Therapeutic strategy for facial paralysis based on the combined application of Si-based agent and methylcobalamin

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ABSTRACT

Facial paralysis results in the decline in the generation of facial expressions and is attributed to several causes. Intractable facial paralysis has a poor prognosis, and new treatments are required. Facial paralysis results in the decline in the generation of facial expressions and is attributed to several causes. Reactive oxygen species can inhibit peripheral nerve regeneration after injury. Therefore, the administration of an appropriate antioxidant can promote nerve regeneration. Silicon (Si)-based agents can react with water to generate antioxidant hydrogen. Oral administration of Si-based agents can effectively alleviate symptoms of disease models associated with oxidative stress. Thus, we orally administered a Si-based agent to a facial paralysis model mice to investigate whether promotion of nerve regeneration occurred. The combined administration of methylcobalamin (MeCbl) with the Si-based agent was also investigated. The Si-based agent improved the clinical score evaluation of facial paralysis. Electroneuronography and immunostaining showed that the Si-based agent promoted myelination and recovery of facial nerve function. Furthermore, in the drug-administered group, oxidative stress associated with facial nerve injury was reduced more than that in the non-administered group. The clinical score evaluation, neuroregeneration effect, and reduction of oxidative stress were improved in the combination group compared to the single administration group. The Si-based agent could rapidly improve the disappearance of facial expressions by promoting myelin sheath formation and alleviating oxidative stress. Combination therapy with a Si-based agent and MeCbl should improve the prognosis and treatment of intractable facial paralysis.

1. Introduction

The facial nerve controls the facial expression muscles, and peripheral facial paralysis causes facial movement dysfunction. Various causes have been identified, such as infection by viruses and bacteria and intracerebral lesions such as cerebral infarction, trauma, and tumors [1]. Bell’s palsy is the most common cause of facial paralysis [2]. Bell’s palsy is purportedly caused by inflammation of the facial nerve due to viral reactivation that damages axons and causes Wallerian degeneration around the damaged area. If the nerve damage is mild, administration of steroids for anti-inflammatory effects will resolve the paralysis [3]; however, if the nerve damage is severe, some facial paralysis is intractable to drug therapy. These patients experience persistent facial contracture and synkinesis as sequelae, which can cause both physical and social distress [4]. Because delayed nerve regeneration affects post-repair deterioration, regenerative medicine is needed to promote regeneration of injured nerves [5].
Oxidative stress is involved in the pathophysiology of facial paralysis. High oxidative stress has been observed in patients with Bell’s palsy due to decreased antioxidant capacity [6]. Moreover, patients with facial paralysis have many autoantibodies against manganese superoxide dismutase, an endogenous antioxidant enzyme [7]. Thus, patients with facial paralysis are subjected to a high degree of oxidative stress, and elimination of oxidative stress leads to treatment because oxidative stress prevent neuroregeneration [8,9]. Antioxidants, such as coenzyme Q10 [10] and polyphenols, such as Ginkgo biloba extract [11], are effective in alleviating facial paralysis symptoms.

Silicon (Si)-based agents can react with water to continuously generate hydrogen, an antioxidant [12]. Furthermore, when a Si-based agent reacts with a weakly alkaline solution, more hydrogen is generated than in the neutral solution. Therefore, oral administration of a Si-based agent can generate hydrogen from the intestinal tract with hydrogen [13]. Si-based agents are demonstrably effective in alleviating the symptoms of various diseases in animal models caused by oxidative stress, such as ulcerative colitis, Parkinson’s disease, and renal failure [13,14]. Since the antioxidant action of hydrogen reportedly promotes nerve regeneration [15], Si-based agents may be effective in alleviating symptoms of facial paralysis and in the provision of early treatment.

However, the treatment method for intractable facial paralysis is also important. The combined administration of steroids and methylcobalamin (MeCbl) is reportedly more effective than the individual administration of each drug [16]. Therefore, the effectiveness of the combined administration of a Si-based agent and MeCbl, in addition to the single administration of a Si-based agent, should be investigated.

MeCbl is an active vitamin B12 that has been reported to promote nerve regeneration and is effective for facial paralysis [16]. To increase its effectiveness, the stable administration of MeCbl was made possible by placing a nanofiber sheet incorporating MeCbl, which can be continuously administered to the nerve injured area. Since the MeCbl administration method using a nanofiber sheet incorporating MeCbl is very effective for nerve regeneration in sciatic nerve injury [17], the effectiveness of MeCbl administration with a biological sheet for facial paralysis is expected.

Therefore, this study investigated the effectiveness of a single administration of a Si-based agent and its combined administration with MeCbl for facial palsy of facial nerve crush-treated mice.

2. Methods

2.1. Animals

C57Bl/6J male mice (8–11 weeks old) were used in all the experiments (CLEA Japan Inc., Tokyo, Japan). They were housed at 23–25 °C and fed special rodent pellets and water ad libitum. All experimental procedures were approved by the Animal Ethics Committee of Osaka University (approval number 02-012-004), in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of experimental animals and optimize their living environments. This study was conducted in compliance with the Animal Research: Reporting of In Vivo Experiments guidelines.

2.2. Si-based agent and diets

Si-based agents were prepared as previously described [12,14]. The Si-based agent was produced from intrinsic (i-type) polycrystalline Si. After crushing using the bead milling method, the surface treatment was conducted to improve the hydrogen generation ability. Special rodent diets (Oriental Yeast Co., Ltd., Tokyo, Japan), AIN93M (control diet), and AIN93M containing 2.5% Si-based agent (Si-based agent-containing diet) were prepared as previously reported [14]. One week before the facial nerve crush procedure, the mice were initiated on both diets.

2.3. Electrospun poly-(ε-caprolactone) (PCL) sheet incorporating MeCbl

As previously described [17], PCL was synthesized by ring-opening polymerization of the terminal hydroxyl group of tetramethyleneglycol, catalyzed by tin octanate. Subsequently, PCL was purified via reprecipitation with hexane and diethyl ether. After electrospinning PCL and MeCbl using an applied voltage of 20 kV (Nanon-01A, MECC Co., Ltd., Fukuoka, Japan) with an 11-cm needle (24-gauge), nanofibers were produced by separation using a collector plate. Characterization of the nanofiber sheet containing 3% MeCbl was performed using FE-SEM (SU8000, Hitachi High-Technologies Corporation, Tokyo, Japan). A nanofiber sheet containing 3% MeCbl (3 × 5 mm) was implanted after compression of the right facial nerve. The sheet was placed on the parotid gland above the facial nerve so that it did not directly contact the facial nerve.

2.4. Preparation of facial paralysis model mice

Mice were deeply anesthetized using an intraperitoneal injection of a mixture of midazolam (2 mg/kg), butorphanol (2.5 mg/kg), and medetomidine (0.15 mg/kg). Under sterile conditions, the main trunk of the bilateral facial nerves was exposed below the parotid gland and released from the surrounding connective tissue (Fig. 1A). The right facial nerve was untouched (sham-operated control side). The facial nerve on the left side was compressed for 10 s three times at 10-s intervals at the proximal 2–3 mm from the bifurcation of the facial nerve trunk using a needle holder (No.12500-12; Fine Science Tools, North Vancouver, Canada) (Fig. 1B). The muscle and skin were then closed. All the surgeries were performed by the same surgeon. After compression, the mice were randomly assigned to four experimental groups: the control diet-fed non-treated group (Con group), Si-based agent-containing diet-fed non-treated group (Si group), control diet-fed MeCbl-treated group (MeCbl group), and Si-based agent-containing diet-fed MeCbl-treated group (Si/MeCbl group).

2.5. Clinical evaluation of facial paralysis

Facial nerve function was determined using total scores for eye blinks, nasal movements, and whisker movements. According to a previous study [18], the clinical signs of facial paralysis were monitored at 3, 6, 8, 10, 13, 15, 17, 20, and 22 days following nerve compression. Eye blinks, nasal movements, and whisker movements were scored separately, and facial paralysis was evaluated with a total score of the three items. For each, a score of 0 was assigned for no detectable movement, 1 for detectable motion, 2 for significant but asymmetric voluntary motion, and 3 for symmetric voluntary motion. The total score was 9 points for healthy mice and 0 for severe facial paralysis.

2.6. Electrophysiological evaluation by electroneurography (ENoG)

The amplitude of the evoked compound muscle action potential of the buccinator muscle was examined for each mouse using conventional procedures with an electromyography monitor (Tucker-Davis Technologies, Alachua, FL, USA) 22 days post-surgery. Under anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), the bilateral
facial nerve was exposed, and the nerve was stimulated supermaximally by single 5-ms electrical pulses with a frequency of 1 Hz at the proximal 7–8 mm from the bifurcation of the facial nerve trunk (proximal to the injury site). The earth electrode was inserted into the crown, and two electrodes were inserted into the buccinator muscle. The amplitude of the compound muscle action potential (CMAP) was measured from the peak to the trough of the evoked response. The CMAP ratio was calculated by dividing the CMAP value on the affected side by the contralateral (unaffected) side.

2.7. Immunofluorescence staining

Under perfusion using 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4), the facial nerve was dissected and postfixed in the above fixative at 4 °C overnight, immersed in 0.1 M PB containing 30% sucrose at 4 °C, and then frozen in dry ice. The frozen samples were cut into 10-μm thick sections, mounted on MAS-coated slide glasses (Matsunami Glass, Osaka, Japan), and stored at −80 °C until use. Immunofluorescence staining was performed as previously described [19]. After air-drying, the sample slides were treated with 0.01 M phosphate-buffered saline (PBS) containing 0.3% Triton-X and 3% bovine serum albumin for 30 min to increase permeability to antibodies and inhibit non-specific staining. The slides were incubated with anti-neurofilament 200 (NF200) rabbit polyclonal antibodies (1:2000; catalog no. N4142; Merck KGaA, Darmstadt, Germany) and anti-myelin basic protein (MBP) rat monoclonal antibodies (1:500; catalog no. MAB386; Merck KGaA) in a blocking buffer at 4 °C overnight. The sections were thoroughly washed in 0.01 M PBS and then treated with Alexa488 conjugated anti-rabbit IgG goat polyclonal antibody (1:2000; catalog no. N4142; Merck KGaA, Darmstadt, Germany) and anti-myelin basic protein (MBP) rat monoclonal antibodies (1:500; catalog no. MAB386; Merck KGaA) in a blocking buffer at 4 °C overnight. The sections were thoroughly washed in 0.01 M PBS and then treated with Alexa488 conjugated anti-rabbit IgG goat polyclonal antibody (1:2000; catalog no. N4142; Merck KGaA, Darmstadt, Germany) and Alexa568 conjugated anti-rat IgG goat polyclonal antibody (1:500; catalog no. ab175476; Abcam) in 0.01 M PBS for 1 h. After washing several times,
the slides were sealed with cover glass using PermaFluor (Thermo Fisher Scientific). The stained samples were analyzed using a Keyence microscope (Keyence Corporation, Osaka, Japan).

2.8. Oxidative stress measurement

Oxidative stress measurement was performed as previously described [13]. Under deep general anesthesia, whole blood was obtained from the right atrium of the following groups: Con, Si, MeCbl, and Si/MeCbl groups. Blood was centrifuged (3000 rpm, 10 min, 4 °C) and serum was collected. The serum was stored at −80 °C until use. To investigate the serum levels of reactive oxygen species (ROS) metabolites and anti-oxidative capacity, the levels of reactive oxygen metabolite-derived compounds (dROMs) and biological antioxidant potential (BAP) were measured by REDOXLIBLA (Wismerll Co. Ltd., Tokyo, Japan). The results of dROM test were shown as arbitrary units (U. Carr); 1 U Carr corresponds to 0.8 mg/L of hydrogen peroxide [20]. BAP indicated the reducing power of blood using the amount of trivalent iron ions (μM) reduced to divalent iron ions as an indicator. Comparative analysis was performed using the dROMs value and the ratio of BAP divided by dROMs.

2.9. Evaluation of immunofluorescence signals and statistical analysis

The percentage of colocalization of NF200 and MBP was measured using a Keyence microscope to estimate facial nerve myelination. Data are expressed as the mean ± standard error of the mean (SEM). The results of the Student’s t-test were considered significant at †p < 0.08, ††p < 0.06, *p < 0.01, and **p < 0.001 vs. the Con group.

3. Results

We investigated the effectiveness of a Si-based agent for facial paralysis in peripheral crushed facial nerve (7NC) mice. First, the degree of recovery from facial paralysis was examined over time based on eye, nose, and whisker movements. As a result of quantification based on the clinical score for facial paralysis, the Con group was 3.5 after 8 days, 6 after 13 days, and finally 8.5 after 20 days. In the Con group, the facial nerve function recovered after approximately 20 days (Fig. 1C). In contrast, the scores of the other groups were low until 10 days later, but after 13 days, the scores of the three groups were higher than those of the Con group. In particular, recovery was observed on day 15 in the Si/MeCbl group. The number of days when the score reached

![Fig. 2. Functional assessment for facial nerve using electroneurography (ENoG). (A–E) The representative waveform of the contralateral side in the Con group (A), of the injury side in the Con group (B), Si group (C), MeCbl group (D), and Si/MeCbl group (E). (F) Table of the compound muscle action potential (CMAP) findings on the contralateral side in the Con group and the injury side in each group. (G) Bar graph of the ratio of the CMAP value (injury side/contralateral side) in the Con (red: n = 7), Si (black: n = 8), MeCbl (green: n = 10), and Si/MeCbl (blue: n = 10) groups. Data are expressed as the mean ± standard error of the mean of seven to 10 mice per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

![Table 1: The value of CMAP on the injured side in each group.](image)

| Group          | Average (mV) | Standard error (mV) |
|----------------|--------------|---------------------|
| Con (N=7)      | 4.98         | 0.94                |
| Si (N=8)       | 2.10         | 0.61                |
| MeCbl (N=10)   | 3.40         | 0.42                |
| Si/MeCbl (N=10)| 2.59         | 0.42                |

![Graph 2: Ratio of CMAP value (affected side/unaffected side).](image)
the perfect score between the groups, the number of days in the Si and MeCbl groups tended to be shorter than those in the Con group. Furthermore, the number of days in the Si/MeCbl group was the shortest among all groups. (Fig. 1D). The difference between the Con and Si/MeCbl groups was significant, and all mice in the Si/MeCbl group showed complete recovery by day 22.

Taken together, it was clarified that the single administration of a Si-based agent tended to accelerate the therapeutic effect on facial paralysis, and in particular, the combined treatment with MeCbl recovered facial expression at an earlier stage.

Next, we performed an evoked myoelectric potential test, ENoG, to examine the functional restoration of the facial nerve. As shown in a representative waveform example of the Con group, the amplitude was small, and the response was delayed on the lesion side compared with the contralateral sham-operated side (Fig. 2A and B). Injury severely reduces facial nerve neurotransmitter function. In contrast, in the waves of the other three groups, although the reaction was similar to that in the Con group, the amplitude was larger than that in the Con group (Fig. 2C–E). The average of the amplitude of the CMAP on the injured side in the other groups was higher in the order of the Si group, MeCbl group, and Si/MeCbl group than that in the Con group (Fig. 2F). Thus, it was found that the decrease in nerve function in the three treated groups was greater than that in the Con group. Subsequently, the ratio of the CMAP value on the affected side to that on the unaffected side was also investigated. The ratio was 40% in the control group. In contrast, the ratio of the other three groups was 50%, which was approximately 10% higher than that of the Con group (Fig. 2G). Taken together, the Si-based agent promoted the recovery of facial nerve function by approximately 1.25 times both in the single and combined administration groups.

Subsequently, to examine the morphology of the facial nerve, we performed double fluorescent immunostaining for MBP (a marker of the myelin sheath) and NF200 (a marker of axons). The co-positive rate of MBP and NF200 in the caudal facial nerve on the lesion side of the other three groups was significantly higher than that in the Con group (Fig. 3A–C). In particular, in the Si group, the myelination rate on the lesion side was almost the same as that on the contralateral side (Fig. 3A–C). No significant difference in the myelination rate of the rostral injured facial nerve was observed among the four groups (Fig. 3B and C). Further, on the contralateral side, no difference was observed in any group, regardless of the rostral or caudal side (Fig. 3D and E). Taken together, it was clarified that myelin sheath formation in nerve regeneration was promoted by the single or combined administration of a Si-based agent.

Finally, we analyzed oxidative metabolites in the serum to determine whether the recovery of facial nerve function in the administration group was due to the alleviation of oxidative stress 2 weeks after facial paralysis. An increase in ROS was detected in the Con group, but no such increase was observed in the other three groups (Fig. 4A). Especially in the Si/MeCbl group, the increase was remarkably suppressed. Furthermore, we also analyzed the antioxidant capacity in the serum. The BAP/ΔROMs ratio was lowest in the Con group and higher in the other groups (Fig. 4B). In the Con group, oxidative stress increased due to decreased antioxidant capacity as well as excess ROS. However, in other groups, the antioxidant capacity was not decreased and eliminated ROS; hence, it was found that oxidative stress was alleviated. Interestingly, at ΔROM values of ≥50, there were four in the Con group, one in the MeCbl and Si groups, and none in the Si/MeCbl group. These results showed that increased oxidative stress associated with facial paralysis was detected in 60%–70% of the Con group, but decreased to 20% of Si or MeCbl group, and was not observed at all in the Si/MeCbl group. In conclusion, it was proved that the Si or MeCbl administration relieved the systemic oxidative stress associated with facial paralysis.

4. Discussion

In the present study, we investigated the efficacy of a Si-based agent for facial paralysis in 7NC mice. The clinical score for facial paralysis revealed that the administration of a Si-based agent accelerated the recovery of the injured facial nerve function. Furthermore, ENoG, immunohistochemistry, and oxidative stress analysis demonstrated that the Si-based agent significantly increased myelination in the injured facial nerve and significantly restored the function of the facial nerve via oxidative stress relief. Surprisingly, it was found that the combined administration of a Si-based agent and MeCbl, which promotes nerve regeneration, significantly accelerated recovery compared to monotherapy. Since the combined administration of MeCbl accelerates the recovery of symptoms, it is expected to become an effective treatment method for intractable facial paralysis in the near future.

4.1. Facial paralysis and oxidative stress

In peripheral nerve injury, oxidative stress is involved in mitochondrial dysfunction, demyelination, neuroinflammation, and apoptosis, leading to worsening symptoms and delayed repair [21]. Cysteine residues in tubulin, tau, and microtubule-associated protein 2 are susceptible to oxidation by nitric oxide and peroxynitrite. Axonal components, microtubules, and microtubule-binding proteins are potential targets for ROS. Oxidative microtubules and microtubule-binding proteins inhibit microtubule polymerization, thereby preventing neurite outgrowth [8, 9]. Conversely, nitric oxide synthase inhibitor promoted nerve regeneration in facial nerve lesion model mice [22, 23]. Additionally, the activation of endogenous antioxidant enzymes, such as superoxide dismutase and catalase, also promoted functional recovery of the sciatic nerve through oxidative stress reduction in sciatic nerve lesion model mice [24]. Although antioxidant administration has been shown to improve oxidative stress-induced cell damage [25], vitamins and polyphenols act on all ROS, which may result in side effects such as a decrease in the immune response. Recent studies have suggested that an overdose of antioxidants interfered with essential defense mechanisms in the body and increased case fatality and cancer incidence [26, 27]. Therefore, antioxidants that eliminate only harmful ROS are required for treatment.

Hydrogen is an excellent antioxidant; unlike other antioxidants, it can specifically eliminate harmful ROS (e.g., hydroxyl radicals) and reactive nitrogen species (e.g., peroxynitrates) [16]. Hydrogen has been reported to be effective in alleviating the symptoms of various nervous system diseases, such as neurodegenerative diseases, psychiatric disorders, and neuropathic pain [28, 29]. In contrast, in an ulcerative colitis mouse model, a Si-based agent generated a large amount of hydrogen in the intestinal tract and alleviated colitis symptoms through anti-inflammatory and antioxidant effects [13]. Since increased systemic oxidative stress is also observed in patients with facial paralysis [6], oxidative stress is closely related to the pathology. In fact, we clarified that systemic oxidative stress associated with facial nerve injury was alleviated by Si-based agent administration (Fig. 4). These results suggest that the Si-based agent reduced the oxidative stress associated with nerve damage, thereby reducing damage to facial nerve cells and promoting nerve repair. Moreover, since MeCbl also has an antioxidant effect [30], oxidative stress was most alleviated in the Si/MeCbl group (Fig. 4). In this study, systemic oxidative stress was observed in facial paralysis model mice, suggesting that the alleviation of oxidative stress is greatly related to the promotion of functional recovery. In fact, the Si/MeCbl group, in which oxidative stress was significantly alleviated, had the shortest number of days for functional recovery among all groups. In summary, it was proved that the antioxidant effects of both Si-based agent and MeCbl are very effective in functional recovery from facial paralysis.

4.2. Good in vivo administration method of hydrogen and MeCbl

In this study, we chose MeCbl for the concomitant administration of a Si-based agent. The combined administration of MeCbl and steroids, an anti-inflammatory agent, has been shown to be more effective than a
Fig. 3. Morphological analysis for facial nerve regeneration using immunofluorescent staining. (A) The cross-section photomicrographs of facial nerve double-stained with NF200 (green) and MBP (red). Lined up in order from the left, Con (red: n = 8), Si (black: n = 8), MeCbl (green: n = 9), and Si/MeCbl (blue: n = 10) and from the upper rostral lesion side, caudal lesion side, rostral contralateral side, and caudal contralateral side. (B–E) High magnification photographs of the facial nerve in the lesion side (B) and the contralateral side (D) double-stained with NF200 (green) and MBP (red), and bar graph of the average of co-positive with NF200 and MBP in the lesion side (C) and contralateral side (E). (B, D) Lined up in order from the left, Con, Si, MeCbl, and Si/MeCbl. Upper panels: rostral lesion side; bottom panels: caudal lesion side. Scale bar: 100 μm (A) and 20 μm (B, D). (C, E) Data are expressed as the mean ± standard error of the mean of seven to 10 mice per group. *p < 0.05, **p < 0.001 vs. Con group, determined by Student’s t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 4. Evaluation of oxidative stress associated with facial paralysis. (A, B) The bar graph of the average of dROMS value (A) and BAP/dROMS ratio (B). Lined up in order from the left, Con (red: n = 6), Si (black: n = 5), MeCbl (green: n = 5), and Si/MeCbl (blue: n = 5). Data are expressed as the mean ± standard error of the mean of five to six mice per group. \[ \frac{\text{p} < 0.06}{\text{vs Con group}.} \] (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

recovery of facial paralysis has a very good prognosis and significantly improves QOL. Hydrogen and MeCbl have already been used in the medical field; hence, it is expected that the combination therapy of this Si-based agent and MeCbl will be clinically applied as a new treatment method for facial paralysis including intractable disease.

Author contributions

Y.K., S.H., and T.S. designed the study, analyzed the data, and wrote the paper. Y.K., S.H., T.S., H.Y., K.O., T.I., Y.O., and T.K. performed the experiments and quantifications. Y.K. and H.K. developed the method for fabrication of Si-based agent. T.I. and H.T. developed the method for fabrication of the nanofiber sheet incorporating methylcobalamine. H.I. and S.S. supervised this study and provided intellectual directions. All authors discussed the findings and commented on this manuscript.

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Declaration of competing interest

The authors report no conflicts of interest.

Data availability

No data was used for the research described in the article.

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5. Conclusion

Facial paralysis is directly linked to facial expression loss, which greatly impairs the patient’s quality of life (QOL). Therefore, early recovery is desired. In the 7NC mice, the Si-based agent showed a tendency to promote recovery of symptoms by promoting myelination via mitigation of oxidative stress. In particular, the combined administration of the Si-based agent and MeCbl significantly accelerated the recovery of symptoms compared to the single administration. Early
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