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Effects of dietary baicalin supplementation on iron overload-induced mouse liver oxidative injury

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Abstract

Iron overload is one of the most common metal related toxicity. Under this circumstance, excessive iron deposition in liver will lead to further injury such as hepatocellular necrosis, inflammation, fibrosis, and in some cases even to carcinoma. In this paper, the effect of a nature flavonoid, baicalin, on iron overload-induced mouse liver oxidative injury has been studied. It was found that when iron–dextran-induced iron overload, mice were fed baicalin-containing diet (0.25% and 1%) for 50 days, hepatic iron, liver-to-body weight ratio, and hepatic lipid peroxidation were dose-dependently decreased; while catalase activity, total antioxidant status, and serum iron content were dose dependently increased. The protective effect of baicalin on liver of iron overload mouse may due to both the antioxidant and iron chelation activities of baicalin. These data provide preliminary experimental support for baicalin as medicine for iron overload diseases.
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Keywords: Baicalin; Iron overload; Oxidative injury; Liver

1. Introduction

Iron overload is one of the most common metal-related toxicity. Progressive iron-induced liver injury in human is most often encountered in diseases of aberrant iron storage resulting from abnormal increase in the gastrointestinal absorption of dietary iron. Under these circumstances, excessive iron deposition in liver will lead to further injury such as hepatocellular necrosis, inflammation (Deugnier et al., 1992), fibrosis (Arezzini et al., 2003; Gardi et al., 2002), and in some cases even to carcinoma (Niederau et al., 1985). Hepatocellular lipid peroxidation of polyunsaturated fatty acids in membranes has been implicated as a mechanism by which iron causes liver damage (Fletcher et al., 1989). Iron-induced peroxidation of intracellular membranes may lead to cellular dysfunction and eventually sideronecrosis (Arthur, 1996).

At present, two chelating agents, i.e. deferoxamine and 1,2-dimethyl-3-hydroxypyrid-4-one (deferiprone, L1) are commonly used in the treatment of iron overload diseases. Although both drugs are very effective, they exert several side effects and disadvantages (Al-Refaie et al., 1992; Richardson, 1999; Kontoghiorghes et al., 2001). Deferoxamine is not orally absorbed and requires slow subcutaneous administration resulting in poor patient compliance (Olivieri and Brittenham, 1997). A non-toxic and orally active iron chelator is needed.

Flavonoids are phenolic compounds widely distributed in plants, which have been reported to exert multiple biological effects, including antioxidant and free radical scavenging abilities (Bors and Saran, 1987; Negre-Salvayre and Salvayre, 1992). They were also suggested to present a strong affinity to iron ions (Boyer et al., 1988; Havsteen, 1983; Afanas'ev et al., 1989; Morel et al., 1993; Borsari et al., 2001). Baicalin is a flavonoid which is found rich in *Scutellaria baicalensis* Georgi, a commonly used traditional antiinflammatory herb medicine in China, Japan, and Korea. Baicalin is an active antioxidant flavonoid confirmed by some researchers (Yoshino and Murakami, 1998) as well as

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us (Gao et al., 1999), it is also found to be an effective iron chelator (Yoshino and Murakami, 1998). Our previous study found that dietary supplementation of baicalin caused a decrease of rat liver iron content (Gao et al., 2003). These results suggest that baicalin might be used as an iron chelator for iron overload. The aim of the present investigation is testing the effect of baicalin on experimental mouse model of iron overload.

2. Materials and methods

2.1. Materials

Baicalin standard was purchased from National Institute for the Control of Pharmaceutical and Biological Products (China). 2,2′ -azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and Trolox were purchased from Sigma. Baicalin was extracted from scutellaria roots according to the method described by Han et al. (1997) and re-crystallized in methanol. The purity of the extraction was tested by polyamine thin-layer chromatography and ultraviolet-visible spectrophotometry; the content of baicalin in the extraction was 98.8%. Other chemicals were purchased from local market. Kunming mice were purchased from Tongji Medical School, Huazhong University of Science and Technology (China).

2.2. Animals

Thirty-six male Kunming mice, initially weighing 15.6±0.9 g, were used. Mice were randomly divided into four groups. Three groups received 6 doses (three doses per week) of 100 mg/kg each (i.p. iron–dextran–saline), the other group received the same volume of saline as a blank (B). The blank group and one iron–dextran group (C) were fed commercial animal chow, the other two groups were fed with the same animal chow supplemented with 0.25% (LBa) or 1% of baicalin (HBa) (wt/wt), respectively (about 0.5 g/kg or 2 g/kg b.w. daily, respectively). The amount of other flavonoids in animal food was not considered. Each group of mice were housed in a large plastic cage and given free access to food and tap water. All groups of animals were kept at 23±2 °C under a 12-h dark/light cycle. The body weight of the mice was measured every 5 days. Animal care in this study conformed to the NIH Guide for Care and Use of Laboratory Animals (NIH publication 86-23, revised 1986).

2.3. Tissue preparation

After 50 days of feeding, mice were fasted overnight. They were anesthetized with ethyl ether, and blood was collected by cardiac puncture. The liver was quickly removed and weighted, then perfused with 4 °C saline to exclude the blood cells and then blotted on filter paper; half of them were cut, weighed and homogenized with a glass homogenizer in 9 volume of ice-cold 50 mM phosphate-buffered saline (PBS). Portions of homogenates were immediately pipetted for measuring the levels of thiobarbituric acid reactive substance. The remaining homogenate was centrifuged (10,000×g), then the supernate was used to measure catalase activity and total antioxidant assay. The protein contents in the homogenate and supernate were measured as Peterson (1977) described. The other half liver was weighed and put into a glass flask, then 5 volumes of mixed acid (nitric acid: perchloric acid=4:1) was added, heated until large amount of white vapors could be seen. The volume of the digested sample was adjusted to 10 ml with double distilled water, and then the obtained solution was used to analyze iron contents.

2.4. Lipid peroxidation and antioxidant enzymes analyses

100 μl of liver homogenate was used to measure the lipid peroxidation by the thiobarbituric acid method (Buege and Aust, 1978). Catalase activity in liver homogenate was assayed at 25 °C by a method based on the absorbency disappearance of 10 mM H_2O_2 at 240 nm (Pedraza-Chaverri et al., 1999).

2.5. Total antioxidant status assay

Total antioxidant status was assayed according to the method introduced by Re et al. (1999) and modified by Gao et al. (2003). Briefly, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by first adding MnO_2 powder in the solution and keeping in the dark at room temperature for more than 12 h, then filtrated with syringe filter and kept in dark for another 6 h. Stock solution of $ABTS^{\cdot+}$ was diluted with PBS, pH 7.4, to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. After adding 1.0 ml of diluted $ABTS^{\cdot+}$ to 10 μl of serum or tissue homogenate supernate, the absorbance reading was taken at 30 °C exactly 2 min after initial mixing. The total antioxidant capacity concentration was compared with equivalent antioxidant capacity of Trolox and was expressed in μmol of Trolox/g of tissue.

2.6. Determination of serum iron and liver iron

Serum iron concentration was determined using the assay based on the generation of an iron–ferrozine complex as described by Galleano and Puntarulo (1992) previously. Iron concentration in the liver digested sample was measured spectrophotometrically at 535 nm, after reaction with 2 mM bathophenanthroline disulfonic acid (Brumby and Massey, 1967).

2.7. Statistical analysis

The results are expressed as means±S.D., (and) statistical significance was determined using a one-way analysis

of variance (ANOVA) with comparisons to a control group. The level of significance was established at $P<0.05$.

3. Results

3.1. Effects of baicalin administration on growing up curve and liver-to-body weight ratio (%) of iron load mouse

Fig. 1 shows the growing up curve of mice. There were no obvious health abnormalities in all groups of the tested animals, but the weight gain of iron load mouse was significant lower than that of blank group after twenty days. However, the weight gain of the mice in both baicalin supplementation dosage groups has no significant difference compared with that of the blank group at first twenty days, and then it became significantly higher than that of blank group after 25 days. This observation may mean that the negative effect of iron overload on mouse can be alleviated by baicalin administration. The liver-to-body weight ratio was significantly increased in all treated animals; however, compared with control group, low baicalin showed moderate protection, and high baicalin showed significant protection (Fig. 2).

3.2. Effects of baicalin administration on catalase in the liver of iron load mouse

Catalase is an important heme containing antioxidant enzyme. Catalase activity in mouse liver was tested, and the results are shown in Fig. 3. From Fig. 3, we can see that iron load caused a significant decrease on catalase activity in mouse liver. Supplementation of baicalin on iron overload mice increases hepatic catalase activity in a dose dependent manner.

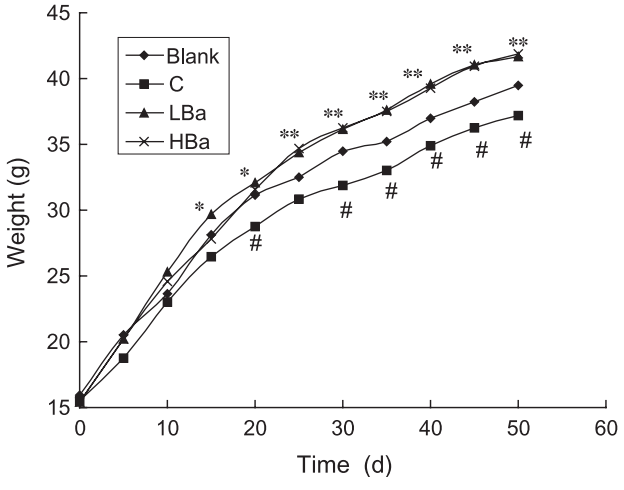

Fig. 1. Growing up curve of different treated mouse. The mice were randomly divided into four groups. Three groups received 6 doses (three doses per week) of 100 mg/kg each (i.p. iron–dextran–saline), the other group received the same volume of saline as a blank. The blank group (B; ◆) and one iron–dextran group (C; ■) were fed commercial animal chow, the other two groups were fed with the same animal chow supplemented with 0.25% (LBa; ▲) or 1% of baicalin (HBa; x) (w/w), respectively. The body weight of the mice was measured every 5 days. Values are means of 9 animals in each group. $^{\#}P<0.05$ compared with Blank; $*P<0.05$ compared with Control, $**P<0.01$ compared with Control.

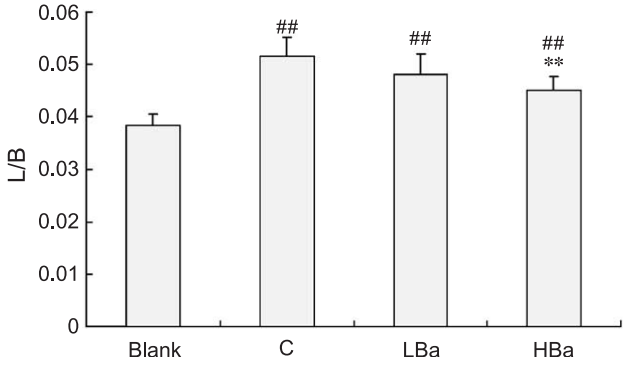

Fig. 2. Liver to body ratio of different treated mouse. Mice were randomly divided into four groups. Three groups received 6 doses (three doses per week) of 100 mg/kg each (i.p. iron–dextran–saline), the other group received the same volume of saline as a blank. The blank group (B) and one iron–dextran group (C) were fed commercial animal chow, the other two groups were fed with the same animal chow supplemented with 0.25% (LBa) or 1% of baicalin (HBa) (wt/wt), respectively. After 50 days of feeding, mice were fasted overnight. They were anesthetized with ethyl ether. The liver was quickly removed and weighted and liver to body weight ratio was calculated. Values are means±S.D. (n=9). $^{\#\#}P<0.01$ compared with Blank; $**P<0.01$ compared with Control.

3.3. Effects of baicalin administration on thiobarbituric acid reactive substance and total antioxidant status levels in the liver of iron load mouse

Thiobarbituric acid reactive substance and total antioxidant status levels are common parameters of oxidative status of tissue. In the liver of iron load mouse, total antioxidant status level was significantly decreased, while

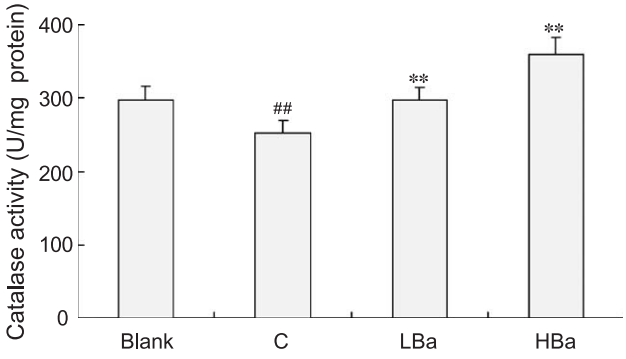

Fig. 3. Catalase activity in different treated mouse liver. Mice were randomly divided into four groups and treated as described in Section 2.2. After 50 days of feeding, mice were fasted overnight, anesthetized with ethyl ether, and the liver was quickly removed. Catalase activity in liver homogenate was assayed at 25 °C by a method based on the absorbency disappearance of 10 mM H_2O_2 at 240 nm (Pedraza-Chaverri et al., 1999). Values are means±S.D. (n=9). $^{\#\#}P<0.01$ compared with Blank; $**P<0.01$ compared with Control.

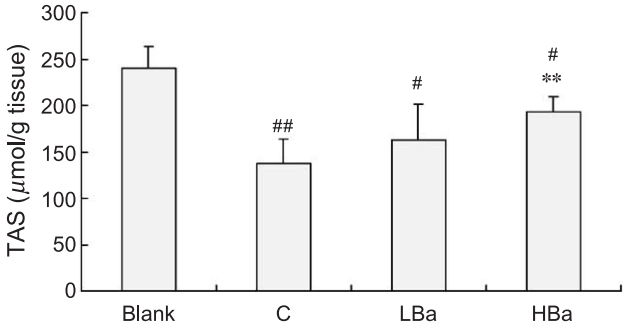

Fig. 4. Total antioxidant status level in different treated mouse liver. Mice were randomly divided into four groups and treated as described in Section 2.2. After 50 days of feeding, mice were fasted overnight, anesthetized with ethyl ether, and the liver was quickly removed. Total antioxidant status was assayed according to the method described in Section 2.5. Values are means±S.D. (n=9). $^{\#}P$<0.05 compared with blank, $^{\#\#}P$<0.01 compared with Blank; *P<0.05 compared with Control, **P<0.01 compared with Control.

thiobarbituric acid reactive substance level was increased compared with those of normal mouse. These results indicated that there was significant oxidative stress on liver. When iron load mice were supplemented with baicalin, there were an increase on total antioxidant status level (Fig. 4) and a decrease on thiobarbituric acid reactive substance (Fig. 5).

3.4. Effects of baicalin on serum iron and hepatic iron of iron load mouse

As we expected, liver iron concentrations were significantly increased in the iron load mice. Baicalin supplementation dose-dependently decreased hepatic iron at our used dosage (Fig. 6). Serum iron was also significantly elevated in iron load mice. When mice were supplemented with

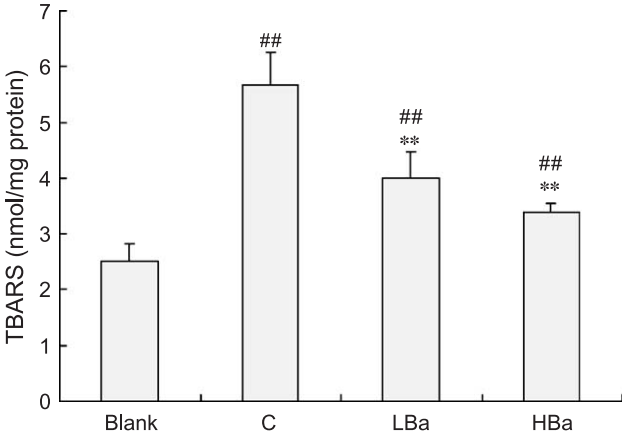

Fig. 5. Thiobarbituric acid reactive substance level in different treated mouse liver. Mice were randomly divided into four groups and treated as described in Section 2.2. After 50 days of feeding, mice were fasted overnight, anesthetized with ethyl ether, and the liver was quickly removed. Thiobarbituric acid reactive substance was assayed according to the method described in Section 2.4. Values are means±S.D. (n=9). $^{\#\#}P$<0.01 compared with Blank; **P<0.01 compared with Control.

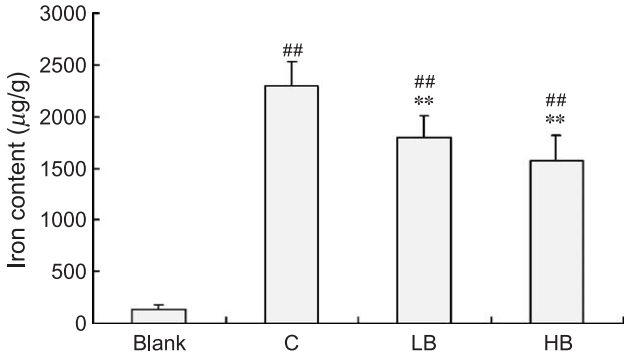

Fig. 6. Liver iron content in different treated mouse liver. Mice were randomly divided into four groups and treated as described in Section 2.2. After 50 days of feeding, mice were fasted overnight, anesthetized with ethyl ether, and the liver was quickly removed and weighted, then digested by nitric acid and perchloric acid mixed in a ratio of 4:1. Iron content was assayed according to the method described in Section 2.6. Values are means±S.D. (n=9). $^{\#\#}P$<0.01 compared with Blank; **P<0.01 compared with Control.

baicalin, the serum iron level was even higher than that of iron load control group (Fig. 7).

4. Discussion

In cases of iron overload, the natural storage and transport proteins such as ferritin and transferrin become saturated and overwhelmed, and then the iron spills over into other tissues and organs. At the same time, oxidative stress arises because of the catalytic activity of the metal ion on producing high reactive oxygen radicals, and finally leads to tissue injury. This injury can be relieved by the administration of an appropriate chelating agent which can combine with the iron and increase its rate of excretion. Some flavonoids can be both antioxidants and iron chelators; it means that flavonoids will be good candidates for curing iron overload disease (Borsari et al., 2001). Here

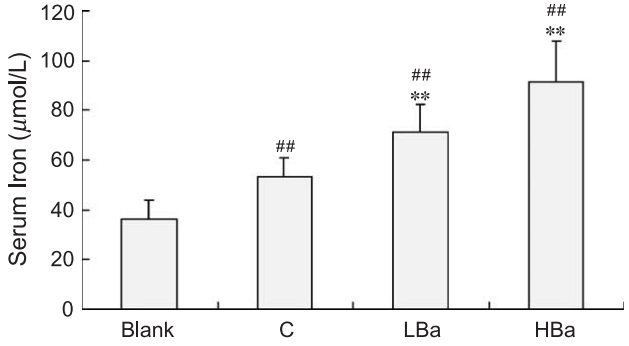

Fig. 7. Non-heme iron content in different treated mouse Serum. Mice were randomly divided into four groups and treated as described in Section 2.2. After 50 days of feeding, mice were fasted overnight, anesthetized with ethyl ether, and blood was collected by cardiac puncture. Non-heme iron content was assayed according to the method described in Section 2.6. Values are means±S.D. (n=9). $^{\#\#}P$<0.01 compared with Blank; **P<0.01 compared with Control.

we used baicalin, an active flavonoid component of commonly used Chinese herb medicine *S. baicalensis* Georgi, to study its effects on iron overload-induced liver oxidative injury.

There are two commonly used animal models for iron overload, one with carbonyl iron as dietary (Park et al., 1987) and the other with i.p. injection of iron dextran (Dillard et al., 1984; Galleano and Puntarulo, 1992). In order to rule out the possibility of iron chelation before adsorption or direct interference of absorption by baicalin in intestine, we chose the model with i.p. injection of iron dextran. As we can see from Fig. 6, after six doses of total 600 mg/kg iron injection within two weeks, hepatic iron concentration in the mouse was increased from 137.8±43.5 μg/g to 2295.9±232.4 μg/g wet weight even after 5 weeks of injection (Fig. 6), liver-to-body weight ratio was also significantly elevated (Fig. 2), thus the treatment could be considered appropriate to study liver damage related to iron overload.

It has been reported that iron overload led to an increase in lipid peroxidation (Galleano and Puntarulo, 1997; Fischer et al., 2002; Sochaski et al., 2002), decrease in total antioxidant status levels (Livrea et al., 1996), the changing of activities of some antioxidant enzymes including catalase (Galleano and Puntarulo, 1997). In our experiment, even 4 weeks after iron–dextran injection, the content of thiobarbituric acid reactive substance in iron load mouse liver was still about two times than that of normal mouse, and hepatic total antioxidant status level was only 60% of that of normal mouse.

Flavonoids can play a double role in reducing the rate of oxidation, one acts as iron chelator (Boyer et al., 1988; Havsteen, 1983; Afanas'ev et al., 1989; Morel et al., 1993; Borsari et al., 2001) and the other acts as radical trap (Pietrangelo et al., 1995; van Acker et al., 1998). The effects of some flavonoids on iron overload animals have been done previously. Afanas'ev et al. (Afanas'ev et al., 1995) found that rutin administration sharply suppressed free radical production in liver microsomes by phagocytes of iron overload rats. Oral administration of silybin was also found to inhibit the iron-induced accumulation of malondialdehyde in liver mitochondria (Pietrangelo et al., 1995). However, few studies have been done on the direct decreasing effect of flavonoid on tissue iron in vivo. Here we observed that when iron load mice were supplemented with baicalin, there was a decrease of hepatic total iron; at the same time, serum non-heme iron was significantly increased. This result indicates that baicalin could gradually combine with hepatic non-heme iron and take it into blood stream and finally excrete it from body. However, this hypothesis needs further experimental support.

Oxidative status depends on the balance between prooxidants and antioxidants (Rice-Evans and Burdon, 1993). Iron is thought to be a prooxidant since it has the ability to catalyze the Fenton or the Haber–Weiss reaction, which produces the hydroxyl radical. Iron overload can destruct the balance between prooxidants and antioxidants, leading to severe loss of total antioxidant status level. This phenomenon can be seen in most iron overload animal models (Dabbagh et al., 1994) as well as iron overload disease such as hereditary haemochromatosis (Young et al., 1994) and thalassemia (Livrea et al., 1996). As nature antioxidants, flavonoids intake may increase total antioxidant status level in living body. In our previous study, we found that supplementation of baicalin or another flavonoid, rutin, could increase hepatic total antioxidant status level (Gao et al., 2003). From Fig. 4, we can see that baicalin supplementation significantly increased hepatic total antioxidant status level of iron overload mice, and this effect may come from two aspects: one is baicalin chelating free iron ion and stopping iron-catalyzed oxidative reaction, the other is baicalin and its metabolites direct increasing antioxidant status by acting as strong antioxidants.

Although catalase is an iron-containing antioxidant enzyme, it has been reported that under iron overload, there was a significant decrease of catalase activity in rat liver (Galleano and Puntarulo, 1997) and testes (Lucesoli and Fraga, 1999). Our previous study found that baicalin supplementation caused a significant decrease of hepatic catalase activity in normal rat (Gao et al., 2003). We wanted to know the effect of baicalin on hepatic catalase activity in iron load animals. Our result showed that iron-dextran injection decreased the hepatic catalase activity (Fig. 3), similar to the profile previously reported in iron overload induced by diets supplemented with 2.5% carbonyl iron (Galleano and Puntarulo, 1997). The supplementation of baicalin on iron overload mice did not cause a further loss of catalase activity as we found in normal rats supplemented with the same flavonoid, conversely, the losing of catalase activity induced by iron overload could be inhibited by baicalin supplementation. It has been reported that iron-induced peroxidation would lead to the degradation of cytochrome *P*-450 heme (Levin et al., 1973). As a heme protein, high catalase sensitivity to iron overload could be related to the destruction of the heme by iron-induced peroxidation (Galleano and Puntarulo, 1997). The supplementation of baicalin introduced a new source of antioxidant and could partly inhibit peroxidation-induced heme destruction, and then provided a protection on catalase.

In conclusion, here we found that baicalin supplementation can increase antioxidative status, decrease iron content and lipid peroxidation in iron overload mice liver. These data provided preliminary experimental support on baicalin as medicine for iron overload diseases. Considering the high content of baicalin in the root of *S. baicalensis* Georgi (more than 10%) and being easy to be extracted, baicalin might be a new source of medicine for iron overload diseases and is worth further study.

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