ashland, USA).

Determination of TEs in HFD rats serum

The standard stock solution (20 μg/mL) containing B, V, Cr, Mn, Fe, Co, Cu, Zn, Se, Sr, and Mo was taken and made into the standard solution with the mass concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 200, and 500 μg/L, respectively. At the same time, Sc, Ge, In, Rh, Re, Bi and other elements of the internal standard storage solution were diluted to 100 μg/L as the internal standard on the machine. The mixed standard solution was injected into the Inductively coupled plasma mass spectrometry (ICP-MS), and the signal response values of the on-line internal standard and the element to be measured were determined (iCAP™ RQ ICP-MS, Thermo Scientific, USA). The standard curve was plotted with the concentration of the element to be measured as the horizontal coordinate and the ratio between the element to be measured and the response value of the corresponding internal standard as the vertical coordinate.

0.5 mL of thawed and mixed rat serum was accurately measured, and the diluent (0.1 mL of Triton X-100 solution, diluted to 1000 mL with 0.1% HNO_3 solution) was used to dilute and bring the volume to 10.0 mL. It was then vibrated sufficiently until homogeneous and centrifuged at 75 g for 5 minutes. The supernatant was taken for determination, while the diluent was simultaneously used to perform the blank test. To eliminate the interference of the carbon element on selenium analysis, 1% isopropanol was added. The experimental conditions of the instrument are shown in Table 1.

Statistical analysis

All values were given as mean ± standard deviation (SD). The experimental data were analyzed using SPSS 26.0 statistical software and plotted using GraphPad Prism 8 software. One-way ANOVA was used to compare data with a normal distribution and homogeneity of variances. Bonferroni test was used for pound-to-group comparisons. Dunnett's T3 test was used to compare two groups with uneven variances. Differences were considered to be significant at $P < 0.05$ (double-tailed). The statistical software MedCalc version 18.0 (MedCalc Software Ltd, Ostend, Belgium) was used to draw figures. Differences were considered to be significant at $P < 0.05$.

Results

Observation of general growth status and weight changes

The rats were divided into four groups. The control group rats exhibited smooth hair, normal eating habits, and mild temperament but displayed a poor mental state during the middle and later stages of the experimental period. In contrast, the HFD group rats showed increased body fat, reduced physical activity, lethargy, decreased food intake, and reduced appetite compared with the control group. The two groups of rats supplemented with TEs demonstrated higher energy levels, regular sleep patterns, liveliness, and better physical fitness compared with their respective non-supplemented groups. No other abnormal conditions were observed, and no animal deaths occurred.

The trend of weight changes in the rats is shown in Figure 1. The differences in body weight of each group for

Table 1: ICP-MS acquisition parameters

Parameter Name	Reference Value
Radio Frequency Power	1550 W
Plasma Gas Flow Rate	15 L/min
Carrier Gas Flow Rate	0.80 L/min
Nebulizer	High-Salt/Concentric Nebulizer
Sampling Cone/Skimmer Cone	Nickel/Platinum Cone
Sampling Depth	8 mm~10 mm
Auxiliary Gas Flow Rate	0.3 L/min
Helium Flow Rate	4 mL/min~5 mL/min
Nebulizer Chamber Temperature	2°C
Sample Injection Rate	0.3 r/s
Collection mode	Peak Shifting
Detection Method	Automatic
Points per Peak	3
Repetition Times	3

each week are shown in Table 2. There was no statistically significant difference in the initial body weight among the groups before the experiment ($P > 0.05$). The weight of rats in all groups increased with prolonged feeding. The HFD group rats exhibited greater weight levels and growth rates compared with the other groups. In the HFD+TE group, there was no statistically significant difference in weight compared with the HFD group only at the end of the 9th and 20th week ($P > 0.05$). However, in all other weeks, the weight of rats in the HFD+TE group was significantly lower than that of the HFD group ($P < 0.01$).

Changes in blood lipid levels

Blood lipid levels could reflect the accumulation of lipids in the body. To investigate the effect of supplementing TEs on lipid metabolism, the TG and TC levels of rats in each group were measured. As shown in Figure 2, the serum triglyceride levels of the TE group rats decreased significantly compared with the control group ($P < 0.05$) at the end of the 4th, 8th, 12th, and 20th week. The serum TG level of rats in the HFD group was higher than that in the control group, and the differences were statistically significant ($P < 0.01$). Compared with the rats in the HFD group, the rats in the HFD+TE group showed a statistically significant decrease in serum TG levels ($P < 0.01$). At the end of the 8th, 12th, and 20th week, the TC levels in the serum of the HFD group rats increased significantly

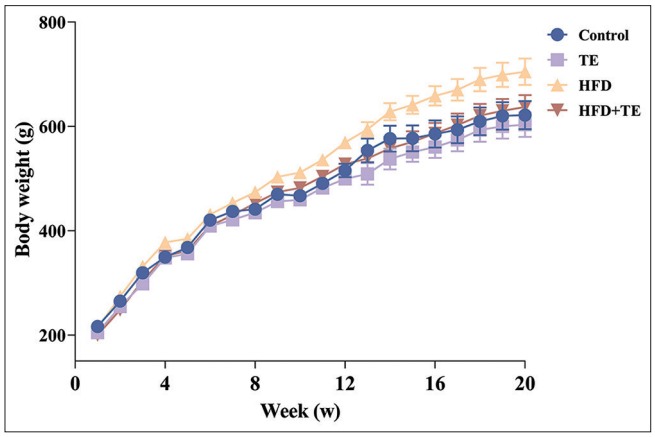

Figure 1: Trend of weight changes in different groups (g)

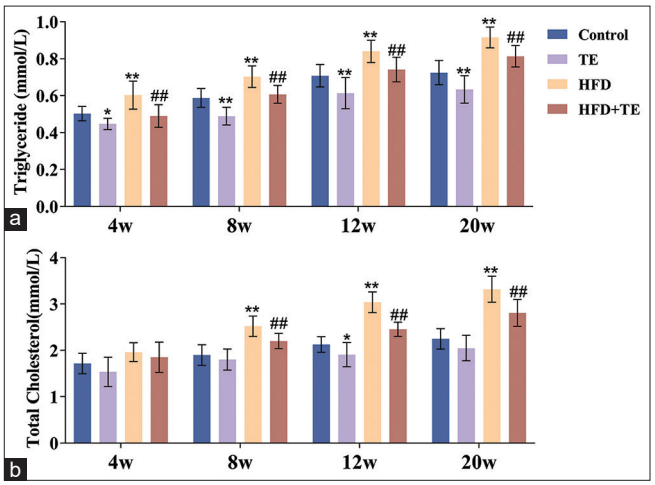

Figure 2: Effects of supplementing multiple TEs on blood lipid levels. (a) Triglycerides. (b) Total cholesterol. The data was presented in the form of $\bar{x} \pm s$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group; $^{\#\#}P < 0.01$ vs HFD group

Table 2: The body weight levels of rats in each group (g)

Group	Control	TE	HFD	HFD+TE
initial body weight	159.42±9.60	159.55±11.30	158.26±9.93	158.38±9.54
1	216.37±11.62	204.47±13.95**	213.27±13.07	199.95±12.26##
2	264.90±13.39	254.39±21.18**	273.91±16.06*	248.27±15.74##
3	319.26±24.70	297.98±33.43**	331.56±23.81*	304.06±23.13##
4	349.44±26.50	347.93±32.42	377.76±27.88**	351.07±26.04##
5	367.75±26.78	355.75±30.61	384.46±28.67*	361.46±26.72##
6	420.19±35.39	408.99±34.33	430.99±35.02	409.20±29.27#
7	437.03±40.57	420.81±33.86	453.34±37.55	429.88±34.72#
8	441.06±42.06	434.67±34.49	473.59±38.65**	452.84±37.07#
9	469.77±53.92	456.06±45.94	503.17±45.12*	473.84±41.35
10	467.04±50.10	459.22±44.62	511.48±46.25**	481.97±44.05#
11	490.59±51.07	481.92±44.90	536.06±45.01**	504.06±45.89#
12	515.37±57.13	499.35±52.36	568.93±47.31**	527.74±54.89#
13	553.78±71.82	508.77±61.99	594.50±43.48	538.36±62.92#
14	576.41±78.94	537.46±60.36	628.03±52.35	557.89±67.90#
15	577.25±78.78	550.88±56.78	641.13±54.97*	570.39±64.12#
16	585.41±82.66	560.41±62.54	658.45±59.10*	586.66±62.91#
17	593.63±81.49	574.21±65.86	670.06±65.10*	602.36±70.04#
18	609.67±84.03	593.09±68.21	689.70±71.02*	620.77±71.39#
19	620.00±83.25	600.14±70.58	698.75±73.84*	629.90±71.53#
20	621.54±84.64	603.49±71.26	704.73±80.61*	636.92±72.84

Data were presented in the form of $\bar{x}\pm s$, $^{*}P<0.05$, $^{**}P<0.01$ vs control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs HFD group

compared with the control group ($P < 0.01$), while the TC levels in the serum of the HFD+TE group rats decreased significantly ($P < 0.01$). Compared with the control group, the serum TC level in the TE group decreased only at the end of the 12th week, and the difference was statistically significant ($P < 0.05$).

Effects of supplementing multiple TEs on antioxidant levels

The levels of T-AOC, GSH, and MDA in serum could reflect the level of oxidative stress in the body. In order to investigate whether the supplementation of TEs could alleviate the oxidative stress of HFD rats, the levels of the aforementioned indicators in the rat serum of each group were measured.

As shown in Figure 3, there was a statistically significant difference ($P < 0.01$) in the levels of T-AOC in the serum among the TE group, the HFD group, and the control group rats at the end of the 4th, 8th, and 12th week. At the end of the 20th week, the serum T-AOC levels in the TE group rats were higher than those in the control group rats ($P < 0.01$), while there was no statistically significant difference between the HFD group rats and the control group rats ($P > 0.05$). Compared with the HFD group, the HFD+TE group exhibited a significant increase in serum T-AOC levels at the end of the 4th, 8th, 12th, and 20th week ($P < 0.01$).

In addition, both the TE group and the HFD+TE group exhibited a significant increase ($P < 0.05$) in serum GSH levels compared with the control group at the end of the 4th, 8th, 12th, and 20th week. At the end of the 20th week, the serum GSH levels in rats from the HFD group were found to be significantly lower than those in the control group ($P < 0.01$).

Furthermore, the level of MDA in the serum of TE group was significantly lower than that of the control group at the end of the 8th, 12th, and 20th week ($P < 0.01$). On the contrary, the MDA levels in the serum of rats in the HFD group were significantly higher than those in the control group ($P < 0.01$). And the serum MDA levels in the HFD+TE group rats showed a significant decrease compared with those in the HFD group at the end of the 4th, 8th, 12th, and 20th week ($P < 0.01$).

By measuring the activities of enzymes (GSH-Px, SOD, CAT, GR, MPO, XOD, etc.) in the serum of rats in each group, the improvement effect of supplementing TEs on the antioxidant levels of HFD rats could be observed. The activity levels of each enzyme were showed in Figure 4. It could be observed that with the prolonged feeding, the GSH-Px enzyme in the serum of rats in the TE group increased in all batches compared with the control group, and the difference was statistically significant ($P < 0.01$). Compared with the HFD group, the serum GSH-Px enzyme activity in the HFD+TE group rats increased significantly in all batches ($P < 0.01$).

As the prolonged feeding, the serum SOD enzyme activity of the HFD group rats showed a significant decrease compared with those in the control group rats ($P < 0.01$). Compared with the HFD group, the HFD+TE group exhibited a significant increase in serum SOD enzyme activity at the end of the 4th, 8th, 12th, and 20th week, and the difference was statistically significant ($P < 0.01$). The SOD enzyme activity in the serum of rats in the TE group were significantly elevated compared with the control group ($P < 0.01$).

The serum CAT enzyme activity of the TE group rats increased at the end of the 4th, 8th, 12th and 20th week compared with those in the control group and the differences were statistically significant ($P < 0.01$). Compared with those in the HFD group, the serum CAT enzyme activity of the HFD+TE group rats showed a significant increased at the end of the 8th, 12th and 20th week ($P < 0.01$).

In addition, the GR activity in the serum of rats in the HFD+TE group were significantly higher than those in the HFD group at the end of the 8th and the 20th week ($P < 0.01$).

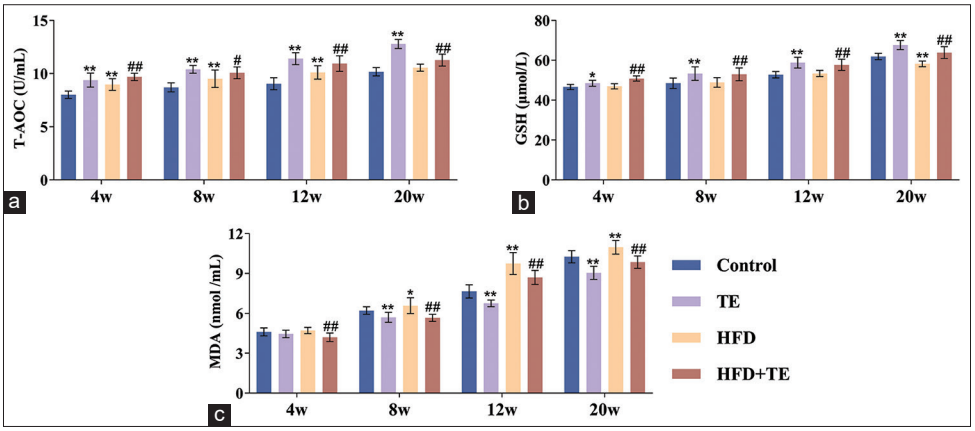

Figure 3: Effects of supplementing multiple TEs on antioxidant levels. (a) T-AOC. (b) GSH. (c) MDA. The data was presented in the form of $\bar{x} \pm s$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs HFD group

As the feeding time increased, compared with the control group, the HFD group exhibited a significant increase ($P < 0.01$) in serum MPO enzyme and XOD enzyme activities. The activities of MPO enzyme and XOD enzyme in the serum of the HFD+TE group rats showed a statistically significant decrease compared with those of the HFD group rats ($P < 0.01$).

To investigate the effects of supplementing TEs on some immune organs of HFD rats and assess their antioxidant levels, the GSH content, GSH-Px enzyme activity, SOD enzyme activity, CAT enzyme activity, MDA content, etc., in the spleens of each group of rats were measured. The results were shown in Figure 5. With the increase in feeding time, the HFD could reduce the GSH level in the spleen of control group rats ($P < 0.01$) and increase the MDA content ($P < 0.01$), respectively. However, supplementing with TEs could increase the GSH level in the spleen of both the control group and HFD group rats ($P < 0.01$) and reduce their MDA content ($P < 0.01$). As the feeding time increased, the activities of SOD, CAT, and GSH-Px enzymes in the spleen of the HFD group rats decreased compared with the control group rats in each batch, and

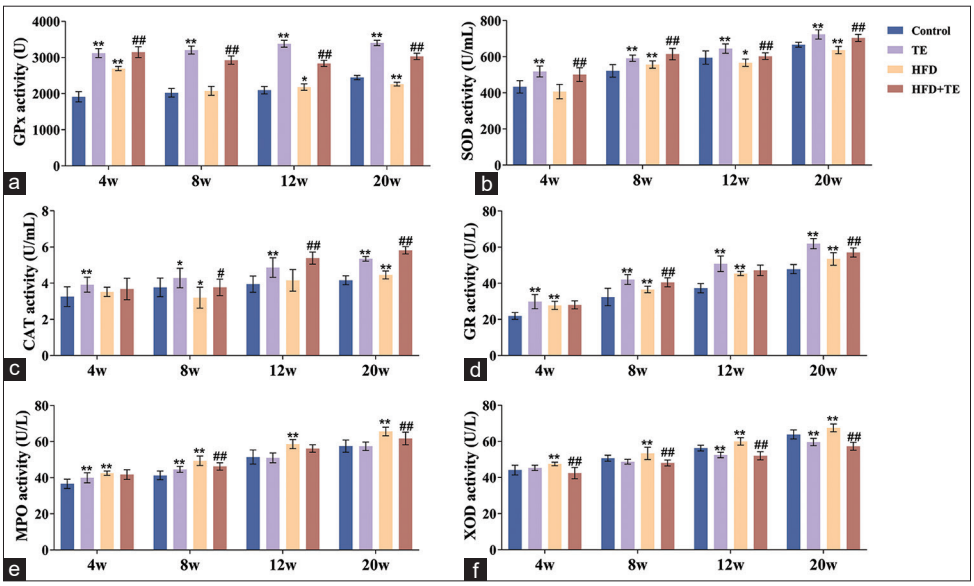

Figure 4: Effects of supplementing multiple TEs on serum antioxidant enzyme activity'. (a) GSH-Px. (b) SOD. (c) CAT. (d) GR. (e) MPO. (f) XOD. The data was presented in the form of $\bar{x} \pm s$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs HFD group

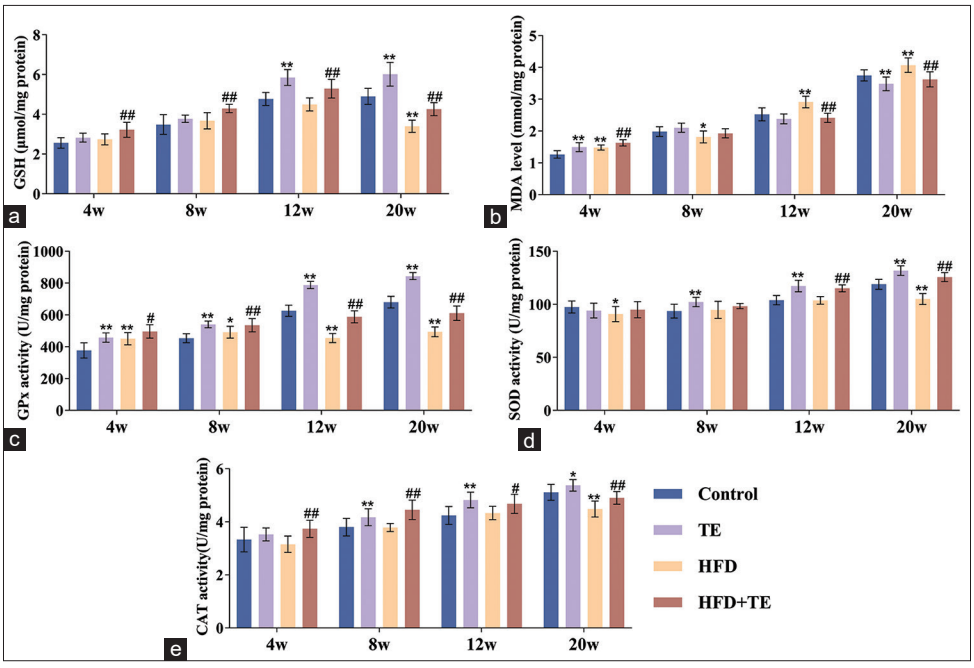

Figure 5: Effects of supplementing multiple TEs on antioxidant levels in the spleen of HFD rats. (a) GSH. (b) MDA. (c) GSH-Px. (d) SOD. (e) CAT. The data was presented in the form of $\bar{x} \pm s$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs HFD group

the differences were statistically significant ($P < 0.01$). Supplementing with TEs could increase the activities of SOD, CAT, and GSH-Px enzymes in the spleen of the control group and HFD group rats in each batch, respectively ($P < 0.01$).

Effect of multiple TEs supplementation on serum levels of inflammatory factors

Serum levels of cytokines such as IL-2, IL-4, IL-6, IL-10, and TNF-α are associated with the body's anti-inflammatory response. In order to investigate the effects of microelement supplementation on the anti-inflammatory ability of HFD rats, the serum levels of cytokines (IL-2, IL-4, IL-6, IL-10, and TNF-α) in each group were measured, as shown in Figure 6.

With prolonged feeding duration, the HFD led to elevated serum levels of IL-2, IL-6, and TNF-α ($P < 0.01$) and reduced levels of IL-4 and IL-10 ($P < 0.01$). Compared with the control group, the content of IL-2 in the serum of the HFD group rats increased at the end of the 8th and 20th week, and the difference was statistically significant ($P < 0.01$); At the end of the 4th week, the serum IL-2 content in the HFD+TE group rats showed a significant decrease compared with those in the HFD group ($P < 0.01$), while there was no statistically significant difference in the other batches ($P > 0.05$). The levels of IL-4 in the serum of rats in the TE group were significantly higher than those in the control group in each batch ($P < 0.01$). The HFD+TE group exhibited a significant increase ($P < 0.01$) in serum IL-4 and IL-6 levels compared with the HFD group at the end of the 4th, 8th, 12th, and 20th week.

Compared with the control group, the IL-10 content in the serum of the HFD group rats decreased significantly in each batch ($P < 0.01$); the IL-10 content in the serum of the TE group rats was significantly higher than that of the control group rats at the end of the 4th and 12th week; At the end of the 12th week, the serum IL-10 content in the HFD+TE group rats were significantly higher than those in the HFD group ($P < 0.01$), while there was no significant difference in the other batches.

Furthermore, the serum TNF-α content of the HFD group was significantly higher than that of the control group at the end of the 4th, 8th, 12th, and 20th week ($P < 0.01$). Compared with the HFD group, the serum TNF-α content of the HFD+TE group rats decreased at the end of the 20th week and the difference was statistically significant ($P < 0.05$), while there was no significant difference in the other batches.

Effect of multiple TEs supplementation on immune function

The proportion of T lymphocyte subsets was used to describe the immune status of the body. Measuring the proportion of $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, and $CD4^+/CD8^+$ T lymphocytes in the spleen helped observe the effect of supplementing TEs on the immune function of HFD rats. Table 3 showed the proportion of T lymphocyte subtypes in the whole blood of each group of rats at the end of the 20th week, while Table 4 showed the proportion of T lymphocyte subtypes in the spleen of each group of rats at the end of the 20th week.

Compared with control rats, the HFD decreased the proportion of $CD3^+$ T lymphocytes in whole blood ($P < 0.01$), decreased the proportion of $CD4^+$ T lymphocytes in the spleen ($P < 0.01$), increased the proportion of $CD8^+$ T lymphocytes in whole blood ($P < 0.01$), and decreased the proportion of $CD4^+/CD8^+$ T lymphocytes in whole blood

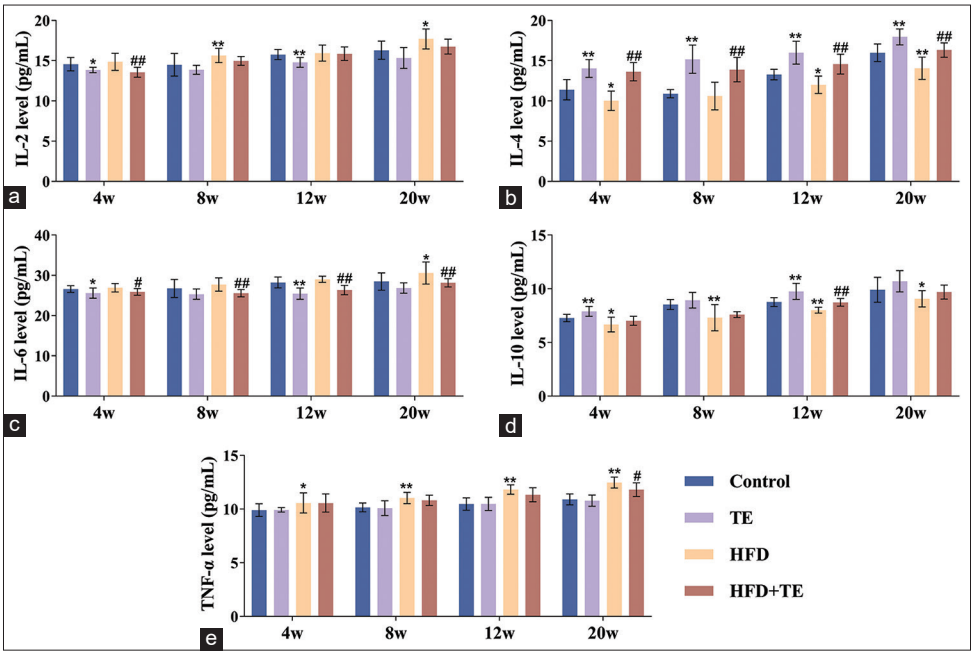

Figure 6: Effects of supplementing multiple TEs on serum inflammatory factor levels. (a) IL-2. (b) IL-4. (c) IL-6. (d) IL-10. (e) TNF-α. The data was presented in the form of $\bar{x} \pm s$, $^*P < 0.05$, $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs HFD group

Table 3: Proportion of T lymphocyte subsets in the whole blood of rats in each group at the end of the 20th week (%)

Group	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Control	42.08±1.37	60.01±2.36	26.70±2.79	2.27±0.26
TE	43.09±2.22	60.74±2.87	24.07±2.11*	2.54±0.22*
HFD	37.16±3.34**	57.76±3.20	34.57±2.49**	1.68±0.19**
HFD+TE	42.28±3.52##	58.20±3.23	30.91±2.30##	1.89±0.20#

Data were presented in the form of $\bar{x}\pm s$, *P<0.05, **P<0.01 vs control group; #P<0.05, ##P<0.01 vs HFD group

Table 4: Proportion of T lymphocyte subsets in the spleen of rats in each group at the end of the 20th week (%)

Group	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Control	48.61±2.68	41.28±2.83	33.90±2.51	1.22±0.13
TE	55.28±3.87**	40.80±3.87	23.35±2.41**	1.76±0.24**
HFD	45.76±2.33	34.70±1.97**	36.43±3.85	0.96±0.13**
HFD+TE	52.22±4.27##	44.82±3.03##	26.08±2.52##	1.73±0.13##

Data were presented in the form of $\bar{x}\pm s$, *P<0.05, **P<0.01 vs control group; #P<0.05, ##P<0.01 vs HFD group

and spleen ($P < 0.01$). Supplementation with TEs reversed the changes in all the indicators in HFD ($P < 0.01$). In detail, TE supplementation reduced the proportion of $CD8^+$ T lymphocytes in whole blood and spleen ($P < 0.01$), increased the proportion of $CD4^+/CD8^+$ T lymphocytes in whole blood ($P < 0.01$), and $CD3^+$ T lymphocytes in spleen ($P < 0.01$) compared with the control group.

Effect of multiple TEs supplementation on serum elemental content

The levels of TEs such as B, V, Cr, Mn, Fe, Co, Cu, Zn, Se, Sr, and Mo in the serum of rats in each group were shown in Figure 7. Compared with the control group, the level of B in the serum of rats in the HFD group decreased at the end of the 8th and 20th week ($P < 0.05$). The content of B in serum of rats in each batch of the TE group was significantly higher than that in the control group and increased with each batch ($P < 0.01$). Compared with the HFD group, the serum B element content in the HFD+TE group significantly increased and exhibited an upward trend at the end of the 4th, 8th, 12th, and 20th week ($P < 0.01$).

Similarly, with increasing feeding time, the HFD could reduce the serum levels of B, V, Cr, Co, Cu, and Zn compared with the control rats, respectively ($P < 0.01$). TE supplementation increased the serum levels of B, V, Cr, Fe, Co, Cu, Zn, Se, and Sr in all batches of rats compared with the control and HFD rats, respectively ($P < 0.01$).

Discussion

In this research, we aimed to modify oxidative stress, inflammatory responses, and immune deficits associated with a HFD through supplementing multiple TEs.

A HFD can lead to excess body weight and the excessive accumulation of blood lipids, such as TG and TC in the body, thereby increasing the risk of many metabolic syndromes.[10] The results showed that the rats supplemented with multiple TEs showed different degrees of weight loss and significant improvement in blood lipid levels. It may be that Fe, Zn, Cu, Cr, Se, and other elements, can inhibit fat accumulation, improve lipid peroxidation and reduce metabolic disorders.[2]

Obesity can lead to excessive production of reactive oxygen species (ROS), elevated levels of oxidative stress products, and decreased antioxidant enzyme activity, ultimately leading to oxidative stress damage. The research conducted by Huang *et al*.[9] demonstrated that the generation of MDA in the body of HFD rats increased, while the levels of T-AOC and GSH were lower than those in the normal diet group rats.[9] MDA was the final product of lipid peroxidation caused by the body's attack on polyunsaturated fatty acids through oxygen free radicals, and was a biomarker of oxidative stress.[12] T-AOC described the overall resistance of antioxidants in the body to oxidants, indicating the ability of the body to maintain synergy and balance between antioxidants.[13] GSH could help clear peroxides in the body, serving as a substrate for GSH-Px and an indicator of the body's oxidative capacity.[14]

In this research, an increase in MDA production and a decrease in GSH were observed in the HFD group; this was reversed by the administration of TEs, reducing the production of MDA and increasing the levels of T-AOC and GSH. As described by Wandt,[3] supplementing with multiple TEs could maintain the overall redox state of the body and immune organs in both early and late stages, reduce the oxidative stress products produced by short-term and long-term HFDs, and enhance the overall antioxidant capacity of the body.

In addition to increasing oxidative stress products, a HFD also reduces the activity of antioxidant enzymes. Many animal and human studies have shown that a HFD leads to a decrease in the enzyme activity of enzymes such as SOD, CAT, GSH-Px, and GR.[15-19] SOD, as a key scavenger of ROS in the body, can protect host cells from oxidative damage.[20] CAT is an important antioxidant enzyme that can decompose hydrogen peroxide in the body and reduce the production of lipid peroxidation.[21] GSH-Px is an enzyme that uses GSH as a substrate and plays an important role in protecting the body from oxidative damage.[12] GR can catalyze the generation of GSH, maintain cell membrane stability, and prevent hemoglobin oxidation.[22] In this research, compared with the HFD group, the HFD+TE group rats were able to simultaneously observe a significant increase in the enzyme activity of SOD, CAT, GSH-Px, GR in the serum and GSH-Px, CAT, SOD and other enzymes in

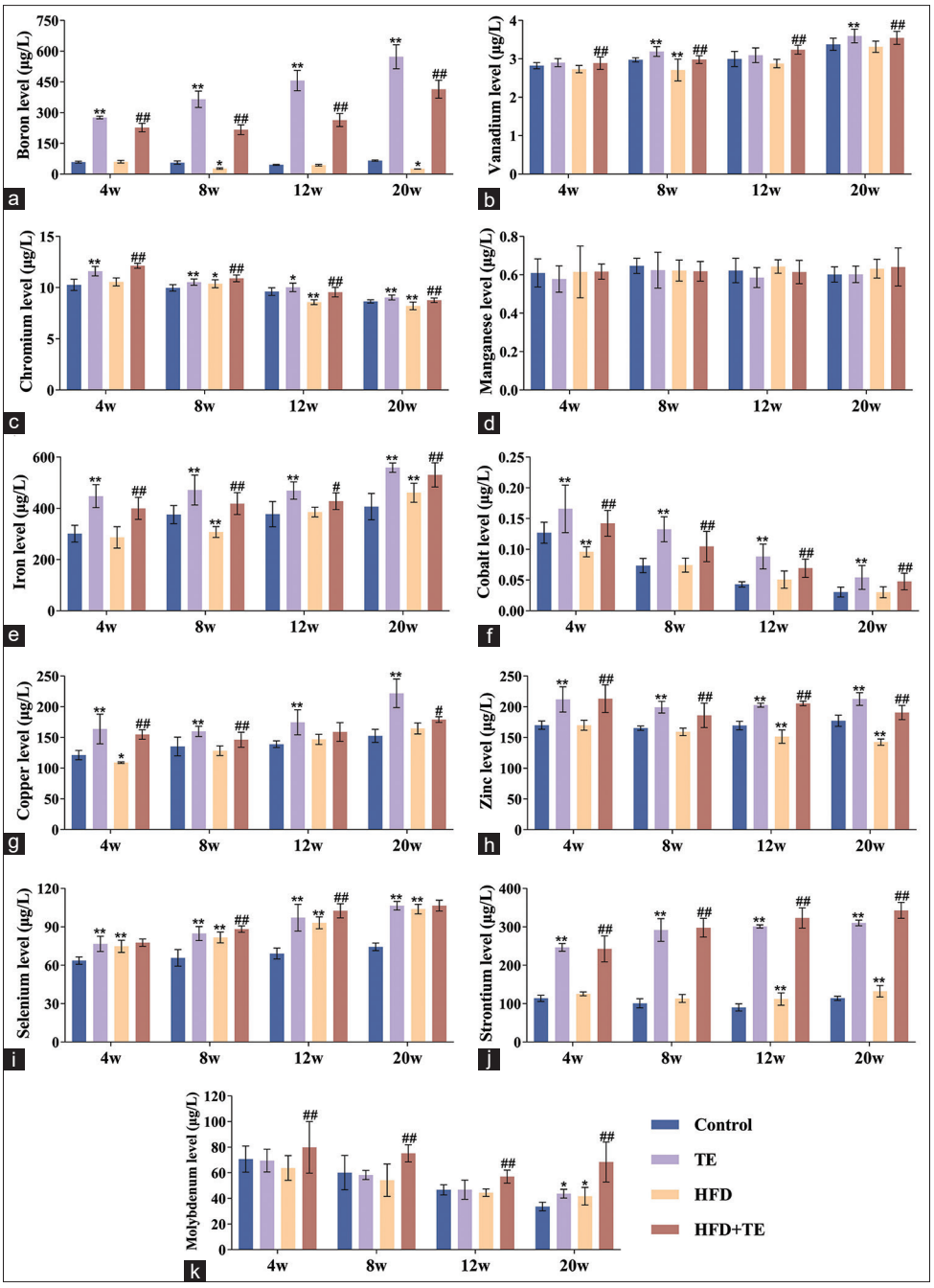

Figure 7: Effect of supplementation of various TEs on serum element content in rats with HFD. (a) B. (b) V. (c) Cr. (d) Mn. (e) Fe. (f) Co. (g) Cu. (h) Zn. (i) Se. (j) Sr. (k) Mo. The data was presented in the form of $\bar{x} \pm s$, *$P < 0.05$, **$P < 0.01$ vs control group; #$P < 0.05$, ##$P < 0.01$ vs HFD group

the spleen in all batches. This result was similar to most existing research results.[3] Our study revealed that, at the end of the experiment, the GSH-Px activity in the serum of the TE group rats increased by 39% compared with the control group, while the GSH-Px activity in the spleen increased by 24%. However, Finke *et al*.[7] did not show a significant effect on GSH-Px activity by supplementing with TEs such as Fe, Mn, Zn, Cu, I, Se.

In contrast, the greater variety of TEs in our study demonstrated that the antioxidant properties of TEs are directly related to their abilities to increase the activity of antioxidant enzymes, which is more beneficial for increasing the antioxidant capacity of the body. XOD is an enzyme responsible for producing hydrogen peroxide and superoxide anions. The resulting products have been shown to decrease the body's levels of antioxidants.[23] The studies of Albrahim *et al*.[24,25] indicated that a long-term HFD produced excessive ROS to induce pro-inflammatory mediators, leading to a significant increase in MPO and XOD activity. Li *et al*.[26] showed that supplementing with Cu and Zn could reduce XOD activity. However, in this study, compared with the control group, the inhibition rate

of XOD enzyme activity in the serum of rats supplemented with multiple TEs was only 15%. It was speculated that this may be attributed to a different modeling method from the reference literature. At the same time, the experimental results showed that supplementing with multiple TEs in a HFD could also affect the XOD action site and effectively modulate XOD enzyme activity. Similarly, supplementing multiple TEs in this experiment could effectively reduce the activity of MPO in rat serum compared with the HFD group. Consistent with the study by El Kebir *et al*., MPO was an inflammatory enzyme mainly produced by neutrophils, and reducing its activity could reduce ROS synthesis, inhibit lipoprotein oxidation, and delay the development of inflammation.[27]

According to literature reports, HFD is also related to low-grade chronic inflammation. Obesity can lead to massive proliferation of adipose tissue and infiltrate cells, secreting TNF-α, IL-6 and other inflammatory cytokines, thus promoting the occurrence and development of inflammation.[10] Finke *et al*.[7] found that a low TE state could induce inflammation, and an increase in IL-6 levels indicated that a mild inflammatory state was a manifestation of insufficient intake of TEs in animals. Inflammation reflected that simultaneously reducing the content of six TEs in the body could damage the homeostasis of other TEs. Hasani's research suggested that supplementing Zn and Se simultaneously could significantly increase enzyme activity and reduce TNF- α compared with supplementing either alone.[1] The content of IL-6 was reduced, and the increase in leptin induced by HFD was weakened. Leptin is related to the increased levels of inflammatory markers observed in the pro-inflammatory response, which can prove the synergistic anti-inflammatory effect between the two TEs.[28]

Similar to our results, balanced supplementation of multiple TEs could significantly reduce serum IL-6 levels and serum TNF-α accumulation during long-term feeding in rats under a HFD, and could significantly increase serum IL-4 levels at all stages throughout the experiment. IL-4 is an anti-inflammatory factor that plays a role in resisting inflammation in the body, due to its inhibitory effect on chemokines in the inflamed area.[29]

This indicator indicates that the synergistic effect of multiple elements can effectively improve the inflammatory state of the body. IL-2, as a chemotactic factor, is capable of stimulating T cell proliferation, inducing T cell differentiation, and mediating activation-induced cell death.[30] IL-10 can participate in the regression stage of inflammation, and the increase in IL-10 levels may be a compensatory mechanism for controlling the excessive development of inflammation in the context of a HFD.[31] The decrease in IL-2 levels in the serum of rats supplemented with TEs was statistically significant compared with the control group. However, in the HFD+TE group, a slight decrease in IL-2 levels and a slight increase in IL-10 levels were observed in the middle and later stages of the experiment, but there were no statistically significant differences. It was speculated that the feeding time of the experimental animals may not be long enough to further reflect their long-term inflammatory homeostasis.

Obesity caused by a HFD can interfere with the normal functioning of the immune system, leading to immune disorders mediated by T cells, including $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells.[32] The experimental data of other researchers[33-35] indicated that the deficiency of TEs such as B, Se, Cr could lead to immune dysfunction, and supplementing these elements could generate the proportion of $CD3^+$ T, $CD4^+$ T, and $CD8^+$ T cells in the body in a direction beneficial to the immune system. Similar to our study, supplementing with multiple TEs significantly increased the proportion of $CD3^+$ T cells in the whole blood and spleen of HFD rats at the end of the experiment, indicating that intake of multiple TEs could increase the proportion of immune cells in the immune organs and circulatory system, thereby enhancing the body's cellular immune function.[36] $CD4^+$ T cells, which are helper T cells, and $CD8^+$ T cells, which are cytotoxic, are both important T cell subsets in the immune system. In our study, the $CD4^+$ T cell ratio of serum in the HFD group was decreased, although this was not statistically significant. Furthermore, the number of $CD8^+$ T cells was significantly increased; this meant that the $CD4^+/CD8^+$ T cell ratio decreased significantly. Simultaneous supplementation with multiple TEs in the context of HFD was effective in reducing the proportion of $CD8^+$ T cells and increasing the $CD4^+/CD8^+$ T cell ratio, thus improving cellular immune function and maintaining immune homeostasis. These results are consistent with those reported in the literature in that multiple TEs (such as Zn, V, Se, Fe and Mn) can improve cellular immunity.[34,37-40]

In order to observe the distribution and content trend of the TEs supplemented in our study in the body of each group of rats, an inductively coupled plasma mass spectrometer was used to determine and analyze the elements in the serum of rats. The results showed that the levels of B, V, Fe, Cu, Se, Sr and other elements in the serum of each group of rats increased significantly with the increase of feeding time, while the levels of Cr, Co, Mo and other elements in the serum of each group of rats decreased with the increase of feeding time. The possible reason was that Cr, Co and Mo, as components of the liver, kidneys, spleen, and bone tissues in animals, gradually accumulate in tissues and organs over time. Therefore, the content of Cr, Co and Mo that flow widely at low concentrations in serum gradually decreased.[41,42] Overall, it could be observed that the levels of all elements in the serum of rats in each group, except for Mn and Mo, were higher in the group supplemented with TEs under short-term and long-term feeding conditions than that in the group without TEs supplement. It was

found that the elements supplemented in this experiment showed differences in different groups, which was similar to previous researches.[43] With the increase of feeding time, the levels of B, V, Cr, Fe, Co, Cu, Zn, Se, Sr and other elements could be effectively improved compared with the serum levels of HFD group and control group rats. It was shown that balanced supplementation of multiple TEs could better promote the normal balance of TEs in the body.[44]

Conclusions

These investigations aimed to explore the effects of various TE supplementation on rats subjected to an HFD. In this study, rats were fed a HFD along with various TE supplements. The levels of blood lipids, antioxidant capacity, and inflammatory factors were measured. In addition, the proportion of immune cells in the blood and immune organs of rats was observed. This study concluded that supplementing various TEs could enhance lipid metabolism, improve dyslipidemia, reduce body weight, and decrease the occurrence of obesity in rats fed an HFD. TEs may serve as the active centers of antioxidant enzymes. Supplementation of various TEs could effectively enhance the activity of antioxidant enzymes and improve the ability to resist oxidative stress. Supplementation of a variety of TEs could improve the body's anti-inflammatory ability, reduce inflammatory response, enhance immunity, and improve immune disorders, which plays an important role in the prevention and control of chronic diseases.

Author contributions

WD: conceptualization, methodology, investigation, visualization and writing - original draft; FS: conceptualization, methodology, software and data curation; XG: supervision, project administration and writing - review and editing. All authors have read and agreed to the published version of the manuscript.

Ethical approval

This study was approved by the Ethics Committee of Shandong University [LL20211206].

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Conflicts of interest

There are no conflicts of interest.

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