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Original Article

Effects of Melatonin and Dexamethasone on Facial Nerve Neurorrhaphy

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OBJECTIVES: To investigate the effects of topical and systemic administrations of melatonin and dexamethasone on facial nerve regeneration.

MATERIALS and METHODS: In total, 50 male albino Wistar rats underwent facial nerve axotomy and neurorrhaphy. The animals were divided into 5 groups: control, topical melatonin, systemic melatonin, topical dexamethasone, and systemic dexamethasone. Nerve conduction studies were performed preoperatively and at 3, 6, 9, and 12 weeks after drug administrations. Amplitude and latency of the compound muscle action potentials were recorded. Coapted facial nerves were investigated under light and electron microscopy. Nerve diameter, axon diameter, and myelin thickness were recorded quantitatively.

RESULTS: Amplitudes decreased and latencies increased in both the melatonin and dexamethasone groups. At the final examination, the electrophysiological evidence of facial nerve degeneration was not significantly different between the groups. Histopathological examinations revealed the largest nerve diameter in the melatonin groups, followed by the dexamethasone and control groups ($p<0.05$). Axon diameter of the control group was smaller than those of the melatonin (topical and systemic) and topical dexamethasone groups ($p<0.05$). The melatonin groups had almost normal myelin ultrastructure.

CONCLUSION: Electrophysiological evaluation did not reveal any potential benefit of dexamethasone and melatonin in contrast to histopathological examination, which revealed beneficial effects of melatonin in particular. These agents may increase the regeneration of facial nerves, but electrophysiological evidence of regeneration may appear later.

KEYWORDS: Facial nerve, axotomy, neurorrhaphy, compound muscle action potential, regeneration

INTRODUCTION

Facial nerve (FN) injury is a relatively common clinical entity that results in both functional and cosmetic consequences [1, 2]. Its extracranial course and superficial location renders the FN susceptible to external damaging factors [3]. Transection of the FN should be repaired surgically by coaptation of the proximal and distal ends, an intervention known as neurorrhaphy [4]. To enhance the regenerating process, many experimental trials have been conducted studying the effects of a variety of agents [3-8].

Melatonin is involved in many physiological processes and has potent anti-inflammatory, antioxidant, and neuroprotective properties [4, 9-11]. Corticosteroids, commonly used in the treatment of nerve injuries, attenuate perineural inflammation and may prevent neuronal death and promote the recovery process [5, 10].

In the present study, we examined the effects of topical and systemic administrations of both corticosteroids and melatonin on nerve regeneration in an FN axotomy and neurorrhaphy model.

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MATERIALS AND METHODS

Fifty male albino Wistar rats (200-250 g) were used in the experiments. The animals were exposed to a 12-h light/dark cycle and had free access to standard rodent diet and water. The study was approved by the Ethics Committee of Bezmialem University Animal Care and Use (2013/14) and conducted according to ethical standards.

The animals were anesthetized using Ketamine 30 mg/kg (Ketalar, Eczacibasi, Istanbul, Turkey) and Xylazine 6 mg/kg (Rompun, Bayer, Istanbul, Turkey), and all of the procedures except for intraperitoneal injections were performed under general anesthesia. After disinfection of the surgical field, a 2-cm horizontal incision below the left auricle was made, and the FN trunk was identified and completely transected midway between the stylomastoid foramen and the point of bifurcation. Immediately thereafter, end-to-end anastomosis of the proximal and distal stumps was performed with 2 8-0 nylon epineural sutures in all animals. The right FN served as an innervated control. The surgical procedures were performed with an operating microscope. Before surgical intervention, all the animals received cefazolin sodium 40 mg/kg (Cefozin, Bilim, Istanbul, Turkey) intraperitoneally.

The rats were randomly divided into 5 groups, each containing 10 animals:

A. Topical administration of a saline-soaked gelfoam over the site of neurorrhaphy+intraperitoneal saline
B. Topical administration of a melatonin (20 mg/mL)-soaked gelfoam over the site of neurorrhaphy+intraperitoneal saline
C. Topical saline+intraperitoneal melatonin (20 mg/kg)
D. Topical administration of a dexamethasone (4 mg/mL)-soaked gelfoam over the site of neurorrhaphy+intraperitoneal saline
E. Topical saline+intraperitoneal dexamethasone (1 mg/kg)

Melatonin (Sigma Aldrich M5250, Milwaukee, WI, USA) solution was freshly prepared before injection by dissolving indoleamine in absolute ethanol and further dilution with normal saline; the final concentration of ethanol was 5%. Melatonin at a daily dose of 20 mg/kg body weight was administered intraperitoneally for 7 consecutive days. Intraperitoneal dexamethasone (Dekort 8 mg/2 mL amp; Deva Holding AS, Istanbul, Turkey) was administered at a dose of 1 mg/kg for 7 days. Topical drugs and saline were applied with a compressed gelfoam over the site of anastomosis.

Electrophysiological Evaluation

Nerve conduction study was performed with a Neuro-MEP 2-channel digital EMG device (Neurosoft, Russia) by one neurologist and evaluated by another in a blinded fashion. Superficial recording electrodes (Plaquette Adhesive Surface Electrodes, TE/K50431-002; Technomed, USA) were placed over the lip muscles, whereas the needle ground electrode (Subdermal Single Needle Electrodes, 13 mm in length-0.4 mm in diameter [27G], TE/S50716-002; Technomed) was inserted into the right posterior thigh region. The FN was stimulated by transcutaneous supramaximal stimulus intensity (10% above the level necessary to achieve maximal amplitude) distal to the site of axotomy and neurorrhaphy. Latency (ms) and the peak amplitude (mV) of the compound muscle action potentials (CMAP) were recorded and compared with those of the healthy control site. Axonal degeneration was calculated using the following formula: 1-[mV (left)/mV (right)], and latency difference was calculated using the following formula: ms (left)-ms (right). Electrophysiological evaluation was performed before FN axotomy and 3, 6, 9, and 12 weeks after the end of drug administration.

Histopathological Examination

At the 12th week after drug administration, the skin incision was reopened and the coapted segment was resected; the animals were sacrificed with sodium thiopental.

Light microscopy: FN samples were fixed in 10% formaldehyde solution for 48 h and then embedded in paraffin blocks for routine histologic processing. Of note, 6-µm-thick sections were prepared from the paraffin blocks, and these were stained with hematoxylin-eosin (H-E). The slides were examined under light microscopy (Nikon Optiphot-2), and images were captured and interpreted using a camera and a vision analysis system (NikonDS-Fi2 camera and Nikon DS L3), respectively. The diameter of the FN was evaluated by the senior histologist who was blinded to the treatment groups.

Transmission electron microscopy (TEM): In this study, 2-mm-long FN tissue samples were taken for TEM (Leica EM AMW Automatic Microwave Tissue Processor; Leica Microsystems GmbH, Wetzlar, Germany) and fixed in 2.5% glutaraldehyde and 1% osmium tetroxide solution. The samples were embedded in araldite blocks by dehydrating with acetone. Sections of 80-nm thickness were prepared with an ultramicrotome on a copper grid. Following contrasting with uranyl acetate and copper citrate, images were captured and examined (Carl-Zeiss, Oberkochen, Germany) by the senior pathologist who was blinded to the treatment groups. The axonal diameter and the thickness of the myelin sheath were measured quantitatively by examining a group of randomly selected 100 nerve fibrils.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 22.0 (IBM Corp.; Armonk, NY, USA) was used for statistical analysis. Mean, standard deviation, and median were used for descriptive statistics. Distribution of the variables was evaluated with Kolmogorov–Smirnov test. For the analysis of quantitative data, Kruskal–Wallis and Mann–Whitney U tests were chosen, and Wilcoxon test was used for continuous measurements. A $p<0.05$ was considered statistically significant.

RESULTS

All the animals tolerated the surgical procedures and the electrophysiological evaluation well. There was no postoperative wound infection, and no animal was lost during the study period.

The mean amplitudes and latencies of each group are given in tables 1 and 2, respectively. Neither the mean amplitude nor the latency variables were significantly different in intergroup comparisons at the postoperative 3rd, 6th, 9th, and 12th weeks ($p>0.05$).

In all the groups, the mean amplitude of postoperative CMAPs measured at all time intervals decreased significantly compared with the preoperative period ($p<0.05$) (Table 1). The change in mean amplitude compared with the preoperative period was not significantly different

Table 1. Compound muscle action potential amplitude values (mV) and the change in amplitude relative to the preoperative period

AMPLITUDE (mV)	Group A	Group B	Group C	Group D	Group E	p
Preop	9.6±1.6	9.1±1.3	9.0±1.3	10.0±1.3	9.4±1.1	0.389
Postop 3rd week	2.8±1.1	2.4±1.1	2.3±1.1	2.7±1.1	2.5±0.9	0.732
Postop 6th week	2.6±0.7	3.2±0.8	3.0±0.8	3.5±0.7	3.5±0.9	0.100
Postop 9th week	5.0±1.3	3.8±0.9	4.2±0.9	4.4±0.5	4.1±0.9	0.057
Postop 12th week	5.2±1.5	4.4±0.8	4.9±1.3	5.5±0.6	4.9±1.1	0.261
Δ preop–postop 3rd week	−0.7±0.1	−0.7±0.1	−0.7±0.1	−0.7±0.1	−0.7±0.1	
	p=0.012*	p=0.005*	p=0.005*	p=0.008*	p=0.005*	0.993
Δ preop–postop 6th week	−0.7±0.1	−0.6±0.1	−0.7±0.1	−0.6±0.1	−0.6±0.1	
	p=0.012*	p=0.005*	p=0.005*	p=0.012*	p=0.005*	0.179
Δ preop–postop 9th week	−0.5±0.1	−0.6±0.1	−0.5±0.1	−0.6±0.1	−0.6±0.1	
	p=0.012*	p=0.008*	p=0.005*	p=0.008*	p=0.008*	0.489
Δ preop–postop 12th week	−0.4±0.2	−0.5±0.1	−0.5±0.1	−0.4±0.1	−0.5±0.1	
	p=0.017*	p=0.008*	p=0.005*	p=0.012*	p=0.012*	0.802

Preop: Preoperative; Postop: Postoperative; Δ: the change between

Table 2. Compound muscle action potential latency values (ms) and the change in latency relative to the preoperative period

LATENCY (ms)	Group A	Group B	Group C	Group D	Group E	p
Preop	1.0±0.1	0.9±0.1	0.9±0.1	0.9±0.1	1.0±0.1	0.051
Postop 3rd week	1.6±0.2	1.5±0.2	1.4±0.1	1.5±0.1	1.5±0.1	0.081
Postop 6th week	1.6±0.3	1.4±0.3	1.3±0.1	1.3±0.1	1.3±0.1	0.063
Postop 9th week	1.3±0.2	1.3±0.3	1.2±0.1	1.2±0.1	1.2±0.1	0.676
Postop 12th week	1.1±0.2	1.2±0.3	1.1±0.1	1.2±0.1	1.2±0.0	0.466
Δ preop–postop 3rd week	0.7±0.3	0.8±0.2	0.7±0.2	0.6±0.2	0.6±0.2	
	p=0.012*	p=0.005*	p=0.005*	p=0.007*	p=0.005*	0.335
Δ preop–postop 6th week	0.7±0.3	0.6±0.4	0.7±0.2	0.6±0.2	0.6±0.2	
	p=0.012*	p=0.005*	p=0.004*	p=0.011*	p=0.004*	0.062
Δ preop–postop 9th week	0.4±0.2	0.5±0.4	0.4±0.2	0.3±0.2	0.2±0.2	
	p=0.011*	p=0.011*	p=0.005*	p=0.007*	p=0.017*	0.241
Δ preop–postop 12th week	0.1±0.2	0.4±0.4	0.2± 0.2	0.3± 0.2	0.2±0.2	
	p=0.340*	p=0.011*	p=0.007*	p=0.026*	p=0.016*	0.112

Preop: Preoperative; Postop: Postoperative; Δ: the change between

between the 5 groups (p>0.05). FN degeneration was not significantly different between the groups at the 3rd, 6th, and 12th weeks (p>0.05), whereas at the 9th week, FN degeneration in the control group was significantly lower than that in the other groups (p<0.05) (Table 3).

In group A, the mean latencies of the postoperative electrophysiological evaluations at the 3rd, 6th, and 9th weeks were significantly longer than that of the preoperative period (p<0.05). However, the mean latency at the 12th week (1.1±0.2 ms) was not significantly different from the preoperative period (1.0±0.1 ms) (p=0.340). In all other groups, the mean latency values at all postoperative evaluations were noted to be longer than those of the preoperative evaluations (p<0.05). The change in mean latency compared with the preoperative period was not significantly different between the 5 groups (p>0.05). Latency differences between the surgery and nonsurgery sides of the groups were not significantly different (p>0.05) except for the findings of the systemic melatonin group at the 12th week, which was −0.2±0.1, indicating a shorter latency of the coapted FN in this group (Table 3).

Histopathological Examination

The nerve diameter, axon diameter, and myelin thickness in each group are given in Table 4. The nerve diameter was significantly smaller in the control group (p<0.05), and animals in the melatonin groups (topical and systemic) had significantly larger nerve diameters than those in the dexamethasone groups (p<0.05). No significant difference was noted between topical and systemic adminis-

Table 3. Facial nerve degeneration and latency difference between the 2 sides measured at postoperative examination periods

		Group A	Group B	Group C	Group D	Group E	p
Postop 3rd week	Deg	0.7±0.2	0.7±0.1	0.7±0.1	0.7±0.1	0.8±0.1	0.729
	LD (ms)	0.6±0.2	0.6±0.2	0.5±0.1	0.5±0.1	0.6±0.1	0.320
Postop 6th week	Deg	0.7±0.1	0.7±0.1	0.7±0.1	0.6±0.1	0.7±0.1	0.846
	LD (ms)	0.7±0.2	0.5±0.4	0.3±0.2	0.4±0.2	0.4±0.1	0.013
Postop 9th week	Deg	0.4±0.2	0.6±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.004
	LD (ms)	0.4±0.2	0.4±0.4	0.2±0.1	0.3±0.2	0.3±0.1	0.284
Postop 12th week	Deg	0.4±0.2	0.5±0.1	0.5±0.2	0.4±0.1	0.5±0.1	0.117
	LD (ms)	0.1±0.3	0.3±0.3	−0.2±0.1	0.3±0.2	0.2±0.1	0.000

Postop: Postoperative; Deg: Facial nerve degeneration; LD: Latency difference

Table 4. Nerve diameter, axon diameter, and myelin thickness of the experimental groups

	Group A	Group B	Group C	Group D	Group E	p
Nerve diameter (μm)	338±15	376±16	382±10	357±8	362±12	0.000
Axon diameter (μm)	3.06±0.4	3.53±0.33	3.37±0.29	3.38±0.25	3.19±0.22	0.025
Myelin thickness (μm)	0.29±0.03	0.34±0.03	0.37±0.02	0.33±0.02	0.35±0.02	0.000

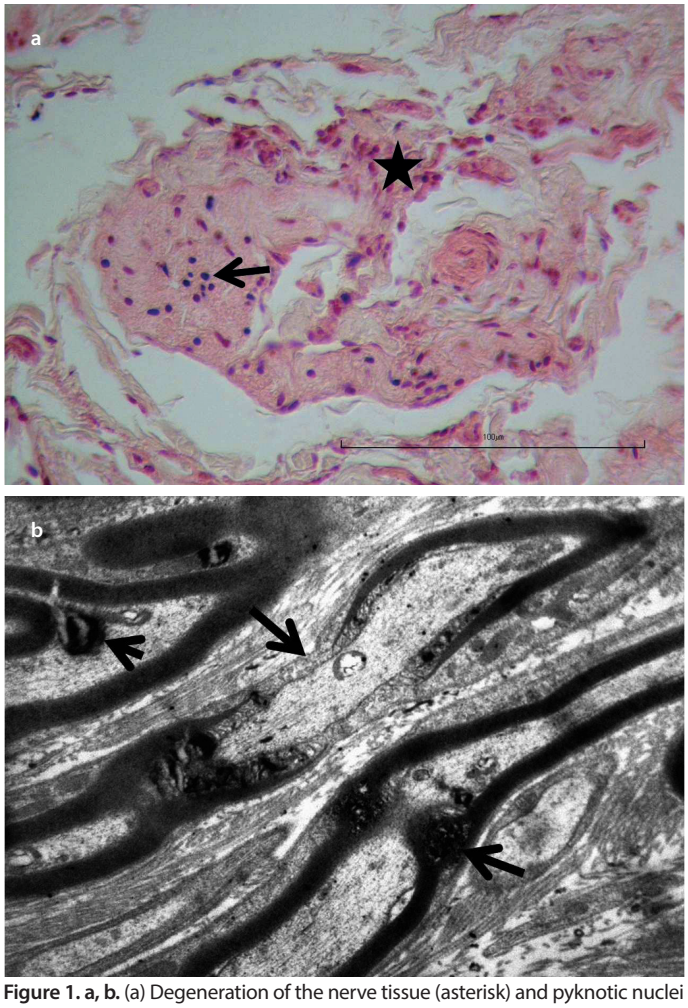

Figure 1. a, b. (a) Degeneration of the nerve tissue (asterisk) and pyknotic nuclei (arrow). Light microscopy, H–E, ×40. (b) Myelin degeneration (arrows) and myelin figures within the axoplasm (arrowhead). Transmission electron microscopy, ×10000. (H-E: Hematoxylin-eosin).

trations of melatonin as well as dexamethasone ($p>0.05$). The axon diameter was significantly smaller in the control group than in the melatonin (topical and systemic) and topical dexamethasone groups ($p<0.05$). No significant difference in axon diameter was detected between groups with topical and systemic administrations of both melatonin and dexamethasone ($p>0.05$); however, the axon diameter was significantly smaller in the systemic dexamethasone group than in the topical melatonin group ($p<0.05$). The control group had a significantly thinner myelin sheath than the other groups ($p<0.05$). The thickness of the myelin sheath was significantly less in the topical melatonin and topical dexamethasone groups than in the systemic melatonin group ($p=0.033$ and $p=0.009$, respectively).

Group A (Control, Figure 1): Prominent inflammatory cell infiltration and local hemorrhagic regions were noted in nerve sections under light microscopy. Axonal degeneration was widespread. Nuclei of the Schwann cells were heterochromatic and pyknotic with prominent cytoplasmic eosinophilia. Widespread degeneration and irregularity of the myelin sheath and prominent vacuolar formations in axoplasma sections were observed under TEM.

Group B (Topical melatonin, Figure 2): Minimal inflammatory cell infiltration was seen in nerve sections under light microscopy. Nuclei of the Schwann cells were moderately heterochromatic and pyknotic. TEM revealed almost normal ultrastructural architecture of the myelin sheaths of the axons. The density of the axoplasmic neurofilaments was normal. Tiny mitotic figures and electron-dense granules were detected in the axoplasm.

Group C (Systemic melatonin, Figure 3): Nerve sections had generally normal histologic structure under light microscopy. However, sporadic axonal undulations were prominent. Nuclei of the Schwann cells were mostly normochromatic, but minimal heterochromatism was noted sporadically. Axons, neurofilaments, and myelin sheaths had normal ultrastructural architecture under TEM.

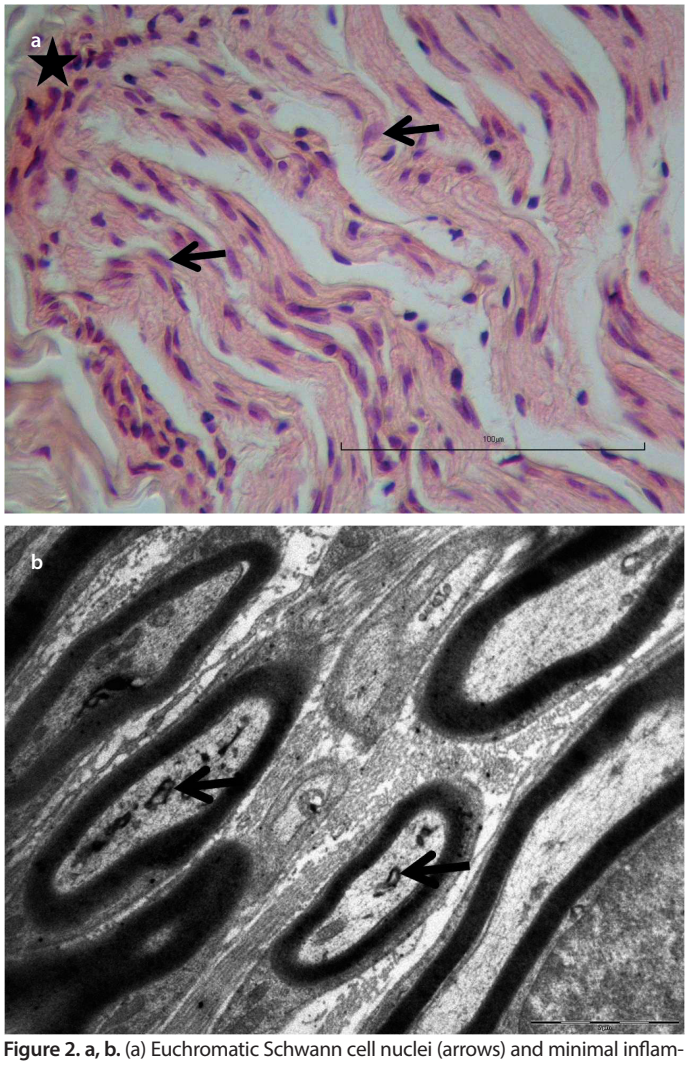

Figure 2. a, b. (a) Euchromatic Schwann cell nuclei (arrows) and minimal inflammatory cell infiltration (asterisk). Light microscopy, H-E, ×40. (b) Tiny myelin figures within the axoplasm (arrow). Transmission electron microscopy, ×10000. (H-E: Hematoxylin-eosin).

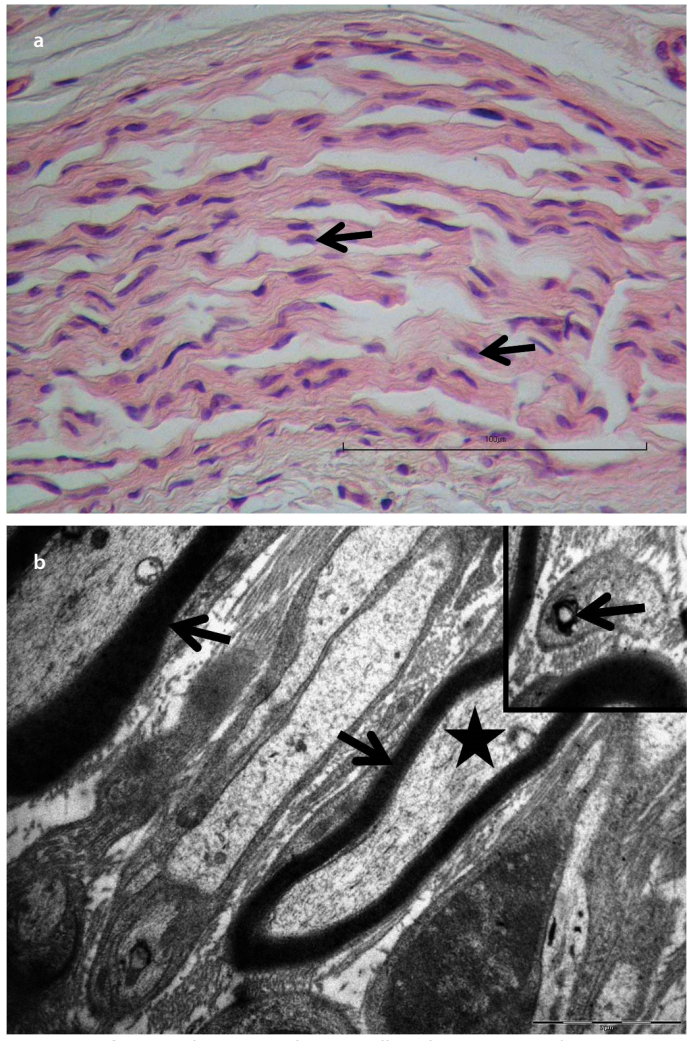

Figure 3. a, b. (a) Euchromatic Schwann cell nuclei (arrows). Light microscopy, H-E, ×40. (b) Myelin sheath (arrow), axoplasm (asterisk), and tiny myelin figures (right upper quadrant, arrow). Transmission electron microscopy, ×10000. (H-E: Hematoxylin-eosin).

Group D (Topical dexamethasone, Figure 4): Nuclei of the endoneurial and perineural connective tissue cells were heterochromatic under light microscopy. Sporadic degeneration and distortion of the myelin sheaths were noted under TEM. Vacuoles comprising myelin figures were detected in axoplasms.

Group E (Systemic dexamethasone, Figure 5): Nuclei of the endoneurial and perineural connective tissue cells and Schwann cells were heterochromatic under light microscopy. Myelin sheaths had normal ultrastructural architecture under TEM. However, myelin figures, vacuoles, and sporadic electron-dense granules were detected in axoplasmic regions.

DISCUSSION

Traumatic FN injury may occur due to accidental trauma or in the surgical practice of otology and head and neck surgery, either as a complication or as a part of the procedure [2, 5, 12, 13]. If both the proximal and distal segments of the transected nerve are available, tension-free end-to-end anastomosis is considered as the gold standard for repair [14]. However, the results following end-to-end anastomosis are not satisfactory mainly due to poor axonal regeneration and synkinesis [1, 7].

Many papers have mentioned the potential action of melatonin against traumatic peripheral nerve injuries [11, 15-17]. Neuroprotective effects of melatonin, particularly at high doses, have been demonstrated in a sciatic nerve injury model [18]. Recently, an increase in the regeneration of the FN after neurorrhaphy was reported with systemic melatonin [4]. Turgut et al. [16, 19] also demonstrated that melatonin has a positive effect on the nerve regeneration process following sciatic nerve surgery. The authors stated that melatonin reduces neuroma formation [16, 19]. The beneficial effects of melatonin on nerve regeneration have been linked to the induction of Schwann cell proliferation, which provides the ideal environment for axonal regrowth [20]. Scar tissue formation between the transected segments can impede axonal growth, and the effects of melatonin in reducing collagen deposition may also contribute to its beneficial effects on recovery [17]. Kaptanoglu et al. [21] studied the effects of methylprednisolone and melatonin in an experimental spinal cord injury model and concluded that melatonin-treated animals show more obvious protection of neurons and subcellular organelles.

The effects of SCs for FN anastomosis are not well known [4]. Can the treatment principles of Bell's palsy be applied to FN transection? This

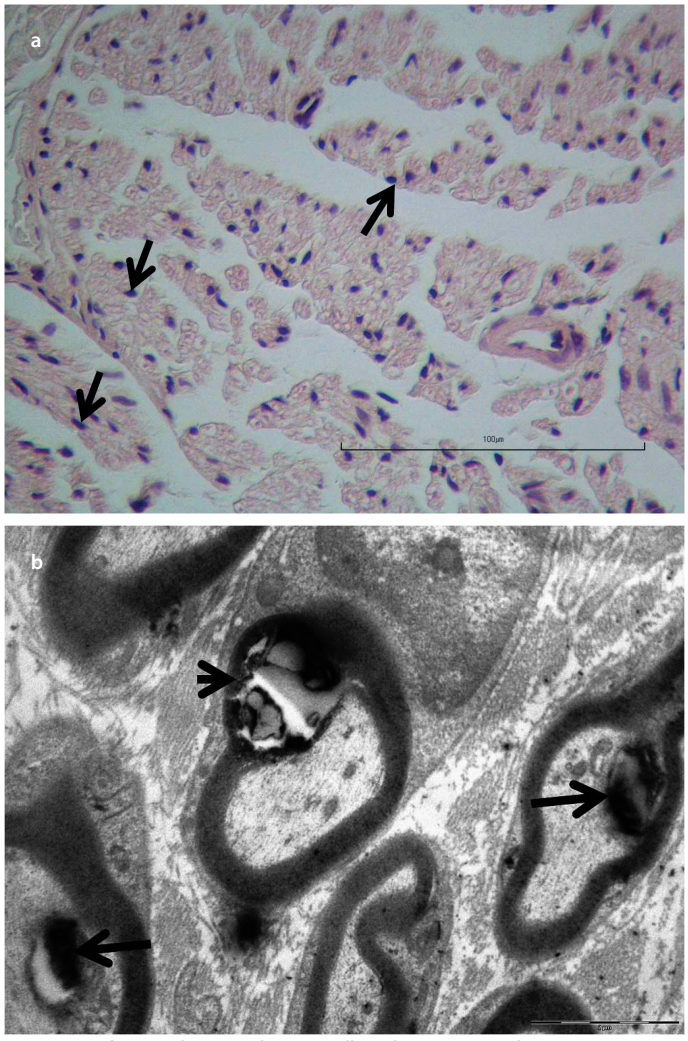

Figure 4. a, b. (a) Pyknotic Schwann cell nuclei (arrow). Light microscopy, H-E, ×40. (b) Myelin sheath degeneration (arrowhead), and vacuolization comprising myelin figures within the axoplasm (arrows). Transmission electron microscopy, ×10000. (H-E: Hematoxylin-eosin).

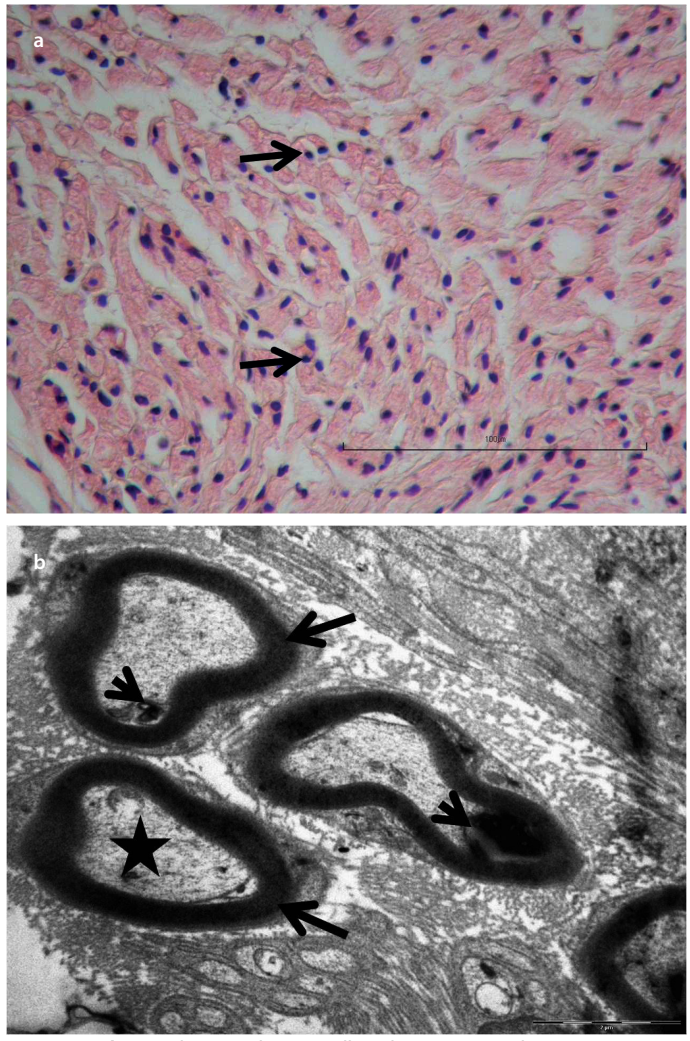

Figure 5. a, b. (a) Pyknotic Schwann cell nuclei (arrows). Light microscopy, H-E, ×40. (b) Myelin sheath (arrow), axoplasm (asterisk), and vacuoles comprising myelin figures (arrowhead). Transmission electron microscopy, ×10000. (H-E: Hematoxylin-eosin).

question has not been examined in detail, and the number of articles is relatively low compared with that for Bell's palsy [4, 5, 22-24]. Yanilmaz et al. [4] and Karlidag et al. [23] demonstrated that methylprednisolone does not provide any beneficial effect on FN regeneration following neurorrhaphy. Seth et al. [5] reported that the improvement of FN recovery might be possible with the use of high-dose systemic dexamethasone, if administered early following neurorrhaphy. Chen et al. [24] stated that high-dose methylprednisolone might enhance the survival time of motor neurons after FN transection. Yildirim et al. [22] concluded that SCs have no effect on the healing process in a nerve transection model. In contrast, in FN compression models, SCs have been reported to have the ability to decrease myelin degeneration, axonal denervation, and edema formation [22, 25]. Similar to nerve compression models, the effectiveness of SCs in the management of Bell's palsy is well known [26, 27]. The main aim of SC administration is the reduction of edema formation [25]. Liberman et al. [28], in contrast, reported that corticosteroids might interfere with functional recovery following crush injury. They stressed that the immunosuppressive state achieved by SCs is responsible for the impaired regeneration process [28]. In our opinion, it is necessary to establish the dose of SCs to have a therapeutic effect following nerve injury.

In animal models of FN injury, the nerve is either crushed or transected (axotomy). Crush injury and transection can be considered as axonotmesis and neurotmesis, respectively [5, 29]. Because crush injuries can produce nonhomogeneous degrees of nerve damage among animals and show different healing mechanisms, we prefer FN transection that produces a more reproducible lesion [1, 5, 29].

It should be kept in mind that the rat FN has a higher regenerative capacity than that of humans [30]. The location and distance of peripheral nerve injury from the central nervous system has also been reported to affect the regeneration process. That is why the results of sciatic nerve injury models may not be applied to the FN [31].

In the present study, we investigated the effects of dexamethasone and melatonin on FN regeneration in an experimental model of axotomy and neurorrhaphy. Both systemic and topical administrations were evaluated. Regarding electrophysiological evaluation, latency and amplitude parameters between the groups did not differ significantly at any examination period. Moreover, a statistically significant decrease in amplitude and increase in latency compared with the preoperative period in all study groups at all examination periods may be interpreted

as follows: neither melatonin nor dexamethasone had any beneficial effect on neural regeneration from the perspective of electrophysiological evaluation. At the 12th week, degeneration did not differ significantly between the groups; however, latency of the surgery side was less than that of the nonsurgery side in the systemic melatonin group. Although systemic melatonin seemed to have a promising effect, this finding was not in compliance with other electrophysiological parameters.

Histopathological results were somewhat different from those of electrophysiological evaluation. One of the most important findings was prominent inflammatory cell infiltration in the control group, which was minimal in the other groups. It was even absent in the systemic melatonin group. The findings of significantly smaller nerve and axon diameters and a thinner myelin sheath in the control group may point to the fact that both melatonin and dexamethasone had important effects in reducing nerve degeneration. Regarding the qualitative comparison of these 3 parameters between the melatonin and dexamethasone groups, the most notable difference was found in nerve diameter, which was significantly larger in the melatonin groups.

The findings of significantly larger axon diameter in the topical melatonin group than in the systemic dexamethasone group may be interpreted as follows: topical melatonin was more effective than systemic dexamethasone in terms of axonal regeneration.

Widespread myelin degeneration in the control group and normal myelin structure in the melatonin groups may reflect the protective effect of melatonin against myelin degeneration. Although the dexamethasone groups also had better myelin ultrastructure than the control group, these effects were not as prominent as in the melatonin groups. Thicker myelin sheath in the systemic melatonin group than in the topical melatonin and topical dexamethasone groups may be interpreted as follows: systemic melatonin had more important effects in preserving the thickness of myelin. We propose that systemic mediators may play a role in myelin regeneration because no statistically significant difference was detected between the systemic melatonin and systemic dexamethasone groups in terms of myelin thickness. However, myelin ultrastructure was also well preserved in the topical melatonin group, despite the presence of a significant difference between the topical and systemic melatonin groups in terms of myelin thickness. Although the dexamethasone groups had some advantages in terms of axon diameter (topical dexamethasone) and myelin thickness (systemic dexamethasone), these were not statistically significant compared with melatonin groups.

CONCLUSION

Electrophysiological evaluation did not reveal any potential benefit of dexamethasone and melatonin. In contrast, histopathological examination revealed beneficial effects of melatonin in particular. This can be interpreted as both corticosteroids and melatonin being able to increase FN regeneration following axotomy and end-to-end anastomosis, but electrophysiological evidence of regeneration may appear later.

Ethics Committee Approval: Ethics Committee Approval was received for this study from the Ethics Committee of Bezmialem University Animal Care and Use (2013/14).

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Original Article

Investigation of Stress Levels before the Onset of Idiopathic Sudden Sensorineural Hearing Loss

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OBJECTIVES: We hypothesized that patients with idiopathic sudden sensorineural hearing loss (ISSHL) would have experienced more stress prior to the onset than they typically did. This study investigated stress levels in patients before the onset of ISSHL.

MATERIALS and METHODS: Forty-two patients with ISSHL were investigated. We used an original questionnaire to evaluate subjective stress levels in 1 week before onset. Serum hemoglobin A1c (HbA1c) and total cholesterol were examined to evaluate biochemical stress markers reflecting the preceding 1 to 2 months. The results on admission were compared with those at the follow-up visit.

RESULTS: Significantly more patients reported greater physical exhaustion, greater mental exhaustion, or a worse physical condition on admission than at follow-up ($p<0.01$, for each variable). On admission, 81% of patients reported greater than normal stress with regard to at least 1 of 3 items. The mean serum HbA1c was slightly but nonsignificantly lower at the follow-up visit ($p=0.10$), while the mean serum total cholesterol was significantly lower at follow-up than on admission ($p<0.01$).

CONCLUSION: The results indicate that patients were under a greater degree of stress before the onset of ISSHL, suggesting that stress plays a role in inducing ISSHL.

KEYWORDS: Idiopathic sudden sensorineural hearing loss, stress, questionnaire, HbA1c, total cholesterol

INTRODUCTION

Even though the cause of idiopathic sudden sensorineural hearing loss (ISSHL) has not been identified, several possible etiologies have been proposed, such as a vascular disorder, viral infection, or membrane breaks [1-3]. Stress is commonly thought to be related to the onset of ISSHL, but there have been few reports investigating the precise relationship between stress and ISSHL. The National Epidemiological Survey in Japan, conducted between 1971 and 1974, reported that 25% of 2.418 patients with ISSHL noted apparent triggers prior to the hearing loss, such as the common cold, physical exhaustion, or mental exhaustion (Results of Nationwide Epidemiological Surveys on Sudden Deafness (NESSD) reported by the Research Committee on Acute Profound Deafness of the Ministry of Health and Welfare in Japan, 1975). We believe that even more patients likely had an identifiable ISSHL trigger, because 47% of the 2,418 responses to the question about a trigger were "uninvestigated." It was not possible to determine a trigger because of a lack of clinical information.

The widely used term *stress* may be ambiguous, but it can be defined as a threat, real or imagined, to the psychological or physiological integrity of an individual [4]. People usually have many stressors in their daily lives. The duration of stress can range from a few seconds to several years. Evaluating stress is done by investigating both stressors and a stress response to them. The latter can be evaluated subjectively using a questionnaire or objectively by evaluating the changes of biophysical measurements and biochemical indicators.

This study was presented at the "62th annual conference of Audiology Japan", "19 October 2017, Fukuoka, Japan".

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We have noted that some patients have developed ISSHL at highly stressful times or immediately thereafter. Thus, we hypothesized that patients with ISSHL experienced more stress prior to the onset of ISSHL than was typical for them. A critical problem in evaluating stress levels before the onset of ISSHL, however, is that the condition itself is highly stressful. Hence physical and psychological stress responses measured after the onset are difficult to separate from conditions leading up to the hearing loss. Many biochemical indicators of stress, such as cortisol, dehydroepiandrosterone, noradrenaline, and adrenaline levels, are influenced by ISSHL. Even when using a subjective questionnaire assessing prior stress levels, the responses could be influenced by the ISSHL itself. We therefore opted to measure longer-term biochemical indicators, hemoglobin A1c (HbA1c) and cholesterol, which are influenced by stress for 1 to 2 months. We also used a simple, original questionnaire about the patient's physical and mental condition before the onset of ISSHL based on the presumption that the duration of stress influencing the onset of ISSHL would range from 1 week to a few months.

MATERIALS AND METHODS

Diagnostic criteria for ISSHL were as follows: (1) a sudden onset of hearing loss or progressive deterioration within 72 h; (2) hearing loss of 30 dBHL or more, over three consecutive frequencies; and (3) sensorineural hearing loss of unknown etiology. Inclusion criteria for the study were as follows: presentation within 7 days of the onset of ISSHL; being admitted to our hospital; age ≥20 years; no history of diabetes mellitus; and consent to participate in the study.

We investigated patient's stress levels subjectively with a questionnaire and objectively with biochemical measurements. We compared the results on admission with those at a follow-up visit 3 months or more after the onset of ISSHL. The follow-up visit results were used as the control data.

Table 1. Sociodemographic characteristics of subjects (n=42)

Gender	15 men/27 women
Age (years)	55.3±17.0 (25-85)
Interval between the onset and first visit to our hospital (days)	3.5±1.6 (1-7)
Arithmetic mean of hearing levels at five frequencies (250-4000 Hz) in the affected ear on admission	77.7±23.0 dB
Arithmetic mean of hearing levels at five frequencies (250-4000 Hz) in the affected ear at the follow-up	36.4±28.3 dB
Treatment before presentation to our hospital	Previously treated, 18 (12 with steroids) Not treated, 24

Table 2. Questionnaire regarding stress levels

We want to know about your stress levels before the onset of your hearing problems.		
Please answer the following three questions by comparing your condition for 1 week before the onset of your hearing problems and your average condition over the entire past year.		
1. Do you think you had greater physical exhaustion?	Yes	No
2. Do you think you had greater mental exhaustion?	Yes	No
3. How was your physical condition?	Normal	Worse

A total of 97 patients with ISSHL were considered for inclusion in the study, of whom 51 did not consent to participate or did not attend their scheduled follow-up visit. Four patients turned out not to have ISSHL, either during or after treatment. Their actual diagnoses were Meniere's disease, acoustic tumor, idiopathic bilateral sensorineural hearing loss, and noise-induced hearing loss.

Of the 42 patients in the study (Table 1), 15 were men and 27 were women, with a mean (±standard deviation) age of 55.3±17.0 years (range, 25-85 years). Their initial visit to our hospital was 3.5±1.6 days after the symptom onset.

The arithmetic mean of hearing levels at five frequencies (250 Hz, 500 Hz, 1,000 Hz, 2,000 Hz, and 4.000 Hz) in the affected ear was 77.7±23.0 dBHL on admission and 36.4±28.3 dB at the follow-up. Before coming to our hospital, 12 patients had been treated elsewhere with steroids, and 6 were treated with adenosine triphosphate and mecobalamin without steroids. The remaining 24 did not receive treatment of any kind before visiting our hospital. The interval between the first day of admission and the follow-up visit ranged from 90 to 388 days, with a mean of 206.3±73.4 days.

The Questionnaire

Patients were instructed on the day of admission to answer three questions regarding physical exhaustion, mental exhaustion, and physical condition. These questions were derived from the NESSD survey. Patients were asked to note their perception of these items in the week before the onset of ISSHL, compared with how they typically experienced them over the previous year (Table 2). At the follow-up visit (≥3 months after the ISSHL onset), we asked them to report on their stress for the 1 week before the visit compared with the preceding year.

Biochemical Data

Serum HbA1c and total cholesterol (TC) levels were used to evaluate relatively long-term physical reactions to stress. Blood samples were collected on admission and at the follow-up visit when the questionnaire was readministered. We compared the mean values of serum HbA1c and TC on admission with those at the follow-up and investigated a relationship between response to the questionnaire and the biochemical data. We also assessed whether the steroid administered to the 12 patients prior to presentation at our hospital influenced serum HbA1c and TC levels on admission.

Statistical Analysis

McNemar's chi-squared test was used to compare the ratio of patients with greater stress between on admission and at the follow-up. A paired t-test was used to compare the values of biochemical indicators. The Statistical Package for the Social Science version 15 (SPSS Inc., Chicago, IL, USA) program was used for statistical evaluation. A $p<0.05$ was considered statistically significant.

This study was conducted between January 2011 and December 2013, and it was approved by and performed in accordance with the ethical standards of the Ethics Committee of Kitasato University, School of Medicine. All patients provided written informed consent to participate in this study. All steps of this study were planned and conducted according to the principles outlined in the Declaration of Helsinki (2008).

RESULTS

Questionnaire

The results of the questionnaire are shown in Table 3. Of the 42 study patients, 26 (62%) reported greater physical exhaustion on admission, and 11 (26%) reported greater physical exhaustion at the follow-up visit. The number of patients with greater physical exhaustion on admission was significantly higher than that at the follow-up visit (McNemar's chi-squared test, p=0.01).

Greater mental exhaustion was reported by 27 (64%) on admission and by 13 (31%) at the follow-up visit. Mental exhaustion was thus reported by a significantly greater number of patients on admission than at the follow-up visit (McNemar's chi-square test, p<0.01).

A worse physical condition was reported by 22 patients (52%) on admission compared with 9 (21%) at the follow-up visit. Again, a significantly greater number of patients gave this response on admission than at the follow-up visit (McNemar's chi-square test, p<0.01).

On admission, 81% of patients reported greater stress for at least one of the three items.

Table 3. Results of the questionnaire (n=42)

	On Admission	At the Follow-Up Visit	McNemar's Chi-Square Test
Greater physical exhaustion	26/42 (61.9%)	11/42 (26.2%)	p<0.01
Greater mental exhaustion	27/42 (64.3%)	13/42 (31.0%)	p<0.01
Worse physical condition	22/42 (52.4%)	9/42 (21.4%)	p<0.01
At least one of the three items	34/42 (81.0%)	19/42 (45.2%)	p<0.01

Table 4. Biochemical data (n=42)

	On Admission	At the Follow-Up Visit	Paired t-test
Serum HbA1c (%)	5.44±0.34	5.38±0.35	p=0.10
Serum TC (mg/dL)	211.5±41.9	197.4±31.3	p<0.01

Data expressed as the mean±standard deviation. TC: total cholesterol.

Biochemical Data

The results of biochemical testing are shown in Table 4. The mean serum HbA1c level was 5.44%±0.34% on admission and 5.38%±0.35% at the follow-up, a slight but not significant difference (paired *t*-test, p=0.10).

The mean serum TC level was significantly higher on admission than at the follow-up visit (211.5±41.9 mg/dL vs. 197.4±31.3 mg/dL; paired *t*-test, p<0.01).

Table 5. Relationship between the questionnaire and biochemical data (n=42)"Physical exhaustion"

		Serum HbA1c (%)			Serum TC (mg/dL)		
		Admission	Follow-Up	Paired t-test	Admission	Follow-Up	Paired t-test
Greater Physical Exhaustion	"Greater" at both visits (n=8)	5.48±0.18	5.41±0.32	p=0.58	218.3±57.9	186.0±21.8	p=0.17
	Admission "greater" Follow-up "no" (n=18)	5.45±0.42	5.39±0.42	p=0.27	204.7±41.7	198.4±33.1	p=0.27
	Admission "no" Follow-up "greater" (n=3)	5.17±0.06	5.17±0.21	p=1	197.3±27.6	190.7±28.0	p=0.06
	"No" at both visits (n=13)	5.47±0.32	5.41±0.32	p=0.22	220.1±35.0	204.5±35.1	p=0.04

Data expressed as the mean±standard deviation. TC: total cholesterol.

Table 6. Relationship between the questionnaire and biochemical data (n=42) "Mental Exhaustion"

		Serum HbA1c (%)			Serum TC (mg/dL)		
		Admission	Follow-Up	Paired t-test	Admission	Follow-Up	Paired t-test
Greater Mental Exhaustion	"Greater" at both visits (n=13)	5.43±0.38	5.36±0.36	p=0.23	205.0±35.0	20.8±27.6	p=0.51
	Admission "greater" Follow-up "no" (n=14)	5.48±0.35	5.40±0.37	p=0.17	222.1±55.5	195.4±38.2	p=0.06
	Admission "no" Follow-up "greater" (n=0)	-	-	-	-	-	-
	"No" at both visits (n=15)	5.41±0.31	5.38±0.34	p=0.64	207.3±32.5	196.3±29.0	p=0.04

Data expressed as the mean±standard deviation. TC: total cholesterol.

Table 7. Relationship between the questionnaire and biochemical data (n=42) "Physical Condition"

		Serum HbA1c (%)			Serum TC (mg/dL)		
		Admission	Follow-Up	Paired t-test	Admission	Follow-Up	Paired t-test
Worse Physical Condition	"Normal" at both visits (n=17)	5.38±0.31	5.37±0.33	p=0.92	210.5±30.0	198.4±26.9	p=0.03
	Admission "normal" Follow-up "worse" (n=3)	5.37±0.38	5.37±0.25	p=1	201.0±25.9	186.7±31.9	p=0.27
	Admission "worse" Follow-up "normal" (n=16)	5.50±0.41	5.36±0.42	p=0.02	203.4±46.3	196.6±39.9	p=0.35
	"Worse" at both visits (n=6)	5.50±0.18	5.47±0.30	p=0.66	241.5±58.8	202.0±20.4	p=0.20

Data expressed as the mean±standard deviation. TC: total cholesterol.

Relationship between Responses to the Questionnaire and Biochemical Data

Among patients denying physical exhaustion at both time points, serum TC values were significantly lower at the follow-up compared with those on admission (paired *t*-test, p=0.04) (Table 5). A similar significant decrease in serum TC levels was seen among patients who denied mental exhaustion at both time points (paired *t*-test, p=0.04) (Table 6). For those who reported mental exhaustion on admission but not at the follow-up, there was a nonsignificant decrease in mean serum TC values (paired *t*-test, p=0.06).

For those who reported a normal physical condition at both time points, the mean serum TC values decreased significantly at the follow-up (paired *t*-test, p=0.03) (Table 7). There was also a significant decrease in the mean serum HbA1c values among those who reported a worse physical condition on admission, but who said it was normal at follow-up (paired *t*-test, p=0.02).

Effect of Previous Treatment on Biochemical Data

We investigated serum HbA1c and TC values on admission in the 12 patients treated with steroids before they were seen at our hospital. Their mean serum HbA1c level was 5.48%±0.31%, and the mean serum TC level was 211.1±53.2 mg/dL. These values did not differ significantly from those of the 30 patients not treated with steroids (HbA1c 5.47%±0.36%, p=0.92; TC 215.6±43.3 mg/dL, p=0.76; t-test).

DISCUSSION

The causes of ISSHL remain unknown, but some etiologic hypotheses have been advanced, including circulatory disorders, viral infections, and membrane breaks [1-3]. It has been widely accepted that stress contributes to ISSHL, but there are few reports investigating the relationship between stress and ISSHL. This may be due to the difficulty in evaluating stress levels before the onset of ISSHL. Possible stressors include many factors, which can be both external or internal. External stressors may include physical or chemical conditions, such as heat, cold, chemicals, noise, and drugs; biological stress, such as over-exercise, infection, and lack of sleep; or social or mental stress, such as anxiety, fear, mental tension, and human relationships. Internal stress may include illnesses, pain, or bleeding. The extent to which a person experiences stress is determined both by the stressor itself and by the individual's response to it [5].

We hypothesized that patients with ISSHL would have experienced more stress before or at the onset of hearing loss than usual. Neuser et al. [6] reported that patients with ISSHL had greater psychological distress and more stressful life events, as measured by the Minnesota Multiphasic Personality Inventory (MMPI) test and the Inventory of Stressful Life-Events (ILE). Fowler [7] reported that 90% of patients with ISSHL had mental problems, and over 70% of patients had a psychosomatic disorder.

Merchant et al. [8] and Adams et al. [9] demonstrated a relationship between ISSHL and stress based on the "stress response hypothesis." They hypothesized that ISSHL may be the result of the pathologic activation of cellular stress pathways involving the nuclear factor kappa B within the cochlea. They investigated this hypothesis using findings in the temporal bone in patients with ISSHL. They also demonstrated that general exposure to stress could activate this cellular reaction.
In this study, we used our own simple questionnaire to evaluate the degree of exhaustion and physical condition before the onset of ISSHL. There are many questionnaires asking about stressors, the response to stressors, or how one coped with a stressor. For example, the Sense of Coherence, MMPI, and ILE have been widely used [6, 10, 11]. For this study, we hypothesized that patients with ISSHL would have had more stress prior to the onset of ISSHL than they typically experienced. We felt that the conventional questionnaires could not be adapted to this purpose. In addition, it seemed difficult to exclude the effects of the ISSHL itself on responses to those instruments. Our goal was to assess stress levels prior to the onset of ISSHL, even though hearing loss had already occurred by the time the patients were seen. Therefore, we made the questions very simple, prompting the patient to compare how they perceived the items at specified periods before the onset of ISSHL. We believed this approach would exclude the influence of the stress from the ISSHL per se.

In this study, patients more frequently reported greater mental or physical exhaustion and physical disorders before the onset of ISSHL than at the follow-up visit. The majority of patients (81%) reported at least one of those stressful conditions. This proportion was remarkably high compared with the 25% reported in NESSD. We consider the results of this prospective study to be more plausible than those from NESSD.

Physiologic reactions to stress can be evaluated using biochemical data. Under stressful conditions, neurotransmitters and neuroregulators are released from the limbic system, stimulating the hypothalamus to release corticotropin-releasing hormone, which in turn stimulates the pituitary gland to release adrenocorticotropic hormone, which stimulates the adrenal cortex to release cortisol. Among other actions, cortisol promotes gluconeogenesis in the liver. Blood sugar combines with serum hemoglobin, which is measured as the percentage of the HbA1 proteins that are glycated. HbA1c is the best indicator of blood sugar levels over the preceding several months. The half-life of hemoglobin is approximately 120 days, and the half-life of serum HbA1c is approximately half of that (approximately 1-2 months). The HbA1c value therefore reflects blood sugar levels in the preceding 1-2 months [12].

Although cortisol, dehydroepiandrosterone, noradrenaline, and adrenaline are well-known indicators of stress, these values can fluctuate immediately or within hours. Therefore, in patients presenting with ISSHL, levels of these substances were likely to have been influenced by the ISSHL alone. Since HbA1c values reflect the average blood sugar levels over the previous 1–2 months, HbA1c can also be used as a marker of stress levels for the same period [12-15]. We considered that the effect of ISSHL alone on the HbA1c would be negligible. We also found that the use of steroids immediately before measurement of HbA1c did not affect the levels. There have been several reports using HbA1c as a stress indicator. Netterstrom et al. [13] reported that HbA1c values were higher during the exam week than they were 4 months later in 23 medical students. However, personal lifestyle and genetic factors can also influence the HbA1c levels. Our study compared the HbA1c levels on admission with those at the follow-up visit, allowing each patient to act as their own control.

We found that mean serum HbA1c levels on admission were slightly higher than those at the follow-up, but the difference was not significant. It is important to keep in mind that the *p*-value was relatively small and that there would have been a possibility of Type 2 error.

We also investigated serum TC levels. The secretion of cortisol is promoted from the adrenal cortex by stressors, as mentioned earlier. Serum-free fatty acid levels increase in response to glucocorticoids, and the liver increases its TC-producing capacity. Therefore, serum TC can also be used to indicate a stress response [4, 15, 16]. The timing of increases in TC with stress is similar to that of HbA1c. In this study, serum TC values on admission were significantly higher than those at the follow-up. This indicates that patients had experienced a higher than usual level of stress for several months before the onset of ISSHL.

We also investigated the relationship between responses to the questionnaire and the serum HbA1c or TC levels. Overall, the relationship between these factors was not strong. One primary reason for this result was that the questionnaire evaluated stress a week before the onset, whereas the HbA1c and TC levels reflected stress in the 1 to 2 months before the onset, a substantial difference in timing. We conducted a preliminary investigation of our questionnaire before this study, creating two questionnaires asking about differing length of time, one for 1 week and the other for 1 month. The correlations between answers for 1 week and for 1 month were 0.39 for physical exhaustion, 0.80 for mental exhaustion, and 0.61 for physical condition (Spearman's rank correlation, data not published). The correlation for physical exhaustion was relatively low, while that for mental exhaustion was high. For this investigation, we selected the questionnaire asking about 1 week because we considered that the stress level closer to onset would more likely contribute to ISSHL, and it seemed more appropriate to compare 1 week rather than 1 month with the average of the preceding 1 year. Consequently, the results of the questionnaire reflected a shorter time period than did the biochemical data, particularly for physical exhaustion. However, there was a large difference in TC values for those who reported mental exhaustion on admission but not at the follow-up. This finding might indicate a relatively strong relationship between the subjective perception of mental exhaustion and the objective evidence indicated by the TC level. Interestingly, for the group reporting no mental exhaustion on either admission or at the follow-up, the mean serum TC values were significantly higher on admission than at the follow-up. This suggests that serum TC values detect a physiological response to stress that could not be detected subjectively with the questionnaire.

We should consider the possibility that patients might take steps to avoid stress after developing ISSHL, which could have influenced the results of this investigation. The mean interval between the first examination and the follow-up visit was approximately 6 months. Although we cannot exclude this possibility, we also assume that many patients resumed their usual lifestyle, such that stress levels at the follow-up would be similar to those they were generally used to.

CONCLUSION

The results of this investigation showed that many patients subjectively perceived greater exhaustion or a worse physical condition a week before the onset of ISSHL than it was usual for them. Patients had relatively higher biochemical stress responses for 1 to 2 months before the onset of ISSHL than usual. We therefore conclude that the patients we studied were under a greater degree of stress before the onset of ISSHL, indicating that stress may play an important role in the onset of ISSHL.

Ethics Committee Approval: Ethics Committee approval was received for this study from the Ethics Committee of Kitasato University, School of Medicine (B10-130).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

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