Effects of Chronic and Acute Stress on Clusterin Secretion of the Rat Submandibular Gland

Sang-Bae Jin, D.D.S.,M.S.D., Yang-Hyun Chun, D.D.S.,M.S.D.,Ph.D.,
Jung-Pyo Hong, D.D.S.,M.S.D.,Ph.D.

Department of Oral Medicine, College of Dentistry, Kyung-Hee University

The aim of this study is to know how the rat submandibular gland changes under various emotional stress condition, using molecular biological methods.

Restraint and chronic unpredictable mild stress (CUMS) experiment is conducted on fifty one 7-week old Sprague-Dawley rats(restraint stress experiment: 21, CUMS: 30). The rats were sacrificed, the submandibular glands were excised immediately at certain time, and examined by the use of immunohistochemistry and western blotting. In CUMS experiment, sucrose preference test, water intake change, weight change were implemented at 1 week interval for the experimental period

The results are as follows:

1. The number of clusterin-secreting cells of restraint stress group compared to control group showed significantly decreasing tendency in all experimental groups except for the 1st hour group ($p<0.001$ in the 9th, 24th, 72nd, 120th, and 168th hour group).
2. The number of clusterin-secreting cells of CUMS group compared to control group showed significantly increasing tendency in the 2nd week group ($p<0.01$), and significantly decreasing tendency in the 4th and 5th week group ($p<0.001$).
3. Sucrose preference test in CUMS experiment showed significant difference between the 5th week experimental group and control group ($p<0.01$).
4. Weight change in CUMS experiment showed significant difference between the 5th week experimental group and control group ($p<0.01$), but water intake change didn't show significant difference compared to control group.
5. In western blot analysis, clusterin expression was decreased on a gradual basis in due time compared to the control group in the restraint stress group. As for CUMS group (chronic unpredictable mild stress group), it was increased till the 2nd week and decreased till the 5th week after that, which is similar to immunohistochemical analysis result and the decreasing tendency of sucrose preference and weigh changes.

Through the test, it was proved that expression of clusterin in saliva glands decreases after receiving either acute or chronic stress, indicating relation with depression caused by chronic stress. Unlike other data, however, apoptotic tendency was hardly found in tissues. Diverse possibilities could be suggested on that: first, the stress was not enough to expedite apoptosis; second, apoptosis-related protein was already being secreted though not detected with microscope; third, clusterin, a major secretion molecule of saliva, decreased with saliva's malfunction due to stress. In the respect, it will be necessary to examine proteins expressed in case of cell death or other heat-shock proteins at the same time, in order to see whether any cellular change or death is caused by decreasing clusterin under high stress, and whether the original state is restored as time goes by under mild stress, through longer-term tests using even higher acute stress.

Key words : Salivary gland, Stress, Clusterin, Immunohistochemistry, Western blot

Corresponding Author : Prof. Jung-Pyo Hong
Dept. of Oral Medicine, College of Dentistry, Kyung-Hee University
1 Hoegi-dong Dong, Daemun-Ku, Seoul 130-701, Korea
E-mail : unicomfort@khu.ac.kr

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I. INTRODUCTION

'Stress,' commonly referred to as physiologic emotional response[1] caused when facing with aversive or threatening situations, is essential to life and can be divided into behavioral response, autonomic nerve response and endocrine response in physiologic terms. Yet this emotional response is designed to mainly respond to short-term stimuli, and chronic stress beyond physiological tolerance limit is likely to deteriorate health severely.

The role of saliva in oral cavity is so important that not only digestion and pronunciation, but also protection and healing promotion for oral tissues depend on it. So, secretion or composition of saliva affects the contraction of most of oral diseases[2], while some systemic diseases, drugs or physiologic mechanism can cause disorder of salivation or saliva compostion[3-6].

In recent days, there have been increasing attempts to identify, treat or prevent diseases by using diverse proteins generated in cells based on advanced molecular biological methods. Clusterin (CLU), among these proteins, is an interesting protein with ubiquitous tissue distribution[7]. In spite of persistent research, clusterin has not to be fully identified in terms of its functions yet[8], but it is believed to play a critical role in survival of cells. In special, clusterin may function as an extracelluar chaperone that stabilizes stressed proteins in a folding-competent state[9]. So, it can be suggested that study of clusterin in saliva or salivary gland helps to elucidate the relation between emotional stress and the change of saliva secretion or composition in the patients that is complaining various oral diseases without apparent cause, and exposed to excessive stressful condition[10].

So, it is necessary to perform stress study for ascertaining the relation between stress and clusterin secretion in salivary gland, but it is very difficult to study stress and stress response, because the concepts of stress is various. Animal models of stress have included simple acute stress model such as induced hunger, exposure to cold, physical restraint, exposure to a socially dominant member of the same species, inescapable electric shocks, or near drowning[11], and more complicated stress models like chronic stress model[12] or learned helplessness[13]. Human stress model are generally more broadly defined to also include complex life events such as adversity in relationship, health, work, finances, and with social structure. Although there is some evident that psychological stress activates the same pathways as observed in animal models, caution must be taken not to overgeneralize.

Given the fact that chronic psychological stress is closely connected to development of oral diseases, this experiment was conducted in order to figure out how rat submandibular gland changes under diverse types of stress, reflected in clusterin secretion, by using acute restraint stress model[10] and chronic unpredictable mild stress model[14,15].

II. MATERIALS AND METHODS

1. Experimental animals

Fifty one 7-week old Sprague Dawley rats (Dae-Han Experimental Animal Research Center, Seoul, Korea), weighing 200~250 g, were used for the experimental procedures. Rats were allowed 1 week to acclimate to the surroundings before beginning any experimentation

2. Restraint stress

Twenty one of them were divided into 2 groups: normal group (n=3), restraint stress group (n=18). They were maintained at 20-23°C and fed *ad libitum* on a normal laboratory diet for the duration of experiment. The restraint stress group was placed in the stress cage throughout the period of the experiment. After that, the rats were sacrificed at 0, 1, 9, 24, 72, 120, and 168 hours after the restraint stress applied and the submandibular glands were excised immediately.

Table 1. The time table of the CUMS experiment

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Water Deprivation	1630 →	0830 (16h)					
Empty Water Bottle		0830-0930					
Tilt Cage (40 degree)		1100-1700 (6h)					
Paired Housing	→	0830 (70h)		1730	→ 1330 (20h)	1030	→
Soiled Cage 300 ml water					1730	→ 1030 (17h)	
Light On	1630 →	0830 (16h)		1730	→ 1030 (17h)		
Strobe Light 300 flashes/min	1100-1600 (5h)			1330-1530 (2h)			
White Noise (90dB)						1030-1330 (3h)	
Sucrose Preference Test			(20h; food and water deprivation) 1630	1230-1330 (1h) → 1230			

3. Chronic unpredictable mild stress (CUMS)

Thirty of them were randomly divided into 2 groups: control group (n=15) and CUMS (n=15) group. Animals were housed in individual cages and fed *ad libitum* on normal laboratory diet for the duration of experiment unless otherwise noted. The CUMS procedure employed here was designed to maximize the unpredictable nature of the stressors [14,15]. The CUMS group was exposed to the following stressors in random order: continuous overnight illumination, 40° cage tilt, paired housing, damp bedding (300 ml water spilled into bedding), exposure to an empty water bottle immediately following a period of acute water deprivation, troboscopic illumination (300 flashes/min), and white noise (approx. 90dB). Details of the CUMS procedure, including time and length of activities, are presented in Fig. 1. The rats were sacrificed, after weekly sucrose preference test during the experiment and the submandibular glands were excised immediately.

4. Sucrose preference test

Sucrose preference tests[15] were used to operationally define anhedonia. Specifically, anhedonia was defined as a reduction in sucrose intake and sucrose preference relative to the intake and preference of the control group. A sucrose preference test consisted of first removing the food and water from each rat's cage (both CMS and control groups) for a period of 20 h. Water and 1% sucrose were then placed on the cages in preweighed glass bottles, and animals were allowed to consume the fluids freely for a period of 1 h. A preference test was also conducted following the 5-week CMS period.

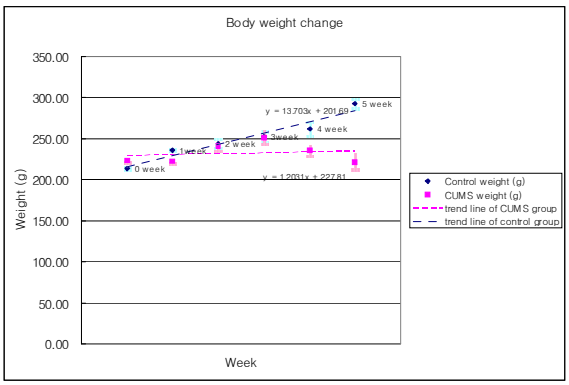

Fig. 1. Body weight change of the CUMS experiment

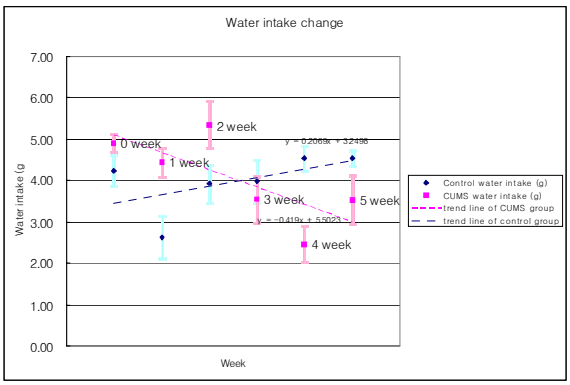

Fig. 2. Water intake of the CUMS experimen

5. Immunohistochemistry

For immunohistochemical analysis the submandibular tissues were placed for fixation in Bouin solution (saturated with picric acid : formaldehyde, 3:1) with 0.1% acetic acid (Sigma, St-Louis, MO, USA) for 24 hours at room temperature, and then the tissues were embedded in paraffin block. Serial paraffin sections (4~6㎛) were cut and placed on poly-L-lysine-coated slides. Immunohistochemical analysis was carried out by ABC (avidin-biotin-peroxidase complex) method[16]. Using primary antibody (goat polyclonal IgG, C-18, 1:200, Santa Cruz Technology; Santa Cruz, CA) and secondary antibody (goat anti-rabbit IgG-B, 1:200 in 0.01M PBS).

6. Morphometry for clusterin secreting cells

For morphological analysis, the tissue sections immunolabeled for clusterin were photographed randomly on each cell of acini over 10 times at a low magnification (x20) and the number of clusterin secreting cells were counted. Three salivary gland tissues were analyzed in each experimental group.

7. Western blot analysis

Western immunoblot analysis was performed using the protocols of BM Chemiluminescence

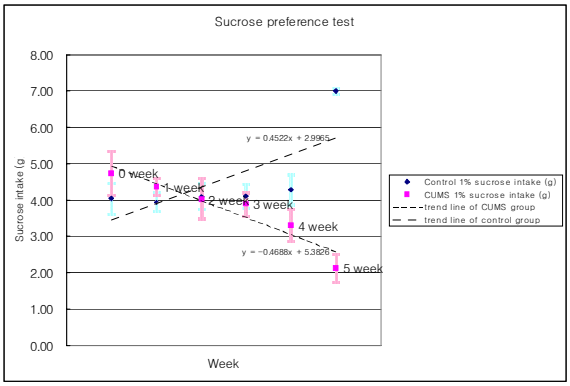

Fig. 3. Sucrose preference test of CUMS experiment

Western Blotting Kit (Boehringer, Mannheim, Germany) using primary antibody (goat polyclonal IgG, C-18, 1:5,000, Santa Cruz Technology; Santa Cruz, CA,) and secondary antibody (rabbit anti-goat IgG HRP, 1:2,000, Santa Cruz Technology; Santa Cruz, CA).

8. Data analysis

Results were statistically analyzed by ANOVA. We considered p values of less than 0.01 as significant. All values are presented as means± standard error of the mean (SEM) and were analyzed using SAS software (version 9.13, SAS institute, Cary, NC.).

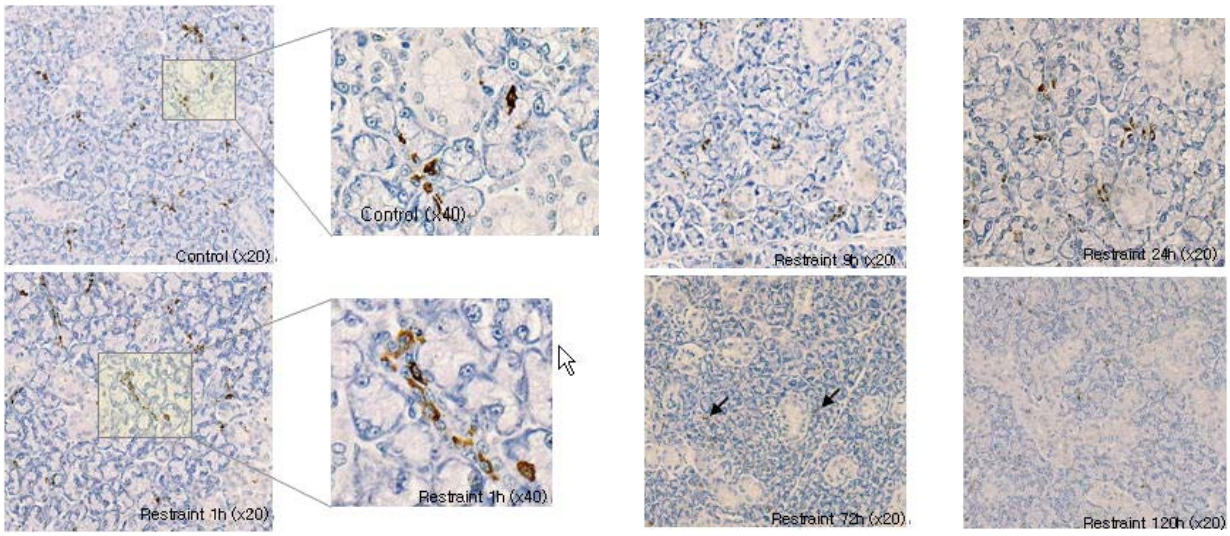

Fig. 5. Immunohistochemical exam of control and the restraint stress group

Ⅲ. RESULTS

1. General Features

In CUMS experiment, body weight of the 5 week group was significantly different to control group, but water intake changes were not significantly different to control group (Fig. 5, 8).

2. Sucrose Preference Test

In sucrose preference test (Fig. 3) carried out during chronic unpredictable mild stress (CUMS) experiment. Experimental group's sucrose preference in the 5th week was significantly decreased compared to the control group ($p<0.001$).

3. General Histology of Normal Submandibular Gland of Rat

The microscopic exam showed that clusterin was expressed abundantly on submandibular gland of control group. Generally clusterin was expressed dominantly in the cells of serous acini compared to mucous acini. In particular, clusterin was expressed intensively in the cells between serous acini and intercalated duct, in other words acini cells like centroacinar cells in pancreas (Fig. 4).

4. Morphometric Analysis

In microscopic exam on submandibular gland of restraint stress group, the number of CLU-

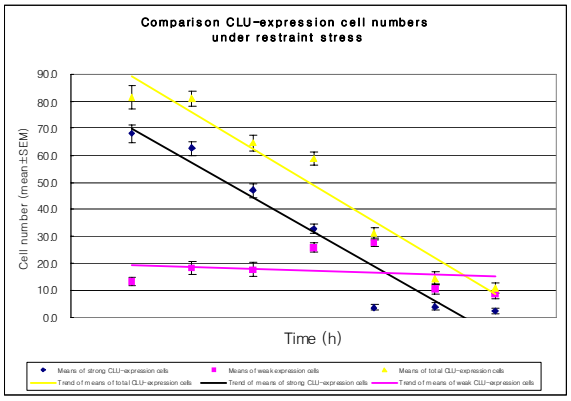

Fig. 6. Morphometric analysis of restraint group

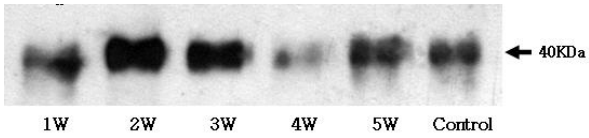

Fig. 7. Immunoblot analysis of restraint group

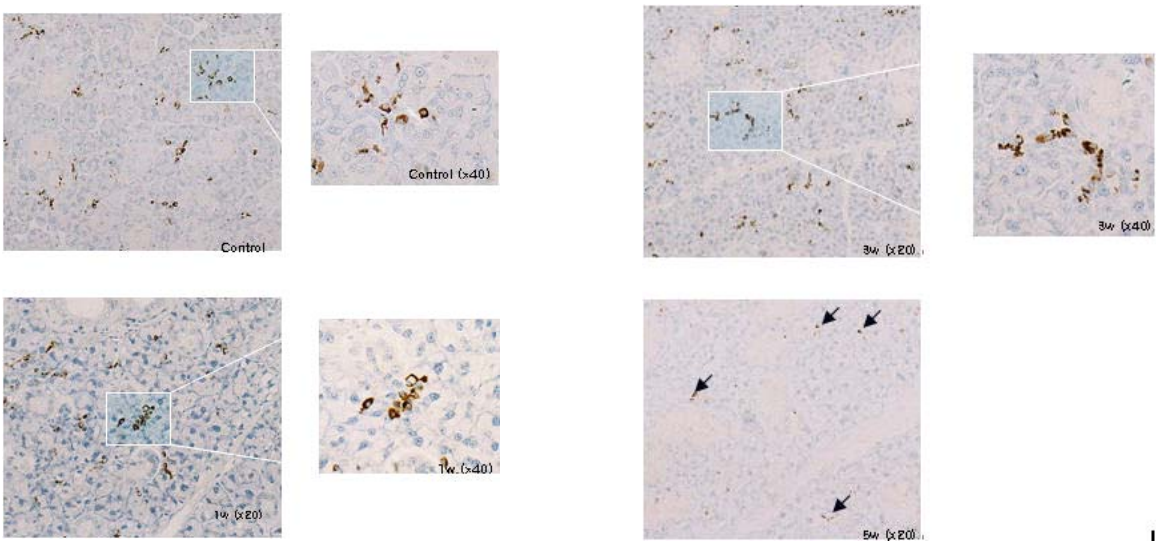

Fig. 8. Immunohistochemical exam of control and the restraint stress group

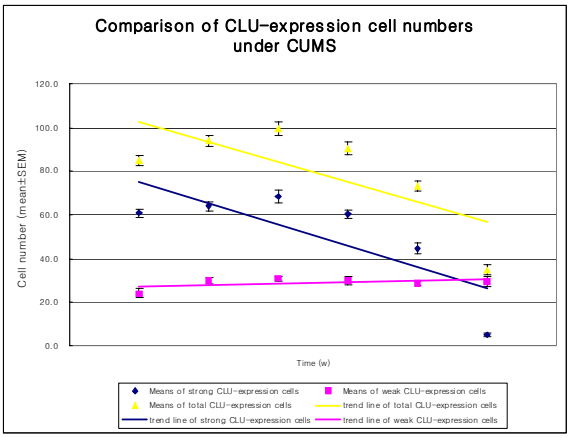

Fig. 9. Morphometric analysis of CUMS group

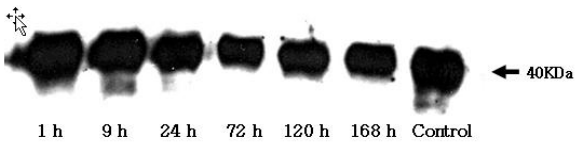

Fig. 10. Immunoblot analysis of restraint group

secretion cells had a change by time. The results were as follows (Fig. 5, 6) :

The number of all clusterin-secreting cells compared to control group showed significantly decreasing tendency in all experimental groups except for the 1st hour group ($p<0.001$ in the 9th, 24th, 72nd, 120th, and 168th hour group).

As for CUMS(chronic unpredictable mild stress) group, the number of CLU-secretion cell numbers showed significant difference compared to control group. The results were as follows (Fig. 8, 9):

The number of all clusterin-secreting cells compared to control group showed significantly increasing tendency in the 2nd week group ($p<0.01$), and significantly decreasing tendency in the 4th and 5th week group ($p<0.001$).

5. Immunoblot Analysis

In the restraint stress group, expression of clusterin was decreased on a gradual basis in due time compared to the control group (Fig. 7). As for CUMS group (chronic unpredictable mild stress group), it was increased till the 2nd week and decreased till the 5th week after that, which is similar to immunohistochemical analysis result and the decreasing tendency of sucrose preference and weigh changes (Fig. 10).

Ⅳ. DISCUSSION

Although it has been believed that there is correlation between stress and diseases for long time, but nothing have been concluded on the assumption. Modern studies about stress began when Bernard[17] mentioned physiological balance in the body, or "*milieu interieur*" for the first time in 1878. And then, Cannon[1] applied the term of stress to aversive response threatening physiological balance. In other words, stress response means fight or flight response to a certain stimulus, which is essential to life and can be divided into behavioral response, autonomic nerve response and endocrine response in physiologic terms.

Yet, this psychological response is designed to respond to short-term stimuli, and chronic stress beyond physiological tolerance limit is likely to severely deteriorate health. As for relation between stress and systemic health, Selye[18] suggested general adaptation syndrome (GAS), mixture of homeostasis and hypothalamus-pituitary gland-adrenal cortex axis (HPA axis), emphasizing that any stimulus exceeding threshold against HPA axis can cause diseases.

In particular, Chrousos and Gold [19] presented two principal components between hypothalamus and brain stem, CRH-neuron and locus ceruleus norepinephrine/sympathetic system, regarding HPA axis and autonomic nervous system as an interacting system, and proved that there is close relation between endocrine response and nerve response.

Given above, it is believed that stress-caused diseases are mainly attributed to chronic nervous and endocrine disorder when exceeding limit of coping[20]. There have been a lot of studies regarding cardiovascular diseases, immune disorder, endocrine disorder and depression, as well as ones on stress[14), 21-23].

One of the most difficult parts in studying stress is that there are too many stressors. Generally, starvation, cold, restraint or unavoidable electric shock are mainly used as stressors in animal tests[11], most of which are artificial acute stressors, while stressors in human society are even more complex, comprehensive and chronic, but lower grade. Animal tests using chronic mild stress are usually applied to depression tests, to give chronic stress for a certain time and verify that the stress can cause depression. There are two types of animal test models using chronic stress, one is "Learned helplessness model"[12] by Seligman and Maier, and the other is Katz's "Chronic stress model"[13]. Willner and his colleagues improved chronic stress model to develop chronic unpredictable mild stress model, and suggested the model was a better analogy of chronic and low-grade stressors and strain, citing decreasing sucrose preference as the evidence [24]. Although there is some doubt as to whether sucrose preference test can be an evidence of chronic stress-caused depression, its effectiveness is generally recognized[25]. This study also used Willner's model, and verified its effectiveness through sucrose preference test.

There has been some argue that stress is closely related to oral diseases as well. Chun and Hong[26] proposed that stress can give rise to diverse oral diseases, and Wimmer and his colleagues[27] asserted that individual stress-coping strategies could make difference in prognosis between before and after treatment of periodontal diseases.

Such oral diseases mainly caused by stress include lichen planus, recurrent aphthous stomatitis, xerostomia, burning mouth syndrome, and halitosis, all of which are believed to be related to secretion or state of saliva[28-32].

Human saliva, complex fluid containing important biomolecules such as mucin, enzyme and immunoglobulin, is secreted from 3 pairs of grand saliva glands and diverse small saliva glands scattered on oral mucosa. Those saliva glands are mainly controlled by autonomic nervous system, easily affected by emotional stress, and often used as a diagnosis index in stress-related tests[33].

Earlier studies often regarded that decreasing saliva and subjective xerostomia usually resulted

from drugs, disregarding emotional factors[34]. Nevertheless, it turns out that physical diseases are considerably emotional changes including depression[35], and that depression is as common as hypertension or diabetes in clinical data[36]. Many studies show that depression patients complain about subjective xerostomia[37], that actually decreasing saliva is found in them[2], and that depression patients treated with electric shock, rather than drugs, see their saliva increasing again[38]. Other studies show that saliva decreases in case of alarming responses including suppression and anxiety, and that exited and relaxed emotions can stimulate saliva secretion[2,39].

Tissues can react in a physiological or pathological way in accordance with grade and type of stress. Stress exceeding physiological tolerance limit of tissues affects diverse organs, leading to transformation in cells and molecules caused by damaged cellular responses; cellular adaptation response, cellular stress response (generation of stress protein), reversible cellular injury, and irreversible cellular injury; apoptosis (programmed cell death)[40,41]. In particular, a few studies presented that stress could expedite apoptosis[42-44], ultimately leading to cell death after failure in cellular proliferation due to transformation in genetic cellular structure. Diverse factors including stress can decrease saliva and increase subjective xerostomia through mechanism similar to that of autonomic nerve system or by directly influencing cells required to salivary secretion[4].

Clusterin is glycoprotein which is found in each tissue and expressed in relation with pathophysiological conditions[45]. It is 75~80 kDa heterodimer, secreted from physiological fluids, and also includes truncated forms targeted to nucleus. Clusterin is highly conservative in almost species. It is identical in amino acid by 79~80% in the mammals, and diverse variants and isomers[46,47] were reported despite their functions unidentified.

Given diverse disorders as well as upregulation of clusterin mRNA and protein detected at in vitro systems, clusterin is believed to play a key role in membrane lipid recycling and apoptotic cell death, and as stress-caused chaperone protein[7].

Prior hypotheses concentrated on specific potential roles, such as cell protection in saliva-tissue boundaries, membrane recycling in development process and response to damage, and complement-mediated membrane attack. More recently, there are further arguments that in light of cell-protecting roles[48] against heat, oxygen or mechanical stress, its diverse ligands, ability to suppress stress-induced protein precipitation, and cytotoxic agents, clusterin is secreted heat-shock protein or chaperone molecule[9,49].

However, it does not mean that all types of clusterin show those benign properties. Recently, 55 kDa form of clusterin induced and truncated by radioactive rays turned out to be targeted to nucleus, serving as death signal[50].The recent study on Knockout mice proved that clusterin exacerbates neuronal death in hypoxia-ischemia, increasing complex of clusterin[51]. Also, Bailey et al.[52] urged that clusterin protects from any cellular damage and helps removal of dead cells.

Through the test, it was proved that expression of clusterin in saliva glands decreases after receiving either acute or chronic stress, indicating relation with depression caused by chronic stress. Unlike other data, however, apoptotic tendency was hardly found in tissues. Diverse possibilities could be suggested on that: first, the stress was not enough to expedite apoptosis; second, apoptosis-related protein was already being secreted though not detected with microscope; third, clusterin, a major secretion molecule of saliva, decreased with malfunction of saliva due to stress. In the respect, it will be necessary to examine proteins presented in case of cell death or other heat-shock proteins at the same time, in order to see whether any cellular change or death is caused by decreasing clusterin under high stress, and whether the original state is restored as time goes by under mild stress, through longer-term tests using even higher acute stress.

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국문요약

급만성 스트레스가 백서 악하선의 Clusterin 분비에 미치는 영향

경희대학교 치과대학 구강내과학 교실

진 상 배 · 전 양 현 · 홍 정 표

구강 내에 발생되는 질환의 대부분이 타액의 영향을 받는다는 사실과 타액에 영향을 주는 전신적인 요소 중에서도 스트레스가 중요하다는 것은 이미 잘 알려져 있으나, 스트레스가 타액선에 미치는 영향에 관해서는 자율신경에 의한 거시적 반응에 대하여만 소개가 되었을 뿐 세포수준의 미시적 변화에 대하여는 별다른 언급이 없었다.. 이에 본 연구에서는 다양한 스트레스 조건하에서 백서의 악하선이 어떠한 변화를 보이는지를 clusterin 의 발현양상을 관찰함으로써 유추해보고자 하였다.

부여할 스트레스 조건을 급성 구속스트레스와 만성 저강도 스트레스의 두 가지로 정하고 7주된 Sprague-Dawley계 웅성백서 51마리를 사용하여 정해진 기간동안 급성 구속스트레스와 만성 저강도 비예측성 스트레스 (CUMS)를 가한 후 희생하여 악하선을 절취하고 역시 면역조직화학법과 웨스턴 면역점적법을 이용하여 악하선에서 clusterin발현의 시간에 따른 변화를 관찰하였으며, 그 결과는 다음과 같다:

1. 급성 구속스트레스 군에서, Clusterin 이 발현된 모든 선포의 합은 1시간 군을 제외한 모든 실험군 (9시간, 24시간, 72시간, 120시간, 그리고 168시간 군) 에서 대조군에 비하여 유의성 있게 감소하는 경향을 보였다 ($p<0.001$).
2. 만성저강도스트레스 군에서, 대조군에 대한 clusterin이 발현된 모든 선포의 합은, 2주군($p<0.01$)에서 대조군에 비하여 유의하게 증가되는 모습을 보여주었고, 4주군($p<0.01$)과 5주군($p<0.001$)에서 대조군에 비하여 유의성 있게 감소하는 경향을 보였다.
3. 만성 저강도 스트레스 실험에서 4주째까지는 대조군과 실험군간의 당선호도 차이에 있어서 유의한 변화가 보이지 않았으나, 5주째에 유의성 있는 감소를 나타내었다($p<0.001$).
4. 만성 저강도 스트레스 부여군은 5주째 대조군에 비하여 유의성 있는 체중변화($p<0.001$)를 보여주었으나, 수분섭취량의 변화는 유의성 있는 상관관계를 보여주지 못하였다.
5. 면역점적검사를 시행한 결과, 구속스트레스 군에서는 clusterin의 발현이 시간에 따라 일정하게 감소하는 것으로 표현되었고, CUMS 군에서는 2주째까지는 증가하다가 3주 이후부터 실험 전기간에 걸쳐 감소하는 것으로 나타났으며 이는 당선호도 및 몸무게 변화의 양상의 변화와 크게 다르지 않았다.

따라서 위의 실험결과를 놓고 볼 때, 타액선 clusterin의 발현이 급성과 만성에 관계없이 스트레스 부여 후에 감소하였지만, 다른 문헌에서 제안된 것처럼 clusterin 의 고갈에 의한 세포자사적 변화는 관찰되지 않았다.

따라서 향후 보다 강화된 강도의 급성스트레스를 부여하는 방법과 더 장기적으로 진행된 연구를 통하여 고강도 스트레스에서 clusterin의 발현감소와 함께 세포의 변성이나 자사가 초래되는지, 저강도 스트레스의 경우 장기간 시간이 경과함에 따라 원래의 상태에 가깝게 회복되는지, 다른 열 충격 단백질이나 세포 사멸 시에 나타나는 단백질들을 동시에 확인하여 보는 것이 필요할 것으로 사료된다

주제어: 타액선, 스트레스, clusterin, 면역조직화학법, 웨스턴 면역점적법