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Research Article

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In vivo antioxidant activity of rabbiteye blueberry (*Vaccinium ashei* cv. 'Brightwell') anthocyanin extracts

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Abstract: Blueberries are rich in phenolic compounds including anthocyanins which are closely related to biological health functions. The purpose of this study was to investigate the antioxidant activity of blueberry anthocyanins extracted from 'Brightwell' rabbiteye blueberries in mice. After one week of adaptation, C57BL/6J healthy male mice were divided into different groups that were administered with 100, 400, or 800 mg/kg blueberry anthocyanin extract (BAE), and sacrificed at different time points (0.1, 0.5, 1, 2, 4, 8, or 12 h). The plasma, eyeball, intestine, liver, and adipose tissues were collected to compare their antioxidant activity, including total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity and glutathione-peroxidase (GSH-PX/GPX) content, and the oxidative stress marker malondialdehyde (MDA) level. The results showed that blueberry anthocyanins had positive concentration-dependent antioxidant activity in vivo. The greater the concentration of BAE, the higher the T-AOC value, but the lower the MDA level. The enzyme activity of SOD, the content of GSH-PX, and messenger RNA (mRNA) levels of *Cu,Zn-SOD*, *Mn-SOD*, and *GPX* all confirmed that BAE played an antioxidant role after digestion in mice by improving their antioxidant defense. The in vivo antioxidant activity of BAE indicated that blueberry anthocyanins could be developed into functional foods or nutraceuticals with the aim of preventing or treating oxidative stress-related diseases.

Key words: Blueberry anthocyanin; In vivo antioxidant activity; Superoxide dismutase (SOD); Glutathione-peroxidase (GSH-PX/GPX); Malondialdehyde (MDA)

1 Introduction

Blueberries are perennial shrubs belonging to the genus *Vaccinium* and family Ericaceae. Numerous studies on the chemical composition and nutritive value of the fruit have led to an increase in the propagation of blueberries worldwide (Norberto et al., 2013). Rabbiteye blueberries (*Vaccinium ashei*), which are collected and consumed throughout North America, were introduced to Nanjing, China, in 1988 by the Institute of Botany, Jiangsu Province, and the Chinese Academy of Sciences. Rabbiteye blueberries are relatively cold-sensitive and are better adapted to cultivation in warmer climatic conditions (Ehlenfeldt et al., 2006). Therefore, rabbiteye blueberries are suitable for growth in Southern China, and have now been distributed to Zhejiang, Jiangsu, Guizhou, Yunnan Provinces, and other places. Appreciated for their appealing taste and contributions to health, blueberries can be eaten as whole or used as ingredients in smoothies, cooking, and baked goods (Chai et al., 2021).

The consumption of blueberries may decrease the risk of several incurable diseases stimulated by oxidative processes, such as diabetes mellitus and cardiovascular diseases (Khoo et al., 2017). These beneficial health functions are related to the presence of important bioactive compounds in blueberries, especially

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anthocyanins (Ma et al., 2018). Anthocyanins are natural water-soluble pigments widely found in plants. They are endogenous, bioactive flavonoid compounds with beneficial activity, including enhancing vision and eliminating eye fatigue, delaying the aging of brain cells and preventing neurodegenerative diseases, treating capillary blockage caused by diabetes and regulating blood glucose and blood lipids, and enhancing cardiopulmonary function and preventing cardiovascular diseases (Wedick et al., 2012; Lee et al., 2017; Huang et al., 2020; Herrera-Balandrano et al., 2021a). These advantageous health effects are based on the superior antioxidant activity of anthocyanins. In 1951, Jacques Masquerier first found that anthocyanins have a high antioxidant capacity by measuring their capacity to absorb oxygen free radicals in the lipophilic and hydrophilic systems (Pertuzatti et al., 2014). Later, numerous reports confirmed the antioxidant activity of anthocyanins (Wang et al., 2018; Attaribo et al., 2021; Pahlke et al., 2021). Thus, blueberries are used as endogenous antioxidant defense ingredients in the modern health-conscious food industry, due to their significant levels of anthocyanins.

The anthocyanin contents of blueberries are conditional on the cultivar, fruit size, ripening stage, and environmental conditions (Mazza and Miniati, 1993). Rabbiteye blueberries have a higher antioxidant capacity than highbush and lowbush types. This might be due to their thicker skin and smaller size, as the skin tends to contain higher levels of anthocyanins than the flesh (Skrovankova et al., 2015; Chai et al., 2021). In our previous survey, we investigated and identified the anthocyanin constituents of the rabbiteye blueberry cultivar ‘Brightwell’ in Nanjing using high-performance liquid chromatography (HPLC)-diode array detection (DAD)-tandem mass spectrometer (MS^n). Delphinidin, cyanidin, petunidin, peonidin, and malvidin glycosides were found, of which malvidin-3-galactoside, malvidin-3-glucoside, and malvidin-3-arabinose were the major components, accounting for nearly 50% (mass fraction) of the total anthocyanin extracts (Hutabarat et al., 2019; Chai et al., 2021). In addition, our findings confirmed that fruit of the rabbiteye blueberry cultivar ‘Brightwell’ have a high level of anthocyanins with various health functions, such as antioxidant, anti-inflammatory, and vasodilatory effects in vitro, as well as hypoglycemic and hypolipidemic effects in vitro and in vivo (Li et al., 2013; Huang et al., 2018a, 2018b; Herrera-Balandrano et al., 2021b). In the present study, the anthocyanins extracted from ‘Brightwell’ rabbiteye blueberries were investigated for their antioxidant activity in vivo.

Anthocyanins are only partially absorbed in human intestines, which affects their bioavailability, resulting in a decrease in the concentration of anthocyanins in the circulatory system (Stalmach, 2014). The content of anthocyanins after digestion and absorption in organisms is very low. Aqil et al. (2014) studied the bioavailability of anthocyanins after the digestion of labeled anthocyanins and emphasized the necessity for in vivo research on blueberry anthocyanins to consider the impact of digestion on their biological activity. Even though the anthocyanin content decreased, antioxidant activity was still maintained. In this study, the content of each blueberry anthocyanin and its antioxidant activity in various tissues (plasma, eyeball, intestine, liver, and adipose) of mice treated with different concentrations of blueberry anthocyanin extract (BAE) at different time points were analyzed systematically. Analyses included the total antioxidant capacity (T-AOC), the activity, contents, and the messenger RNA (mRNA) levels of antioxidant enzymes, and the oxidative stress marker malondialdehyde (MDA) level. The effects of different BAE concentrations and treatment time were also compared to explore the antioxidant activity of blueberry anthocyanins after digestion in mice.

2 Results

2.1 Evaluation of organ indexes in mice

Indexes of various organ tissues from the mice treated with different concentrations of BAE for different treatment time are shown in Table S1. After treatment with BAE for 0.1 to 12 h, the weights of the eyeball, liver, kidney, lung, spleen, thymus, and adipose (mesenteric fat) tissues showed no significant differences from those of the control group ($P>0.05$). No significant differences were found in the eyeball, lung, or thymus index among the mice treated with 100, 400, or 800 mg/kg of BAE for different treatment time ($P>0.05$). Other organ indexes showed two or three different levels ($P<0.05$), but the changes did not seem related to the treatment time or concentrations. The liver index of mice was the highest of these organ indexes (from 6.54% to 11.21%), followed by the kidney

index (from 1.27% to 2.05%), while the thymus index was the lowest (<0.30%). The spleen index (from 0.36% to 1.32%) and adipose index (from 0.45% to 1.37%) varied by three or four folds.

2.2 Anthocyanins in BAE and mice treated with BAE

Our previous study identified 13 major anthocyanins in rabbiteye blueberry fruits, including (in order of the HPLC chromatogram) delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-arabinoside, cyanidin-3-glucoside, petunidin-3-galactoside, petunidin-3-glucoside, peonidin-3-galactoside, petunidin-3-arabinose, peonidin-3-glucoside, malvidin-3-galactoside, malvidin-3-glucoside, and malvidin-3-arabinose (Hutabarat et al., 2019). In this study, 12 peaks were detected in BAE (Fig. S1a), with petunidin-3-glucoside and peonidin-3-galactoside presenting in one peak. These two anthocyanins could not be separated, which may have been due to the elution gradient time decreasing from 90 to 36 min. The anthocyanin contents in the tissues of mice treated with BAE were very low, and most could not be detected by HPLC analysis. Even when the HPLC chromatogram showed the anthocyanin peaks, it was difficult to quantify their contents. For example, Fig. S1b showed the extremely low levels of anthocyanins extracted from the mice liver after treatment with 800 mg/kg BAE for 2 h.

2.3 T-AOC of different tissues from mice treated with BAE

The T-AOC was detected using a kit based on the 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) method, which can be used to evaluate both lipophilic and hydrophilic antioxidant capacities (Wu et al., 2019). The T-AOC values of plasma, eyeball, intestine, liver, and adipose tissues from mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) at various time points varied (Fig. 1, P<0.01). The control group without BAE treatment had a significantly lower T-AOC value than most BAE treatment groups (P<0.05), whose T-AOC values of plasma, eyeball, intestine, liver, and adipose were (5.29±0.93) mmol/L, and (1.55±0.36), (1.47±0.98), (2.07±0.84), and (3.51±0.73) mmol/g protein, respectively (Table S2).

In the plasma of mice treated with 100 mg/kg BAE, the T-AOC value was the lowest (only (5.16±1.24) mmol/L at 0.1 h) and similar to that of the control (P>0.05). With increasing treatment time, the T-AOC first increased steadily, and then suddenly decreased

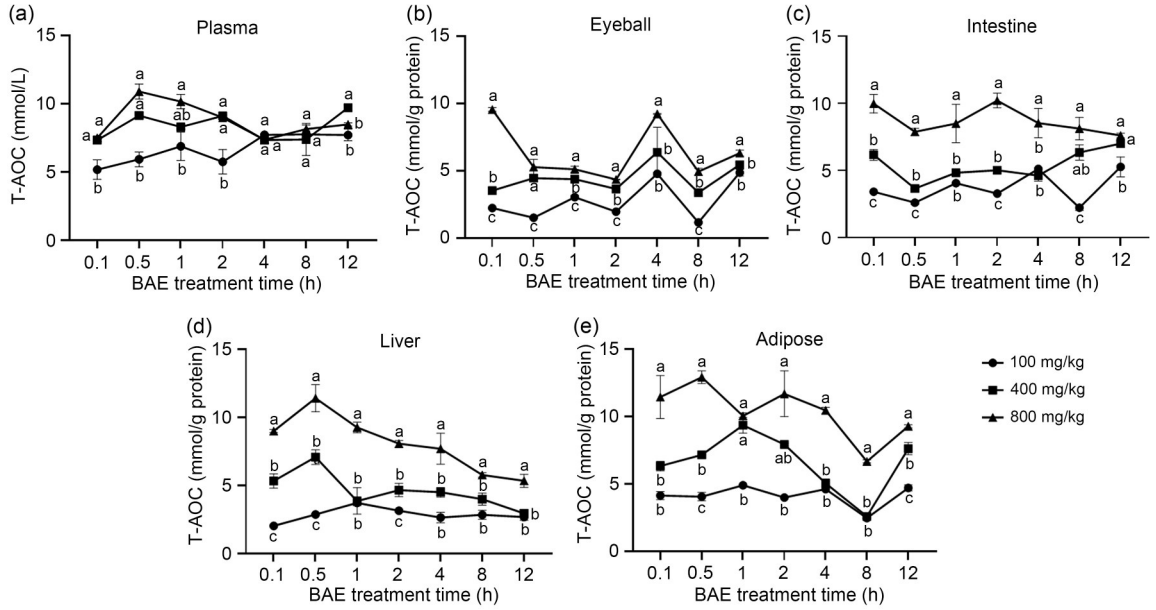

Fig. 1 Changes in the T-AOC of plasma (a), eyeball (b), intestine (c), liver (d), and adipose (e) tissues from mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) for different time (0.1, 0.5, 1, 2, 4, 8, and 12 h). All the data are expressed as mean±standard deviation (SD), n=3; different lowercase letters indicate significant differences among different treatment concentrations of BAE at the same time (P<0.05). T-AOC: total antioxidant capacity; BAE: blueberry anthocyanin extract.

at 2 h and was stable at about 7.70 mmol/L from 4 to 12 h. In general, there was no significant difference between the 400 and 800 mg/kg BAE treatments (P>0.05). Their T-AOC values quickly increased from about 7.40 mmol/L at 0.1 h for both treatments to (9.12±0.42) mmol/L for the 400 mg/kg BAE treatment and (10.88±0.94) mmol/L (the highest value of all, and significantly higher than those of most groups treated with low concentrations; P<0.05) for the 800 mg/kg BAE treatment at 0.5 h. Later, their T-AOC values seemed to decrease slowly and tended to stabilize after 4 h, which was similar to the 100 mg/kg BAE treatment (P>0.05). However, the T-AOC in the plasma of mice treated with 400 mg/kg BAE reached a higher value at 12 h than those of the other two concentrations (Fig. 1a, P<0.05).

In the eyeball, the T-AOC values significantly increased with increasing BAE concentration at each time point (P<0.05). Strangely, the 800 mg/kg BAE treatment at 0.1 h had the highest T-AOC value ((9.58±0.23) mmol/g). However, T-AOC values showed an overall increasing trend, but were unstable over time. All T-AOC values peaked at 4 h ((4.80±0.19), (6.41±3.19), and (9.26±0.05) mmol/g for 100, 400, and 800 mg/kg, respectively; Fig. 1b). Similarly, in the intestine, the T-AOC values following treatment with a high concentration of BAE were always higher than those with a low concentration (P<0.05), but the values were not stable. The T-AOC of 100 mg/kg BAE treatment reached high values at 4 and 12 h, but decreased significantly at 8 h (P<0.05). The 400 and 800 mg/kg BAE treatments each began with a high T-AOC value. For the 400 mg/kg BAE treatment, the T-AOC values first decreased from (6.16±0.70) to (3.65±0.05) mmol/g, and then gradually increased significantly to (7.01±0.37) mmol/g (P<0.05). For the 800 mg/kg BAE treatment, the T-AOC values first decreased, and then increased, peaking at 2 h with (10.21±0.94) mmol/g, and at last decreased again (Fig. 1c, P<0.05). In the liver, the T-AOC values of the treatment with 100 mg/kg BAE did not change greatly, reaching a peak of (3.70±0.33) mmol/g at 1 h (P<0.05). However, both the 400 and 800 mg/kg BAE treatments had an overall decreasing trend, peaking at 0.5 h ((7.07±0.93) and (11.39±1.72) mmol/g, respectively; Fig. 1d, P<0.05). A similar decreasing trend was found in the adipose. The T-AOC values reached a peak at 1 h for the 100 and 400 mg/kg BAE treatments, but at 0.5 and 2 h for the 800 mg/kg BAE treatments. All three concentrations of BAE treatments showed the lowest values at 8 h, but there were no significant differences among the treatments at that time (Fig. 1e, P>0.05).

2.4 Antioxidant enzyme levels of different tissues from mice treated with BAE

The levels of antioxidant enzymes superoxide dismutase (SOD) and glutathione-peroxidase (GSH-PX/GPX) in plasma, eyeball, intestine, liver, and adipose tissues from mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) at various treatment time points also changed (Fig. 2). Similarly, all the tissues of the control group exhibited significantly lower SOD activity and GSH-PX content than those of 400 or 800 mg/kg BAE treatment group (P<0.05). In particular, the GSH-PX content ((0.16±0.05) mmol/g) of control group for intestine was much lower than those of all other groups (Table S2, P<0.05).

No significant differences were found in SOD activity of plasma among mice treated with BAE for different time (P>0.05), but the BAE concentration significantly affected their SOD levels (P<0.001). In the plasma of mice treated with 100 mg/kg BAE, the SOD activity was the lowest, reaching its highest value ((38.4±4.23) mU/L) at 4 h and the lowest value ((18.87±2.05) mU/L) at 8 h (P<0.05). The SOD activity of plasma from mice treated with 400 mg/kg BAE at first increased with increasing treatment time, but suddenly decreased to (45.22±16.80) mU/L at 8 h (P<0.05). However, the SOD activity in the plasma of mice treated with 800 mg/kg BAE did not change greatly, reaching a peak of (52.21±14.49) mU/L at 4 h (Fig. 2a, P<0.05). In the eyeball, SOD activity showed an increasing trend, but was unstable over time, reaching higher values at 4 h ((52.78±3.67), (71.87±7.89), and (73.09±8.04) U/g for 100, 400, and 800 mg/kg BAE treatments, respectively; Fig. 2b, P<0.05). In the intestine, after treatments with 100 and 400 mg/kg BAE, SOD activity first increased, and decreased, and then increased, and finally decreased again, with two peak values at 1 and 4 or 8 h, respectively. However, the SOD activity value of 800 mg/kg BAE treatment showed only one peak of (97.91±3.18) U/g at 4 h (Fig. 2c, P<0.05). In the liver treated with 100 and 400 mg/kg BAE, SOD activity first increased and then decreased, reaching peaks of (52.66±1.27) and (69.27±13.38) U/g at 2 h, respectively (P<0.05). However, the SOD activity for 800 mg/kg BAE-treated liver had maximum

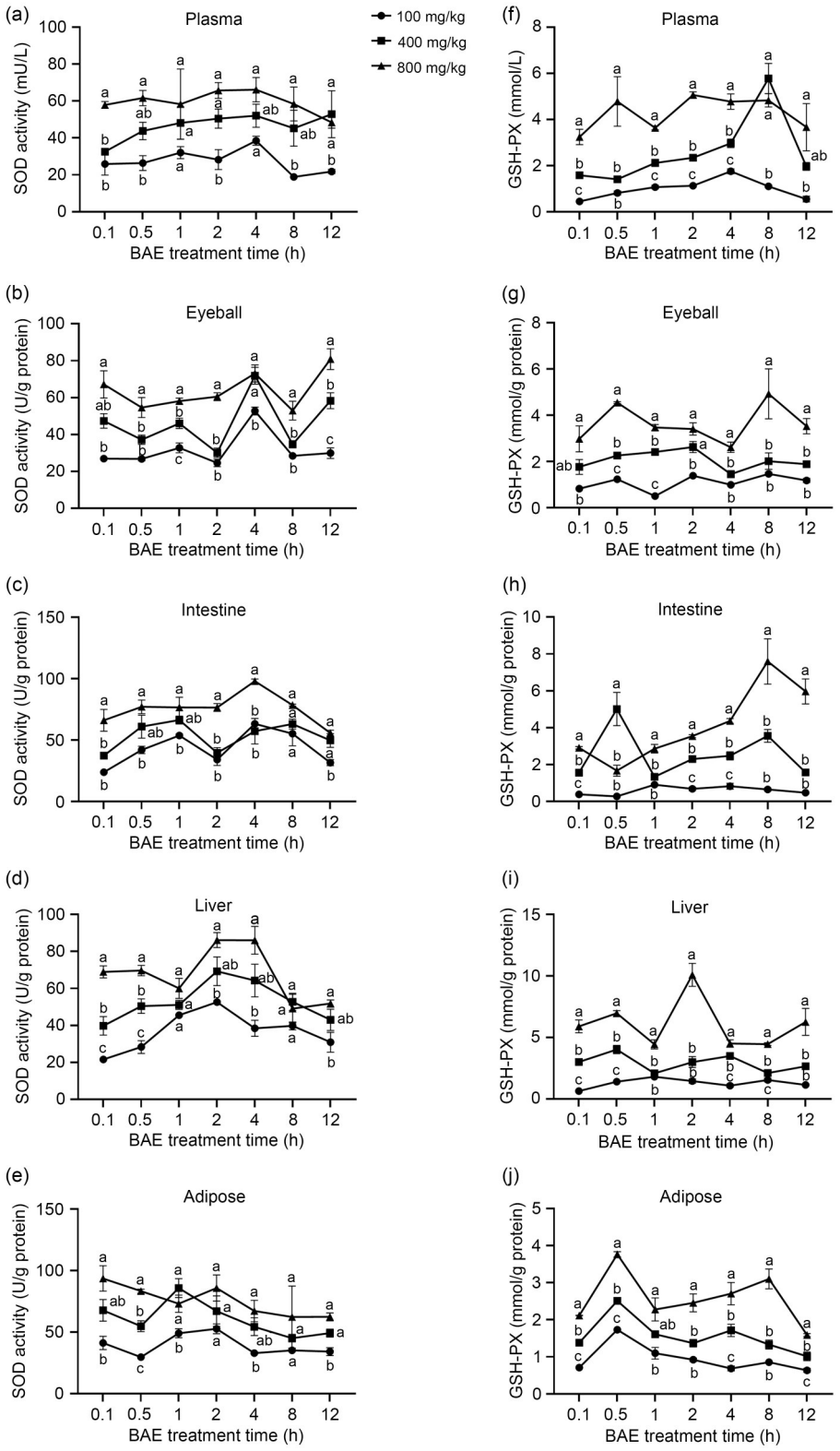

Fig. 2 Changes in the antioxidant enzymes (SOD activity and GSH-PX content) of plasma (a, f), eyeball (b, g), intestine (c, h), liver (d, i), and adipose (e, j) tissues from mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) for different time points (0.1, 0.5, 1, 2, 4, 8, and 12 h). All the data are expressed as mean±standard deviation (SD), *n*=3; different lowercase letters indicate significant differences among different treatment concentrations of BAE at the same time (*P*<0.05). SOD: superoxide dismutase; GSH-PX: glutathione-peroxidase; BAE: blueberry anthocyanin extract.

values (about 86 U/g) at 2 and 4 h, and a minimum value of (49.14±13.39) U/g at 8 h (Fig. 2d, $P<0.05$). In the adipose tissue, after treatment with 100, 400, and 800 mg/kg BAE, SOD activity showed a downward trend, but with intermittent spikes at 0.1, 1, or 2 h (Fig. 2e, $P<0.05$ for 100 and 400 mg/kg BAE treatments, but $P>0.05$ for 800 mg/kg BAE treatment).

Similar results were found for GSH-PX levels. In the plasma of mice treated with 100 mg/kg BAE, the GSH-PX content did not change significantly, reaching a peak of (1.77±0.19) mmol/L at 4 h ($P<0.05$). However, for the 400 mg/kg BAE treatment, the GSH-PX value showed an increase, and then a decrease with a sharp peak of (5.78±1.23) mmol/L at 8 h ($P<0.05$). Under the 800 mg/kg BAE treatment, the GSH-PX contents were unstable within time, reaching higher values at 0.5, 2, 4, and 8 h (Fig. 2f, $P<0.05$). In the eyeball, the lowest GSH-PX value was detected in 100 mg/kg BAE treatment at 1 h, while the highest two values were found in the 800 mg/kg BAE treatment at 8 and 0.5 h ((4.93±1.87) and (4.54±0.07) mmol/g, respectively, $P<0.05$). For the treatments with 100 and 400 mg/kg BAE, the GSH-PX values both peaked at 2 h (Fig. 2g, $P<0.05$). In the intestine, the GSH-PX values peaked at 1 h for the 100 mg/kg BAE treatment, 0.5 and 8 h for the 400 mg/kg BAE treatment, and 8 h ((7.59±2.13) mmol/g, the highest value of all, $P<0.05$) for the 800 mg/kg BAE treatment (Fig. 2h). In the liver, the peaks were found at 1 h for 100 mg/kg BAE treatment, 0.5 and 4 h for 400 mg/kg BAE treatment, and 2 h (highest value (10.90±1.61) mmol/g) for 800 mg/kg BAE treatment, respectively (Fig. 2i, $P<0.05$). In adipose tissue, the GSH-PX value of a high-concentration of BAE treatment was always higher than that of a low-concentration BAE treatment ($P<0.05$). The GSH-PX values first peaked at 0.5 h, and then peaked again at 8 h for 100 and 800 mg/kg BAE treatments, but for the 400 mg/kg BAE treatment, the peak was reached again at 4 h (Fig. 2j, $P<0.05$).

2.5 MDA levels of different tissues from mice treated with BAE

MDA is the most important product of the oxidative degradation of lipids, and can therefore be used as an index to measure the degree of lipid peroxidation (Tsikas, 2017). It was reported that type 2 diabetes mellitus is related to increased plasma lipid peroxidation, which is expressed as an increase in MDA (Marjani, 2010). Therefore, antioxidants might also regulate lipid metabolism. The MDA changes in the plasma, eyeball, intestine, liver, and adipose tissues from mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) at various time points are shown in Fig. 3. The tissues analyzed from the control group contained more MDA than tissues from most of the BAE treatment groups ($P<0.05$) except for the plasma treated with 100 mg/kg BAE at 1 h, which showed a higher value ((9.41±0.31) μmol/L vs. the control (6.71±1.94) μmol/L, $P>0.05$; Table S2).

In contrast to the T-AOC and antioxidant enzymes, higher concentrations of BAE were linked to lower MDA levels ($P<0.05$). In the plasma, the maximum MDA value of (9.41±0.31) μmol/L was found in mice treated with 100 mg/kg BAE at 1 h ($P<0.05$). For the 400 and 800 mg/kg BAE treatments, the MDA values fluctuated from 2.71 to 4.67 μmol/L, and 1.16 to 3.93 μmol/L, respectively (Fig. 3a, $P<0.05$). In the eyeball, the overall trend of MDA showed an initial decrease and then an increase. Initial concentrations at 0.1 h were very high ((7.32±1.71), (4.17±0.88), and (3.32±0.45) μmol/g for 100, 400, and 800 mg/kg BAE treatments, respectively, $P<0.05$). The 100 mg/kg BAE treatment also had two peak values at 2 and 8 h, while the 400 and 800 mg/kg BAE treatments both slowly increased to a maximum at 12 h (Fig. 3b, $P<0.05$). In the intestine, the MDA values of mice treated with 100 and 400 mg/kg BAE showed an overall upward trend, reaching peaks of (8.10±1.74) and (5.82±0.47) μmol/g at 8 h, respectively, but having a sudden decrease at 4 h ($P<0.05$). For the 800 mg/kg BAE treatment, the MDA values were all maintained at a low level (less than (2.02±0.36) μmol/g; Fig. 3c, $P>0.05$). In the liver, the MDA values showed a downward trend, but the value of the 100 mg/kg BAE treatment suddenly increased at 2 h, reaching a maximum of (6.43±1.44) μmol/g. However, the MDA values of both the 400 and 800 mg/kg BAE treatments peaked at 1 h (Fig. 3d, $P<0.05$). Similar downward trends were found in the adipose tissue, but the MDA values all peaked at 8 h ((8.69±1.21), (4.67±1.22), and (1.84±0.26) μmol/g for 100, 400, and 800 mg/kg BAE, respectively; Fig. 3e, $P<0.05$).

2.6 Antioxidant enzyme mRNA levels of different tissues from mice treated with BAE

As a metalloenzyme existing in vivo, SOD can be roughly divided into three categories according to its

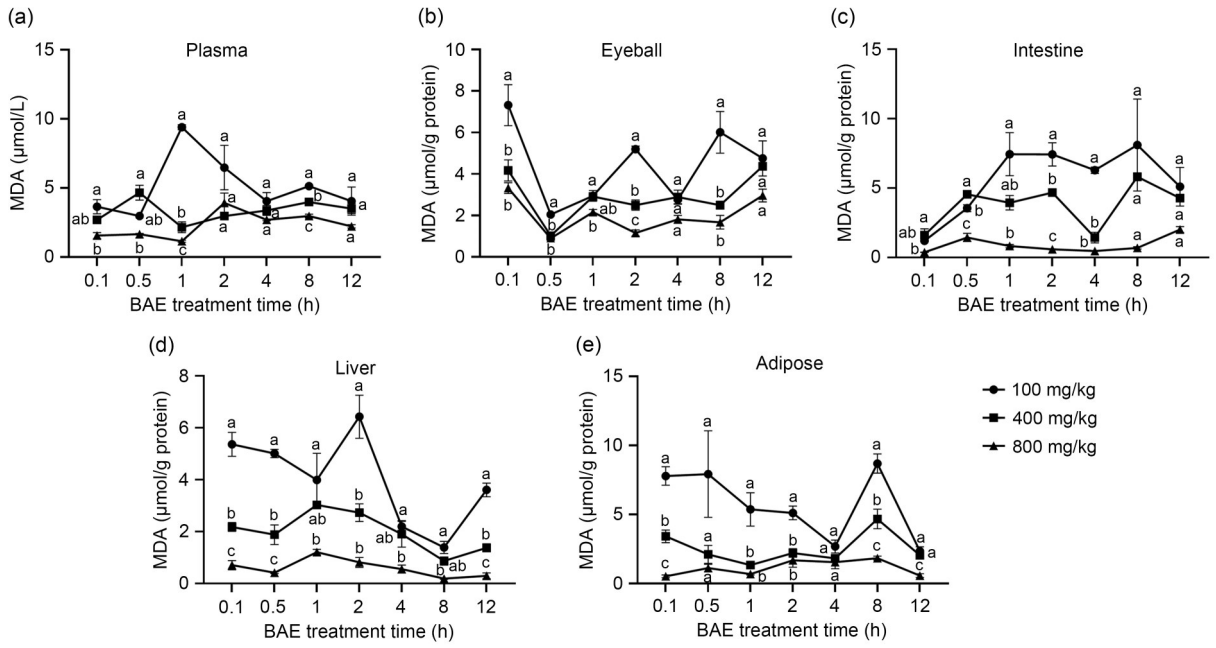

Fig. 3 Changes in the oxidative stress marker MDA contents of plasma (a), eyeball (b), intestine (c), liver (d), and adipose (e) tissues from mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) for different time (0.1, 0.5, 1, 2, 4, 8, and 12 h). All the data are expressed as mean±standard deviation (SD), *n*=3; different lowercase letters indicate significant differences among different treatment concentrations of BAE at the same time (*P*<0.05). MDA: malondialdehyde; BAE: blueberry anthocyanin extract.

different metal auxiliary groups, namely Cu,Zn-SOD, Mn-SOD, and Fe-SOD (Sanyal et al., 2018). The most widely distributed type, Cu,Zn-SOD, is blue-green in color and exists mainly in the cytoplasm of eukaryotic cells. Mn-SOD is pink and exists mainly in the mitochondria of eukaryotes and prokaryotes, while Fe-SOD is yellowish-brown and is found mostly in prokaryotic cells (Hanini et al., 2017). In the present study, levels of Cu,Zn-SOD and Mn-SOD, which can be found in eukaryotes, were investigated in the tissues of mice treated with different concentrations of BAE (100, 400, and 800 mg/kg) for different time. The mRNA levels of the antioxidant enzyme GSH-PX were also evaluated. Generally, treatment with BAE resulted in higher *Cu,Zn-SOD*, *Mn-SOD*, and *GPX* mRNA levels than the control ($P<0.05$) with only a few of the low concentrations of BAE treatments resulting in values similar to the control ($P>0.05$). The data are shown in Fig. 4, while those of the control can be found in Table S3. Due to the insufficient quantity in some of the tissue samples, data were not available for all time points. *Cu,Zn-SOD* and *Mn-SOD* mRNA levels were not always consistent with the SOD activity in the tissues, or with each other. This may be due to their presence in different parts of eukaryotic cells, one in the cytoplasm and the other in mitochondria. Similarly, *GPX* mRNA levels did not perfectly mirror the GSH-PX contents in various tissues.

In the eyeball, *Cu,Zn-SOD* mRNA levels changed little ($P>0.05$ for all), while *Mn-SOD* mRNA values of the different treatments showed significant differences ($P<0.05$). This was especially apparent in mice treated with 800 mg/kg BAE for 8 h, where the *Mn-SOD* mRNA values were much higher than others ((26.07± 1.95) times of the control, $P<0.05$). The *Cu,Zn-SOD* and *Mn-SOD* mRNA values of mice with the 400 or 800 mg/kg BAE treatments both reached a main peak at 8 h, much later than that of SOD activity (4 h). However, for the 100 mg/kg BAE treatment, their peak time points were not the same (Figs. 4a and 4b). On the other hand, *GPX* mRNA values changed markedly ($P<0.05$). For the 100, 400, and 800 mg/kg BAE treatments, the *GPX* mRNA values reached a maximum at 1, 4, and 2 h, respectively ($P<0.05$). They all decreased to low values at 8 h, which was the opposite of the GSH-PX content (Fig. 4c). In the intestine, the 100 and 400 mg/kg BAE treatments had only two time points with similar mRNA values (Fig. 4d–4f, $P>0.05$). For the 800 mg/kg BAE treatment, the *Cu,Zn-SOD* and *Mn-SOD* mRNA values each had two peaks at 1 and

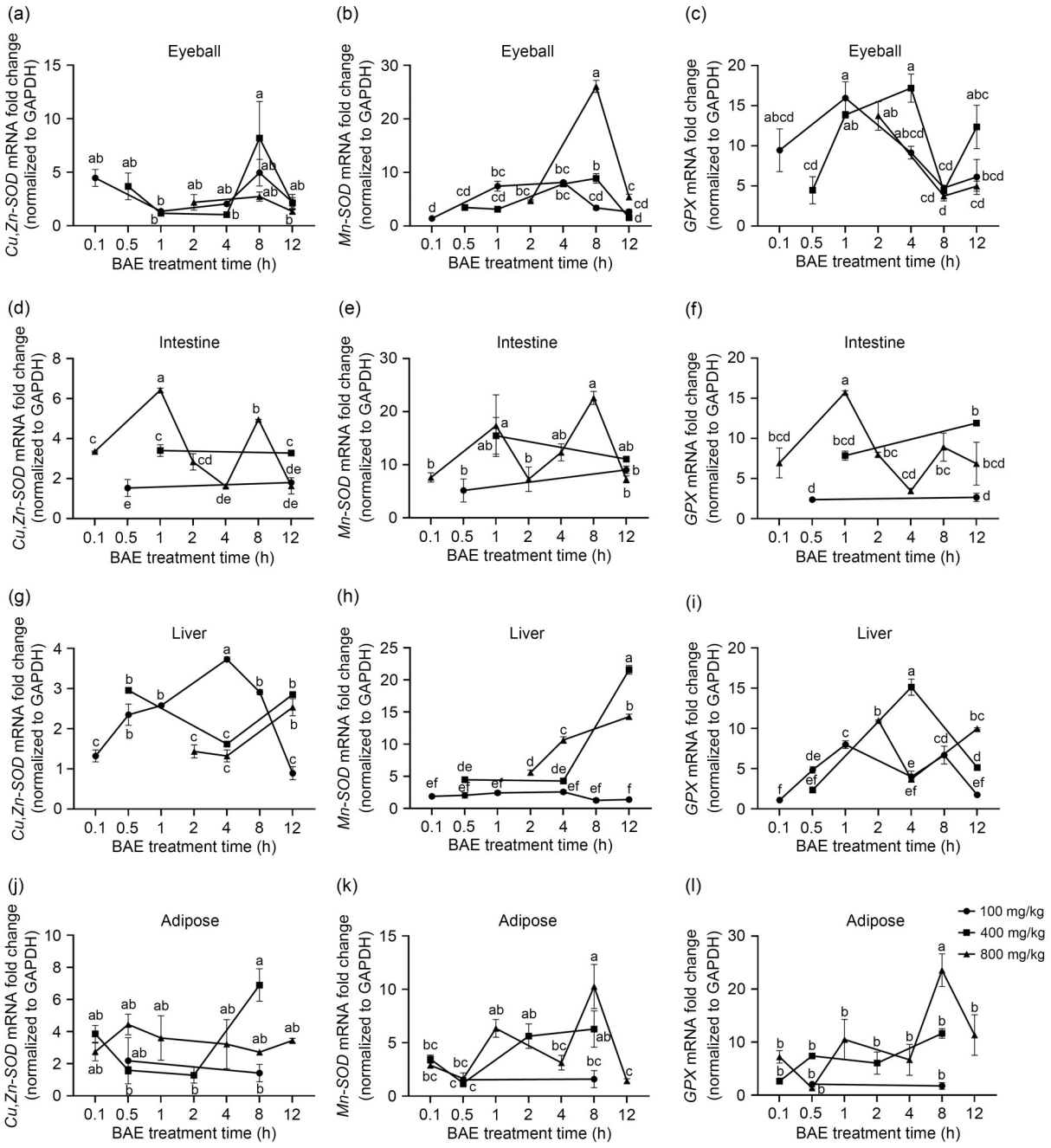

Fig. 4 Changes in the mRNA concentrations of three antioxidant enzymes (*Cu,Zn-SOD*, *Mn-SOD*, and *GPX*) of eyeball (a–c), intestine (d–f), liver (g–i), and adipose (j–l) tissues from mice treated with different concentrations of BAE (100, 400, or 800 mg/kg) for different time (0.1, 0.5, 1, 2, 4, 8, or 12 h). All the data are expressed as mean±standard deviation (SD), *n*=3. Different lowercase letters indicate significant differences among mice treated with different concentrations of BAE at all time points (*P*<0.05). mRNA: messenger RNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SOD: superoxide dismutase; GPX: glutathione-peroxidase; BAE: blueberry anthocyanin extract.

8 h, unlike the SOD activity (at 4 h) (Figs. 4d and 4e), and the *GPX* mRNA values also reached peaks at 1 and 8 h, and the value at 1 h was much higher than that at 8 h (Fig. 4f, $P<0.05$). In the liver, the *Cu,Zn-SOD* mRNA values showed a similar trend to the SOD activity except for the unusually high value of 400 mg/kg BAE treatment at 12 h (Fig. 4g). The *Mn-SOD* mRNA level ((21.56±1.16) times of the control, $P<0.05$) of the 400 mg/kg BAE treatment at 12 h was also much higher than others. The *Mn-SOD* mRNA levels of the

100 mg/kg BAE treatment were always very low, and the 800 mg/kg BAE treatment showed an overall upward trend (Fig. 4h). The *GPX* mRNA values of the 100, 400, and 800 mg/kg BAE treatments reached their maximum at 1, 4, and 2 h, respectively (Fig. 4i, $P<0.05$). In the adipose tissue, the *Cu,Zn-SOD* mRNA values of the 400 mg/kg BAE treatment were lower than those of the 100 and 800 mg/kg BAE treatments at 0.5 and 2 h, but its peak at 8 h reached a much higher value than others ((6.90±1.75) times of the control, Fig. 4j, $P<0.05$). The *Cu,Zn-SOD* mRNA in mice treated with the 100 or 800 mg/kg BAE treatment reached the maximum at 0.5 h. However, for both *Mn-SOD* and *GPX* mRNAs of the 800 mg/kg BAE treatment reached maximum values at 8 h. Similar effects of BAE concentrations over time were observed for *Mn-SOD* and *GPX* mRNAs, but not for SOD activity or GSH-PX concentration (Figs. 4k and 4l).

2.7 Effects of different BAE concentrations on different tissues

The antioxidant activity of blueberry anthocyanins is notably dose-dependent. In plasma, eyeball, intestine, liver, and adipose tissues, T-AOC values, SOD activity, and GSH-PX contents increased significantly with increasing BAE concentrations, while MDA levels decreased significantly (Figs. 5a–5d, $P<0.05$). These data are the means of seven time points with large standard errors, but there was still a significant difference among the different BAE concentrations. The plasma of mice treated with various concentrations of BAE showed smaller changes in T-AOC values than other tissues, whose values at low concentrations of BAE (100 or 400 mg/kg) were significantly higher than those of the eyeball, intestine, liver, and adipose ($P<0.05$). The adipose tissue of mice treated with a high

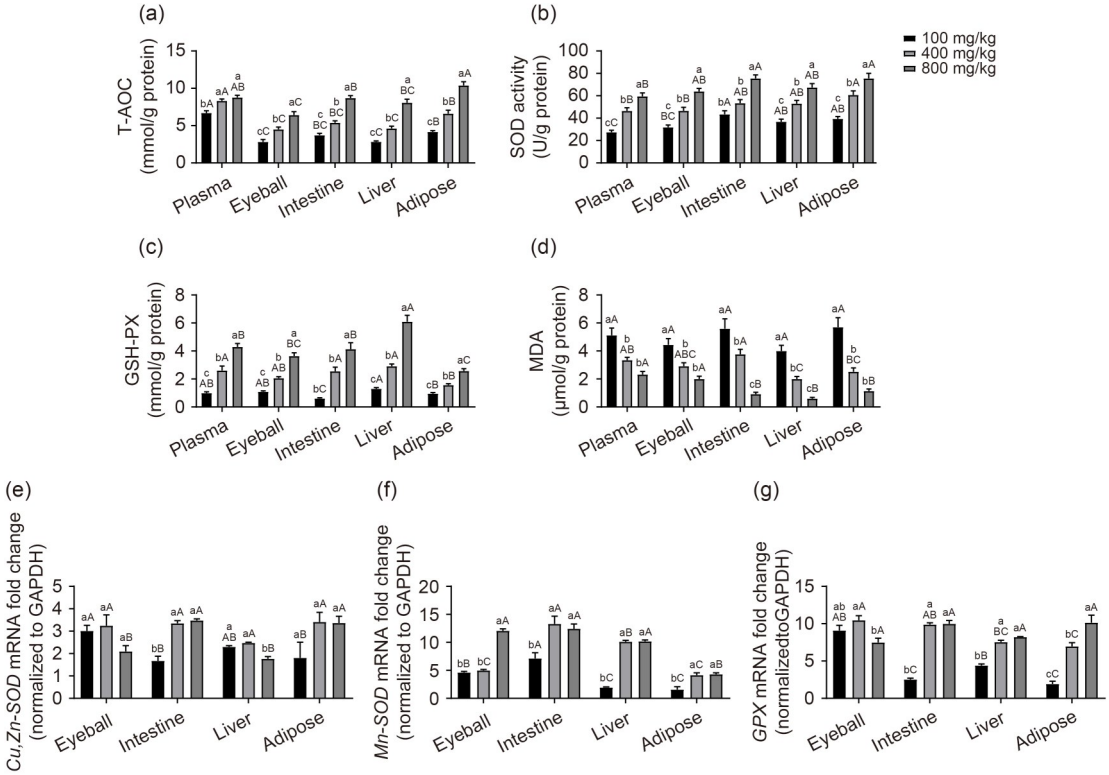

Fig. 5 Effects of different concentrations of BAE (100, 400, and 800 mg/kg) on the T-AOC value (a), SOD activity (b), GSH-PX content (c), MDA level (d), and *Cu,Zn-SOD* (e), *Mn-SOD* (f), and *GPX* (g) mRNA levels of different tissues in mice. All the data are expressed as mean±standard deviation (SD) (n=21 for T-AOC, SOD, GSH-PX, and MDA, each triplicated for seven time points; n=6–18 for *Cu,Zn-SOD*, *Mn-SOD*, and *GPX* mRNAs, each triplicated 2–6 time points). Different lowercase letters indicate significant differences among mice treated with different concentrations of BAE for the same tissue ($P<0.05$); different uppercase letters indicate significant differences among different tissues (plasma, eyeball, intestine, liver, and adipose; no plasma for mRNA) for mice treated with the same concentration of BAE ($P<0.05$). BAE: blueberry anthocyanin extract; T-AOC: total antioxidant capacity; SOD: superoxide dismutase; GSH-PX/GPX: glutathione-peroxidase; MDA: malondialdehyde; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

concentration of BAE (800 mg/kg) presented the highest T-AOC value and the strongest SOD activity, but the lowest GSH-PX content among all tissues treated with the same concentration of BAE (Figs. 5a–5c, $P<0.05$). The MDA value of 800 mg/kg BAE treatment was lower than those of the plasma and eyeball (Fig. 5d, $P<0.05$).

Changes in the mRNA levels of antioxidant enzymes seem to match the BAE concentrations (Figs. 5e–5g), but this may be due to incomplete data (data are not available for all the time points). The *Cu,Zn-SOD* mRNA value of adipose tissue treated with 400 or 800 mg/kg BAE was higher than that treated with 100 mg/kg BAE ($P<0.05$), and much higher than that all the other samples (Fig. 5e). The eyeball treated with three different concentrations of BAE had similar *Cu,Zn-SOD* and *GPX* mRNA values ($P>0.05$). Its *Cu,Zn-SOD* mRNA remained at a low level while its *GPX* mRNA values were significantly higher than those of other tissues when treated with 100 mg/kg BAE ($P<0.05$). However, the *Mn-SOD* mRNA changes seemed unusual. The eyeball treated with 800 mg/kg BAE had higher *Mn-SOD* mRNA level than that with 100 or 400 mg/kg BAE treatment. The liver had low *Cu,Zn-SOD* and *GPX* mRNA levels, but high *Mn-SOD* mRNA levels with 400 or 800 mg/kg BAE, which might be due to its distribution in mitochondria.

2.8 Effects of different BAE administration time on tissues

The antioxidant activity of blueberry anthocyanins seemed to fluctuate with treatment time. However, because of the significant differences among the different BAE concentrations, the mean values of the three concentrations had an extremely big standard error and nearly no significant differences were found in T-AOC values, SOD activity, GSH-PX contents, or MDA levels among plasma, eyeball, intestine, liver, and adipose tissues treated with BAE at seven time points (Figs. 6a–6d, $P>0.05$). Plasma and adipose tissues had higher T-AOC values than eyeball, intestine, and liver, particularly when treated with BAE for 0.5, 1, 2, or 12 h, with maximum values of (8.64±2.29) mmol/g at 0.5 h and (8.12±2.48) mmol/g at 1 h, respectively (Fig. 6a). After BAE treatment (0.1 to 2 h), adipose tissue had a higher SOD activity (Fig. 6b), while the liver had a higher GSH-PX content (maximum of (4.85±4.09) mmol/g at 2 h; Fig. 6c). However, the highest SOD activity was found in the intestine at 4 h, with a value of (72.77±21.11) U/g (Fig. 6b). Intestine (at 0.1 h), liver (at 8 and 12 h), and adipose (at 12 h) tissues had the lowest MDA levels, indicating superior antioxidant capacity (Fig. 6d, $P<0.05$).

Adipose *Cu,Zn-SOD* mRNA levels remained higher than those of eyeball, intestine, and liver when treated with BAE for 4 and 12 h (Fig. 6e). All samples had low *Mn-SOD* mRNA levels shortly after BAE administration (0.1 and 0.5 h), but the eyeball at 8 h and the liver at 12 h had much higher levels, with (12.78±1.38)-fold and (12.41±0.67)-fold changes, respectively (Fig. 6f, $P<0.05$). However, for *GPX* mRNA, the eyeball treated with BAE for 1, 2, and 4 h reached the highest values, with more than 10-fold changes and significantly higher than the other time points (Fig. 6g, $P<0.05$).

3 Discussion

Anthocyanins are hydrophilic macromolecular substances that are thought to be difficult to absorb in the intestine. However, a previous study has found that anthocyanins can enter the circulatory system of organisms after being directly absorbed, and are excreted through urine after oral treatment, but their bioavailability and concentration in the human circulatory system remain very low (Stalmach, 2014). After entering the organism, one part of the anthocyanins is directly excreted, and the other part is transferred into the plasma, where it is metabolically converted by methylation of hydroxyl groups and binding to glucuronic acid or sulfuric acid (Felgines et al., 2005). Ichiyanagi et al. (2006) studied the effect of structural diversity on the bioavailability of blueberry anthocyanins in rats and found that the plasma levels of anthocyanins with the same anthocyanidin were in the order of galactoside>glucoside>arabinoside. Aqil et al. (2014) also found that anthocyanins could exert their effects beyond the gastrointestinal tract. Although blueberry anthocyanins could not be detected directly in lung tissues of female athymic mice following a 10-d diet including 5% (mass fraction) blueberry powder, when extracted anthocyanins were converted to their aglycone form by acid hydrolysis, delphinidin and cyanidin were detected. In this study, anthocyanin peaks were detected in the liver tissue of mice following BAE administration, but in very low quantities. Due to the sensitivity and detection limit of

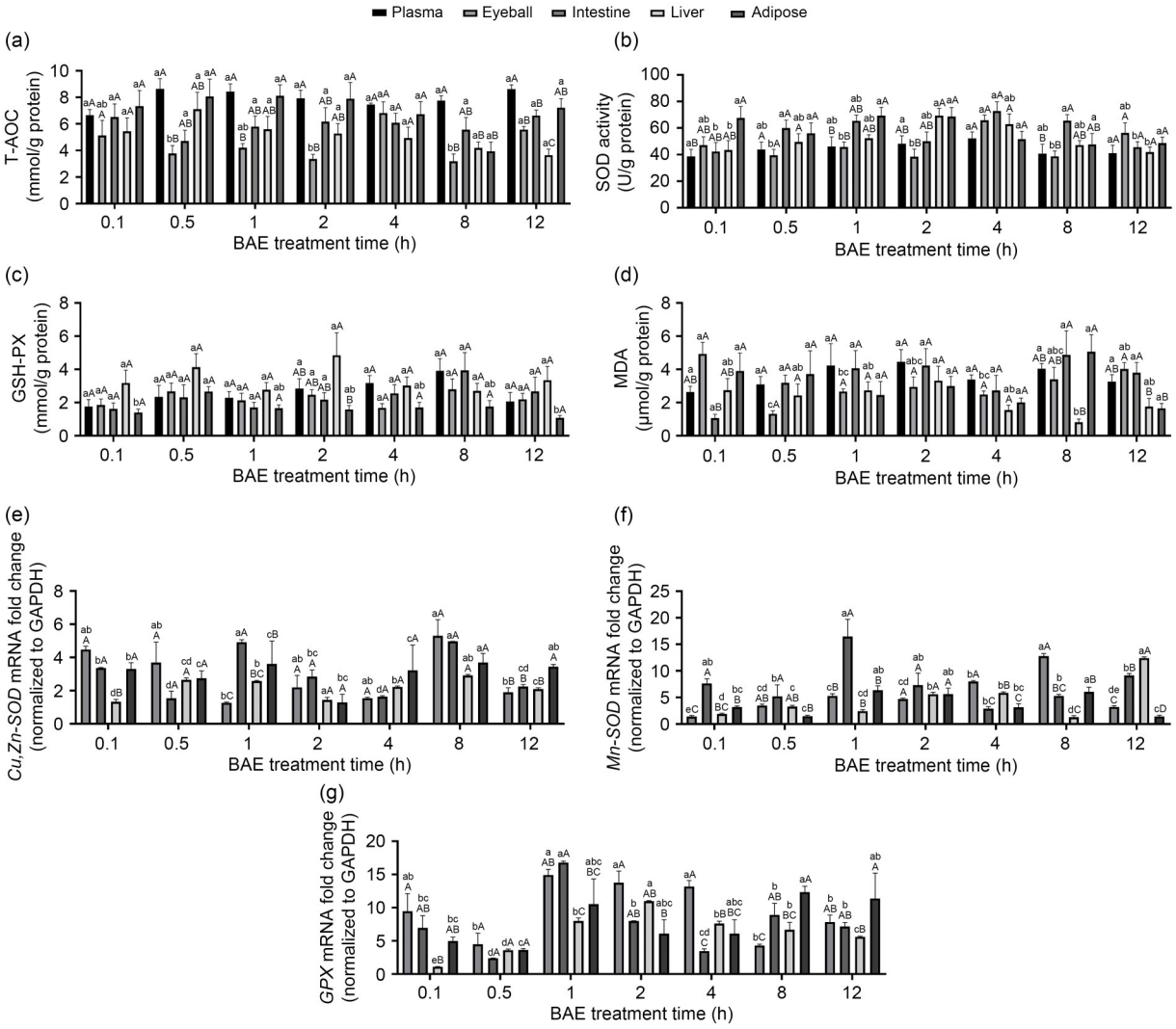

Fig. 6 Effects of different treatment time (0.1, 0.5, 1, 2, 4, 8, and 12 h) of BAE on T-AOC value (a), SOD activity (b), GSH-PX content (c), MDA level (d), and *Cu,Zn-SOD* (e), *Mn-SOD* (f), and *GPX* (g) mRNA levels of different tissues in mice. All the data are expressed as mean±standard deviation (SD) (*n*=9 for T-AOC, SOD, GSH-PX, and MDA, each triplicated for three concentrations; *n*=3–9 for *Cu,Zn-SOD*, *Mn-SOD*, and *GPX* mRNAs, each triplicated 1–3 concentrations). Different lowercase letters indicate significant differences in mice treated with different time of the same tissue (*P*<0.05); different uppercase letters indicate significant differences in different tissues (plasma, eyeball, intestine, liver, and adipose; no plasma for mRNA) of mice treated with the same time (*P*<0.05). BAE: blueberry anthocyanin extract; T-AOC: total antioxidant capacity; SOD: superoxide dismutase; GSH-PX/GPX: glutathione-peroxidase; MDA: malondialdehyde; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

the HPLC system, the anthocyanin contents of other tissues could not be quantified.

Anthocyanins, the largest group of water-soluble pigments in the plant kingdom, have been reported to have much pharmacological activity, such as antioxidant activity (Tsuda et al., 1994; Hribar and Ulrih, 2014). Like other polyphenols, anthocyanins exhibit antioxidant activity both in vivo and in vitro. In in-vivo antioxidant assays, anthocyanins inhibit reactive oxygen species (ROS) formation in phagocytes and regulate the balance between oxidants and antioxidants (Bunea et al., 2013; da Silva Souza et al., 2020), unlike in-vitro assays in which they scavenge free radicals because of the presence of hydroxyl groups. Yang et al. (2021) found that blueberry crude extract had better antioxidant properties in vivo than purified extract. The purified extract exhibited superior in vivo antioxidant properties after the crude extract of blueberry anthocyanin and polyphenols was added to it. Mueller et al. (2017) pointed out that multiple mixtures of anthocyanins, rather than a single

anthocyanin, were more beneficial to health. Crude extracts may contain a much richer range of antioxidant substances, which may act synergistically to impart greater benefits. Anthocyanins from purple highland barley exhibited high antioxidant activity in PC12 cells by maintaining cell viability, restoring cell morphology, reducing ROS levels, and enhancing antioxidant enzyme activity (Zhang et al., 2021). Anthocyanins extracted from 'Brightwell' rabbiteye blueberries have been shown in our previous studies (Li et al., 2013; Huang et al., 2018a) to have strong antioxidant activity in various cells and advantageous scavenging ability against various free radicals. This strong antioxidant capacity has also been demonstrated in vivo in the present study.

Known as first-line defense antioxidants, SOD and GSH-PX provide cellular protection against free radicals and ROS (Ighodaro and Akinloye, 2018). These enzymes catalyze the breakdown of hydrogen peroxides and hydroperoxides into harmless molecules. Therefore, they are an indispensable part of the entire defense strategy based on antioxidants and have been widely investigated for the prevention and treatment of diseases resulting from oxidative damage. Puiggròs et al. (2009) provided evidence that grape seed procyanidin extract increased Cu,Zn-SOD activity in rats. Ma et al. (2016) showed that anthocyanins effectively regulate the lipid metabolism of rats with hyperlipidemia, increasing their T-AOC levels and SOD activity in vivo. Chatterjee et al. (2021) illustrated the ability of black carrot anthocyanin-loaded chitosan nanoparticles to increase SOD and catalase enzyme activity levels in serum samples of experimental rats in vivo. Their results indicated that the nanoencapsulation process improved the stability and bioavailability of anthocyanins. In the present study, high enzyme activity, contents or mRNA levels of these antioxidant enzymes were detected in various tissues of mice treated with blueberry anthocyanins, indicating that anthocyanins are linked to high antioxidant capacity in vivo. Therefore, the antioxidant role of blueberry anthocyanins in mice may be exerted by improving the activity of antioxidant enzymes. In addition, the low contents of MDA in various tissues indicated that BAE effectively reduced lipid peroxidation in mice. In this regard, Lau et al. (2007) found that blueberry extract inhibited the activity of enzymes associated with the production of free radicals and chelating trace metals, thereby inhibiting the formation of ROS, in addition to scavenging free radicals.

Anthocyanins and polyphenols might improve the health of mice since they can significantly enhance final body weights in experimental animals (Kang et al., 2003). Yang et al. (2021) found that different types of blueberry extracts administered by gavage for 20 d significantly increased the thymus and spleen indexes of experimental mice, indicating that blueberry extracts could promote the growth of immune organs. However, in our study, a short-term (less than 12 h) administration of blueberry anthocyanins affected neither the body weights nor the tissue indexes of mice, but still increased antioxidant defense. The BAE exhibited excellent dose-dependent antioxidant activity in vivo, consistent with the report by Yang et al. (2021). In general, the antioxidant content of a test substance positively correlates with its total reducing power. Several studies have reported that natural antioxidants display concentration-dependent reducing power and scavenging effects, and that in vivo results are consistent with those in vitro (Maciel et al., 2018; Muceniece et al., 2019). The administration time of BAE had no significant influence on antioxidant activity in vivo, which might be because that these molecules are rapidly absorbed through the gastric wall and have a fast-urinary clearance (McGhie and Walton, 2007). While passing through the gastrointestinal tract, the chemical and bioactive properties of anthocyanins are susceptible to change because of their sensitivity to pH, temperature, oxygen, enzymes, and other environmental factors (Herrera-Balandrano et al., 2021a). Czank et al. (2013) previously investigated the absorption, distribution, metabolism, and elimination (ADME) of a $^{13}C_5$-labeled anthocyanin in humans, and found that anthocyanins are more bioavailable than previously perceived, and their metabolites are present in the circulation for less than 48 h after ingestion. Further research is required to determine the method by which their metabolites, such as phenolic, hippuric, phenylacetic acids, and phenylpropanoid, as well as the parent anthocyanins, may all contribute to the antioxidant capacity.

4 Conclusions

Anthocyanins extracted from 'Brightwell' rabbiteye blueberries had advantageous antioxidant activity

in vivo in a concentration-dependent manner, although only a very low level of anthocyanins could be detected in various tissues after digestion in mice. During short-term administration of BAE from 0.5 to 12 h, the tissue indexes of mice did not change. However, the tissues soon exhibited antioxidant activity which fluctuated with time. The plasma, eyeball, intestine, liver, and adipose tissues of mice administrated with different concentrations of BAE exhibited a degree of increased T-AOC values. The higher the concentration of BAE, the stronger the antioxidant activity. The effect of blueberry anthocyanins on antioxidant activity in vivo might be exerted through improving the first-line antioxidant defense enzymes SOD and GSH-PX, since these enzymes showed increased activity and mRNA levels in a manner corresponding to the concentrations of BAE during different treatments. In addition, the level of the oxidative stress marker MDA decreased, indicating that blueberry anthocyanins could also reduce the lipid peroxidation reaction in mice. Consistent with our previous findings that blueberry anthocyanins had high antioxidant activity in vitro, the antioxidant activity of blueberry anthocyanins in vivo reported here provides important evidence that they could be used to treat diseases, especially those associated with oxidative stress.

Materials and methods

The detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Conceptualization: Jing WANG, Wuyang HUANG, and Zhongquan SUI; Methodology and formal analysis: Jing WANG and Xiaoxiao ZHANG; Data curation: Xingyu ZHAO; Investigation: Jiawei ZHENG and Xiaoxiao ZHANG; Writing – original draft: Jing WANG, Xingyu ZHAO, and Daniela D. HERRERA-BALANDRANO; Writing – review, editing, and supervision: Wuyang HUANG; Project administration and funding acquisition: Wuyang HUANG and Zhongquan SUI. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Jing WANG, Xingyu ZHAO, Jiawei ZHENG, Daniela D. HERRERA-BALANDRANO, Xiaoxiao ZHANG, Wuyang HUANG, and Zhongquan SUI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. The animal experiments were carried out according to the guidelines of the Laboratory Animal Research Center of Jiangsu Province and approved by the Research Animal Care and Use Committee at Jiangsu Academy of Agricultural Sciences, Nanjing, China (No. SYXK 2020-0024).

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Supplementary information

Materials and methods; Tables S1–S3; Fig. S1