Effect of tonicity of saline irrigant on mucin secretion and morphology of human nasal epithelial cells *in vitro*

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ABSTRACT

Effect of tonicitiy of saline irrigant on mucin secretion
and morphology of human nasal epithelial cells *in vitro*

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In order to determine the most physiologically beneficial saline solution concentration for nasal irrigants evidenced by mucus secretion and cellular morphology, the effect of saline solutions of different tonicity was studied on normal human nasal epithelial (NHNE) cells. Secondary diploid NHNE cells were treated with diethyl pyrocarbonate treated water (DEPC water), hypo-, iso-, and hypertonic saline solutions (0.3%, 0.9%, and 3%). In the levels of mRNA of the major airway mucin genes, MUC5AC and MUC5B, assayed by reverse transcriptase polymerase chain reaction, the differences were not significantly varied by the different solutions treated. However, DEPC water treatment of the NHNE cells significantly increased the amount of total mucin (17Q2) as well as MUC5AC and MUC5B mucins in the supernatant, and treatment with hypotonic (0.3%) and hypertonic (3%) saline solutions resulted in significant increase of MUC5B mucin. Morphologic analysis showed extensive damage in NHNE cells treated with DEPC water but not in the cells treated with isotonic saline solution. It was assumed that isotonic saline solution is the most beneficial irrigant on the basis of mucus secretion and the cellular morphology of nasal epithelial cells.

Key words : irrigation, mucin, mucus, saline

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I. INTRODUCTION

Mucociliary clearance is one of the most important host defense mechanisms and plays a critical role in protecting against external stimuli and infections. The mucus of the airway epithelium is composed of two layers: the upper mucus layer and the lower periciliary fluid layer.[1] Mucociliary clearance is primarily regulated by the amount and rheologic properties of the secreted mucin and by ciliary activity; its dysfunction can result in the development or aggravation of various nasal diseases. In airway diseases, reduction in the depth of the periciliary fluid layer, resulting from ion transport abnormalities, together with excessive production of mucus with abnormal viscoelastic and adhesive properties leads to reduced clearance of mucus and its accumulation in the airways.[2]

Although mucociliary clearance is known to be decreased in chronic rhinosinusitis[3], this change of function is reversible. Nowadays, saline nasal irrigation is used in various nasal diseases, such as chronic sinusitis[4], allergic rhinitis[4,5], and

cystic fibrosis.[6] Isotonic saline has commonly been used in nasal irrigation. However, the use of hypertonic saline in nasal irrigation has increased since the effects of hypertonic saline, such as reduction of edema due to osmotic pressure-induced water transport through the mucosal epithelial membrane and improvement in nasal mucociliary clearance have been reported.[6-8] Improved mucociliary clearance by hypertonic saline has been also reported in patients with cystic fibrosis and asthma, as well as in those with nasal diseases.[6,7] In addition, several *in vivo* studies have reported that the topical administration of hypertonic saline to human nasal mucosa evoked mucin secretion.[9,10] Donaldson et al[11] reported that the administration of hypertonic saline *in vitro* produced a sustained increase in the volume of airway surface liquid in cultures from patients with cystic fibrosis. Currently, only few reports exist that deal with the effect of varying tonicities of saline solutions on mucin secretion and nasal mucosal morphology.

In this study we investigated the effects of varying tonicities of saline solutions on mucin gene expression and mucin secretion in cultured human nasal epithelial cells. We also examined the morphological properties of the cells using scanning electron microscopy and light microscopy.

II. MATERIALS AND METHODS

1. Air-liquid interface culture

Secondary diploid normal human nasal epithelial (NHNE) cells (1×10^5 cells/culture) were seeded in 0.5 ml of culture medium onto the surface of rat tail, collagen type 1 gel-coated (3.0g/mL; Collaborative Research, New Bedford, MA, USA), 24.5-mm, 0.45-mm pore-sized Transwell clear culture inserts (Costar Co., Cambridge, MA, USA). Cells were cultured in media made of 1:1 mixture of bronchial epithelial cell growth medium (Clonetics Corp., San Diego, CA, USA) and Dulbecco's modified Eagle's medium containing insulin (5.0μg/ml), hydrocortisone (0.5μg/ml), epinephrine (0.5μg/ml), triiodothyronine (6.5ng/ml), transferrin (10ng/ml; Clonetics Corp.), epidermal growth factor (0.5ng/ml; Collaborative Research), all-trans retinoic acid (50nM; Sigma, St. Louis, MO, USA), bovine pituitary extract (1% vol/vol; Pel Freez Biologicals, Rogers, AR, USA), gentamycin; amphtericin (50 μg/ml:50μg/ml; Clonetics Corp.), bovine serum albumin (1.5μg/ml; Sigma).[12] Culture cells were grown submerged for the first 9 days, during which time the culture medium was changed on the first day and every other day thereafter. On the ninth day, an air-liquid interface (ALI) was made by removing the apical medium and feeding the cultures only from the basal compartment. The culture medium was changed daily after creating the ALI. Two weeks after confluence, the NHNE cells were treated with saline solutions for 30 minutes.

2. Total RNA in the culture supernatant of NHNE cells treated with DEPC water and hypo-, iso-, and hypertonic saline solutions

Cultured cells were treated with 1 ml of diethyl pyrocarbonate treated water

(DEPC water) and with various concentrations of saline solutions (0.3%, 0.9%, 3%); the solution was collected in 30 minutes after the treatments. Total RNAs were then extracted from the saline-treated NHNE cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA).

3. Reverse transcriptase polymerase chain reaction for secreted mucins

Oligonucleotide primers were designed according to published sequences for major secretory mucins, MUC5AC (Genbank accession No. U06711, 680 base pair (bp), 5' primer TCCGGCTCCATCTTCTCC; 3' primer ACTTGGGCACTGGTGC-TG) and MUC5B (Genbank accession No. Z72496, 338 bp, 5' primer ACTCCAGA-GACTGTCCACAC; 3' primer TACCACTGGTCTGTGTGCTA).[13] Oligonucleotide amplimers for β2- microglobulin (β2M; Clontech Laboratories Inc., Palo Alto, CA, USA) were used as a control and generated a 335-bp polymerase chain reaction (PCR) fragment. Reverse transcriptase (RT)-PCR reactions were performed using a Cetus DNA Thermal Cycler (Perkin-Elmer Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. Annealing was performed for 1 minute at 558C for MUC5B and at 608C for MUC5AC and β2M. We used comparative kinetic analysis to compare the mRNA levels for each gene under each set of culture conditions. PCR products (MUC5AC, 580 bp; MUC5B, 338 bp) were separated by electrophoresis on a 2% Seakem agarose gel (FMC, Rockland, ME, USA) containing 50 ng/ml ethidium bromide and were photographed with Polaroid Type 55 film.[12] Each experiment was repeated three times.

4. Detection and quantitation of mucin produced by the cells

The amount of total mucin (17Q2) and MUC5AC and MUC5B mucins present in

the supernatant solutions was assayed using an immunoblot assay.[12] The amount of total mucin (17Q2) was detected using 17Q2 antibody (Covance Research Product Inc., Berkeley, CA, USA), a monoclonal antibody against human mucin, MUC5AC mucin was detected using a polyclonal anti-MUC5AC antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and MUC5B was detected using a monoclonal MUC5B antibody (Santa Cruz Biotechnology Inc.). Aliquots of the supernatant were applied to a nitrocellulose membrane, which were then incubated with the appropriate primary antibody. These were then treated by a reaction with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. The signals were recorded by use of chemiluminescence assay kit (ECL kit; Amersham, Little Chalfont, UK), and a standard curve was generated by linear regression analysis to determine the concentrations of the individual samples. The data were presented as the mean ± SD of the same experiments performed three times. Statistical analysis was performed by use of Student's t-test and $p<0.05$ was considered to be significant.

5. Morphological examination

Cultured cells were examined routinely by use of a phase contrast light microscope (Vanox S-type Light Microscope; Olympus, Japan). For the examination of saline-induced morphologic changes, NHNE cells treated with saline solutions on the permeable membrane were fixed in 10% buffered neutral formalin, embedded in paraffin and sectioned. The cells were then stained with hematoxylin-eosin. For scanning electron microscopy studies, the treated cells on the porous membrane were fixed with chilled 2.5% glutaraldehyde for 4-6 h and washed with 0.1 M PBS. The cells were treated with 1% osmium tetroxide for 2 hours. Specimens were examined using a scanning electron microscope (H-800; Hitachi, Japan).

III. RESULTS

1. Expression of secretory mucin genes and quantitation of mucin in the culture supernatant

MUC5AC and MUC5B mRNA levels were not affected by treatment with DEPC water or various concentrations of saline solutions (Fig. 1).

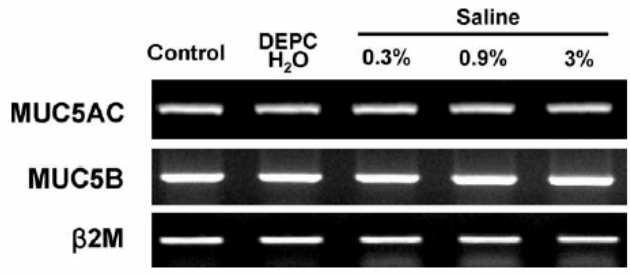

Figure 1. mRNA levels of major secretory mucin genes (MUC5AC, MUC5B) after treatment with DEPC water and 0.3%, 0.9% and 3% saline solutions. Control: NHNE cells without treatments.

Total mucin (17Q2) detected in the supernatant were increased significantly with DEPC treatment (4.0±0.3 times greater than control; $p<0.05$; Fig. 2A). However, the concentrations of saline in the solutions used for the experiment significantly affected the amount of total mucin regardless of its tonicity (Fig. 2A). Subsequently, the amount of MUC5AC and MUC5B mucins, the two major secretory mucins in human airway, was measured in the culture supernatant. The amount of MUC5AC mucin was found to be increased in cells treated with DEPC water (5.6±1.4 times greater

than those of control; $p<0.05$) whereas the amount of MUC5AC mucin was not changed significantly by the concentrations of saline solutions (Fig. 2B). MUC5B mucin was identified to be increased in the supernatant following treatment with DEPC water (19.7±2.1 times greater than control; $p<0.05$), 0.3% (4.2±0.1 times greater than control; $p<0.05$) and 3% (10.9±0.5 times greater than control; $p<0.05$) saline solutions, but not following treatment with 0.9% saline solution (Fig. 2C).

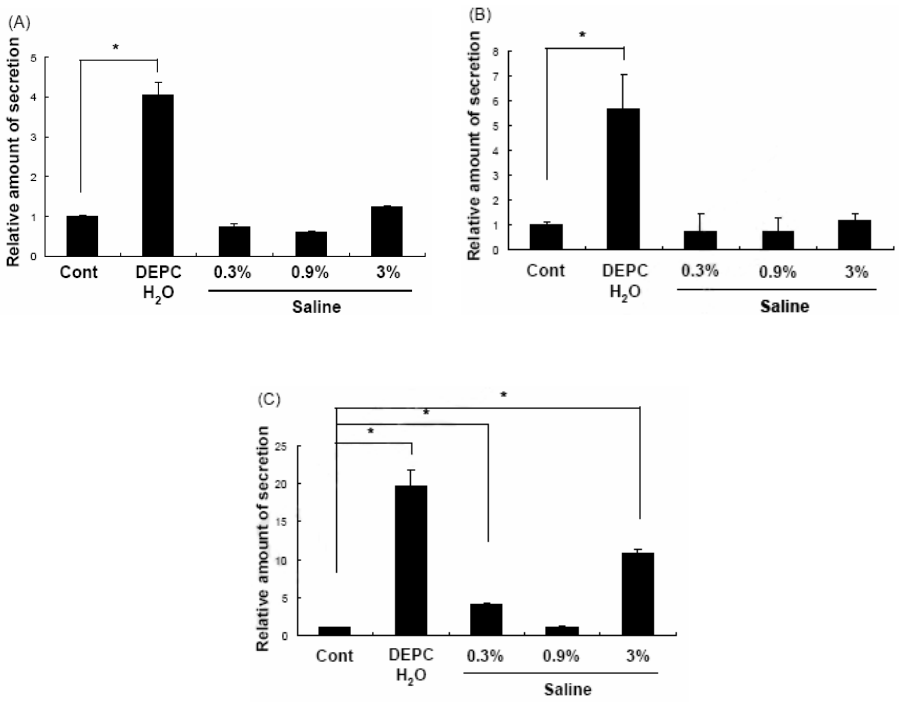

Figure 2. Mucins in the supernatant of NHNE cells after treatment with DEPC water and 0.3%, 0.9% and 3% saline solutions: (A) total mucin; (B) MUC5AC; and (C) MUC5B. *$p<0.05$. Control: NHNE cells with growth media only.

2. Morphologic changes of NHNE cells

Morphologic changes of NHNE cells were examined using scanning electron microscopy and light microscopy. NHNE cells for the control (growth media only) had well-differentiated cilia covering up to 50% of the cell surface and maintenance of intact cell-to-cell integrity was examined (Fig. 3A, 4A). In the cultures treated with DEPC water, the cells were extensively damaged and cell-to-cell integrity was destroyed. Numerous extracellular mucous droplets were observed outside of the secretory cells, and the total number of ciliated cells significantly decreased (Fig. 3B, 4B). In the 0.3% saline solution treated group, the damage was moderate and the number of ciliated cells was also obviously decreased. Extracellular mucous droplets which were obvious in the DEPC water treated group, were not significant in this group (Fig. 3C, 4C). In the 0.9% saline treated group, the cell morphology appeared to be normal which was covered with cilia. Cell-to-cell integrity appeared to be maintained and mucous droplets in extracellular fluids were not observed (Fig. 3D, 4D). In cultures of cells treated with 3% saline, a few plaques were noticed, which indicate detachment of the cells, but the cell-to-cell integrity was maintained otherwise (Fig. 3E, 4E).

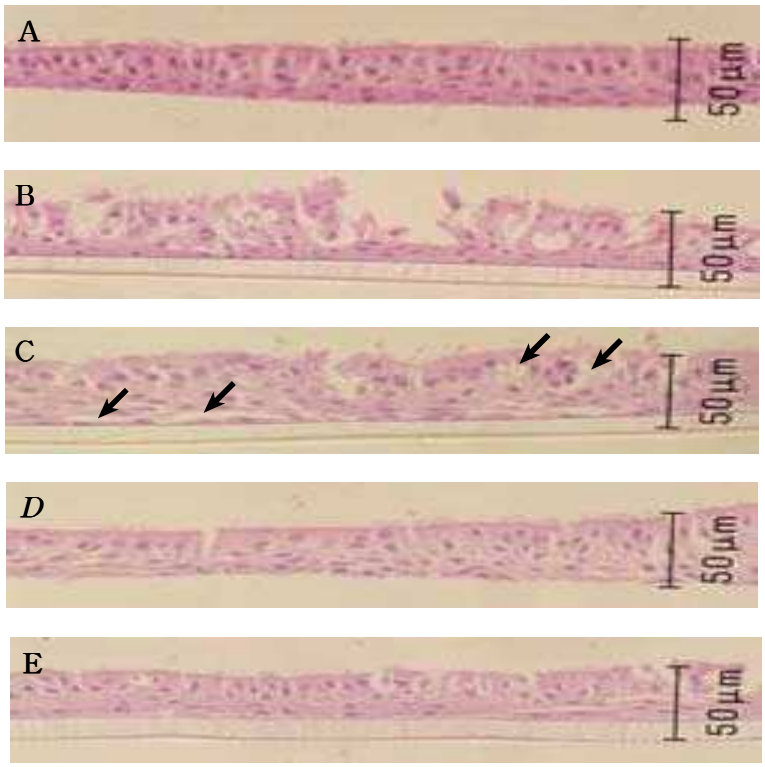

Figure 3. Light microscopy (original magnification ×200) of NHNE cells treated with DEPC water and 0.3%, 0.9%, 3% saline solutions. (A) Ciliated NHNE cells in the control group (growth media only) are noticed without damage. (B) In the DEPC water treated group, extensively damaged NHNE cells are noted. (C) In the 0.3% saline solution treated group, NHNE cells are moderately damaged and the cytoplasm of NHNE cells looks edematous (arrows). (D) In the 0.9% saline solution treated group, the epithelium appears normal. (E) In the 3% saline solution treated group, some detached cells are seen in the epithelium.

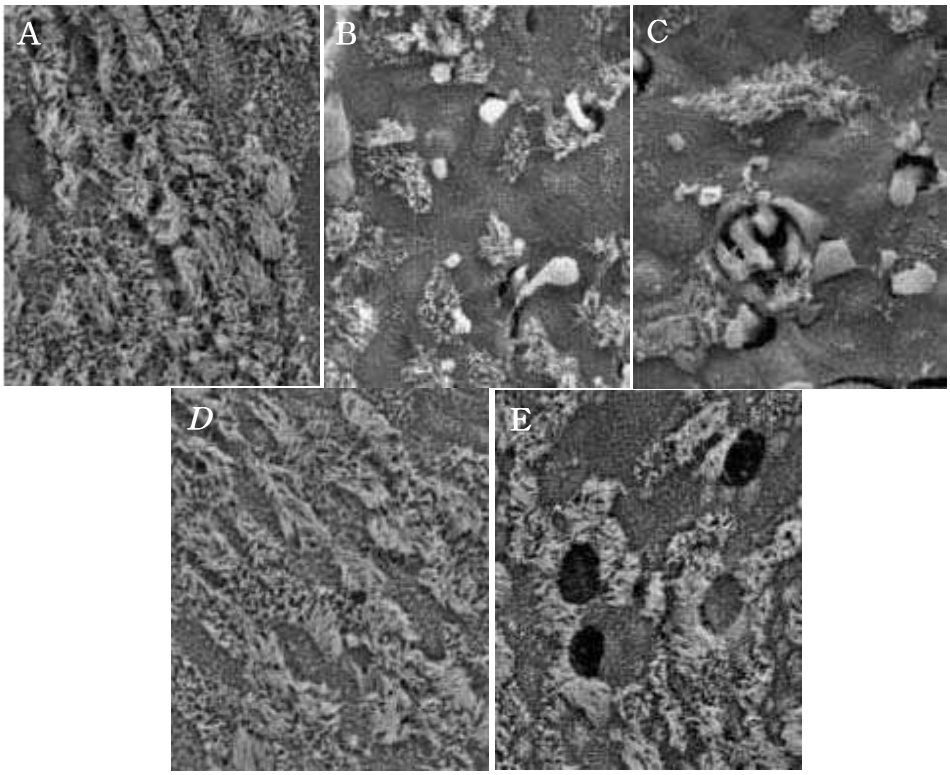

Figure 4. Scanning electron microscopy (original magnification ×/1500) of NHNE cells after treatment with DEPC water and 0.3%, 0.9%, 3% saline solutions. (A) In the control group (growth media only), NHNE cells have many well-differentiated cilia. (B) In the DEPC water treated group, NHNE cells are extensively damaged and numerous mucous droplets are observed outside of the secretory cells. The total number of ciliated cells are decreased. (C) In the 0.3% saline solution treated group, NHNE cells are moderately damaged. (D) In the 0.9% saline solution treated group, the epithelium appears normal and is covered with cilia. (E) In the 3% saline solution treated group, a few plaques are found to be scattered in the epithelium which is mostly covered by cilia.

IV. DISCUSSION

Mucociliary clearance of the airway epithelium is primarily regulated by ciliary activity and the amount and rheologic properties of secreted mucus. It is known that the ciliary beat frequency is reduced in various airway diseases.[3] With this in mind, efforts have been made to improve ciliary activity and mucociliary clearance using hyper-, iso- or hypotonic saline irrigation. However, considering reports on the lack of a direct correlation between ciliary beat frequency and mucociliary clearance[14] and contradictory results regarding the effect of irrigation with various concentrations of saline on ciliary activity[4-9], it can be speculated that the most important factors determining mucociliary clearance are the amount and rheologic properties of the secreted mucus. Therefore, in this study, the effect of varying tonicities of saline solution on mucin gene expression and mucin secretion was investigated in cultured human nasal epithelial cells. We also examined the morphological changes of the nasal epithelium after treatment with various concentrations of saline solution, in order to determine the most physiologically optimal concentration of saline solution for nasal irrigation.

In NHNE cells, expression levels of two major mucin genes in the airway, MUC5AC and MUC5B,[15] were not affected by treatment with DEPC water or hypo-, iso- or hypertonic saline solutions. Thus, it can be assumed that if there is a change in mucin secretion with saline solution treatment, then mucus secretion is primarily regulated by the secretagogue effect of preformed mucus, and not by genetic modulation of mucin genes.

The amount of total mucin and MUC5AC mucin in the supernatant of NHNE cells increased as a result of treatment with DEPC water but not with saline solutions

of various concentrations (Figure 2A, 2B). However, MUC5B mucin was increased following treatment with DEPC water and hypo- and hypertonic saline solutions (Figure 2C). Interestingly, treatment with hypertonic saline solution induced a 10.9-fold increase in the amount of MUC5B mucin detected in the supernatant compared to the control whereas no increase was seen for total mucin and MUC5AC mucin. Since the alteration in MUC5B mucin level had little affect on the amount of total mucin, it may be assumed that the amount of MUC5B mucin released from NHNE cells was too minimal to technically detect the difference. Based on these results, it is suggested that MUC5AC may be the most predominant major mucin secreted by the airway epithelium. Accordingly, these results demonstrate that isotonic saline treatment may be the most appropriate treatment because isotonic saline did not affect the secretion of total mucin or MUC5AC mucin in NHNE cells.

Until now, no studies have been performed in which morphologic changes of the nasal epithelium were analyzed after treatment with saline solutions. In our laboratory, an NHNE cell culture system has been established[16], and it was speculated that by treating the apical surface of cultured nasal epithelial cells with various concentrations of saline solutions, this system could mimic the real situation of saline irrigation. Morphologic observation showed that DEPC water treatment of NHNE cells resulted in severe cellular damage and rupture, as well as active secretion of mucus from the mucous cells (Figure 4B). According to secretion and morphologic data, we speculated that the increase in total mucin secretion following DEPC water treatment could be explained by the degranulation of mucous droplets from the mucous cells, which could be caused by either the secretagogue effect or by the excretion of mucus by mucous cell destruction. In the 0.3% (hypotonic) saline solution treated group, cellular damage was minimal, but cellular edema and a

moderate degree of ciliary damage were observed (Figure 3C, 4C). These effects were thought to be due to the osmotic pressure gradient between the saline solution and the intracellular cytoplasm. In the 0.9% (isotonic) saline solution treated group, healthy cilia covered >50% of the epithelial surface (Figure 4D), indicating minimal cellular damage, which suggested that isotonic saline could be the most applicable irrigant. In the 3% (hypertonic) saline treated group, a few plaques were apparent in places where secretory cells had been detached (Figure 4E); however, it is not clear at present what caused this detachment. The number of cilia-containing cells was slightly decreased.

Although it was assumed that treating the apical surface of cultured NHNE cells with saline solutions of various tonicities could mimic the real situation of saline irrigation, there are some limitations. Diverse factors have influence on the mucociliary clearance system in the nasal cavity of humans and therefore, the culture system used in this study cannot be considered to represent the exact physiologic condition of the nasal cavity. The anatomical factors with the complex structures of the nasal cavity and environmental factors such as temperature and humidity are presumed to have much influence when nasal irrigation is performed in the nasal cavity. Also, ciliary beating movement is another important component to be considered in the regulation of mucociliary clearance when determining the actual effect of nasal irrigation. In this study, the aspect of the amount and rheologic properties of secreted mucin was focused on and despite the limitations of using the cell culture system, identifying the effect of tonicity of saline irrigant on mucin secretion and cell morphology independent from the influence of other factors may considered to be an advantage of this study.

V. CONCLUSION

The ideal nasal irrigant should not affect mucus secretion or cause morphologic changes of the nasal epithelial cells in the nasal cavity while serving its purpose of improving mucociliary clearance. In this study, it was demonstrated that by treatment with DEPC water, the amount of total mucin (17Q2) as well as MUC5AC and MUC5B mucins was significantly increased in NHNE cells and that treatment with hypotonic (0.3%) and hypertonic (3%) saline solutions resulted in a significant increase of MUC5B mucin in the culture supernatant. However, by treatment with varying tonicities of saline solutions, the gene expression of the major airway mucin genes, MUC5AC and MUC5B were not significantly varied. Morphologic analysis showed extensive damage in NHNE cells treated with DEPC water and minimal to moderate changes in the cells treated with hypo- or hypertonic saline solutions but not in the cells treated with isotonic saline solution.

In conclusion, the *in vitro* results of this study suggests that isotonic saline solution may be the most beneficial nasal irrigant in terms of mucin secretion and the cellular morphology of nasal epithelial cells.

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ABSTRACT(IN KOREAN)

저장성, 등장성, 고장성 식염수 세척이 배양된 사람 코점막 상피세포의 점액 분비와 세포 형태에 미치는 영향

<지도교수 윤주헌>

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송 미 현

고장성, 등장성, 또는 저장성의 식염수를 이용한 코 세척을 통해 섬모 운동을 증진시키고 점액 섬모 청소를 향상시키려는 노력이 이루어져왔다. 본 연구에서는 배양된 정상 사람 코점막 상피 세포를 이용하여 다양한 농도의 식염수 용액이 점액 분비와 세포 형태에 어떠한 영향을 미치는지를 살펴봄으로서 가장 생리적인 식염수 농도를 알아보고자 하였다.

정상 사람 코점막 상피 세포를 *D*EPC (*diethyl pyrocarbonate*) 함유물과 0.3%(저장성), 0.9%(등장성), 3%(고장성) 식염수로 30분간 처치한 후 RT-PCR(*reverse transcriptase polymerase chain reaction*)을 이용하여 기도 상피에서 분비되는 주요 점소인 *MUC*5AC와 *MUC*5B의 *m*RNA 발현을 측정하였고 면역조직화학염색법을 이용하여 *MUC*5AC와 *MUC*5B, 그리고 전체 점액의 분비량을 알아보았다. 세포 형태의 변화는 전자 현미경과 *hematoxylin-eosin* 염색 후 광학 현미경을 이용하여 관찰하였다.

*D*EPC 함유물이나 여러 농도의 식염수 처치 후 *MUC*5AC나 *MUC*5B의 *m*RNA 발현 정도에는 유의한 차이가 나타나지 않았다. 전체 점액 양과 *MUC*5AC 양은 *D*EPC 함유물로 처치한 후에 증가되는 양상을 보였고 여러 농도의 식염수 용액으로 처치한 후에는 변화를 보이지 않았다. *MUC*5B는 *D*EPC 함유물, 저장성, 그리고 고장성 식염수의 처치에 의해 그 양이

증가되었다. 형태학적으로는 *D*EPC 함유물로 처치한 경우 정상 사람 코점막 상피 세포에 심한 손상을 유발하였고 등장성 식염수만이 정상 세포 형태에 영향을 미치지 않는 것으로 나타났다.

결론적으로 등장성 식염수를 이용한 코 세척이 점액 분비나 세포 형태학적인 면에서 가장 생리적이라 하겠다.

핵심되는 말 : 세척, 점소, 점액, 식염수