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Effects of Chorda-lingual Denervation on NOS Expression in the Rat Submandibular Gland

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Nitric oxide (NO) gas has been recognized to diffuse readily across the membrane and bind directly to molecule (s) inside target cells. In the salivary gland, eNOS and nNOS are constitutively expressed. iNOS was also reported to express in neoplastic salivary tissues. Regarding the role of NO in the salivary gland, it has been suggested that it may control blood flow to the glands and furthermore involve in growth and development of the gland.

The present study hypothesized that denervation of parasympathetic secretomotor fibers may lead to salivary secretion dysfunction, changing NOS expression.

The gland weight on the denervated side significantly decreased from 3 days after the denervation, comparing the control ($p < 0.01$). Some atrophic and hyperchromatic changes, but no inflammatory reactions were found for the whole period of the experiment. All three kinds of NOS were mainly expressed in the ducts of the gland in both the control and experimental sides. Immunoreactivities of nNOS and eNOS were not noticeably different from those of the control. However, iNOS was also detected in ducts in the normal submandibular gland by immunohistochemical staining. The iNOS expression increased more than 2 times at denervated side of the gland than the control.

These results suggest that NOS isoforms, especially iNOS following chorda-lingual denervation may lead to matrix loss or cell death in the salivary gland.

Key words : Chorda-lingual denervation, NOS, Submandibular gland

Introduction

Salivary secretion from acinar cells in salivary glands is achieved by various signalling molecules in addition to norepinephrine and acetylcholine, secreted from adrenergic and cholinergic nerve endings respectively. Among these molecules, nitric oxide (NO) has been recognized to readily diffuse across the membrane and bind directly to an enzyme inside target cells. NO is produced from L-arginine by the action of nitric oxide synthase (NOS). At least three isoforms of NOS have been documented thus far : Type I NOS or nNOS (calcium dependant and constitutive form markedly expressed in the brain), Type II or iNOS (calcium independent and inducible form expressed in macrophages by appropriate immunological and inflammatory stimuli) and Type III or eNOS (calcium dependant and constitutive form mainly expressed in vascular endothelial cells).

The expression and function of NOS vary in glan-

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dular tissues (Lohinai et al. 1995, Ember et al. 1997, Konttinen et al. 1997, Xu et al. 1997, Klotz et al. 1998, Rettori et al. 2000, Jeong et al. 2001). In salivary glands, eNOS is mainly expressed in intercalated and striated ducts, in addition to endothelial cells of blood vessels. nNOS is expressed in the submandibular ganglion, postganglionic fibers, and striated and intercalated ducts. iNOS is also reported to be expressed in neoplastic salivary tissues (Brennan et al. 2000a, b, Massi et al. 2001). The wide distribution of NOS isoforms in the salivary gland suggests that NO may have several dynamic roles in the glands.

Regarding the role of NO in salivary glands, NO regulates blood flow to the glands by releasing vasoactive intestinal polypeptide (VIP) from the parasympathetic nerve ending (Edwards and Garrett 1993), by activating kallikrein-kinin system (Damas 1994) or by mediating Ca^{++} concentration via cGMP pathway (Xu et al. 1997). NO may also control the diameter of duct lumen and reabsorption and secretion of electrolytes in the glands (Kim 1995, Kim et al. 2000). Furthermore, recent studies suggested that NO is related with growth and development of tissues, where it can be an inhibitory or promotive (Wink et al. 1998, Brennan et al. 2000b, Baltaci et al. 2001).

In general, parasympathetic activation provides the main drive for the secretion of fluid by salivary glands, and sympathetic stimulation leads to secretion of protein. For example, electrical stimulation of the sympathetic nerve to the rat parotid gland causes a very small flow of saliva with a high concentration amylase associated with extensive degranulation of the acinar cells. If the same level of stimulation is applied to the parasympathetic nerve, a considerable volume of saliva which was low in amylase content associated with insignificant degranulation (Proctor et al. 1989).

The submandibular gland is a major salivary gland in secretion. It secrets about 70% of total saliva in humans. An experimental design to block whole secretory function in salivary glands can be a direct model to study a functional role of a molecule. Parasympathetic fibers to the submandibular gland exit the brain stem and join the chorda tympani which then, merges with the lingual nerve. The fibers then reach the submandibular ganglion, and their postganglionic fibers distribute to the submandibular and sublingual glands.

Chorda-lingual nerve damages have been frequent complications in oromaxillofacial surgery such as mandibular osteotomy, lingual and inferior alveolar nerve block anesthesia, surgical cancer therapy and wisdom tooth extraction. The present study hypothesized that denervation of parasympathetic secretomotor fibers can change NOS expression and distribution. Distribution and expression of NOS isoforms in the submandibular gland were examined following surgical cut of the chorda-lingual nerve to elucidate functional roles of NO.

Materials and Methods

Forty female adult Sprague-Dawley rats weighing about 200 gm, aging about 3 month were used.

1. Chorda-lingual nerve excision

An intraperitoneal injection of ketamine (50 mg/kg) was given for a brief anesthesia. All procedures were carried out under aseptic conditions, and the right chorda-lingual nerves were cut. The contralateral nerves were sham-operated and left intact. Vertical skin incision in the neck was done and the underlying muscles were bluntly dissected to reach the mylohyoid muscle with the help of an operating microscope. The chorda-lingual nerve was exposed deep to the mylohyoid muscle, being excised as extensively as possible so that preganglionic and partial postganglionic secretomotor nerve fibers were cut. After full recovery from the anesthesia, regular diet was freely given to the animal.

The animals were sacrificed at 1, 3, 7, 14 and 30 days after the cut. They were sacrificed for RT-PCR and perfusion fixed for histological and immunohistochemical analyses.

2. Immunohistochemical staining

For immunohistochemistry, rats were perfusion fixed with 4% paraformaldehyde. The submandibular glands were removed and immersion-fixed in the same fixative. The tissues were then dehydrated, paraffin-embedded and cut into 5 μm thick sections. Immunohistochemical staining was carried out using Vectastain Elite ABC Kit (Vector Lab. Burlingame, CA, USA). Purified monoclonal mouse anti-endothelial NOS, monoclonal mouse anti-neuronal NOS and polyclonal rabbit anti-inducible NOS (Transduction Lab., Lexington, KY, USA) were used as primary antibodies. Normal horse serum was used for negative control instead of these antibodies. Sections were deparaffinized with xylene and rinsed in PBS. Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in water for 30 minutes. They were incubated in 10% horse serum to block non-specific reactions for 1 hours and reacted in the primary antibodies diluted to 1 : 200 in PBS overnight. The tissues were then incubated in biotinylated anti-rabbit IgG secondary antibody for iNOS and biotinylated anti-mouse IgG secondary antibodies for eNOS and nNOS for 2 hours. Sections were developed with 3-amino-9-ethylcarbazole and counterstained with Mayer's hematoxylin.

3. RT-PCR

Relative quantitation of the NOS isoforms mRNA expression was assessed by RT-PCR. The deeply freezed submandibular glands were homogenized in RNAse-free tubes and total RNAs were extracted using Trizol reagent (Life Technology, USA) according to the manufacture's instructions. cDNA synthesis was carried out using Superscript II (Gibco BRL, MD, USA). No RT controls were carried out in all cases using the same RT reaction mix excepting substituting DEPC-H_2O for Superscript II. The NOS isoforms (Bobadilla et al. 1998) and GAPDH primers (da Silva et al. 1994) for amplification were summerized at Table 1. The relative expression of NOS isoforms between experimental and control sides was measured by normalization using GAPDH as a reference.

Table 1. Primer sequences to amplify each fragment of NOS isoforms and GAPDH

Isoforms	Primer sequences	Amplicon size
GAPDH	5′ CCA TGG AGA AGG CTG GGG 3′ 5′ CAA AGT TGT CAT GGA TGA CC 3′	175 bp
Neuronal NOS	5′ GAA CCC CCA AGA CCA TCC 3′ 5′ GGC TTT GCT CCC ACA GTT 3′	308 bp
Inducible NOS	5′ GTG TTC CAC CAG GAG ATG TTG 3′ 5′ CTC CTG CCC GCT GAG TTC GTC 3′	576 bp
Endothelial NOS	5′ TAC AGA GCA GCA AAT CCA C 3′ 5′ CAG GCT GCA GTC CTT TGA TC 3′	813 bp

4. Statistical analysis

The weight of the submandibular glands was measured. Values were given mean ± standard deviation and t tested for statistical significance.

Results

1. Changes in gland weights

The wet weights of the right and left submandibular glands were 186.0 ± 12.0 mg and 185.6 ± 14.9 mg respectively and not significantly different between them ($p > 0.01$). The weights of the submandibular glands after denervation were shown in Table 2. The weights in the control side did not change significantly from the normal glands over the whole experimental period ($p > 0.01$), indicating that there were no compensatory weight change in the control side. In contrast, the weights in the experimental side were signi-

Table 2. Wet weights (mean±SD) of the submandibular glands in the control and the experimental sides

Group	n	Weight (mg)		% change
		Control (Lt)	Experiment (Rt)	
Normal	6	185.6±14.9	186.0±12.0	4.1±1.2
1 day	5	171.0±27.9	172.4±25.7	1.8±3.1
3 days	5	175.2±10.2	141.4±12.2*	19.1±7.9+
7 days	4	172.8±18.1	129.5±21.3*	25.0±10.2+
14 days	4	217.0±21.6	141.8±16.6*	34.5±6.4+
30 days	4	185.5±27.6	120.3±18.3*	33.2±14.0+

* $p < 0.05$ between the control and the experiment at the same day
+ $p < 0.01$ between the normal and the experiment

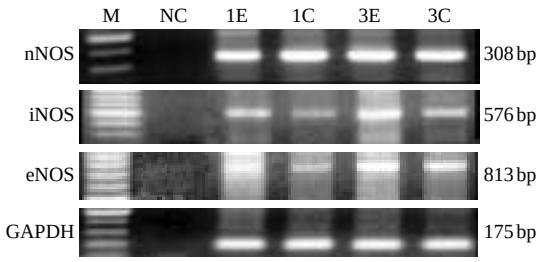

Fig. 9. Expression of each NOS isoforms in the control side. E: nerve cut side, C: control side, M: 100 bp DNA ladder.

ficantly reduced from postdenervation day 3 ($p < 0.05$). Percent changes of weights in each day of the experiment were calculated by the below formula.

$$\frac{(\text{control side of the glands} - \text{experimental side of the glands})}{\text{control side of the glands}} \times 100\%$$

2. Immunohistochemical findings

There were no inflammatory reactions and notable connective tissue growth in all experimental groups. The glands of experimental sides at postdenervation day 1 appeared similar to normal glands (Fig. 1). But the glands at the denervated side appeared hyperchromatic and atropic at day 3. At 30 days after the denervation, ducts and acini became smaller, but appeared pyknotic (Fig. 2). The submandibular glands were stained using immunohistochemistry for e-NOS, n-NOS and iNOS at postdenervation day 3 when the first decrease in weight of this gland was detected. Negative controls were always negative (Not shown in this study).

1) Endothelial nitric oxide synthase

eNOS-immunoreactivities were strongly demonstrated in the vascular endothelial cells and most of the excretory, striated and intercalated ductal epithelia (Fig. 3). At postdenervation day 3, immunoreactivities in the experimental sides, were similar to those of the control (Fig. 4).

2) Neuronal nitric oxide synthase

At postdenervation day 3, nNOS-immunoreactivity in the ducts in the control (Fig. 5) was a little decreased, but generally was maintained after the nerve cut (Fig. 6).

3) Inducible nitric oxide synthase

iNOS was also strongly immunostained in the ductal epithelium of the normal submandibular glands (Fig. 7). In the experimental side, the immunoreactivities were much increased in intensity and area at day 3 (Fig. 8).

3. Gene expression of NOS isoforms

Expression of NOS isoforms mRNA were measured by RT-PCR at postdenervation days 1 and 3, when the weight of glands at the experimental side was significantly reduced. The expressions were normalized using GAPDH expression as a reference (Fig. 9).

Both nNOS and eNOS expression levels were relatively constant at both postdenervation days 1 and 3. However, iNOS expression increased more than 2 fold at both day 1 and 3.

Discussion

Salivary secretion, a main function of the salivary

gland, is known to be controlled entirely by both sympathetic and parasympathetic nervous stimuli. Generally, cholinergic stimulation regulates water and electrolyte release via a breakdown of phosphatidylinositol-4, 5-biphosphate and release free Ca^{2++} from endoplasmic reticulum, whereas β-adrenergic sympathetic stimulation induces protein secretory mechanism through cyclic adenosine monophosphate and protein kinase cascade mechanism. Sympathetic stimulation is intermittent and tend to modulate salivary composition. On the contrary, parasympathetic impulses are much more prevalent and evoke most of the salivary secretion (Ten Cate 1998). The present study blocked parasympathetic impulses by surgical cutting of the chorda tympani nerve, resulted in salivary secretion dysfunction.

The function of NO in exocrine glands were documented in the salivary gland and insulin secretion in the pancreas (Belvisi et al. 1995, Ember et al. 1997, Xu et al. 1997). NO could control VIP secretion in the parasympathetic nerve endings (Fisher et al. 1996). In addition, it might dilate the respiratory tract by increasing cGMP concentration in the respiratory smooth muscle and control mucin secretion in tracheal epithelium (Adler 1995, Ward et al. 1995, Rosbe et al. 1996). In the salivary gland, NO has been regarded as a putative NANC neurotransmitter, which is supported by the reports that NOS immunoreactive nerve fibers were found in the glands (Jeong et al. 2001).

One of the recent researches in the salivary gland is towards the role of NO in the growth and development of tissues (Wink et al. 1998, Scott et al. 1999, Brennan et al. 2000b). Functional changes in the salivary gland can be achieved using drugs. Since the drug effects are not complete to block salivation and can also affect other functions than salivation, direct denervation was used in the present study. It also provides easy comparison between the control and the experimental sides of the gland. The surgical denervation procedure itself had no effect on the general growth and development of the gland in rats (Scott et al. 1999).

Atropic changes are considered to be either physiological or pathological. The physiological atropy can be induced by disuse and aging etc., resulting in the morphological changes and loss of function. The pathological atropy can be brought about by the consequences of diseases such as inflammation, autoimmune disease, raised intraductal or external pressure, irradiation or direct physical injury or ductal ligation, leading to the structural changes and loss of function subsequently (Scott et al. 1999). Chorda tympani nerve cut in the present study resulted in weight loss and atropy of glandular acinar cells. However, there were no inflammatory reactions. The glandular weight loss in experimental side was notable after 3 days of denervation, then arriving at the maximum 34.5% at 14 days after the denervation. This result was consistent with the previous report that parasympathetic denervation resulted in about 30% weight loss in the salivary gland (Kyriacou and Garrett 1988), indicating that the gland has other functions than salivation.

The distributions of eNOS and nNOS have been documented in the salivary gland. But reports on iNOS distribution in the normal salivary gland are scanty. The present study showed that iNOS immunoreactivity profusely existed in the glandular ducts mainly as in eNOS and iNOS. The presence of iNOS even in the normal gland which had no inflammatory signs suggests that iNOS may play a role in functional alterations of the gland (Tschaikowsky et al. 2000).

In the present study, the expression of NOS isoforms in the experimental side was varied from immuohistochemistry and RT-PCR. Both nNOS and eNOS expression appeared constant. These findings were in accord with the previous reports that the eNOS expression could not be related to hypofunction or hyperfunction of the thyroid gland (Colin et al. 1997, Kayser et al. 2000). iNOS has been known to be related with inflammatory reactions and need a cytokine for its expression (Tschaikowsky et al. 2000). It also plays an

important role in tumor growth and angiogenesis and affects cytotoxicity and tumor induced immunosuppression (Klotz et al. 1998, Gavilanes, 1999, Brennan et al. 2000a). Both eNOS and nNOS are constitutively expressed and regulated through calcium/calmodulin-dependant second messenger pathway. NO concentrations produced by this pathway are usually relatively small (in the picomolar range). In contrast, activation of iNOS occurs at the level of gene transcription and is relatively slow, but leads to long lasting and significant increases in NO production (giving rising to nanomolar local concentrations of NO) (Moncada and Higgs 1993, Nathan and Xie 1994).

It has been also reported that NO has a double-edge action on tumor growth, depending on the local concentration of the molecule. Low concentrations of NO protect many cell types from apoptosis, whereas high concentrations of NO inhibit various iron-containing DNA synthases and mitochondrial enzymes, and thus inhibiting cell growth and division. High concentrations of NO induce wild-type p53 protein accumulation and apoptosis in an attempt to minimize NO-induced DNA damage (Stamler 1994, Chinje and Stratford 1997, Dimmeler et al. 1997, Nicotera et al. 1997, Massi et al. 2001). iNOS expression increase in pleomorphic adenoma of the salivary gland (Brennan et al. 2000b).

In the present study, there were no inflammatory signs during the whole period of experiments. iNOS expression was notably increased at day 3. These results were in agreement with the finding that the weight of the gland in experimental side was significantly reduced at 3 days after denervation, suggesting that iNOS expression may reflect functional changes of the gland itself, which was supported by the reports that iNOS activities and functional changes or diseases development of the tissue are related (Chinje and Stratford 1997, Brennan et al. 2000a, b, Massi et al. 2001). Apoptotic cells appeared most at 7 days of chorda-lingual denervation in the submandibular gland (Lee et al. 1999). Thus, high expression of iNOS at day 3 in the present study may bring about apoptosis, subsequently leading to weight loss of the gland.

All together, chorda tympani denervation brought about weight loss. In the sequential events, NOS isoforms, especially iNOS, have diverse functions such as apoptosis than salivation.

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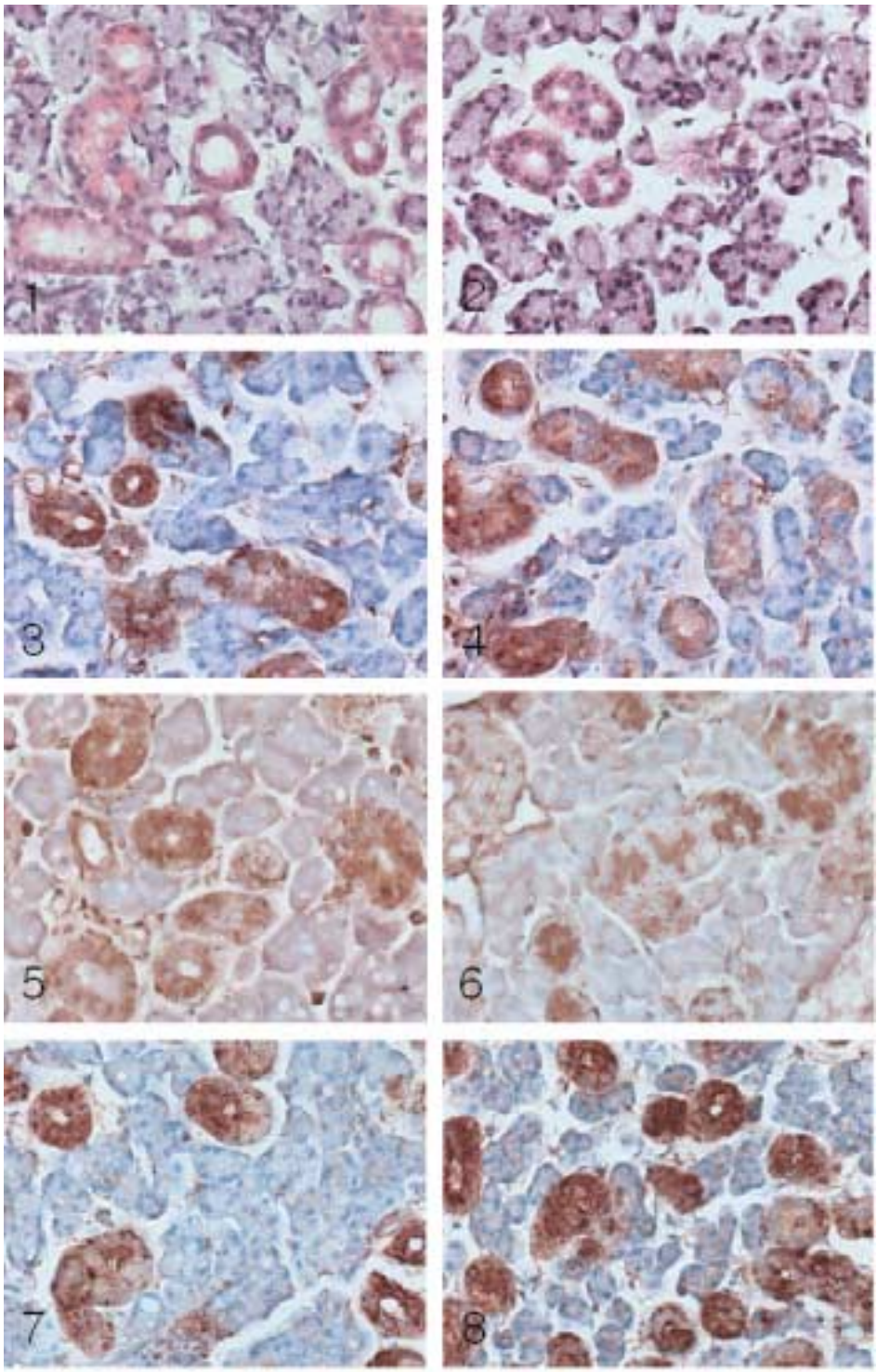
Legends for Figures

Fig. 1. At day 1 after the denervation, no conspicuous changes in the submandibular gland are found. H-E stain, ×300.
Fig. 2. At day 30 after the denervation, ducts and acini became smaller and nuclei are hyperchromatic. H-E stain, ×300.
Fig. 3. eNOS immunoreactivities are mainly seen in ducts and blood vessels in the normal salivary gland. ×300.
Fig. 4. At day 3 after the denervation, eNOS immunoreactivities are similar to those of the normal gland in Fig. 3. ×300.
Fig. 5. nNOS immunoreactivities in the normal salivary gland are mainly seen in ducts. ×300.
Fig. 6. At day 3 after the denervation, nNOS immunoreactivities are similar to those of the normal gland in Fig. 3. ×300.
Fig. 7. iNOS imunoreactivities are also seen in ducts in the normal salivary gland. ×300.
Fig. 8. At day 3 after the denervation, iNOS imunoreactivities much increased in reaction intensity and area. ×300.



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흰쥐 턱밑샘에서 고실끈-혀신경 절단이 NOS 발현에 미치는 영향

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간추림 : 침샘에서 NO의 역할에 관하여 혈류 및 분비도관의 직경 조절 등이 제시되고 있다. 그러나 NOS가 침샘 종양 등에서 발현됨은 NO가 상기 작용 외에도 침샘의 발육과 성장에 관여함을 제시한다.

본 연구는 부교감신경의 절단이 침분비의 차단을 초래하고 이 결과 NOS의 발현과 분포가 변화된다는 가정 하에 시행되었다. 오른쪽 고실끈-혀신경을 절제하고 절제 후 1일, 3일, 7일, 14일, 30일에 희생시킨 후, 면역조직화학염색 및 RT-PCR을 통하여 NOS 발현을 관찰하였다.

턱밑샘 무게는 신경절제 3일 경과 후부터 유의하게 감소하였다. 반대측 대조군에서 침샘 무게의 보상성 변화와 조직학적 변화가 없었으며, 절제 후 염증반응도 관찰되지 않았으나 실질세포 핵이 hyperchromatic 변화를 보였다. NOS 아형은 혈관내피세포와 도관 및 신경에서 발현되었다. 신경절단 1일과 3일 후 면역조직화학염색에서 대조군의 nNOS와 eNOS의 조직면역성은 실험군과 유사하였다. 그러나 iNOS는 실험 1, 3일 후 크게 발현이 증가되었으며 이는 RT-PCR에서 확인되었다.

이상의 결과는 흰쥐에서 고실끈-혀신경 절단이 침샘의 무게 감소를 초래하고 이 과정에서 iNOS가 침샘의 실질 변화와 관련되어 있음을 제시하였다.

찾아보기 낱말 : 고실끈-혀신경, 신경절단, 턱밑샘, NOS

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