Flavonoids Sophoranone Promotes Differentiation of C2C12 and Extraocular Muscle Satellite Cells

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Keywords

Sophoranone · Strabismus · C2C12 cells · Extraocular muscle · Myogenic differentiation

Abstract

Introduction: Paralytic strabismus involves a functional loss of extraocular muscles resulting from muscular or neuronal disorders. Currently, only a limited number of drugs are available for functional repair of extraocular muscles. Here, we investigated the effects of a novel drug, flavonoids sophoranone, on the differentiation of extraocular muscles as assessed in both in vivo and in vitro models. **Materials and Methods:** The effect of flavonoids sophoranone on C2C12 cells was examined in vitro as evaluated with use of apoptosis, reactive oxygen species (ROS), and cell viability assays. Then, both in vivo and in vitro effects of this drug were examined on the differentiation of C2C12 and satellite cells within extraocular muscles in rabbits. For these latter experiments, RT-PCR and Western blot assays were used to determine expression levels of markers for myogenic differentiation. **Results:** With use of flavonoids sophoranone concentrations ranging from 0 to 10 μM , no effects were observed upon cell apoptosis, ROS, and cell cycle in C2C12 cells. Based

on MTT assay results, flavonoids sophoranone was shown to increase C2C12 cell proliferation. Moreover, flavonoids sophoranone promoted the differentiation of C2C12 and satellite cells within extraocular muscles in rabbits, which were verified as based on cell morphology and expression levels of mRNA and protein markers of myogenic differentiation. Finally, flavonoids sophoranone treatment also increased gene expressions of Myh3, Myog, and MCK. **Conclusion:** The capacity for flavonoids sophoranone to upgrade the differentiation of both C2C12 and satellite cells within extraocular muscles in rabbits at concentrations producing no adverse effects suggest that this drug may provide a safe and effective means to promote repair of damaged extraocular muscles.

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Introduction

Strabismus is a common eye misalignment disorder which often results in amblyopia or loss of depth perception. This condition affects 3–5% of all children, making

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it an important public health problem [1, 2]. The major cause of paralytic strabismus involves a dysfunction in extraocular muscles (EOMs). Accordingly, muscular regeneration/repair represents an important process for a functional recovery of this condition. EOM satellite cells (ESCs), located between the basal lamina and plasma membrane of muscle fibers, are crucial facilitators in muscular repair [3]. Following injury, satellite cell activation is triggered by chemokines, as released from the extracellular matrix or secreted by supporting inflammatory and interstitial cells. The activated satellite cells (myoblasts) will proliferate or self-renew, thereby maintaining a dormant satellite population for future repair [4, 5]. At 3–7 days after injury, myoblasts either fuse to damaged myofibers or fuse together to form myotubes (immature myofibers), with these newly formed myofibers then growing into new mature myofibers [6]. The process of muscle regeneration is regulated by proteins in the basic helix-loop-helix (bHLH) family, and include MyoD, myogenin, Myf4, and Myf5. Pax7 and the Notch pathway are also crucial regulators during muscle regeneration. The development of new drugs which can target these proteins would have the potential to improve or advance muscle regeneration/repair in these dysfunctional EOMs and thus significantly enhance the therapeutic outcomes for these patients.

Flavonoids comprise a class of chemical compounds extracted not only from various plants, such as orange peel, red bayberry, and liquorice, but also vegetables, tea, grain, and wines. The basic structure of flavonoids consists of a C6-C3-C6 skeleton [7], but variations in the configuration of the three carbon chains and location of the phenyl connexion (the 2- or 3-position) generate a number of subgroups, including flavones, flavonols, dihydrogen yellow ketone, dihydrogen flavonol, and different yellow ketones. Results from recent reports have revealed that flavonoids show therapeutic effects on various disorders, such as anti-inflammatory and antispasmodic effects [8, 9], cancer cell inhibition [7], and an amelioration of diabetes [10]. The traditional Chinese medicine *Sophora tonkinensis* is a herbal medicine used for the treatment of diseases related to inflammation. It was reported that the flavonoids from *S. tonkinensis* modulate inflammatory cytokines [11]. In our previous study, we discovered flavonoids from *S. tonkinensis* showing potent anti-neuroinflammatory effects [12]. Given the wide variety of biological effects of flavonoids on antioxidant, apoptosis, necrosis, and cell cycle arrest, we considered it important to investigate the effects of flavonoids sophoranone on muscle regeneration. In particular, the findings that

chronic or excessive inflammatory conditions are considered harmful for muscle regeneration [13] suggest that the potential for anti-inflammatory effects of this flavonoid may serve as a particularly effective agent in the treatment of EOM dysfunction in strabismus. As the effects of flavonoids sophoranone on muscle regeneration are unknown, in this report we investigated the effects of flavonoids sophoranone on muscle regeneration.

Materials and Methods

Reagent

Flavonoids sophoranone was isolated from *S. tonkinensis* by one of our coauthors (Q.G.) from the School of Pharmaceutical Sciences, Sun Yat-sen University. The intermediate acids were synthesized through a Perkin reaction [14] with commercially available phenylacetic acids and benzaldehydes or cinnamaldehydes. The antibodies used for Western blot assays included the myosin heavy chain antibody (ab124205, 1:1,000, Abcam, China), β -tubulin antibody (1:1,000, Affinity, USA), and the secondary rabbit antibody (KC-RB-035, 1:6,000, KangChen Bio-Tech, China).

Cell Culture and Differentiation

DMEM medium was used to culture C2C12 cells (ATCC). Culture and differentiation conditions were according to procedures previously described [15, 16].

Real-Time PCR

Real-time PCR was used to determine relative expression levels of the myogenic differentiation markers: Myog (myogenin), Myh3 (myosin heavy chain 3), MCK (muscle creatine kinase), and GAPDH (as the internal control). Primers were synthesized by Sangon Biotech (Guangzhou, China). Forward and reverse sequence chains were as described previously [15–17].

Apoptosis Measurement, Reactive Oxygen Species Assay, and Cell Viability

As described previously [15–17], the Multicaspase Kit (Millipore) was used to measure apoptosis of C2C12 cells treated or not with flavonoids sophoranone. Reactive oxygen species (ROS) was detected by dihydroethidium (DHE; Millipore, Hayward, CA, USA) and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell viability.

Acute EOM Injury Model

Based on methods described previously [18], a total of 3 New Zealand rabbits, 4–8 weeks old, obtained from Zhongshan Ophthalmic Laboratory Animal Center were used in these experiments. After systemic anesthesia, the belly of medial rectus muscles in both eyes was clamped for 10 min using hemostatic forceps. A subconjunctival injection of flavonoids sophoranone (10 μ M) was then administered into the right eye while an injection of normal saline into the left eye served as a control. These procedures were repeated daily for 3 days. Rabbits were then euthanized on day 4 and the medial rectus muscles from both eyes were removed for RT-PCR and Western blot assay.

Statistical Analysis

Each experiment was repeated at least three times, and statistical analysis was performed using the IBM SPSS Statistics 20 software program. Results are presented as the means and standard deviations. Statistically significant differences between groups were determined by multiple LSD multiple comparison test (one-way analysis of variance). *p* values are shown as * *p* < 0.05, *** *p* < 0.001, and # *p* > 0.05.

Results

Flavonoids Sophoranone Induces C2C12 Cell Proliferation

After 3 days of treatment all C2C12 cells were apoptotic in response to a 20- μ M concentration of flavonoids sophoranone, but not to concentrations ranging from 0 to 10 μ M (Fig. 1a). Therefore, doses of 0–10 μ M of flavonoids sophoranone were used in subsequent experiments. With use of Multicaspase reagent and 7-AAD staining of the cell nucleus, apoptosis within C2C12 cells was assessed in response to a 24-h exposure of flavonoids sophoranone with doses ranging from 0 to 10 μ M and assayed with use of flow cytometry (Fig. 1b). No apoptosis was observed in these C2C12 cells in response to this flavonoids sophoranone treatment, nor were the numbers of ROS+ cells (Fig. 1c) or cell cycle of C2C12 cells (Fig. 1d) significantly altered by this 0- to 10- μ M exposure of flavonoids sophoranone. With use of the MTT assay, we next examined OD values in C2C12 cells at 0, 24, or 48 h after treatment with 0–10 μ M of flavonoids sophoranone. The results of this experiment revealed that flavonoids sophoranone increased C2C12 cell proliferation (Fig. 1e).

Flavonoids Sophoranone Promote Differentiation of C2C12 Cells

The effects of flavonoids sophoranone on differentiation of C2C12 cells was assessed using different approaches. First, we evaluated the morphology of photographed C2C12 cells on the third day of differentiation. In the control group, most C2C12 cells remained as monocytes and were not fused. However, an increase in the number of differentiated cells was observed in response to the 5- μ M sophoranone dose and a further increase was obtained with 10 μ M of sophoranone (Fig. 2a). Then, RT-PCR was performed to verify that mRNA expression levels of myogenic differentiation markers were associated with C2C12 differentiation. As determined on the third day of treatment, sophoranone promoted gene expressions of Myh3, Myog, and MCK in a concentration-dependent manner (Fig. 2b). Finally, at the protein level, as performed using

Western blot analysis, we verified that Myh3, a differentiated marker protein, was increased in response to sophoranone (Fig. 2c).

Sophoranone Promoted Repair of EOMs in Rabbits

The effect of sophoranone on ESCs was examined in vivo in a rabbit model of acute medial rectus muscle injury. Injured medial recti were obtained on day 4 after 3 days of treatment with 10 μ M of sophoranone injected into the right and saline (control) into the left medial rectus muscle. Results from RT-PCR revealed that mRNA levels of Myog, Myh3, and MCK were increased in medial rectus muscles treated with sophoranone versus that obtained in the control (Fig. 3).

Discussion

At present, the most common treatment for strabismus is surgery to re-establish ocular alignment and binocular vision. However, the long-term success rate of 62% following surgery is relatively low and the resultant conjunctival scars and changes in rotational forces following surgery may limit ocular motility [19]. For this reason, drugs such as botulinum toxin A (BTXA) and bupivacaine (BPX) have been used as clinical alternatives to surgery as a means to chemically weaken and strengthen EOMs, respectively. Such drug treatments may promote changes in muscle biomechanics and muscle satellite cell proliferation not attainable with surgery [20, 21]. Other approaches used in the treatment of strabismus include cell growth factors which have been investigated both in vitro and in animal models [22–28]. Despite these advances in drug therapy for strabismus that have been developed over the past 2 decades, these treatments remain insufficient in producing a complete restoration. Currently, no other drugs have been used for the treatment of strabismus.

Interestingly, considerable clinical evidence has accumulated indicating the benefits of herbs for the treatment of a number of human disorders. Due to their lower inci-

Fig. 1. Flavonoids sophoranone induces C2C12 cell proliferation. **a** C2C12 cells were treated with varying concentrations of flavonoids sophoranone (0, 5, 10, 20 μ M) for 3 days. Flow cytometry was used to assess: the viability of C2C12 cells (**b**), intracellular ROS (**c**), and cell cycle (**d**). **e** OD values in C2C12 cells treated with varying concentrations of flavonoids sophoranone (0, 5, 10, 20 μ M) for 12, 24, or 48 h as determined with the use of MTT assay (* *p* > 0.05, * *p* < 0.05, *n* = 3, bars represent SD).

(For figure see next page.)

Fig. 2. Flavonoids sophoranone promote C2C12 cell differentiation. **a** Differentiation of C2C12 myoblasts was induced in response to varying concentrations of flavonoids sophoranone (0, 5, 10 μ M). Photos were taken at 3 days after flavonoids sophoranone treatment. **b** Transcript levels of muscle-specific genes including

Myog, Myh3, and MCK as analyzed with use of qRT-PCR. RNA samples were all collected at differentiation as determined 3 days after flavonoids sophoranone treatment. **c** Protein levels of Myh3 were analyzed with use of Western blot ($n = 3$).

Fig. 3. Flavonoids sophoranone promotes EOM injury repair in rabbits. Flavonoids sophoranone (Soph) at 10 μ M was injected into the right medial rectus and saline (Cont) into the left medial rectus in this rabbit model of EOM injury. Medial recti were obtained on day 4 after treatment and subjected to qRT-PCR assay. **a** Tran-

script levels of muscle-specific genes including Myog, Myh3, and MCK were analyzed with qRT-PCR. RNA samples were all collected at differentiation as determined 3 days after flavonoids sophoranone treatment. **b** Protein levels of Myh3 as analyzed by Western blot ($\# p > 0.05$, $*** p < 0.001$, $n = 3$).

dence of adverse side effects, herbs have become attractive agents for treatments, especially in China. As skeletal muscles, including EOMs, represent the most abundant tissue in the human body and have great regenerative capacity, we considered the potential for use of herbs in the treatment of EOM injury. Specifically, in this study, we evaluated effects of the naturally derived substance sophorane on muscle regeneration as assessed in vitro with the cell line C2C12 and in vivo with use of a rabbit model of EOM injury. The cell line C2C12 is a muscle myoblast from mice and is commonly used in in vitro studies of muscle disorders [29].

When evaluating the effects of any new drug, such as sophorane, a fundamental issue to establish is its capacity for adverse side effects. Any drug can be harmful at high concentrations and flavonoids are no exception [30, 31]. Some flavonoids have been shown to stimulate the generation of ROS, especially hydroxyl radicals, which are responsible for concurrent lipid peroxidation and DNA damage [30]. Other possible adverse effects associated with various plant flavonoids include: (1) inhibition of 5-lipoxygenase and oxygenase activities in peritoneal leukocytes of rats, (2) inhibition of lipid peroxidation in rat liver microsomes, and (3) an acceleration in DNA degradation in the presence of ferric ions [31]. Accordingly, we first needed to determine a range of sophorane doses which proved to be safe when applied to C2C12 cells. We found that with 24 h of sophorane treatment at concentrations of 0–10 μM , three critical parameters of C2C12 cell viability were maintained: (1) no apoptosis was observed, (2) no significant changes in ROS+ cells were present, and (3) the cell cycle was not affected. These findings suggest that sophorane is safe at concentrations lower than 10 μM . More importantly, results from our MTT assays indicated that sophorane promoted cell proliferation at this concentration. Therefore, sophorane may play an important role in treating muscle disorders. In support of this conclusion are results from a previous study demonstrating that various flavonoids reduced the severity of nutritional muscular dystrophy [32], which indicates that flavonoids can influence muscle stem cell differentiation.

The first stage in muscle regeneration is the activation and proliferation of muscle satellite cells. Subsequently, myoblasts stop proliferating and differentiate into myotubes. As sophorane can promote C2C12 proliferation, this may represent one of its beneficial effects in muscle regeneration. With regard to the effects of sophorane on the differentiation of C2C12 cells, we show here that: (1) the ability for differentiation of C2C12 cells was con-

centration dependent on sophorane, (2) mRNA levels of genes associated with C2C12 differentiation (such as Myh3, Myog, and MCK) showed a concentration-dependent increase in response to sophorane, and (3) high levels of Myh3 expression were observed in response to sophorane treatment. Thus, at concentrations of 5 or 10 μM , sophorane increased the expression of differentiation-related genes. These findings led us to study the effect of sophorane on muscle differentiation in EOM stem cells, which contain greater concentrations of enriched satellite cells and more multipotent precursor cells than those in skeletal muscles of the limb [22–24]. Indeed, the EOM provides a particularly effective opportunity and a rich area in which to examine the development of strategies for the potential treatment of muscle pathology [22–24].

To establish whether sophorane may exert any effects on EOM, we tested the effect of this drug in a rabbit model of injured EOM. Our results showed that in rabbits treated with sophorane, the medial recti expressed higher mRNA levels of the Myog, Myh3, and MCK genes than were obtained in the control group. These findings confirmed that sophorane can promote regeneration within injured EOM in vivo and provide evidence suggesting that damaged muscle can be strengthened by sophorane. Therefore, these data strongly indicate that sophorane have a dramatic effect on muscle satellite cells. Based on the results of this preliminary study, we hypothesize that sophorane may promote myosin formation which helps to optimize actin-myosin overlap and repair of injured EOMs. Future work involving the potential use of sophorane in the treatment of strabismus will require investigation into such issues as changes in muscle lengths, muscle fiber size, muscle force, and other compensatory extraocular biomechanics.

There are limitations to this study. First, experiments conducted in the C2C12 cell line may not represent the ESCs. Second, we did not isolate and culture the EOM satellite cells in these rabbits. Only RT-PCR and Western blot assays were used to assess expression levels of Myog, Myh3, and MCK genes in medial rectus muscles in response to sophorane, as associated with muscle regeneration. Third, the display of muscle formation in the EOM of rabbits may not necessarily indicate a functional recovery of the EOM. In this way, the potential effect of sophorane on strabismus and the force change in EOM remains unclear. Finally, the exact molecular mechanisms underlying the effects of sophorane need further study. While our current results show a promising new approach for the treatment of strabismus, much more

work will be required prior to the use of sophorane in clinical practice.

In conclusion, we have shown that flavonoids sophorane can facilitate muscle regeneration as demonstrated in both in vitro and in vivo models at concentrations which produce no apparent adverse effects. Such findings suggest the potential for development of a safe, new drug for use in the treatment of strabismus in patients with EOM damage. However, further basic and clinical research is warranted to establish the overall efficacy and safety of sophorane in patients with strabismus.

Statement of Ethics

No human participants were involved in this study. All animal experiments conformed to the institutional guidelines for the use and welfare of laboratory animals. All experimental procedures were conducted in line with the Guide for the Care and Use of Laboratory Animals and were approved by Zhongshan Ophthalmic Center's ethical guidelines.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the Nature Science Foundation of China (grant No. 81670885). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Author Contributions

Qiong Gu, Huangxuan Shen, and Jianhua Yan conceived this study. Qing Xia, Xiangtian Ling, Minghao Chen, Danyi Mao, Xinqi Ma, Jie Ning, Han Zhang, and Dongli Chen designed and performed the experiments. Qing Xia and Xiangtian Ling wrote the manuscript. Huangxuan Shen and Jianhua Yan edited and improved the final manuscript.

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