of the endolymphatic sac (Figure 1F). None of the BMDCs were integrated into the epithelial layer or concentrated at the dark cell area in the vestibular end organs. Observation of BMDCs expressing EGFP in the vestibular end organ with a confocal microscope revealed their morphological variety. The vast majority of EGFP-positive cells exhibited a spindle shape with several processes (Figure 1G), suggesting that BMDCs in the vestibular end organs and the endolymphatic sac morphologically follow the microglia that correspond to resident tissue macrophage in the central nervous system.

To investigate the phenotype of BMDCs in the vestibular stroma and the endolymphatic sac,

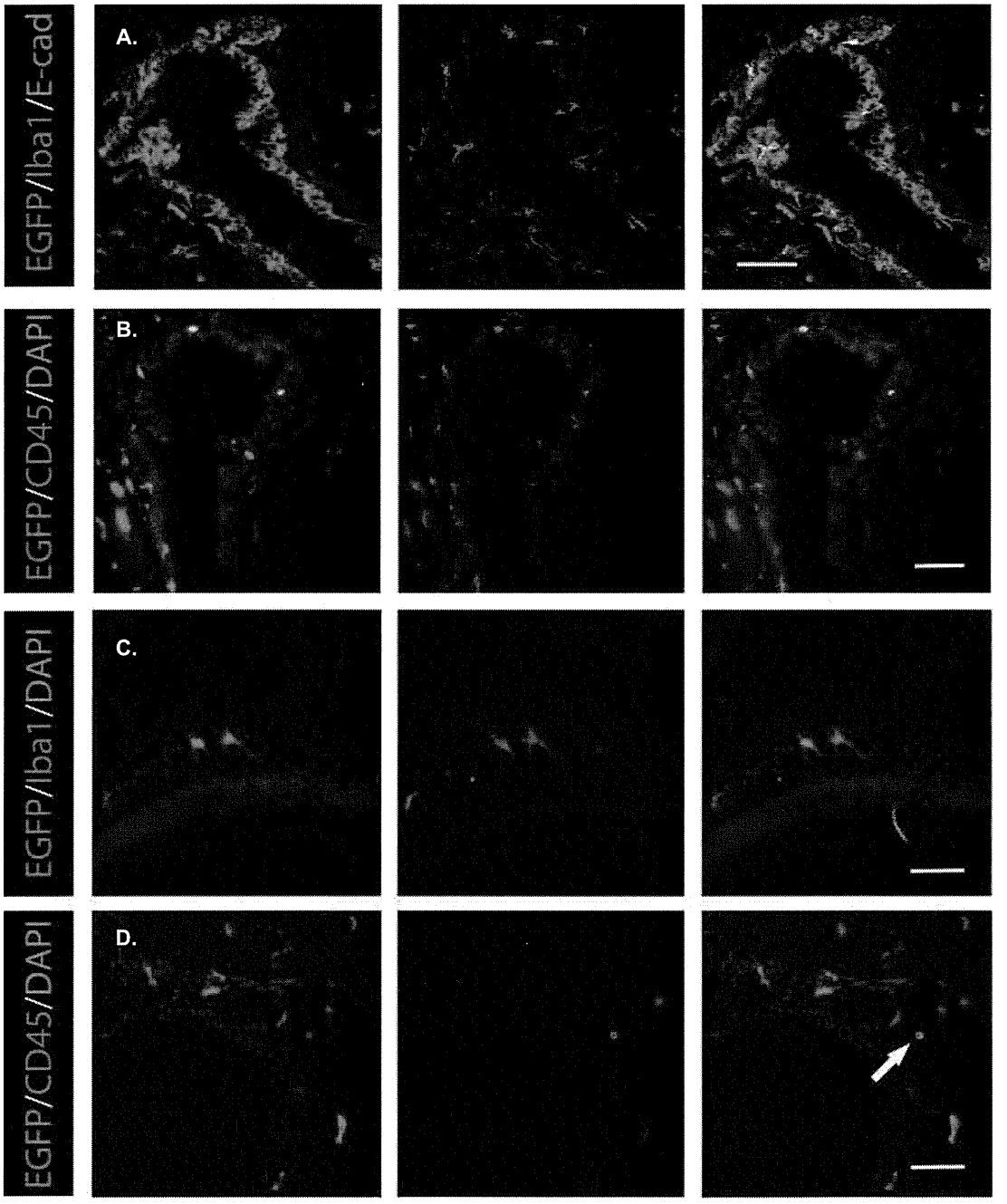

Figure 2. Bone marrow-derived cells (BMDCs) show the phenotype of macrophage in the vestibular end organ and in the endolymphatic sac. Approximately two-thirds of EGFP-positive cells in the endolymphatic sac demonstrated the phenotype of macrophage expressing Iba1 (A), while about 40% express a common leukocyte marker CD45 (B). In contrast, more than 70% of BMDCs in the vestibular end organ are Iba1-positive (C), whereas about 14% of EGFP-positive cells show CD45 immunoreactivity (D). Scale bars represent 50 μm.

immunohistochemistry for CD45 and Iba1 was performed. In the endolymphatic sac, 66.2 ± 8.9% of EGFP-positive cells expressed macrophage-specific protein Iba1 (Figure 2A). In addition, EGFP-positive cells, especially those with round shape, expressed common leukocyte antigen CD45 (40.5 ± 6.0%) in the epithelial layer of the intermediate or distal portion of endolymphatic sac, where direct access to the endolymph was available (Figure 2B). In contrast to the endolymphatic sac, 74.5 ± 4.1% of EGFP-positive cells in the vestibular stroma were labeled with Iba1 (Figure 2C). While three-quarters of BMDCs in the vestibular stroma showed Iba1-immunoreactivity, EGFP-positive cells in the vestibular end organ were occasionally positive for leukocyte marker CD45 (14.8 ± 6.0%) (Figure 2D). These findings demonstrate that BMDCs in the adult vestibular end organ are predominantly distributed as macrophages.

BMDCs have characteristics of immunocompetent cells

Immunostaining for antigen-presenting protein MHC class II was performed to further analyze the function of BMDCs in the vestibular end organ and the endolymphatic sac. Subpopulations of EGFP-positive cells in the vestibular stroma and the endolymphatic sac were observed to express antigen-presenting protein MHC class II (Figure 3A), indicating that a part of BMDCs function as antigen-presenting cells and play a role in initiating immune reactions in the vestibular end organs and the endolymphatic sac. We also investigated the response of BMDCs to local mechanical stress. Seven days after saline injection, EGFP-positive cells in the stroma underlying vestibular sensory epithelia were observed more densely in operated specimens than in controls (Figure 3B and C). While there was no significant difference between the numbers of total nuclei in each group (control 32.3 ± 1.5 cells/10 000 μm^2 vs operated 34.9 ± 2.2), the ratio of EGFP-positive cells to total cell nuclei was significantly higher in operated specimens than in controls, which amounted to 10.1 ± 1.1% and 5.1 ± 0.7%, respectively (Figure 3D). These results demonstrate that the vestibular macrophages increased in number in response to local mechanical stress, suggesting that BMDCs contribute to the immune reactions in the vestibular end organs and the endolymphatic sac.

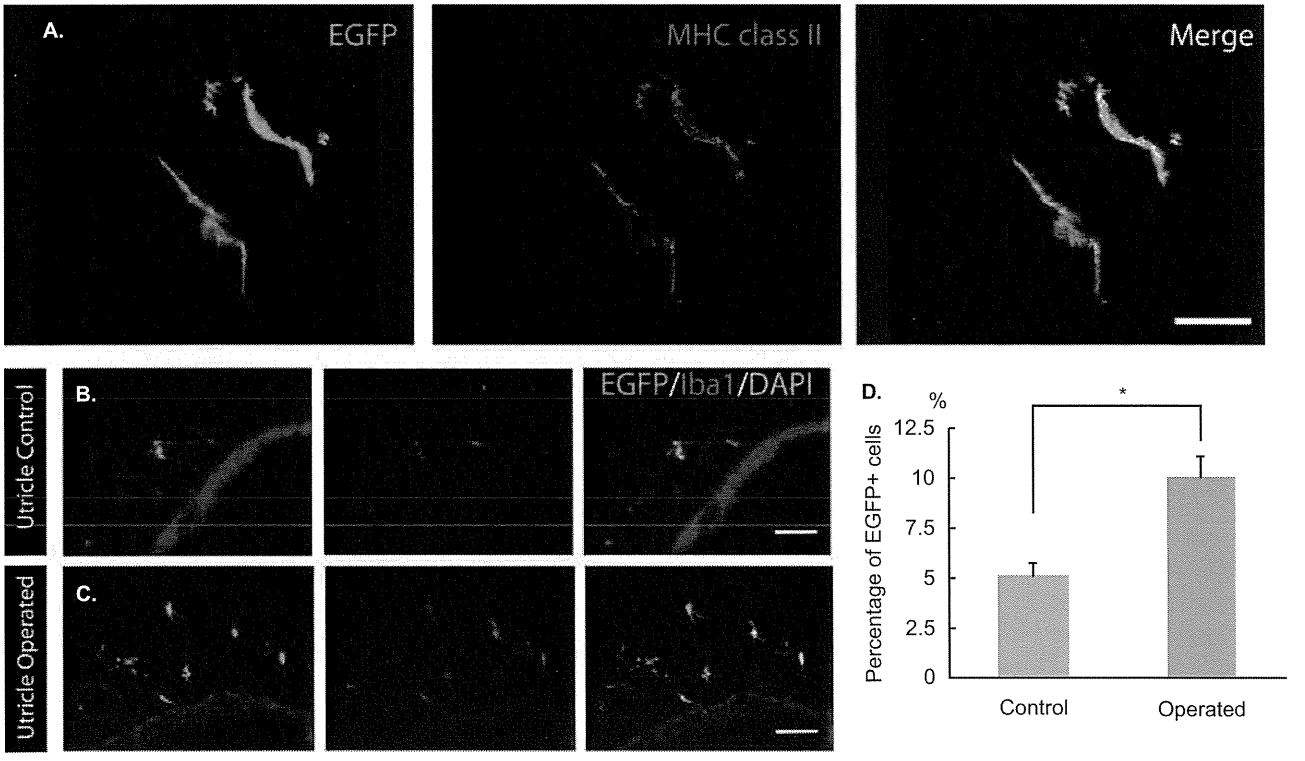

Figure 3. Bone marrow-derived cells (BMDCs) contribute to the immune reaction in the vestibular end organ. A subpopulation of BMDCs expresses antigen-presenting protein MHC class II in the utricle (A). Moreover, BMDCs expressing Iba1 remarkably increase in response to surgical stress. The distribution of EGFP-positive cells in the utricle of the control group (B) and the operated group (C) is shown. The ratio of EGFP-positive cells to total cells in the vestibular stroma was significantly higher in the operated group than in the control group (D, $*p < 0.01$, unpaired t test). Bars represent 50 μm.

Discussion

The endolymphatic sac has been thought to be the only organ in the inner ear where immune reactions take place [7]. Through serial studies, a possible inner ear immune response in the endolymphatic sac has been investigated using a locally immune-activated model by systemic immunization with keyhole limpet hemocyanin [14]. In the present study, leukocytes and macrophages derived from bone marrow were seen to reside at the epithelial layer of the endolymphatic sac under normal conditions, which is compatible with previous studies [6], and these findings convinced us of active immune processing in the endolymphatic sac. The difference in distribution and characteristics of BMDCs between the vestibular end organs and the endolymphatic sac also indicates the specific role of the endolymphatic sac in the inner ear immunology.

Kupffer cells in the liver or microglias in the brain are well known as resident tissue macrophages. Resident tissue macrophages are originated from bone marrow, migrate and settle, and reside in the organ for a long period – playing roles such as antigen presentation, phagocytosis, and release of chemokines [15,16]. Our study on the morphology and phenotype of BMDCs provides the first evidence that cells derived from hematopoietic bone marrow differentiate towards resident tissue macrophages in the adult vestibular end organ. Moreover, the resident macrophages appear to be novel constitutive cells in the vestibular stroma that consists of fibrocyte, blood vessel or capillary, and nerve fibers. Considering the role of Kupffer cells in liver cirrhosis or microglias in Alzheimer's disease [16,17], the resident macrophages in the vestibular end organs may play a role in the pathogenesis of inner ear immune disorders. The resident macrophages can be therapeutic targets by controlling their ability for phagocytosis, migration, or the release of cytokines. Future studies using a model of immune disorder in the inner ear will likely reveal the functional properties of resident macrophages in the vestibular end organs and the endolymphatic sac.

In conclusion, the present results demonstrate that BMDCs are present in the vestibular end organs and the endolymphatic sac in normal conditions, and that the majority have the characteristics of tissue-specific macrophages. In addition, some BMDCs in the vestibular end organs and the endolymphatic sac express MHC class II. Moreover, the number of BMDCs increases in response to local surgical stress. These findings indicate roles of BMDCs in the vestibular end organs and the endolymphatic sac in immune reactions and initiation of subsequent immunologic cascades.

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ORIGINAL ARTICLE

Multivariate analysis of hearing outcomes in patients with idiopathic sudden sensorineural hearing loss

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Abstract

Conclusions: Contralateral hearing loss is significantly correlated with poor hearing outcomes in patients with idiopathic sudden sensorineural hearing loss (ISSNHL). *Background:* The hearing outcome in patients with ISSNHL was analyzed using multiple variables. *Methods:* A retrospective chart review was conducted using 89 patients with ISSNHL. Patients within 40 dB HL of average hearing levels and/or patients whose hearing loss was restricted to low frequencies were excluded. The influence of pre-existing conditions on hearing outcome was analyzed using a polytomous universal model. Pre-existing conditions analyzed included hyperglycemia, hypercholesterolemia, hypertension, and contralateral hearing loss. In addition, the severity of hearing loss, age group, and the existence of vertigo were analyzed concomitantly. *Results:* Hearing recovery was significantly reduced in patients with a past history of contralateral hearing loss.

Keywords: *Contralateral hearing loss, pre-existing conditions, polytomous universal model*

Introduction

Idiopathic sudden sensorineural hearing loss (ISSNHL) is defined as inner ear hearing loss that develops abruptly without definitive causes. Although ISSNHL is one of the few sensorineural hearing disorders that can be cured, the hearing outcome differs greatly among cases. This diverse outcome is usually attributed to the heterogeneity of ISSNHL. No single pathophysiology can fully explain this diversity and various pathogeneses have been hypothesized to explain this disorder, including viral infections [1,2], genetic factors [3,4], and microvascular disturbance [1]. Given that different pathogeneses often result in different outcomes, prior studies have tried to correlate pre-existing pathological conditions with the hearing outcome of ISSNHL. For example, metabolic (e.g. hyperglycemia and hypercholesterolemia) and circulatory disorders (e.g. hypertension) may suggest underlying microvascular disorders as a cause of ISSNHL [5,6], while prior hearing disturbance in the contralateral ear may be suggestive of genetic factors [3]. However, the prognostic value of these factors differed from study to study. One reason for the inconsistencies may be the analytic procedures used. In previous studies aimed at evaluating prognostic values, the effects of single factors were analyzed. However, the results obtained from analyzing a single factor can be distorted by the existence of multiple background factors. Hearing level before treatment, age of the patient, and the existence of vertigo have all been reported to affect the prognosis of ISSNHL [7]; therefore, these factors should be analyzed concomitantly to more accurately assess the effect of pre-existing conditions on the hearing outcomes of ISSNHL. Since some of these factors are ordinal and the others are nominal, multifactorial analyses that can handle ordinal or nominal factors are needed. An analysis with a polytomous universal model is used to test for the effects of multiple independent factors on an ordinal dependent variable, which, in this case, is hearing outcome. These independent factors can be ordinal or nominal. Using this model it is possible to determine the significance of

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these independent variables. Thus, in this study we analyzed the influence of pre-existing pathological conditions on the post-treatment hearing results of ISSNHL using a polytomous universal model.

Material and methods

Patients

We conducted a retrospective chart review of patients who received initial treatment between January 2002 and December 2009 from the Department of Otolaryngology-Head & Neck Surgery, Kyoto University Graduate School of Medicine. One hundred and five ISSNHL patients who were more than 18 years of age visited the hospital within 2 weeks after the onset of hearing loss. Patients within 40 dB HL of average hearing levels at five frequencies between 250 and 4000 Hz and/or patients whose hearing loss was restricted to low frequencies were excluded from this study, because such patients were reported to have better hearing prognoses [8]. Some patients declined to receive the standard treatment provided by our hospital and they were also excluded from the analysis. In total, 89 patients with ISSNHL were included in the analysis. There were 50 men and 39 women ranging from 19 to 83 years of age (mean 53.0 years). All patients were hospitalized and administered corticosteroid intravenously (starting with 200 mg of prednisolone, tapered over 9 days), vitamin B12 perorally (1500 μg per day, during the follow-up) and vasodilators perorally (kallidinogenase 150 IU/mg per day, during the follow-up). Some patients were administered hyperbaric oxygen. Patient follow-ups were conducted until they showed complete recovery or their hearing level was stabilized for more than 1 month (fixed stage).

Analytic procedure

Auditory function was determined by pure-tone audiometry and was expressed by the pure-tone average (PTA in decibels) hearing level at five frequencies (250, 500, 1000, 2000, and 4000 Hz). The PTA before treatment was obtained at the first visit and the post-treatment PTA was obtained at the fixed stage.

Hearing outcome was analyzed based on the criteria prepared by the Acute Severe Hearing Loss Study Group [9]. Using these criteria, the outcome was graded into four classes: (1) complete recovery, recovery to a hearing level within 20 dB HL at all five frequencies between 250 and 4000 Hz, and/or recovery to the same hearing level as the 'good' side; (2) marked recovery, more than 30 dB recovery in the mean hearing level at the five frequencies tested; (3) slight recovery, recovery of 10–29 dB in the mean hearing level at the five frequencies tested; and (4) no response, recovery < 10 dB in the mean hearing level at the five frequencies tested. The hearing outcomes based on these criteria were analyzed using a polytomous universal model.

We recorded the following conditions for this analysis: hyperglycemia, hypercholesterolemia, hypertension, and a past history of contralateral hearing loss. In addition, severity of hearing loss, age group, and the existence of vertigo were also included in the analysis [7]. Hyperglycemic patients were defined as those who had been treated for hyperglycemia and/or whose fasting glucose at the first visit exceeded 100 mg/dl. The hypercholesterolemic patients included those previously treated for this condition and those with total serum cholesterol exceeding 240 mg/dl at the initial visit. Hypertensive patients were defined as those who had been treated for hypertension and/or whose blood pressure continued to measure 140/90 mmHg or higher before the administration of steroids. Patients with a past history of contralateral hearing loss were defined as those who had displayed distinct symptoms in the non-affected ear including hearing loss, tinnitus, and ear fullness before the onset of ISSNHL and whose PTA was above 40 dBHL. Sensorineural hearing loss compatible with normal age-related changes and/or hearing loss without subjective symptoms were not included.

In accordance with the criteria prepared by the Acute Severe Hearing Loss Study Group [9] the severity of hearing loss was described using the PTA at five frequencies (250, 500, 1000, 2000, and 4000 Hz): (1) moderate, PTA of 40–59 dB HL; (2) severe, PTA of 60–89 dB HL; (3) profound, PTA of > 90 dB HL. The patients were classified into three age groups: the young group consisted of those who were younger than 30, the middle-aged group was between 30 and 60, and those in the old group were over 60 years of age. The presence of vertigo was defined as rotatory sensation with nystagmus. Statistical analysis was conducted using SPSS software.

Results

The grand-averaged pretreatment hearing level was 79.1 dB HL, while the post-treatment hearing level was 44.6 dB HL. The averaged absolute hearing gain was 34.5 dB. Based on the criteria set by the Acute Severe Hearing Loss Study Group, 23 patients were evaluated as showing complete recovery, 35 patients

as marked recovery, 16 patients as slight recovery, and 15 patients as showing no response. The treatment results are shown in Figure 1.

The severity of the hearing loss before treatment was moderate in 18 patients, severe in 40 patients, and profound in 31 patients. Sixteen patients were classified as young, 37 patients were classified as middle-aged, and the remaining 36 patients were classified as old. The hearing outcomes according to pretreatment severity and age group are shown in Tables I and II, respectively. Among the 89 patients, 10 patients had hyperglycemia, 27 patients had hypercholesterolemia, 20 patients had hypertension, and 12 patients had contralateral hearing loss. Twenty-eight patients had accompanying vertigo.

The analysis of these treatment outcomes using the criteria prepared by the Acute Severe Hearing Loss Study Group showed that a past history of

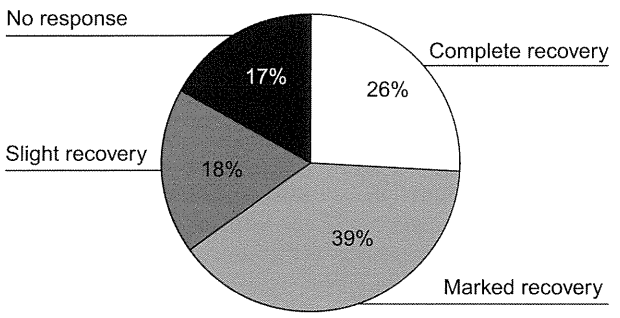

Figure 1. Overall treatment results for patients with idiopathic sudden sensorineural hearing loss. The hearing outcome was graded into four classes: complete recovery, recovery to a hearing level within 20 dB HL at all five frequencies between 250 and 4000 Hz, and/or recovery to the same hearing level as the 'good' side; marked recovery, more than 30 dB recovery in the mean hearing level at the five frequencies tested; slight recovery, recovery of 10–29 dB in the mean hearing level at the five frequencies tested; and no response, recovery < 10 dB in the mean hearing level at the five frequencies tested.

Table I. Summary of hearing outcome according to the pretreatment severity of hearing loss.

	Outcome				
Severity	Complete recovery	Marked recovery	Slight recovery	No response	Total
Moderate	7	2	5	4	18
Severe	13	13	7	7	40
Profound	3	20	4	4	31
Total	23	35	16	15	89

The severity was graded into three classes: moderate, pure-tone average (PTA) of 40–59 dB HL; severe, PTA of 60–89 dB HL; profound, PTA of greater than 90 dB HL.

Table II. Summary of hearing outcome by age group.

	Outcome				
Age group	Complete recovery	Marked recovery	Slight recovery	No response	Total
Young	5	9	2	0	16
Middle aged	7	12	11	7	37
Old	11	14	3	8	36
Total	23	35	16	15	89

The patients were classified into three age groups: the young group consisted of those who were younger than 30, the middle-aged group was between 30 and 60, and those in the old group were over 60 years of age.

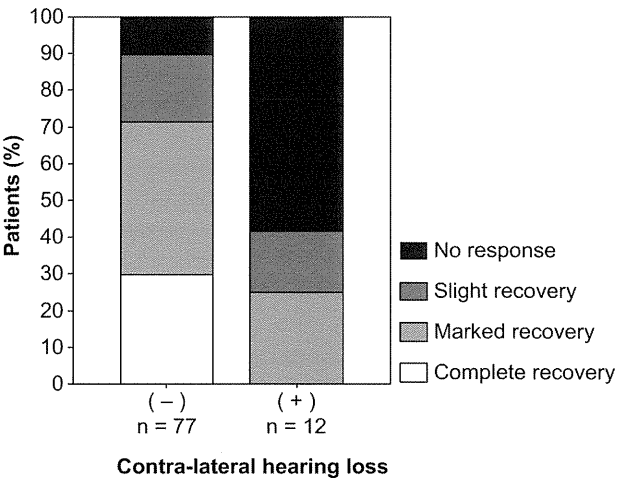

Figure 2. Hearing treatment results for patients with and without a past history of contralateral hearing loss. The treatment results from the groups with a past history of contralateral hearing loss were significantly worse than those without a past history of contralateral hearing loss.

contralateral hearing loss is significantly correlated ($p < 0.01$) with reduced recovery from ISSNHL. Figure 2 shows a statistically significant difference ($p < 0.01$, Mann-Whitney test) between the treatment results from the groups with and without a past history of contralateral hearing loss. The overall results of the analysis are shown in Table III.

Discussion

The effect of a past history of contralateral hearing loss on hearing outcomes following ISSNHL remains controversial. Stahl and Cohen have reported that the hearing outcome of ISSNHL is the same in patients with or without good hearing in the opposite ear [10]. In contrast, when Cvorović et al. analyzed 541 cases

Table III. Multivariate analysis of the results of treatment on hearing outcome (polytomous universal model).

Parameter	B	SEM	Wald	*p* value
Severity				
Moderate	−0.28	0.60	0.22	0.64
Severe	−0.11	0.50	0.05	0.82
Profound	0.00			
Hypercholesterolemia				
−	0.09	0.45	0.04	0.85
+	0.00			
Age group				
Young	−0.18	0.64	0.08	0.78
Middle-aged	0.69	0.49	1.97	0.16
Old	0.00			
Hyperglycemia				
−	−0.20	0.67	0.09	0.77
+	0.00			
Hypertension				
−	−0.26	0.52	0.24	0.62
+	0.00			
Vertigo				
−	−0.91	0.48	3.71	0.05
+	0.00			
Contralateral hearing loss				
−	−2.51	0.66	14.33	<0.001*
+	0.00			

The analysis of these treatment outcomes showed that a past history of contralateral hearing loss was significantly correlated with reduced recovery. B, regression coefficient; SEM, standard error of the mean; Wald, Wald statistics.
*$p < 0.01$.

they concluded that patients with previously diagnosed hearing loss in the opposite ear had poor hearing results [7] and the correlation between hearing recovery in the affected ear and hearing in the opposite ear was significant. In our study, similar to the study of Cvorović et al., we demonstrated poor hearing outcomes in ISSNHL patients with prior contralateral hearing loss. This discrepancy between studies may be explained by the patient selection criteria. In the present study we excluded those with low-frequency hearing loss in order to exclude patients with delayed endolymph hydrops. Patients with previous hearing loss often develop endolymph hydrops in the opposite ear (delayed endolymph hydrops), and short-term hearing recovery is good in such patients. Cvorović et al. included only a small number of patients with low-frequency hearing loss (4.7%) [7], whereas in the study by Stahl and Cohen, five of nine patients showed up-slope hearing loss and two patients experienced the recurrence of hearing loss [10], suggesting that these patients were affected by endolymph hydrops. These data could explain the more favorable hearing results reported in that study for patients with contralateral hearing loss.

Microvascular disease is one candidate for the pathophysiology of ISSNHL. Metabolic and circulatory diseases including hyperglycemia, hypercholesterolemia, and hypertension are well known risk factors for microvascular diseases. The levels of triglycerides, total cholesterol, and lipoprotein A are often significantly higher in patients with sudden deafness than in control subjects [11]. However, the influence of such metabolic diseases on the hearing outcomes of ISSNHL is still controversial. Orita et al. reported that ISSNHL patients with hyperglycemia and hypercholesterolemia had improved hearing results [5]. In the present study, however, we did not find that such diseases were correlated to the outcomes of ISSNHL. This discrepancy may be explained by the different treatment regimens employed. Orita et al. used prostaglandin and hyperbaric oxygen administration, which may be beneficial in patients with microvascular diseases [5]. Another study using a similar treatment regimen to ours reported that ISSNHL patients with hyperglycemia, hypercholesterolemia, and hypertension showed poor hearing results [12]. These results suggest that modification of the treatment regimen may be preferable in ISSNHL patients with risk factors for microvascular diseases.

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Local hypothermia in the treatment of idiopathic sudden sensorineural hearing loss

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Abstract

Objective: The additive effects of local hypothermia and restricted activity in the treatment of idiopathic sudden sensorineural hearing loss (ISSHL) were investigated by case-matched study as a multicenter (13 hospitals) pilot trial.

Patients and methods: In a preliminary experiment, we evaluated the effects of cooled water pillow (15 °C). Cooling the neck and mastoid with the pillow decreased the tympanic membrane temperature for 1.4 °C in 2 h without causing uncomfortable sensation or frostbite. In this study, 86 patients with ISSHL were enrolled in the hypothermic group, which received hypothermic treatment with restricted activity in addition to medication, and 86 ISSHL patients constituted the control group, which received the same medication but without cooling and rest. Control patients were selected retrospectively from case records by matching the experimental patients with respect to age, gender, days until the start of treatment, hearing loss, shape of the audiogram, and accompanying vertigo. The patients in the hypothermic group were admitted and treated with a cooled water pillow for 48 h, in addition to conventional drug treatment (*e.g.*, 60 mg of prednisone) for 7 days. The water pillow was cooled to 15 °C and was changed 4–5 times per day. The patients used the water pillow for the first 48 h after admission, with restricted activity. The control patients received only the medications.

Results: Hearing results were evaluated using criteria proposed by the Sudden Sensorineural Hearing Loss Research Group of the Japanese Ministry of Health and Welfare. The recovery rates were judged 6 months after onset. The recovery rate in the hypothermic group was significantly ($p < 0.05$) better than that in the control group. When the comparison was limited to younger patients, the use of the cooled water pillow was effective in facilitating the recovery of hearing.

Conclusions: Hearing restoration in ISSHL may be improved by adding mild hypothermia and restricted activity to the conventional treatment.

Keywords: Local hypothermia; Cooling therapy; Idiopathic sudden sensorineural hearing loss

1. Introduction

Idiopathic sudden sensorineural hearing loss (ISSHL) usually presents as an acute idiopathic unilateral deafness. It affects 27,000 patients annually in Japan and involves otherwise healthy people, mainly between 50 and 60 years of age [1]. Various therapeutic options have been attempted, both alone and in combination; these include steroids, vasodilators, diuretics, anticoagulants, contrast medium, and carbogen inhalation. According to recent double-blind randomized control studies [2–4], no specific agent or treatment dramatically restored hearing in this disease. The natural course of ISSHL is fairly good, although the rate of complete recovery remains 24–63% [5] even when treatment starts within 14 days after onset. Better treatment modalities are needed to improve the results, especially in cases of severe hearing loss.

The etiology of ISSHL remains unknown; theoretical causes of ISSHL include viral infection [6,7], immune-mediated disorder [8,9], rupture of the labyrinth membrane, and disturbed vascular circulation [10,11]. Given that ISSHL

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occurs suddenly without any preceding signs or symptoms, we believe that an acute interruption of the blood supply to the inner ear is the primary cause of ISSHL. Previously, we showed that occluding both labyrinthine arteries for 15 min caused profound deafness and a substantial loss of hair cells in Mongolian gerbils [12]. We found that post-ischemic hypothermia could reduce the profound cochlear damage induced by transient ischemia in the animal model [13]. Recent studies of ischemic damage in the brain have proven hypothermia to be effective for preventing the sequelae of ischemia-induced brain damage. These findings suggest that reducing the cochlear temperature may prevent the progression of hearing loss; however, the effects of hypothermia in ISSHL remain unclear.

The present study evaluated the effect of hypothermic treatment with restricted activity, when applied as an adjunct to conventional treatment, on the recovery of hearing in ISSHL.

2. Background and preliminary experiments

Unlike in other organs, localized cooling of the inner ear is technically difficult. The inner ear is located deep in the skull, within the pneumatic mastoid cavity, and is nourished solely by the labyrinthine artery, a branch of the basilar artery. Irrigation of the external ear canal with cool water decreases the temperature in the inner ear, but it causes severe rotational vertigo. Therefore, indirect procedures such as cooling a wide area of the neck and mastoid would be more suitable for clinical application. We used a cooled water pillow to reduce the inner ear temperature. The effects of a cooled pillow on body temperature and sleep have been studied extensively. Kawabata and Tokura [14] showed that the use of a pillow filled with a special cool medium consisting of sodium sulfate and ceramic fiber significantly lowered the forehead skin temperature and heart rate during sleep at night. They noted that mild cooling of the head decreased wakefulness at night and induced deeper sleep. Okamoto-Mizuno et al. [15] demonstrated that a cooled pillow decreased the temperature of the tympanic membrane, which is correlated with the temperature of the inner ear [16].

Therefore, we evaluated the effectiveness of using a cooled water pillow to reduce the ear canal temperature in seven volunteers, 29–48 years old, including six males and one female. A water pillow was filled with water at 15 °C, as this temperature is not thought to cause frostbite. The subject was asked to lie on a bed in the supine position with the pillow behind the neck and mastoid on one side. Using an ear thermometer (MC510, Omron), the tympanic membrane temperature was measured every 10 min for 2 h. The axillary temperature was monitored as a control. Fig. 1 shows the changes in the tympanic membrane temperature over time. With the water pillow, the tympanic membrane temperature decreased by 1.4 °C in the seven subjects, from an average of 36.5 °C to 35.1 °C, while the axillary temperature remained

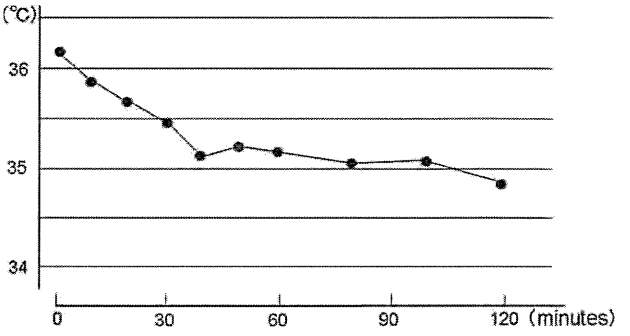

Fig. 1. Changes in the tympanic membrane temperature after using water pillow filled with water at 15 °C. Subject was asked to lie on a bed in the supine position with the pillow behind the neck and mastoid on testing side. The tympanic membrane temperature was decreased by 1.4 °C at 2 h after the local cooling.

stable. In two subjects, the temperature was monitored for 48 h. They were asked to stay in bed as much as possible but were allowed free access to a bathroom and to a bedside table for eating. The temperature decrease remained within the range of 1.0–1.5 °C for the 48 h. Encouraged by this result, we conducted the following clinical trial as a pilot study.

3. Patients and methods

The effects of hypothermia and restricted activity in the treatment of ISSHL were evaluated using a case-matched study, as a blind-control study was impossible for this experiment. Between April 2006 and January 2008, we recruited 86 patients with ISSHL at 13 public and private hospitals in Japan, including Ehime University Hospital, who met the following selection criteria: (a) older than 15 years of age, (b) hearing loss of more than 40 dB in pure tone average, (c) excluded acute low-tone sensorineural hearing loss, (d) treatment initiated within 14 days after onset, (e) accepted treatment by being admitted to a hospital, (f) followed until complete recovery or longer than 6 months, and (g) no systemic disease or contraindications to steroid use, including diabetes, peptic ulcer, pregnancy, and psychosis. These patients constituted the hypothermic group. As the control group, another 86 patients were selected by using case records to find patients matching those in the hypothermic group with respect to age, gender, starting day of treatment, hearing loss, shape of the audiogram, and accompanying vertigo. Detailed profiles showing the similarities between the hypothermic and control groups are presented in Table 1. In the hypothermic group (40 males and 46 females), patient age ranged from 15 to 82 years (mean, 59.9 years), the average time between the onset of hearing loss and the start of treatment was 3.7 days, and the average hearing level was 73.8 dB for the pure tone average of five frequencies (0.5, 1, 2, 4, and 8 kHz). No significant difference in age was found between the hypothermic group and the control group. Furthermore,

Table 1
Characteristics of two groups with or without cooled water pillow.

	Hypothermic group ($n = 86$)	Control group ($n = 86$)
Average age (years old)	59.9	59.0
Age range (years old)	15–82	18–78
Average hearing level (dB)	73.8	73.6
Period between onset and initial treatment (days)	3.7	4.2

there were no statistical differences in average hearing level, or the period from onset and the initial treatment between the two groups. The data were analyzed statistically using Mann–Whitney test.

As many studies have indicated that steroids are effective for restoring hearing in ISSHL, ethical considerations dictated that all of the patients in both groups be treated with steroids. Each patient received 60 mg of prednisone for 3 days, which was tapered subsequently over 7 days, and 60 mg of ATP plus 150 mg of methylcobalamin for 7 days. In the hypothermic group, hypothermia was applied by placing a cooled (15 °C) water pillow behind the neck and ipsilateral mastoid while the patient was in the supine position in bed. The patients used the water pillow for 48 h after hospital admission, and the water pillow was changed 4–5 times per day, as needed. The patients were asked to stay in bed as much as possible during the 48 h. They were allowed to free access to a bathroom, although bathing was not permitted. Great care was taken to avoid skin damage, which can result from long exposure to hypothermia below 15 °C. The control group was treated with the same drug regimen but not with a cooled water pillow and rest.

The hearing results were evaluated using the criteria proposed by the Sudden Sensorineural Hearing Loss Research Group of the Japanese Ministry of Health and Welfare [17]. At 6 months after the onset of ISSHL, each patient's hearing was evaluated as complete recovery, substantial recovery, slight recovery, or unchanged. Complete recovery was defined as hearing level recovered within 20 dB at five frequencies (0.5, 1, 2, 4, and 8 kHz) or hearing level recovered to that of the intact ear. Substantial recovery, slight recovery and unchanged were defined as more than 30 dB improvement in average hearing level at the five frequencies, 10–29 dB improvement of the average hearing level, and within 10 dB improvement of the average hearing level, respectively. The data were also analyzed by grading the degree of hearing loss based on the pure tone average of five frequencies (0.5, 1, 2, 4, and 8 kHz), as follows: grade 1 (<40 dB), grade 2 (40 to <60 dB), grade 3 (60 to <90 dB), and grade 4 (90+ dB). Possible complications related to the use of the cooled water pillow were also evaluated. The data were analyzed statistically using the χ^2 test or Fisher's test.

4. Results

No side effects related to the use of the cooled water pillow were noted. Most of the patients in the hypothermic group stated that the cool pillow made them feel sleepy and comfortable.

Fig. 2 summarizes the hearing results in the two groups. The complete recovery rate was 41.9% in the hypothermic group ($n = 86$) and 25.6% in the control group ($n = 86$). The recovery rate (*i.e.*, complete recovery plus substantial recovery) differed significantly ($p < 0.05$) between the hypothermic (65.1%) and control groups (50.0%).

For patients treated within 24 h after onset, the complete recovery rates were 48.1% and 34.5% in the hypothermic ($n = 27$) and control groups ($n = 29$), respectively, and the recovery rates were 63.0% and 51.7%, respectively. These rates were not significantly different between the two groups. Similar results were seen for patients who were treated more than 2 days after onset.

The rates were also compared according to the degree of hearing loss. Among those with mild hearing loss (grades 1 and 2), the recovery rates were 47.8% ($n = 23$) and 34.8% ($n = 23$) in the hypothermic and control groups, respectively, and the respective complete recovery rates were 47.8% and 30.4%; the differences in rates were not statistically significant. For those with severe hearing loss (grades 3 and 4), the recovery rates were 52.3% ($n = 63$) and 40.1% ($n = 63$) in the hypothermic and control groups, respectively, and the respective complete recovery rates were 39.7% and 23.8%; the differences in these rates were not statistically significant.

The rates in patients older than 60 years also did not differ significantly between the hypothermic and control groups; the respective recovery rates were 60.8% and 53.5%, and the respective complete recovery rates were 37.2% and 25.6%. The complete recovery rate in patients younger than 59 years did not differ significantly between the hypothermic (48.6%) and control groups (25.6%). However, the recovery rate in

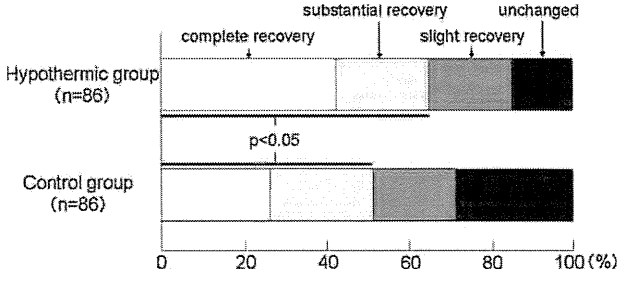

Fig. 2. Hearing results in local hypothermic group and control group. The recovery rate (*i.e.*, complete recovery plus substantial recovery) differed significantly ($p < 0.05$) between the hypothermic (65.1%) and control groups (50.0%).

patients younger than 59 years was significantly ($p < 0.05$) different between the hypothermic (71.4%) and control groups (46.5%). Thus, hypothermia and restricted activity was effective in helping to restore hearing in younger patients.

5. Discussion

Hypothermia is a proven strategy for preventing ischemia-induced damage and is currently applied clinically in various fields, including brain and cardiac surgery. Although deep hypothermia causes complications such as reduced blood flow, increased bacterial infection, and decreased cellular immunity [18], mild hypothermia (lowering the body temperature by 1–2 °C) was recently shown to be effective in preventing ischemic damage to the brain [19,20] and in facilitating the recovery of comatose survivors of cardiac arrest [21]. Mild hypothermia also reduced damage to the cochlea upon electrode insertion in cochlear implants or from exposure to loud noise [22,23]. However, no report has addressed the effect of hypothermia in ISSHL. Here, we demonstrated that some patients treated with a cooled pillow and rest in addition to drugs achieved better recovery of hearing, compared with patients treated only with drugs. Although the effects were limited, our findings are promising and warrant further studies investigating the best indications, optimal conditions, and appropriate medication for the treatment of ISSHL using mild hypothermia.

Historically, cooled water pillows have long been used in the palliative treatment of febrile disease. Another advantage of a cooled pillow is that it often causes a sleepy sensation by reducing brain function, and sleep may facilitate recovery from disease. We consider a water pillow to be an easy, inexpensive, and safe way of inducing mild hypothermia in the ear. This therapeutic option may be acceptable even to pediatric patients. Further clinical study in a large population is necessary to confirm the efficacy of mild hypothermia and restricted activity in the treatment of ISSHL.

The beneficial effects of hypothermia in preventing ischemic injury can be attributed to various mechanisms, including decreased metabolic rate, reduced tissue oxygen consumption, decreased metabolic acidosis, suppressed calcium influx into neurons, and reduced production of superoxide anion radicals. Recent experimental studies on brain ischemia have suggested that reduced glutamate toxicity is an important mechanism underlying the protective effects of hypothermia. Glutamate released from the ischemic core flows into the surrounding region, where it is toxic to neuronal tissues. The neuroprotective efficiency of hypothermia was influenced by the varying levels of age-related glutamate release [24]. Therefore, hypothermia was especially effective in younger patients. As glutamate is a neurotransmitter at the synapses between the inner hair cells and primary afferent auditory neuron, it is released extensively in ischemia/reperfusion injury of the inner ear. Hyodo *et al.* [25] showed that hypothermia can reduce glutamate efflux into the perilymph and prevent ischemic neural damage that would otherwise spread in the inner ear. As mild hypothermia works non-specifically, it may be useful in the treatment of various causes of sensorineural hearing loss, including aminoglycoside ototoxicity, traumatic inner ear damage, and noise-induced hearing loss.

6. Conclusion

Hypothermic therapy is widely accepted in many fields of medicine, although its clinical application in the treatment of ISSHL has never been tested. We investigated the effects of mild hypothermia with restricted activity in the treatment of ISSHL by cooling the ear using a water pillow. In this pilot study, mild hypothermia and restricted activity was effective in helping to restore hearing in patients with severe hearing loss and in patients younger than 59 years. Although the effects were limited in some patients, we believe that the use of a cooled water pillow in the treatment of ISSHL is a promising approach with no apparent side effects.

Acknowledgement

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RESEARCH ARTICLE Open Access

Delayed neuronal cell death in brainstem after transient brainstem ischemia in gerbils

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Abstract

Background: Because of the lack of reproducible brainstem ischemia models in rodents, the temporal profile of ischemic lesions in the brainstem after transient brainstem ischemia has not been evaluated intensively. Previously, we produced a reproducible brainstem ischemia model of Mongolian gerbils. Here, we showed the temporal profile of ischemic lesions after transient brainstem ischemia.

Results: Brainstem ischemia was produced by occlusion of the bilateral vertebral arteries just before their entry into the transverse foramina of the cervical vertebrae of Mongolian gerbils. Animals were subjected to brainstem ischemia for 15 min, and then reperfused for 0 d (just after ischemia), 1 d, 3 d and 7 d (n = 4 in each group). Sham-operated animals (n = 4) were used as control. After deep anesthesia, the gerbils were perfused with fixative for immunohistochemical investigation. Ischemic lesions were detected by immunostaining for microtubule-associated protein 2 (MAP2). Just after 15-min brainstem ischemia, ischemic lesions were detected in the lateral vestibular nucleus and the ventral part of the spinal trigeminal nucleus, and these ischemic lesions disappeared one day after reperfusion in all animals examined. However, 3 days and 7 days after reperfusion, ischemic lesions appeared again and clusters of ionized calcium-binding adapter molecule-1(IBA-1)-positive cells were detected in the same areas in all animals.

Conclusion: These results suggest that delayed neuronal cell death took place in the brainstem after transient brainstem ischemia in gerbils.

Background

In the central nervous system, certain areas are selectively damaged even after a brief ischemic insult, and this topographical heterogeneity is known as "selective vulnerability of the brain". Hippocampal CA1 and neocortical III, V, and VI are extremely vulnerable to ischemia and hypoxia [1]. The mechanism responsible for this vulnerability is of particular importance to establish therapeutic procedures, because elucidation of the mechanism may lead to the development of novel therapy to ameliorate ischemic damage.

Pathologic aspects and the topographic distribution of ischemic lesions after transient ischemia have been extensively studied in the rodent forebrain [2,3]. However, little is known about the distribution of ischemic lesions after transient brainstem ischemia because of the lack of reproducible brainstem ischemia models in rodents. Previously, we established a brainstem ischemia model in gerbils by occlusion of the bilateral vertebral arteries, and demonstrated selective vulnerability after permanent brainstem ischemia [4]. This gerbil model has the following advantages: (1) it produces brainstem ischemia without intracranial injury, (2) it produces severe, reproducible brainstem ischemia, and (3) it allows reperfusion.

In the present study, using this animal model, we investigated the temporal profile of ischemic lesions in the brainstem after transient brainstem ischemia in gerbils. We demonstrated ischemic lesions by immunostaining for microtubule-associated protein 2 (MAP2) in the lateral vestibular nucleus and the ventral part of the spinal trigeminal nucleus three days after transient brainstem ischemia, while these ischemic lesions were not found one day after ischemia. This delayed neuronal

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damage in the brainstem is reminiscent of the delayed neuronal cell death in the hippocampus after transient forebrain ischemia [5].

Methods

Animals and surgical procedure

Adult 12-16 week-old male Mongolian gerbils, weighing 60-80 g, were used in this study. All experiments were approved by the Ethics Committee of Ehime University Graduate School of Medicine and were conducted according to the Guidelines for Animal Experimentation at Ehime University Graduate School of Medicine. The gerbils were housed in an animal room with a temperature of 21 to 23°C and a 12-hour light/dark cycle (light on: 7 a.m. to 7 p.m.). The animals were allowed free access to food and water until the end of the experiment.

The gerbils were randomly divided into four groups, which were subjected to brainstem ischemia for 15 min and reperfused for 0 d (just after ischemia), 1 d, 3 d and 7 d (n = 4 in each group). Sham-operated animals (n = 4) were used as control. Animals were anesthetized with 1% halothane in 70% N_2O and 30% O_2. Anesthetized animals were orotracheally intubated with a ventilation tube. To facilitate access to the vertebral arteries, animals were placed in the supine position on a table tilted at approximately 30° to the horizontal. An anterior midline cervical incision was made, and the musculi longus colli were dissected to expose the vertebral arteries just before their entry into the transverse foramina of the cervical vertebrae. Both vertebral arteries were looped with 4-0 silk sutures. Then, the suture around each vertebral artery was pulled by a 5-g weight to occlude the circulation for 15 min. Consequently, apnea was observed within 1 min after occlusion, and subsequent convulsions were observed in all four limbs for about 1 min. After convulsions had ceased, all animals became unresponsive and lost their corneal reflex. Mechanical ventilation was initiated immediately after apnea was elicited during ischemia. The tidal volume was set to 1 ml and the rate was set to 70 breaths per minute. After 15 min of ischemia, the sutures were cut and removed to allow recirculation, which was confirmed by visual observation through an operating microscope. Within 10 min after reperfusion, spontaneous breathing reappeared and mechanical ventilation was ceased within 15 min after reperfusion.

Rectal temperature was maintained between 36.5 and 37.0°C by a heating lamp and a heating pad connected to a thermistor (ATB-1100, Nihon Koden, Tokyo, Japan) during surgery and until 1 h after reperfusion. After resuscitation, the animals were maintained in an air-conditioned room at about 22°C.

Histological procedures

After deep anesthesia with a lethal dose of sodium pentobarbital (0.1 g/kg), the gerbils were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and the brain was dissected out. After fixation with the same fixative for overnight the brain was dehydrated and embedded in paraffin. To investigate the temporal profile of ischemic lesions in the brainstem, we performed immunostaining for MAP2, IBA-1 and GFAP at the level of the lateral vestibular nucleus in the brainstem (5.5 mm caudal to the bregma) since this area has been reported to be most vulnerable to ischemia [4]. Coronal 5-μm-thick sections were examined by immunostaining for microtubule-associated protein 2 (MAP2), IBA-1 and glial fibrillary acidic protein (GFAP). Sections were immunostained using a Vectastain ABC Elite Kit (Vector Laboratories; Burlingame, Calif) with polyclonal anti-MAP2 (donated by Dr. Niinobe, Osaka University), polyclonal anti-IBA-1 (019-19741, Wako, Osaka, Japan) or monoclonal anti-GFAP (G9369, Sigma, St. Louis, USA) antibodies. Endogenous peroxidase in deparaffinized tissue sections was blocked for 10 minutes with 3% H_2O_2 in deionized water, followed by blocking with 10% goat serum diluted in 0.2% Tween-20 in phosphate buffered saline at room temperature for 1 hour. The tissues were then incubated with primary antibody (anti-MAP2, 1:1000; anti-IBA-1, 1:500; anti-GFAP, 1:500) at 4°C overnight. Tissue sections were washed and incubated with secondary antibody (1:1000) for 1 hour at room temperature. After washing, sections were incubated with ABC complex for 30 minutes at room temperature, and then stained with the chromogenic substrate 3, 3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2, until optimal staining was obtained.

Measurement of ischemic lesions

MAP2-stained sections were analyzed and images were viewed using a microscope (ECLIPSE E800, Nikon, Tokyo, Japan). The ischemic lesions detected by the loss of immunoreaction for MAP2 were measured and quantification was performed on images using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistics

All values are given as mean ± SD. Statistical analysis was performed with the Statistical Package for the Social Sciences, release 15 (SPSS ver. 15). Differences were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test. A p value of less than 0.05 was considered to indicate statistical significance.

Results

Immunohistochemical investigation

Four gerbils each were used for the reperfusion periods of 0, 1 d, and 3 d. As for the reperfusion period of 7 d, we evaluated three animals because one animal died of respiratory failure 5 days after ischemia. Sham-operated animals (n = 4) were used as control. Loss of immunoreaction for MAP2 in neuropils, nerve cell bodies, and dendrites was used as the criterion for the presence of ischemic lesions. The findings were compared with those in sham-operated controls. Each brain section was examined by two investigators; and whenever there was any uncertainty, a third investigator examined the specimen without any prior information.

Just after brainstem ischemia

Ischemic lesions detected by immunostaining for MAP2 were found in the lateral vestibular nucleus (LVe; blue arrows in Figure 1B) and the ventral part of the spinal trigeminal nucleus (Sp5; red arrows in Figure 1B) in all 4 animals (100%). Higher magnification photomicrographs of ischemic lesions showed loss of immunoreaction for MAP2 in neuropils and nerve cell bodies in LVe (blue arrows in Figure 2B) and the ventral part of Sp5 (red arrows in Figure 3B). Compared with sham-operated controls, there was no change in IBA-1 (a marker of microglia and monocytic lineage) and GFAP (a marker of astrocytes) expression (Figure 1G and 1L).

One day after brainstem ischemia

No ischemic lesion was detected by MAP2 staining (Figure 1C). Furthermore, there was no change in IBA-1 and GFAP expression, compared with that in sham-operated controls (Figure 1H and 1M).

Three days after brainstem ischemia

Ischemic lesions in LVe (blue arrows in Figures 1D and 2D) and the ventral part of Sp5 (red arrows in Figures 1D and 3D) appeared again in all 4 animals (100%). Compared with the ischemic lesions just after brainstem ischemia, ischemic lesions in LV expanded ventrally to include the spinal vestibular nucleus (SpVe) in 2 out of 4 animals (50%). De novo ischemic lesions were detected in the dorsal part of Sp5 (blue arrowheads in Figures 1D and 3D) and ventral cochlear nucleus (VC) (red arrowheads in Figures1D and 4D) in 2 out of 4 animals (50%).

In addition, IBA-1 immunoreactivity was markedly up-regulated in the central part of the ischemic lesions where MAP2 immunostaining was lost. Up-regulation of IBA-1 immunoreactivity was detected in LVe (blue arrows in Figures 1I and 2I) and the ventral part of Sp5 (red arrows in Figures 1I and 3I) in 3 out of 4 animals (75%). Up-regulation of IBA-1 immunoreactivity was also detected in the dorsal part of Sp5 (blue arrowheads in Figures 1I and 3I) and ventral cochlear nucleus (VC) (red arrowheads in Figures 1I and 4I) in 2 out of 4 animals (50%). Higher magnification photomicrographs demonstrated strongly IBA-1-positive cells in these areas. These IBA-1-positive cells displayed an amoeboid shape including only small perisomal lamellopodial expansions or a few unbranched processes. They were morphologically easily distinguishable from ramified microglial cells, which were recognized by their thick processes and large cell bodies.

Furthermore, immunoreactivity for GFAP disappeared in ischemic lesions where immunostaining for MAP2 was lost, whereas immunoreactivity for GFAP increased in the neighboring areas around ischemic lesions. A reduction of GFAP staining was detected in LVe (blue arrows in Figures 1N and 2N) and the ventral part of Sp5 (red arrows in Figures 1N and 3N) in 3 out of 4 animals (75%). A reduction of GFAP staining was also detected in the dorsal part of Sp5 (blue arrowheads in Figures 1N and 3N) and the ventral cochlear nucleus (VC) (red arrowheads in Figures 1N and 4N) in 2 out of 4 animals (50%). Higher magnification photomicrographs showed that GFAP-positive astrocytes were not observed in ischemic lesions where immunostaining for MAP2 was lost. Reactive astrocytes with thick, long GFAP-positive processes were distributed around ischemic lesions.

Seven days after brainstem ischemia

Ischemic lesions detected by immunostaining for MAP2 expanded further (Figure 5A-D). Ischemic lesions in LVe (blue arrows in Figures 1E and 2E) and the ventral part of Sp5 (red arrows in Figures 1E and 3E) appeared in all 3 animals (100%). Ischemic lesions were also detected in the dorsal part of Sp5 (blue arrowheads in Figures 1E and 3E) and the ventral cochlear nucleus (VC) (red arrowheads in Figures 1E and 4E) in 1 out of 3 animals (33%).

IBA-1 immunoreactivity was markedly up-regulated in ischemic lesions where MAP2 immunostaining was lost. Compared with the profile of IBA-1 staining three days after brainstem ischemia, strongly IBA-1-positive cells with an amoeboid shape were distributed more peripherally in ischemic lesions as well as in the center of ischemic lesions. Up-regulation of IBA-1 immunoreactivity was detected in LVe (blue arrows in Figures 1J and 2J) and the ventral part of Sp5 (red arrows in Figures 1J and 3J) in all three animals (100%). Up-regulation of IBA-1 immunoreactivity was also detected in the dorsal part of Sp5 (blue arrowheads in Figures 1J and 3J) and ventral cochlear nucleus (VC) (red arrowheads in Figures 1J and 4J) in one out of three animals (33%).

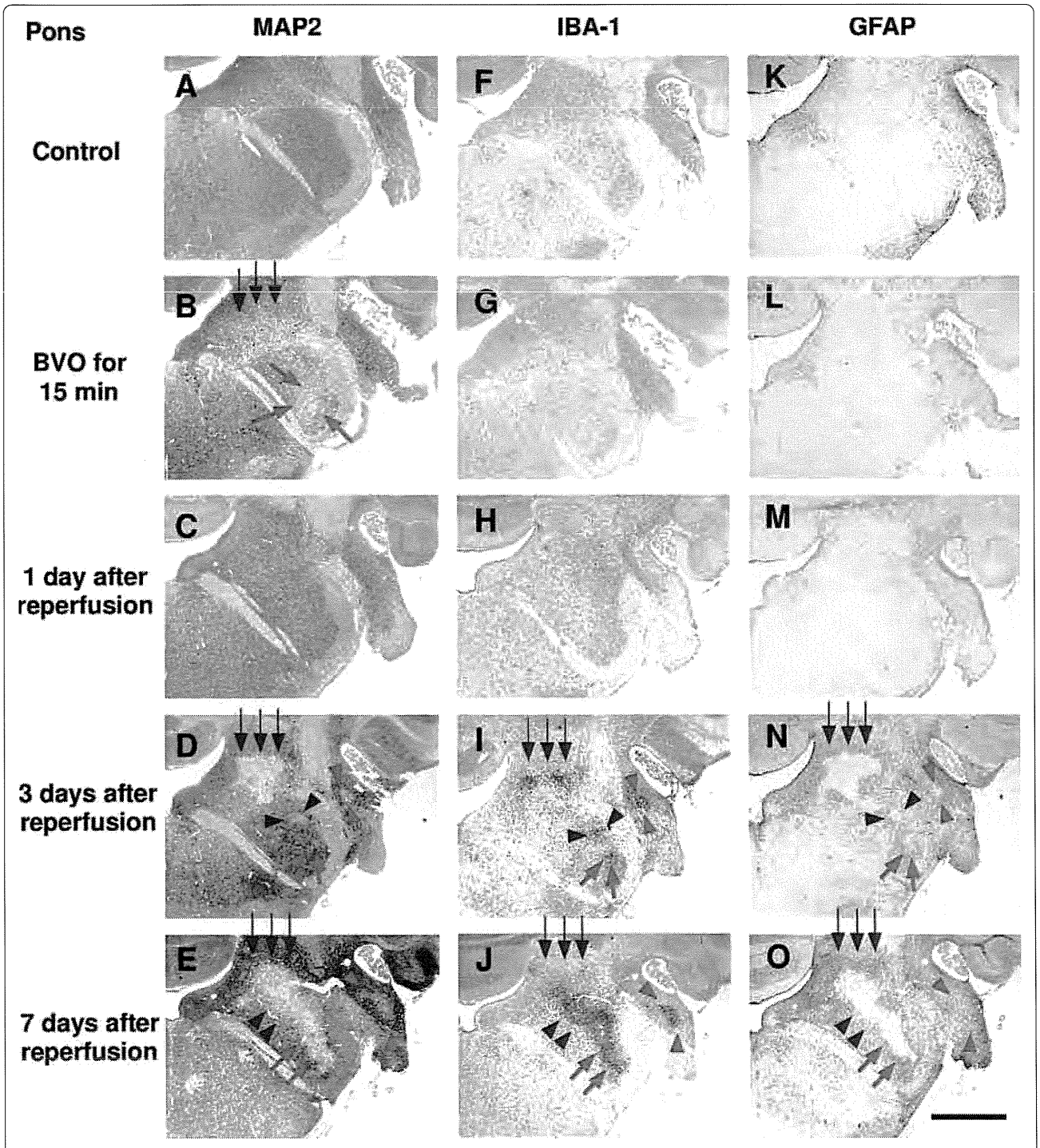

Figure 1 Representative photomicrographs of immunostaining after transient brainstem ischemia for 15 min (pons). Left column shows immunoreactivity for MAP2 (A-E); middle column shows immunoreactivity for IBA-1 (F-J); right column shows immunoreactivity for GFAP (K-O). Ischemic lesions with loss of MAP2 staining were detected in the lateral vestibular nucleus (LVe: B; blue arrows) and the ventral part of the spinal trigeminal nucleus (Sp5: B; red arrows) after bilateral vertebral artery occlusion (BVO) for 15 min. These ischemic lesions had disappeared at 1 day after reperfusion (C). At 3 days and 7 days after reperfusion, ischemic lesions appeared again and expanded further (D and E; blue and red arrows). New ischemic lesions were detected in the dorsal part of Sp5 (D and E; blue arrowheads) and ventral cochlear nucleus (VC) (D and E; red arrowheads). At the same time, clusters of amoeboid microglia/macrophages were detected in the same areas (I and J; blue and red arrows/arrowheads). At 3 days and 7 days after reperfusion, immunoreactivity for GFAP was lost in the ischemic lesions, and increased immunoreactivity for GFAP was detected around ischemic lesions (N and O; blue and red arrows/arrowheads). Scale bar = 1 mm.

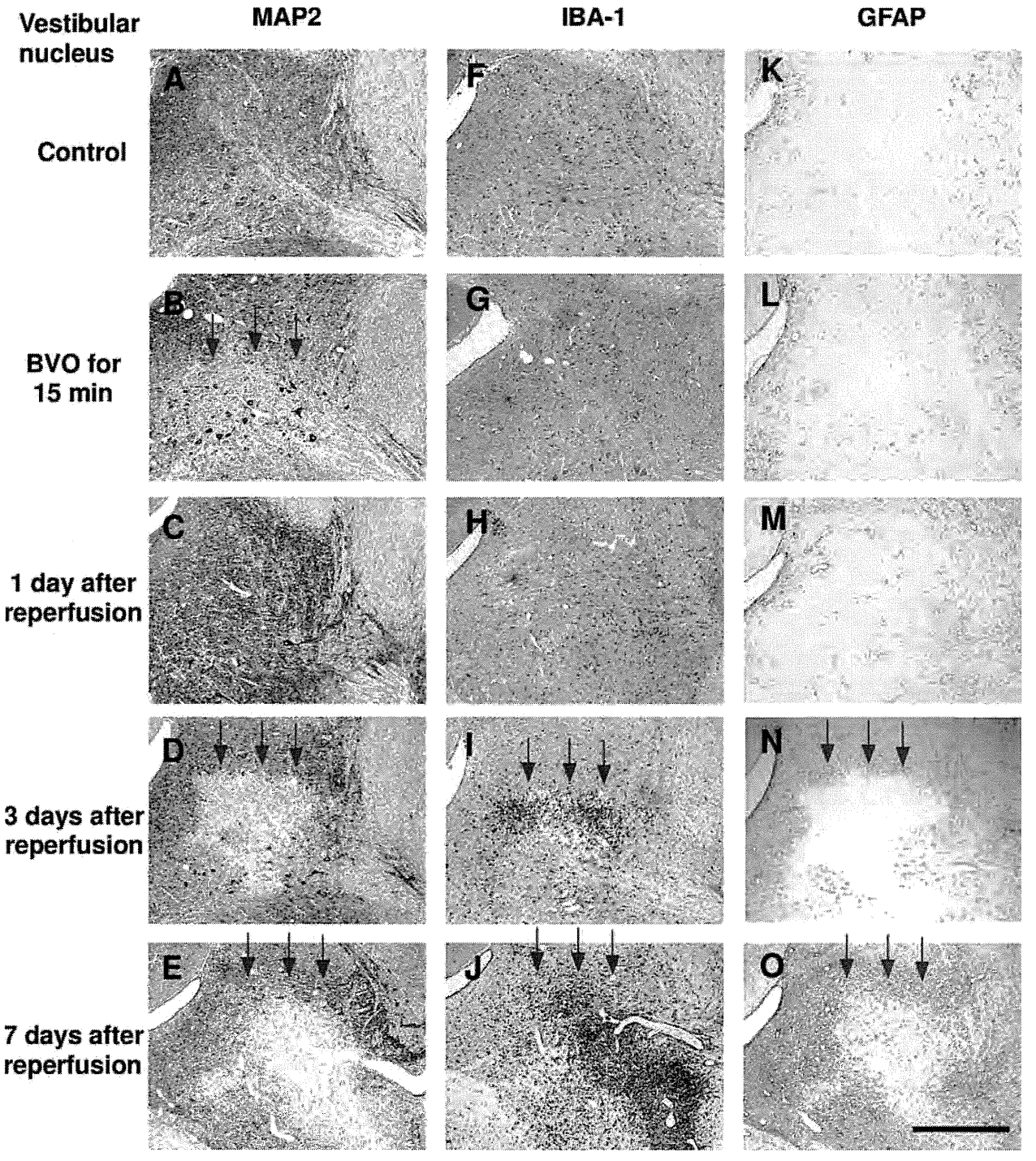

Figure 2 Representative photomicrographs of immunostaining after transient brainstem ischemia for 15 min (vestibular nucleus). Left column shows immunoreactivity for MAP2 (A-E); middle column shows immunoreactivity for IBA-1 (F-J); right column shows immunoreactivity for GFAP (K-O). Ischemic lesions with loss of immunoreactivity for MAP2 were seen in the lateral vestibular nucleus after bilateral vertebral artery occlusion (BVO) for 15 min (B; blue arrows), and these lesions had disappeared at 1 day after reperfusion (C). However, ischemic lesions had reappeared and expanded further at 3 days and 7 days after reperfusion (D and E; blue arrows). Clusters of IBA-1-positive amoeboid microglia/macrophages (I and J; blue arrows) and loss of expression of GFAP (N and O; blue arrows) were detected in the same areas where MAP2 expression was markedly lost at 3 days and 7 days after reperfusion. Increased immunoreactivity for GFAP (N and O; blue arrows) was also detected around ischemic lesions at 3 days and 7 days after reperfusion. Scale bars = 0.5 mm.

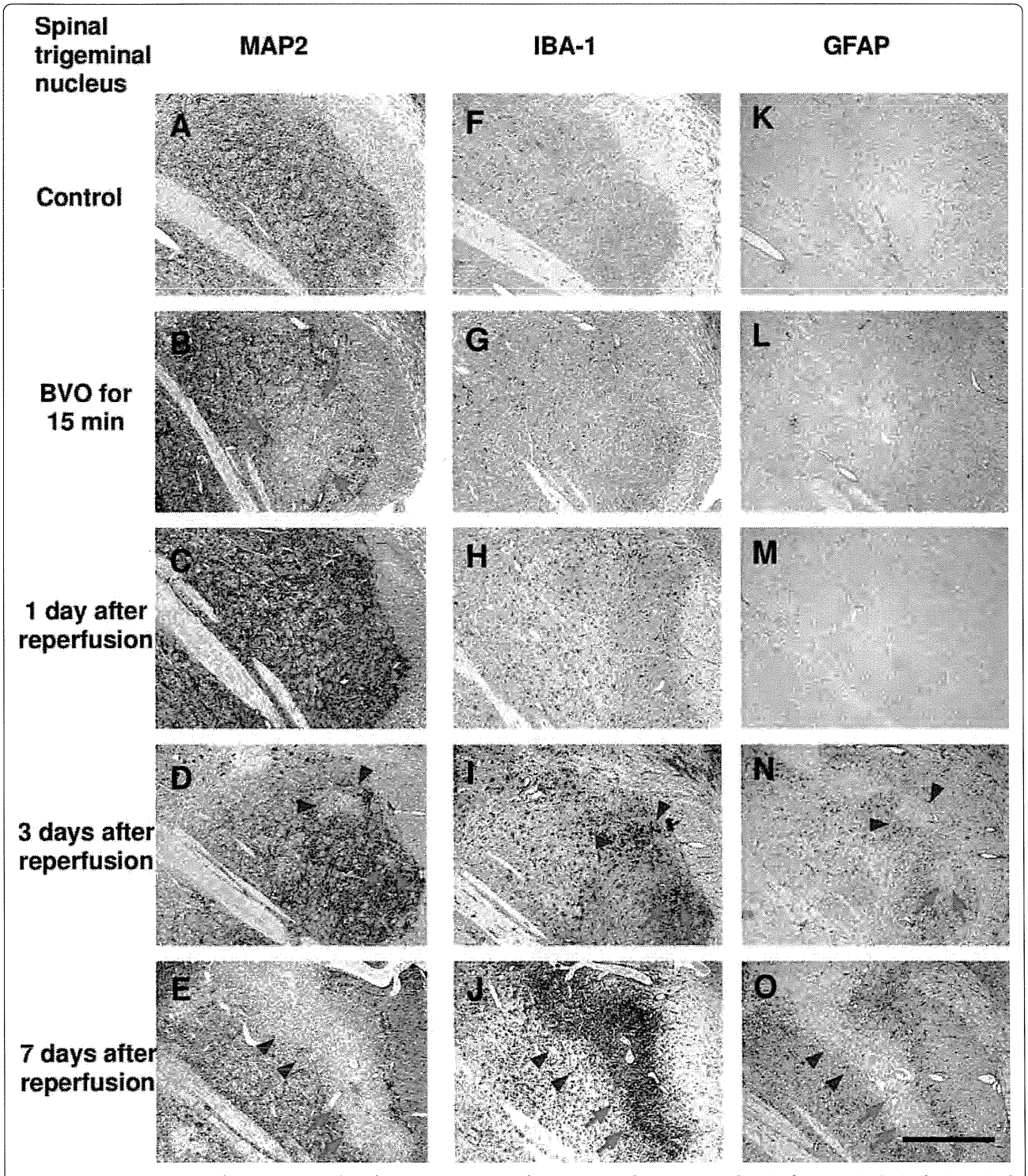

Figure 3 Representative photomicrographs of immunostaining after transient brainstem ischemia for 15 min (spinal trigeminal nucleus). Left column shows immunoreactivity for MAP2 (A-E); middle column shows immunoreactivity for IBA-1 (F-J); right column shows immunoreactivity for GFAP (K-O). Ischemic lesions with loss of immunoreactivity for MAP2 were seen in the ventral part of Sp5 after bilateral vertebral artery occlusion (BVO) for 15 min (B; red arrows), and these lesions had disappeared at 1 day after reperfusion (C). However, ischemic lesions had reappeared and expanded further (D and E; red arrows) and new ischemic lesions were detected in the dorsal part of Sp5 (D and E; blue arrowheads) at 3 days and 7 days after reperfusion. Clusters of IBA-1-positive amoeboid microglia/macrophages (I and J; red arrows and blue arrowheads) and loss of GFAP expression (N and O; red arrows and blue arrowheads) were detected in the same areas where MAP2 expression was lost at 3 days and 7 days after reperfusion. Increased immunoreactivity for GFAP (N and O; red arrows and blue arrowheads) was also detected around ischemic lesions at 3 days and 7 days after reperfusion. Scale bars = 0.5 mm.