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Pharmacological Investigation on the Qi-Invigorating Action of Schisandrin B: Effects on Mitochondrial ATP Generation in Multiple Tissues and Innate/Adaptive Immunity in Mice

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Abstract

Schisandrae Fructus, containing schisandrin B (Sch B) as its main active component, is recognized in traditional Chinese medicine (TCM) for its Qi-invigorating properties in the five visceral organs. Our laboratory has shown that the Qi-invigorating action of Chinese tonifying herbs is linked to increased mitochondrial ATP generation and an enhancement in mitochondrial glutathione redox status. To explore whether Sch B can exert Qi-invigorating actions across various tissues, we investigated the effects of Sch B treatment on mitochondrial ATP generation and glutathione redox status in multiple mouse tissues ex vivo. In line with TCM theory, which posits that Zheng Qi generation relies on the Qi function of the visceral organs, we also examined Sch B's impact on natural killer cell activity and antigen-induced splenocyte proliferation, both serving as indirect measures of Zheng Qi. Our findings revealed that Sch B treatment consistently enhanced mitochondrial ATP generation and improved mitochondrial glutathione redox status in mouse tissues. This boost in mitochondrial function was associated with stimulated innate and adaptive immune responses, marked by increased natural killer cell activity and antigen-induced T/B cell proliferation, potentially through the increased generation of Zheng Qi.

Keywords

Zheng Qi, Schisandrin B, Mitochondria, ATP Generation, Glutathione Redox, Innate Immunity, Adaptive Immunity, Natural Killer Cell Activity, Splenocyte Proliferation

1. Introduction

Traditional Chinese Medicine (TCM) offers a promising approach to disease prevention and treatment. TCM theory posits that the human body is an organic whole, where different organs (Liver, Heart, Spleen, Lung, Kidney, or Zang in Chinese) are interconnected and function together harmoniously [1] [2] [3] [4]. The interaction between Yin and Yang within the body produces Zheng Qi, the vital energy that circulates through Meridians to nourish organs [1] [5] [6]. A complete lack of Zheng Qi results in death, while an imbalance between Yin and Yang can lead to Qi deficiency, causing diseases or a suboptimal health state [7] [8]. TCM uses Qi-invigorating herbs to address Qi deficiencies [9]. Our studies have demonstrated that Yang and Qi herbs boost mitochondrial ATP production, enhancing organ function, whereas Yin and Blood herbs improve adaptive immunity and red blood cell production/microcirculation, respectively [10] [11] [12]. Since cellular functions largely depend on ATP, increasing mitochondrial ATP production with Yang/Qi herbs supports organ health [9] [13]. Generating Zheng Qi, essential for maintaining vitality, combines Acquired Qi (from air and food) with Primordial Qi (inherited from parents), necessitating healthy organ function [2] [3] [9]. Qi-invigorating herbs can thus restore organ Qi, facilitating Zheng Qi production. Zheng Qi comprises Wei Qi, which protects against external pathogens (similar to innate immunity), and Ying Qi, which provides essential nourishment to organs, including the adaptive immune system [2] [9] [14].

Schisandra chinensis Turcz (Ballion) is a widely used Chinese herb known for its astringent and liver-protecting properties. Sun Simiao, a renowned Chinese herbalist in the Tang Dynasty, claimed that Wu-Wei-Zi (Schisandrae Fructus, the fruit of *Schisandra chinensis*, transliterally meaning the fruit of five tastes) can invigorate the Qi of the five visceral organs [15]. Schisandrin B (Sch B), the primary active component in Schisandrae Fructus, has shown various beneficial effects, including antioxidation, anti-inflammation, immunomodulation, neuroprotection, and anti-cancer properties, as evidenced by animal and cell model assays [16] [17] [18]. The wide-ranging action of Sch B may be due to its supposed Qi-invigorating effect in different tissues. Our findings suggest that Qi invigoration involves increased mitochondrial ATP generation, which is linked to improved mitochondrial glutathione redox status [19] [20] [21]. We investigated the impact of Sch B treatment on mitochondrial ATP generation and glutathione redox status in multiple mouse tissues, including the liver, heart, brain, spleen, lungs, and kidneys. These organs correlate, albeit not entirely, with the five visceral organs in Traditional Chinese Medicine (TCM). Furthermore, based on TCM theory that Zheng Qi generation relies on the Qi function of various organs, we also explored Sch B's effects on natural killer cell activity (representing innate immunity) and antigen-induced splenocyte proliferation (representing adaptive immunity), which indirectly measure Zheng Qi.

2. Materials and Methods

2.1. Reagents

Fetal bovine serum (FBS) was obtained from Life Technologies Corporation (Carlsbad, CA). Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), adenosine triphosphate (ATP), adenosine diphosphate (ADP), RPMI-1640 medium (without phenol red), penicillin, streptomycin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), nitroblue tetrazolium chloride (NBT), lithium lactate, phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide hydrate (β-NAD), and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO). The luciferase kit was obtained from Perkin Elmer (Boston, MA). Concanavalin A (Con A) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Sch B was isolated from the fruit of Schisandra chinensis, as described by Ip and colleagues [22].

2.2. Animal Care

Adult female ICR mice were maintained under a 12-hour dark/light cycle at an ambient temperature of approximately 22˚C with *ad libitum* access to food and water. Preliminary studies indicated that Sch B had a similar effect on mitochondrial ATP generation in both male and female mice. Experimental protocols were approved by the Research Practice Committee at the Hong Kong University of Science and Technology (AEP-2023-0062).

2.3. Animal Treatment

Animals were randomly divided into groups, with four animals each. In the treatment groups, mice were intragastrically administered Sch B (suspended in water) at a daily dose of 0.3 or 1 g/kg for 3 days. Control animals only received water. Twenty-four hours after the last dose, animals were euthanized by cervical dislocation, and organs (liver, heart, brain, spleen, lung, and kidneys) were harvested for biochemical analysis.

2.4. Preparation of Mitochondrial Fractions and Measurement of ATP Generation Capacity (ATP-GC) *ex Vivo*

Tissue samples were excised and rinsed with ice-cold isolation buffer (0.25 M sucrose, 0.1 mM EDTA (free acid), 5 mM Tris base, pH 7.4). Mitochondrial fractions were prepared through differential centrifugation in the isolation buffer at 4˚C. A 10% (w/v) tissue homogenate was produced by homogenizing the minced tissue using a Teflon-glass homogenizer at 2000 - 4000 rpm for 10 - 20 complete strokes. The homogenate was centrifuged at 600 ×g for 10 min to remove nuclei and cell debris. The supernatant was further centrifuged at 9200 ×g for 30 min to pellet the mitochondria. The pellets were resuspended in 1 mL of respiration buffer (125 mM KCl, 20 mM MOPS, 10 mM Tris base, 5 mM EDTA (free acid), 2 mM KH2PO$_4$, pH 7.2) to reconstitute the mitochondrial fractions.

The protein concentration of mitochondrial fractions was determined using the Bradford method [23].

2.5. ATP Generation Capacity

Before measuring mitochondrial ATP generation capacity (ATP-GC), commercially sourced ADP was enzymatically treated to remove contaminated ATP molecules that could interfere with the accurate measurement of ATP produced by mitochondria. Mitochondrial fractions were prepared by adjusting to 1 mg protein/mL for brain, heart, kidneys, and liver fractions, and 0.5 mg protein/mL for lung and spleen fractions. For the assay, 100 µL of these fractions were mixed with 100 µL of a substrate solution (containing 3 mM pyruvate and 3 mM malate) and 50 µL of pretreated ADP (15 mM for brain, heart, kidneys and liver fractions; 7.5 mM for lung and spleen fractions) solution. The mixtures were incubated for varying durations (0 - 15 minutes for brain, heart, kidneys, and liver fractions; 0 - 5 minutes for lung and spleen fractions) at 37˚C. The reaction was stopped by adding 50 µL of perchloric acid (30%, w/v), followed by centrifugation at 2150 ×g for 10 minutes at 4˚C. An aliquot (120 µL) of the supernatant was neutralized with 90 µL of 1.4 M $KHCO_3$, centrifuged again at 600 ×g for 10 minutes at 4˚C, and the supernatants were analyzed for ATP content using a bioluminescence assay. The mitochondrial ATP-GC for untreated animals was estimated by calculating the area under the curve (AUC1) of ATP generated (nmol/mg protein) plotted against time (0 - 15 or 0 - 5 minutes) and expressed in arbitrary units. For samples from Sch B-treated mice, AUC1 values for increasing incubation times (7.5 and 15 minutes or 3 and 5 minutes) were normalized to the mean control value from untreated mice and expressed as percent control. The area under the curve (AUC2) plotting percent control values against incubation time (7.5 - 15 minutes or 3 - 5 minutes) was calculated and expressed in arbitrary units. Data from Sch B-treated groups were expressed as a percentage of the control using the formula: [AUC2 (Sch B-treated)/AUC2 (untreated)] × 100%. This two-step data processing aimed to minimize inter-animal and inter-assay variability under the experimental conditions [23].

2.6. Measurement of Mitochondrial Glutathione Redox Status

Mitochondrial GSH and GSSG levels were determined enzymatically using DTNB and GR, as previously described [23]. A 210 µL aliquot of the mitochondrial fraction was mixed with 90 µL of 10% SSA, and the supernatant was used to measure GSH and GSSG. The mitochondrial glutathione redox status was expressed as the GSH/GSSG ratio.

2.7. Isolation of Splenocytes

Splenic tissue from mice was processed by pressing through a stainless steel mesh sieve with a glass pestle in 25 mL RPMI-1640 medium to achieve a single-cell suspension. This suspension was centrifuged at 400 ×g for 5 minutes,

and the resulting pellet was washed once with RPMI medium. Red blood cells in the pellet were lysed using 4.5 mL of water, and the lysis was halted by adding 0.5 mL of 10X Hank's Balanced Salt Solution (HBSS: 1.4 M NaCl, 53 mM KCl, 4.4 mM KH_2PO_4, 55.6 mM Glucose and 3.36 mM Na_2HPO_4) and 5 mL RPMI-medium supplemented with 5% (v/v) heat-inactivated (HI) FBS. After another round of centrifugation, the pellet was resuspended in RPMI-1640 medium with 5% HIFBS for cell counting using 0.4% trypan blue. Finally, the splenocytes were diluted to a final concentration of 1×10^7 cells/mL in RPMI-1640 medium supplemented with 5% HIFBS to serve as effector cells for NK cell activity assays. For the Con A/LPS-induced splenocyte proliferation assay, aliquots of cells were prepared at a final concentration of 5×10^6 cells/mL in RPMI-1640 medium supplemented with 10% HIFBS [24].

2.8. NK Cell Activity Assay

YAC-1 cells, used as target cells (T), were seeded in 96-well U-bottom culture plates at a density of 2×10^4 cells/well in RPMI-1640 medium supplemented with 5% HIFBS. Splenocytes, prepared as described earlier, served as effector cells (E) and were added at 1×10^6 cells/well to give an E/T ratio of 50:1. The cell mixture was then incubated for 24 h at 37˚C in atmospheric air containing 5% CO_2. Following incubation, lactate dehydrogenase (LDH) activity in the culture medium was measured as previously detailed. NK cell activity was estimated using the following equation and expressed as the percentage of target cells killed [24].

$$\text{NK cell activity}(\%) = \left[\left(A_{ii} - A_i - A_{iii}\right) \times \left(A_{iv} - A_i\right)\right] \times 100\%$$

where A equals absorbance value of the respective experimental sample at 600 nm; *i*, denotes basal LDH release from target cells; *ii*, denotes LDH release from a mixture of target cells and effector cells; *iii*, denotes basal LDH spontaneously released from effector cells; *iv*, denotes total LDH from target cells.

2.9. Con A/LPS-Induced Splenocyte Proliferation *ex Vivo*

Mouse splenocyte concentration was adjusted to 5×10^6 cells/mL with RMPI-medium supplemented with 10% HIFBS. Then, 90 μL of this cell suspension was seeded in each well of a 96-well plate, either with or without Con A/LPS, to a final volume of 100 μL. Con A/LPS was added at final concentrations of 0, 0.5, 1, 2, 4, or 7.5 μg/mL. The splenocytes were cultured for 72 hours at 37˚C in a humidified atmosphere containing 5% CO_2. Cell proliferation was assessed using MTT as previously described.

The extent of Con A/LPS-stimulated proliferation of isolated splenocytes was determined by calculating the area under the curve (AUC) from a graph that plotted the percentage of initial absorbance (mean absorbance of cells stimulated with Con A or LPS/mean absorbance of cells not stimulated with Con A or LPS × 100%) against the Con A or LPS concentration. The increase in Con A or LPS-stimulated splenocyte proliferation in Sch B-treated mice was estimated by

comparing it with the untreated control and expressed as a percentage of the control [24].

2.10. Statistical Analysis

Expressed as mean ± SD, data were analyzed using one-way ANOVA followed by Tukey's test to detect significant differences between groups when $p < 0.05$.

3. Results

As shown in Figure 1, Sch B treatment had a non-dose dependent or biphasic effect on ATP-GC in various mouse tissues when administered orally at daily doses of 0.3 or 1.0 g/kg. At the lower dose of 0.3 g/kg, Sch B significantly increased ATP-GC across different tissues compared to the untreated control, with the degree of stimulation in descending order: liver > heart > lungs > brain > kidneys > spleen. However, at the higher dose of 1.0 g/kg, while Sch B similarly increased ATP-GC in the brain, kidneys, lungs, and spleen as at the lower dose, the extent of stimulation in the liver and heart tissues decreased from 40% to 11% and 31% to 13%, respectively.

Moreover, Sch B treatment at 0.3 g/kg improved mitochondrial glutathione redox status in the tested tissues, with the improvement ranked from highest to lowest as follows: spleen > liver, heart, lungs, kidneys > brain (Figure 2). At a dose of 1.0 g/kg, Sch B similarly enhanced mitochondrial glutathione redox status in the brain, kidneys, and lung tissues compared to the lower dose. However,

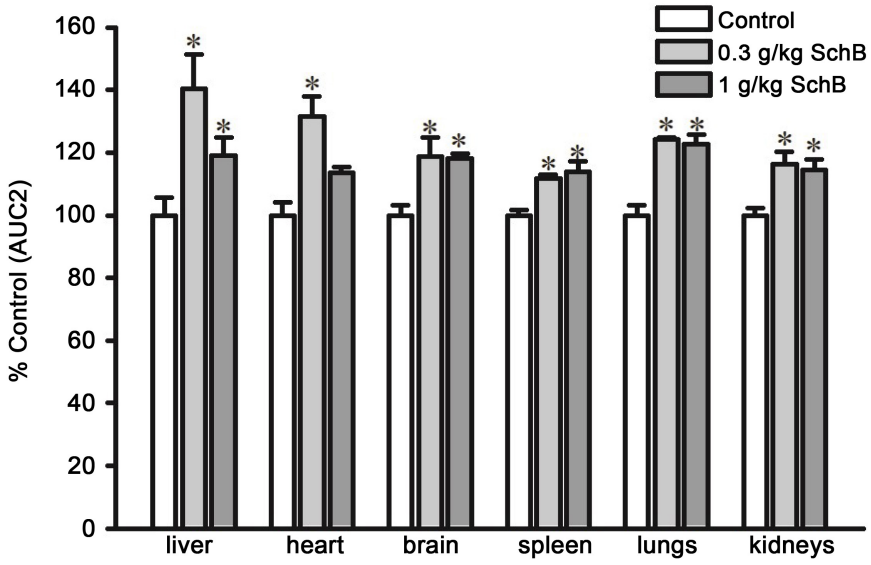

Figure 1. The effect of Sch B on mitochondrial ATP-GC in various mouse tissues was evaluated. Mitochondrial ATP-GC levels were measured as outlined in the Materials and Methods section. The results are presented as a percentage of the control, with values expressed as mean ± SD (n = 4). The control values (AUC_2) were: liver = 500 ± 28.7; heart = 500 ± 23.4; brain = 500 ± 14.7; spleen = 200 ± 8.6; lungs = 200 ± 6.3; kidneys = 500.0 ± 15.3. *Values that are significantly different from control, with a p-value < 0.05, were determined using one-way ANOVA followed by a Tukey test.

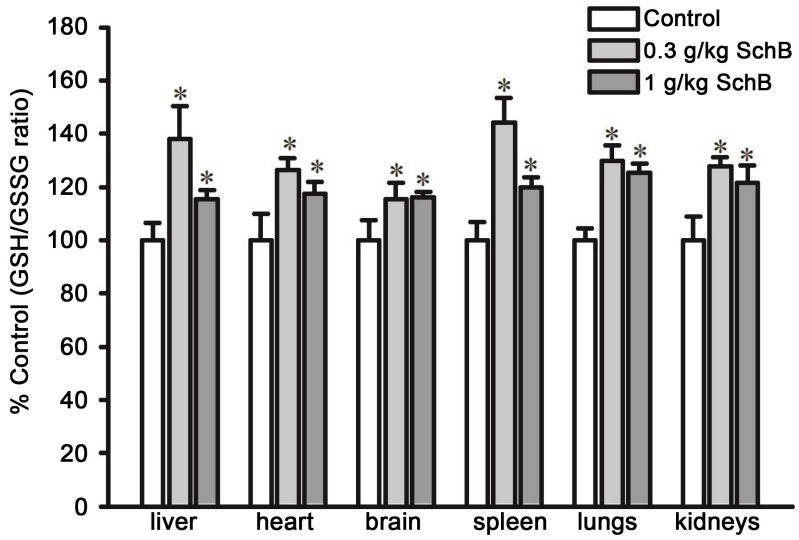

Figure 2. The effect of Sch B on the mitochondrial glutathione redox status in various mouse tissues was investigated. The mitochondrial glutathione redox status was measured as outlined in the Materials and Methods section. The values are presented as percent control compared to untreated controls and are expressed as mean ± SD (n = 4). The control values (GSH/GSSG ratio) were as follows: liver = 17.2 ± 1.2; heart = 20.8 ± 2.8; brain = 30.5 ± 4.1; spleen = 16.9 ± 1.1; lungs = 7.9 ± 0.7; kidneys = 24.1 ± 3.9. Values that are significantly different from control, with a p-value < 0.05, were determined using one-way ANOVA followed by a Tukey test.

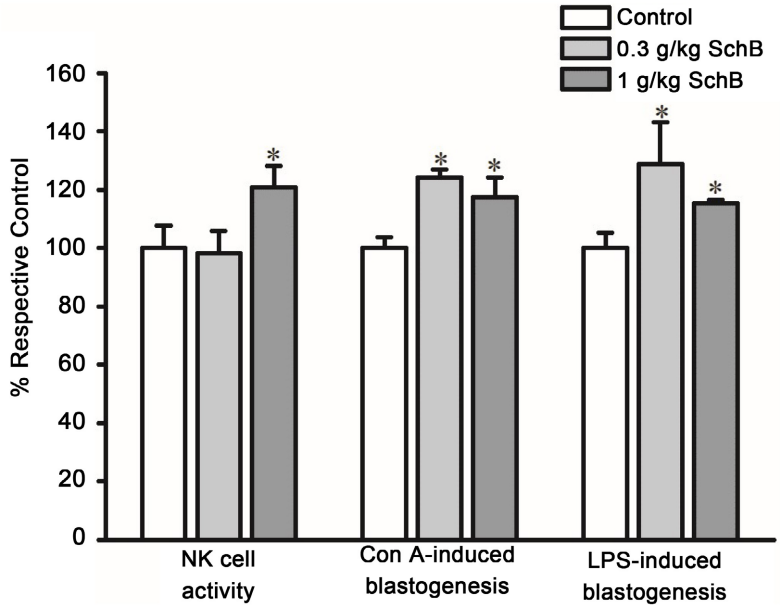

Figure 3. The immunomodulatory effect of Sch B on mice was evaluated by measuring NK cell activity and Con A/LPS-induced splenocyte blastogenesis as described in the Materials and Methods section. The results are presented as a percentage of the respective control, compared with the untreated control, and expressed as mean ± SD (n = 4). The control values were: NK cell activity (%) = 4.5 ± 0.4; Con A-induced blastogenesis (AUC): 3396 ± 445; LPS-induced blastogenesis (AUC): 2263 ± 289. Values that are significantly different from control, with a p-value < 0.05, were determined using one-way ANOVA followed by a Tukey test.

the extent of enhancement decreased in the heart, liver, and spleen tissues from 26% to 17%, 38% to 16%, and 44% to 20%, respectively.

Sch B treatment at a higher dose of 1 g/kg increased NK cell activity by 20% compared to the untreated control. At a lower dose of 0.3 g/kg, Sch B significantly increased Con A/LPS-induced splenocyte proliferation by 24% - 29%. However, at a higher dose of 1.0 g/kg, Sch B had a smaller effect on Con A/LPS-induced splenocyte proliferation, reducing the potentiation from 25% to 15%/29% to 15% (**Figure 3**).

4. Discussion

Schisandrae Fructus has been recognized for its Qi-invigorating effects on the five visceral organs in Traditional Chinese Medicine (TCM) [15]. The study showed that treatment with Schisandrin B, the primary active component of Schisandrae Fructus, increased mitochondrial ATP production in various organs of mice, including the liver, heart, brain, spleen, lungs, and kidneys. This supports the traditional use of Schisandrae Fructus for Qi-invigorating actions in the five visceral organs (liver, heart, spleen, lung, and kidney) according to TCM theory [25] [26]. Moreover, the increase in ATP production was accompanied by an improvement in mitochondrial glutathione redox status, particularly in the liver and heart. This enhancement in antioxidant capacity likely reduces reactive oxygen species produced during mitochondrial respiration, leading to increased ATP generation [21] [27] [28]. This finding aligns with our previous studies, demonstrating that the stimulatory effect of Qi-invigorating Chinese tonifying herbs on ATP-GC is directly linked to an increase in cellular reduced glutathione levels in H9c2 cardiomyocytes [28]. In addition, the protection provided by Sch B treatment against oxidant-induced tissue injury was linked to the improvement of cellular/mitochondrial glutathione redox status [29]-[33]. The nonlinear relationship between Sch B dose and ATP production observed in this study may result from the self-regulating mechanism of mitochondrial ATP generation, which limits the maximum level of stimulation [34] [35] [36].

Sch B treatment enhanced both NK cell activity and antigen-induced T/B cell proliferation in mice, indicating a boost in both innate and adaptive immunity. This increase in NK cell activity may be partly due to the elevation of cellular ATP through enhanced mitochondrial ATP-GC [37]. The increase in antigen-induced T/B cell proliferation was likely related to the enhancement of cellular/mitochondrial glutathione redox status in Sch B-treated mice [38]. A non-linear relationship was also observed between Sch B dose and antigen-induced T/B cell proliferation. This may also be related to the self-limiting mechanism of lymphocyte proliferation [39]. According to Traditional Chinese Medicine (TCM) theory, Zheng Qi is closely linked to the functioning of both innate and adaptive immunity [40]. By invigorating the Qi function of visceral organs, Schisandrin B (Sch B) can enhance the function of these organs and help generate Acquired Qi from the Lung and Spleen, as well as extract Primordial Qi

from the Kidney. These processes are essential for the generation of Zheng Qi. This theory is supported by our previous study, which showed that long-term treatment with Sch B increased the lifespan of mice, indicating improved Zheng Qi generation [20].

5. Conclusion

In conclusion, the present study showed that Sch B treatment consistently increased mitochondrial ATP-GC and improved mitochondrial glutathione redox status in various tissues of mice. This enhancement in mitochondrial ATP generation capacity could lead to the improvement of both innate and adaptive immune responses, possibly by boosting the generation of Zheng Qi according to Traditional Chinese Medicine (TCM) theory.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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