

Cranial hyperthermia resulting from decreased parotid gland salivation induced by ischemic degeneration of glossopharyngeal network in subarachnoid hemorrhage

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ABSTRACT

Objective: We speculated that subarachnoid hemorrhage (SAH) induces ischemic lesions in the cranial parasympathetic nerves, which may decrease saliva secretion and lead to hyperthermia. We tested this hypothesis by examining histologic features of parotid glands and glossopharyngeal nerves (GPNs) in a rabbit model of SAH.

Material and Methods: Rabbits (n = 25) were divided into control (n = 5), sham (n = 5), and SAH (n = 15) groups. Animals in the sham and SAH groups were examined over a 3-week period before sacrifice. Salivation score (SC) was determined by measuring the mean wetted area of an orally inserted cotton ball. Sections of parotid glands and intracranial and intraparotid branches of the GPNs were stained with hematoxylin and eosin and SAH-induced damage was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling of apoptotic cells. Specimens were stereologically examined to determine saliva-filled total vesicle volume (TVV) per cubic meter; vasospasm index (VSI) based on wall/lumen ratio of parotid glands arteries, and degenerated neuron density (DND) of glossopharyngeal ganglia.

Results: The mean oral temperature was 36.9°C. In the control group, mean values were as follows: SC, 46±8 mm²; DND, 19±4/mm³; VSI, 1.065±0.049; and TVV, (780±1187) × 106/μm³. In the sham group, mean values were as follows: SC, 31±6 mm²; DND, 98±23/mm³; VSI, 1.67±0.32; and TVV, (617±110) × 106/μm³. In the low hyperthermia SAH group, mean values were as follows: SC, 16±5 mm²; DND, 1520±261/mm³; VSI, 2.12±0.21, and TVV, (314±98) × 106/μm³. In the high hypothermia SAH group, mean values were as follows: SC, 9±2 mm²; DND, 3210±912/mm³; VSI, 3.18±0.30; and TVV, (432±99) × 106/μm³.

Conclusions: Decreased salivary secretion due to secretory gland atrophy originated from ischemia-induced GPN network degeneration at the brainstem, which may be responsible for cranial hyperthermia following SAH.

Keywords: Hyperthermia, subarachnoid hemorrhage, Glossopharyngeal Nerve, Parotid Gland

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INTRODUCTION

Fever is a common clinical complication in patients with subarachnoid hemorrhage (SAH) and is usually related to poor prognosis in early stages (1, 2) fever following SAH is linked to increased mortality (3). The parotid gland is the largest salivary gland and is an important secretory and digestive organ (4).

The glossopharyngeal nerves (GPNs) provide parasympathetic innervation of this gland (5), controlling both secretion and vasodilation (6). Parasympathetic innervation controls the secretion of large volumes of watery saliva, while sympathetic innervation contributes to the production of dense, low-volume, but enzyme-rich saliva (7). Saliva is essential for taste perception; the initiation of salivation is regulated by brainstem gustatory-salivary reflex networks (8).

The present study investigated whether salivary gland dysfunction induced by SAH causes hyperthermia. This is the first study to demonstrate decreased salivation of the parotid and other glands as causing the development of hyperthermia following SAH.

MATERIAL and METHODS

Animal selection and experimental groups

A total of 25 rabbits were randomly assigned to one of three groups (Group I, control, $n = 5$; Group II, sham, $n = 5$; and Group III, SAH, $n = 15$). Animals were maintained in individual metal cages at room temperature and 50% relative humidity on a 12:12-h light/dark cycle and were under veterinary supervision. The animals had free access to a standard laboratory diet and water. The study protocol was reviewed and approved by the Ethics Committee for Animal Experiments of the Faculty of Medicine, Ataturk University, Turkey. Animal care and experimentation were according to guidelines set forth by the Ethics Committee. Anesthesia was first induced with isoflurane administered via a face mask, followed by subcutaneous injection (0.2 ml/kg; total volume, 1 ml) of a cocktail consisting of ketamine HCl, 150 mg/1.5 ml; xylazine HCl, 30 mg/1.5 ml; and distilled water before surgery. Blood (1 ml) was collected from the auricular artery of each animal. While the head of the animal was held in a hyperflexed position, the posterior notch of the foramen magnum was identified, the cisterna magna penetrated with a needle, and cerebrospinal fluid was aspirated. A 1-ml volume of blood (SAH group) or saline (sham group) was injected using a 22-gauge needle into the cisterna magna over a period of about 1 min. Control animals were not injected.

All rabbits were monitored for 10 min twice daily for 3 weeks. Oral temperature was measured using a standard hospital thermometer; the mean oral temperature was measured as 36.8°C; 37.8°C and 38.7°C were taken as low and high hyperthermia, respectively. Oral salivary secretion was assessed as follows. A rolled cotton ball (0.5 cm in diameter) was inserted into the oral cavity of rabbits for 30 s, then placed on a paper and compressed by with a 250-g iron weight with a 3-cm² base surface area for 20 s. The wetted area on the paper was measured and used for salivation score (SC). After 3 weeks, animals were decapitated under general anesthesia and their bodies were immersed in 10% formalin solutions for 7 days. The parotid glands, GPNs, and their ganglia along with other brainstem nerves were dissected, dehydrated through a graded alcohol series, and embedded in liquid paraffin.

Histopathological analysis

The parotid glands were sectioned at a thickness of 5 µm separated by a distance of 30 µm. Every 30th and 31st section

was used for saliva follicle counts. The total number of saliva follicles in the parotid glands was estimated by the fractionator method. Tissue sections from each block were collected on glass slides for histopathological examination and stained with hematoxylin and eosin (H&E) (Fig. 1).

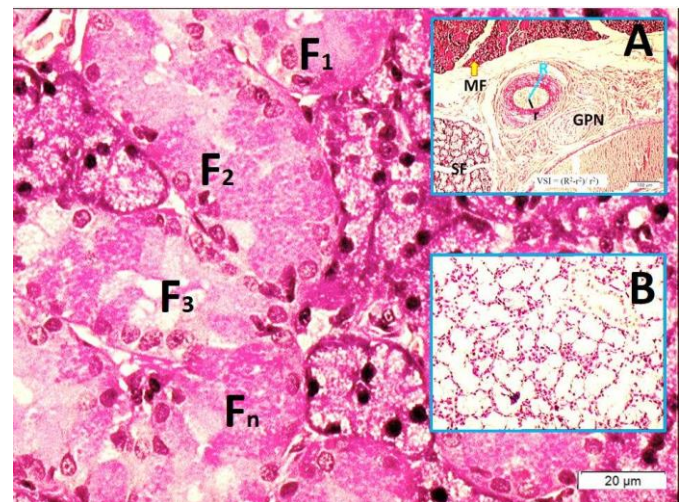


Figure 1. Histopathological analysis of parotid gland. **A:** Serous follicles (SF), mucous follicles (MF), glossopharyngeal nerve (GPN) axons, and parotid artery are shown along with formula for calculation of vasospasm index (VSI) (LM, H&E, 10×). **B:** Secretory material-filled follicles (LM, TUNEL, 10×) and follicles almost completely filled with saliva (Fn) (LM, PAS, 10×).

For the analysis of GPN lesions, brain specimens were sectioned parallel to the long axis for the examination of both axons and ganglia. To determine neuronal density in the GPN ganglion (GPNG), the whole tissue along with the extensions was embedded in paraffin blocks in a longitudinal orientation to observe all the roots during the histopathological examination. Sections were cut and stained with H&E and analyzed by the TUNEL assay, and examined under a light microscope. Images were acquired at 10× and 100× magnification (Fig. 2). The Cavalieri method was used to evaluate GPNG density.

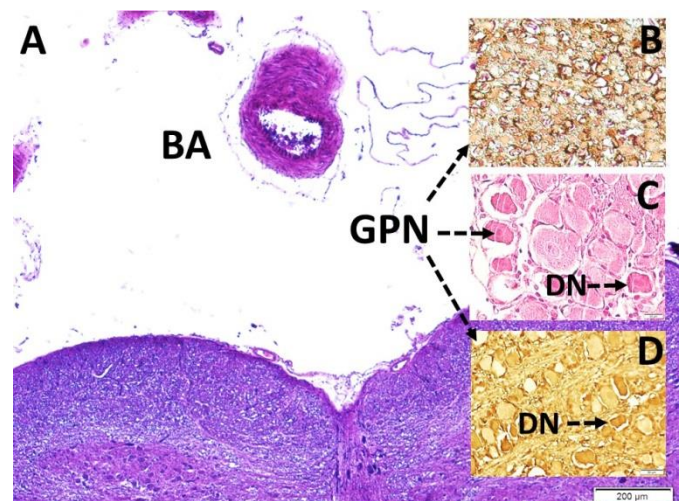


Figure 2. A: Histopathological appearances of constructed basilar artery (LM, H&E, x4/A), normal (dark) and degenerated axons of parotid branches of glossopharyngeal nerves (GPN; LM, S-100/B), normal and degenerated neurons (DN) (LM, H&E, x10/C); and, normal and apoptotic neurons (DN) of petrosal ganglion are seen (LM, Tunel, x20/D) of a study animal..

Stereological analysis

Stereological methods are tools that enable the estimation of bulk parameters, such as the total volume of a structure and the number and size of cells. The first sampled pair of sections were randomly selected from a starting point within the first 20-section interval. Thereafter, every 20th section and its neighbor were sampled. Therefore, the section sampling fraction (f_1) was $f_1 = 1/30$. Section pairs not containing the parotid glands and ganglia were discarded. The sampling fraction yielded on average 10 to 11 section pairs. The area of the sampling fraction, f_2 , was $1/1$. We used the physical dissector method to evaluate the number of saliva follicles in the parotids since it is simple; free from assumptions about particle shape, size, and orientation; and unaffected by overestimation errors and truncation. Two consecutive sections (dissector pairs) obtained from tissue samples with a named reference were mounted on each slide. The order of reference and look-up sections were reversed to double the number of dissector pairs without having to cut new sections. The number of counted follicles was designated ΣQ . The total number of saliva-filled follicles (N) in the parotid glands was estimated from the equation $N = \Sigma Q \times 1/f_1 \times 1/f_2$. In all groups, the number of follicles did not show a normal distribution according to Kolmogorov–Smirnov, and Shapiro–Wilk tests ($P < 0.05$). Before scores were separately compared using the post-hoc Mann–Whitney U test for pairwise comparisons of independent samples, followed by the Kruskal–Wallis test. The P-value used for multiple comparisons (0.05, 95% confidence interval) was divided by 6 with a Bonferroni correction (i.e., P values were considered statistically significant at ≤ 0.0083).

Vasospasm index (VSI) was calculated as follows: $2R$ and $2r$ are external and internal diameters, respectively, of parotid arteries. VSI was determined as a proportion of the external surface to lumen ratio [$\pi R^2 - \pi r^2 / \pi r^2 = (R^2 - r^2 / r^2)$] (Fig. 1-3 A). After counting, total vesicle volume (TVV) was estimated by summation of VV. Each vesicle was considered as a sphere with a volume of $V_n = 4/3\pi r_n^3$. TVV was estimated as: $TVV = N = 1N = NN \times V_n$

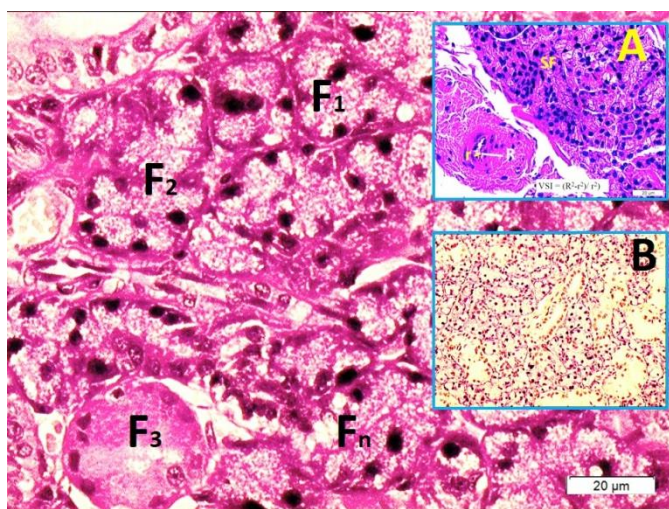


Figure 3. A: Histopathological analysis of parotid gland with follicles (F) and constructed parotid artery, with the formula for calculating vasospasm index (VSI) (LM, H&E, 10 \times). **B:** The number of secretory material-filled small follicles and saliva-filled small follicles (Fn) (LM, PAS, 10 \times) with neuronal apoptosis and nerve degeneration detected animals (LM, TUNEL, 10 \times).

The physical dissector method was used to evaluate the numbers of living and degenerated GPNG neurons. Two consecutive sections (dissector pairs) obtained from reference tissue samples were mounted on each slide. The order of paired reference sections was reversed to double the number of dissector pairs without having to cut new sections. The mean GPNG neuronal density (N_v/G_v) per mm³ was estimated with the following formula:

$$N_v/G_v = \Sigma Q - N / \Sigma A \times d$$

where $\Sigma Q - N$ is the total number of counted neurons appearing only in reference sections, d is section thickness, and A is the area of the counting frame. The most effective way of estimating ΣA for the set of dissectors is by $\Sigma A = \Sigma P \times a$, where ΣP is the total number of counting set frame points and a is a constant area-associated set point. A and B are the areas of the counting frames. The Cavalieri volume estimation method was used to determine the total number of neurons in each specimen, which was calculated by multiplying the volume (mm³) and numerical density of neurons in each GPNG. Differences between salivary follicle number and degenerated neuron density in the GPNG were compared statistically. Neuronal angulation, cytoplasmic condensation, nuclear shrinkage, and apoptotic changes were used as criteria for neuronal degeneration. Data were analyzed using SPSS for Windows v.12.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed with the Kruskal–Wallis and Mann–Whitney U tests. Differences were considered significant at $P < 0.05$.

RESULTS

Four animals in the study group and one in the sham group died within 7 days of surgery. Neck stiffness, unconsciousness, convulsive attacks, fever, apnea, cardiac arrhythmia, and breathing disturbance were observed in all hyperthermic animals. In control animals, heart rate was 280 ± 15 /min, respiratory rate was 35 ± 9 /min, and blood oxygen concentration was $95\% \pm 7\%$. Soon after inducing SAH, heart rate decreased to 150 ± 30 /min, respiratory rate to 18 ± 5 /min, and blood oxygen concentration to $78\% \pm 10\%$. Electrocardiographic changes were also observed, including ventricular extrasystole, ST depression, QRS separation, bi- or trigeminal extrasystole, and fibrillation. However, in the late phase of fatal SAH, heart rate increased to 350 ± 40 /min. A decrease in respiration frequency (bradypnea) (14 ± 3) and increase in respiration amplitude were observed in the first few hours following SAH. However, after longer intervals, an increase in respiration frequency (tachypnea) and a decrease in respiration amplitude were observed, resulting in shortened inspiration and longer expiration times, apnea-tachypnea attacks, diaphragmatic breath, and respiratory arrest. Massive subarachnoid hemorrhage was observed in the basal cisterns of animals in Group III, which showed meningeal irritation signs and cardiorespiratory dysrhythmia. The oral temperature was measured as $36.80C \pm 0.3$, $37.80C \pm 0.4$, $38.70C \pm 0.2$, $38.70C \pm 0.3$ were considered as low and high hyperthermia, respectively. The mean GPNG neuronal density was estimated as 12.230 ± 1065 /mm³ in normal animals (Group I), 11.606 ± 975 /mm³ in the sham group (Group II), and 9.940 ± 732 /mm³ and 7.500 ± 650 /mm³ in the low and high hyperthermia groups, respectively (i.e., Group IIIA and IIIB, respectively).

Table 1. TVV was decreased while DND and VSI were increased in hyper thermic animals after SAH

	Group I (Control)	Group II (Sham)	Group IIIA (low hyperthermia)	Group IIIB (high hyperthermia)	P value
SC/mm ²	46±8	31±6	16±5	9±2	< 0.05
DND/mm ³	19±4	98±23	1520±261	3210±912	< 0.05
VSI	1.065±0.049	1.67±0.32	2.12±0.21	3.18±0.30	< 0.05
TVV (× 10 ⁶)/μm ³	780±1187	617±110	314±98	432±99	< 0.05
Temp. V.	36.8 ⁰ C±0,3	37.8 ⁰ C±0,4	38.7 ⁰ C±0,2	38.7 ⁰ C±0,3	

Data were analyzed with the Kruskal–Wallis and Mann–Whitney U tests ($P < 0.05$).

DND, degenerated neuron density; SC, salivation score; TVV, total vesicle volume; VSI, vasospasm index, Temp. V; Temperature Value.

In Group I, mean values were as follows: SC, 46±8 mm²; DND, 19±4/mm³; VSI, 1.065±0.049, and TVV, (780±1187) × 106/μm³. In Group II, mean values were as follows: SC, 31±6 mm²; DND, 98±23/mm³; VSI, 1.67±0.32; and TVV, (617±110) × 106/μm³. In Group IIIA, mean values were as follows: SC, 16±5 mm²; DND, 1520±261/mm³; VSI, 2.12±0.21; and TVV, (314±98) × 106/μm³. In Group IIIB, mean values were as follows: SC, 9±2 mm²; DND, 3210±912/mm³; VSI, 3.18±0.30; and TVV, (432±99) × 106/μm³. TVV decreased markedly with increased DND and VSI in hyperthermic animals ($P < 0.05$). The total volume of saliva-filled vesicles per cubic meter was reduced in these animals, especially in Group IIIA ($P < 0.05$) (Table 1). TVV also decreased with increased DND and VSI in Group IIIB ($P < 0.005$); this trend was moderately significant between Groups II and IIIB ($P < 0.0005$); highly significant between Groups II and IIIA ($P < 0.005$); and most significant between Groups I and IIIB ($P < 0.0001$). VSI of parotid arteries and follicles volumes were correlated in Group I vs. III ($P < 0.0001$) and Group II vs. III= ($P < 0.0001$).

DISCUSSION

Fever is common in neurocritical care patients and has a negative impact on neurological outcome (9); it is also linked to increased in-hospital mortality after SAH (1, 3). Fever is associated with worse outcomes and late recovery in high- and low-grade patients, respectively (10). Functional improvement after SAH is associated with cumulative fever burden, admission neurological grade, aneurysm obliteration procedure, admission computed tomography score, vasospasm, and external ventricular drainage (11). Hyperthermia is a potential risk factor for vasospasm (12).

In our study, brain edema, stiffness, leptomeningeal thickness, brain swelling, and increased brain weight were observed in all animals that developed hyperthermia. Basal cisterns and occasionally fourth and lateral ventricles were filled with blood, while arachnoidal membranes in the lower cranial parts adhered to lower cranial nerve roots.

Organs have dual autonomic innervation that is critical for regulating gland function (4). GPNs have secretory and vasodilator effects in the parotid gland (6). The tympanic branch of the GPN known as Jacobson's nerve is located immediately after the jugular foramen and contributes to the tympanic plexus on the promontory, providing secretory innervation to the parotid gland (13). Parasympathetic, cholinergic innervation modulates the secretion of a large volume of watery serous saliva while sympathetic and adrenergic innervation stimulates the production of dense, low-volume, but enzyme-rich saliva (7).

Saliva is essential for taste perception and is required for initiating salivation, which is regulated by gustatory-salivary reflex networks in the brainstem (8) that include afferent limbs, salivary nuclei within the medulla, and an efferent limb comprising both sympathetic and parasympathetic secretomotor nerves. In addition, gustatory, visual, olfactory, psychic, masticatory, thermoreceptive, and possibly nociceptive stimulants induce saliva secretion (14). The GPN innervates taste receptors located on the epithelial folds of the foliate and circumvallate papillae in the posterior part of the tongue (15). The GPN as well as facial and vagal nerves have important roles in salivary secretion from salivary, respiratory, and intestinal glands. The GPN and vagus nerve are mixed nerves containing sensory, gustatory, motor, and autonomous (parasympathetic) fibers (16).

GPN lesions result in difficulty swallowing; impairment of taste over the posterior one-third of the tongue and palate; impaired sensation over the posterior one-third of the tongue, palate, and pharynx; loss of a gag reflex; and dysfunction of the parotid gland (17). Post-ganglionic parasympathectomy of rat parotid glands caused reductions in gland weight, acinar cell size, and glandular amylase expression (18).

In this study, parotid gland size was decreased in SAH animals. This was accompanied by loss of acinar, tubular, and supporting cells due to apoptosis, a decreased number of saliva filled vesicles, reduction in the total volume of saliva-filled vesicles, ductal epithelial cell injury related to ductal closing, and degenerative changes in intraparotid parasympathetic ganglia neurons (Fig. 3).

Unilateral parasympathetic denervation of ovine parotid glands in sheep 21–28 days after nerve resection reduced the mass of the ipsilateral gland while increasing that of the contralateral gland, to the extent that total gland mass was greater than in sheep with normally innervated glands. These results demonstrate that parasympathetic innervation to the parotid gland has important trophic effects (19). Bilateral resection of the glosso-pharyngeal nerves resulted in a 40%–50% decrease in enzyme activity of parotid glands over 7 days (20).

The results of this study reveal that unilateral parasympathetic denervation of ovine parotid gland induces substantial changes in parotid size, basal salivary flow, and protein expression. Both the size reduction and morphological changes observed following denervation were compatible with previously reports (21). In particular, nerve root- and ganglia-supplying arteries were more vasospastic in Groups IIIA and IIIB; these groups also showed greater neuronal apoptosis and GPN degeneration.

CONCLUSION

Understanding the origin of fever in SAH patients may improve their functional outcomes. Decreased salivary secretion due to secretory gland degeneration resulting from GPN ischemia at the brainstem likely causes hyperthermia in SAH. However, it is also possible that ischemic degeneration of the GPN and facial and vagal nerves per se leads to decreased salivation, and that morphological changes in the parotid and other glands may be responsible for subsequent hyperthermic sequelae. This is the first study demonstrating decreased salivation by the parotid and other glands as causative to the development of hyperthermia following SAH

Author contributions: ME, IY, OT, NA, CO, MÇ, AE, MDA; Study design, Animal experiments, Literature search, AE; Writing article and revisions

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Ethical issues: All authors declare originality of research.

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