The protective role of musk on salivary glands of mice exposed to chronic unpredictable mild stress

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Abstract: This study assessed the impact of chronic unpredictable mild stress (CUMS) on the structure of mouse salivary glands and the role of musk in alleviating this impact. Forty male albino mice were distributed equally into four groups; control (untreated), CUMS (exposed to CUMS for 4 weeks), CUMS+fluoxetine (FLU) (exposed to CUMS then treated with FLU, CUMS+musk (exposed to CUMS then treated with musk). Behavioral changes and serum corticosterone levels were assessed at the end of the experiment. The submandibular and parotid glands were dissected out and processed for histopathological and immunohistochemical examination using antibodies against alpha smooth muscle actin (ASMA) and brain-derived neurotropic factor (BDNF). Exposure to CUMS significantly (P < 0.001) increased the serum corticosterone level and induced depression. CUMS also induced vacuolation in acinar cells along with a significant (P < 0.001) reduction of ASMA immunoexpression, indicating an effect on myoepithelial cells, and a significant (P < 0.001) increase of BDNF expression in the gland ductal system. Both FLU and musk alleviated the CUMS-induced behavioral, biochemical and histopathological changes in the salivary glands. In conclusion, musk ameliorates stress-induced structural changes in mouse salivary glands. This effect might be mediated through up-regulation of BDNF secretion by the glands.

Keywords: stress; BDNF; submandibular; parotid; musk; fluoxetine.

Introduction

The salivary glands play an important role in both oral and systemic health, secreting saliva and a number of biologically active substances such as nerve growth factor (NGF), produced specifically by the submandibular glands (SMGs) (1).

Stress has been described as a state of threatened homeostasis that results in different physiological and pathological changes, triggering coping reactions in the body, a phenomenon known as “adaptation”. Failure to do so leads to various psychological disorders, such as anxiety or depression (2). Chronic unpredictable mild stress (CUMS) is an animal model of depression characterized by predictive validity, face validity and construct validity (3). This model has been shown to display pathophysiological alterations relevant to depression, such as...
activation of the hypothalamic-pituitary-adrenal (HPA) axis and decreased levels of brain-derived neurotrophic factor (BDNF), which can be reversed by antidepressant drugs (4).

It has been reported that the salivary glands are affected by both acute and chronic stress. The SMGs in rats and humans produce BDNF, a member of the neurotrophin family that is heavily implicated in stress-coping mechanisms (5,6). Tsukinoki et al. have reported that acute stress triggers an increase of BDNF expression in salivary glands of the rat (7), and in fact it has been confirmed that SMGs are the major source of plasma BDNF in rats during acute immobilization stress (8). Since BDNF is able to pass through the blood-brain barrier (9), an increased level of plasma BDNF is considered to be a crucial neuroprotective response under conditions of acute immobilization stress, and may play an important role in homeostasis during exposure to stress (5). However, details of the mechanism by which BDNF affects the salivary glands during exposure to stress are still unknown (8).

Aromatherapy is now being used in psychotherapy to improve emotional affect in patients with various neurodegenerative diseases (10). Musk is a powerful odoriferous material obtained from the Siberian musk deer (*Moschus moschiferus*), and is widely used throughout the world, especially in the Gulf region and Arab countries (Khan IA et al., Leung’s Encyclopedia of Common Natural Ingredients, 455-465, John Wiley & Sons, Inc. Hoboken, 2010). Musk has been reported to exert an antidepressant-like effect (11). The aim of the present study was to assess the impact of exposure to CUMS on the structure of the salivary glands in mice and the role of musk in alleviating this impact.

### Materials and Methods

#### Drugs

Musk from *M. moschiferus* was obtained from a market in Jeddah, Saudi Arabia, and was approved by the biomedical research ethics committee at the Faculty of Medicine, King Abdulaziz University (KAU), Jeddah (reference number 48-16). Forty male Swiss albino mice were purchased from the animal unit at the KFMRC and left for 1 week to acclimatize to the laboratory conditions (temperature 22 ± 3°C, relative humidity 44-55%, with a 12 h dark/light cycle). The mice were weighed before the start and after finishing the experiment. They were divided into 4 groups: an untreated control group (*n* = 10) and three other groups (*n* = 10 each) exposed to CUMS for 4 weeks, as described by Doro et al. (13). The latter three groups were exposed, respectively, to CUMS plus 5% amyl acetate (CUMS group), CUMS and FLU (CUMS+FLU group) and CUMS and musk (CUMS+M group). FLU was administered by intragastric gavage at a dose of 20 mg/kg (14). All therapies were continued for 2 weeks. Mice were exposed to amyl acetate or musk daily for 2 weeks through inhalation for 15 min immediately after exposure to the CUMS procedure in a special odor-isolated inhalation chamber, according to the method of Chioca et al. (15).

At the end of the experiment, the behavioral changes in the mice evoked by CUMS were assessed in order to ensure the development of depression. For this purpose, the elevated plus maze (EPM) and the open field test (OFT) were used. The EPM was performed as described by Carobrez and Bertoglio (16). The numbers of closed arm entries in 6 min and the time spent by each mouse inside the open and closed arms were observed, recorded and expressed in seconds. The OFT was performed according to Mineur et al. (17). The number of rearing exhibited by mice in 25 min was recorded manually and the distance traveled by each mouse during this period was also measured using a video tracking system (Columbus Instruments, Columbus, OH, USA).

On the morning after the behavior tests, the mice were subjected to light ether anesthesia. Blood samples were obtained from the retro-orbital venous plexus, centrifuged for 10 min (2,200 × g, 4°C) and kept in a refrigerator until estimation of the serum corticosterone level using RIA (ELISA kits, ALPCO Diagnostics, Orangeburg, NY, USA).

After the blood samples had been obtained, the mice were decapitated and the submandibular and parotid glands were immediately dissected out and fixed in 10% neutral buffered formalin, dehydrated, cleared, and embedded in paraffin. Sections 4 μm thick were prepared and stained with hematoxylin and eosin (H&E) according to Bancroft and Gamble (Bancroft JD et al., Theory and
practice of histological technique, 112, Churchill Livingstone, 2008). Another two sets of 4-μm-thick paraffin sections were processed for immunohistochemical examination using the streptavidin-biotin-peroxidase technique according to Gu and Herrera (18). Nonspecific protein binding was blocked using a blocking solution (PBS + 10% normal goat serum). An antibody against alpha smooth muscle actin (ASMA) (Dako Cytomation, Heverlee, Belgium; dilution 1/1,000), was used for identification of myoepithelial cells (MECs) according to Takahashi et al. (19). An antibody against BDNF (Santa Cruz Biotechnology, Dallas, TX, USA; dilution 1/400) was also utilized. Sections incubated without the primary antibody but with PBS were used as negative controls for color development on the same slide.

For photography, an Olympus BX-51 (Tokyo, Japan) Microscope with an attached digital camera was used. Pro Plus image analysis software version 6.0 was used to assess immunoreactivity of ASMA and BDNF. The extension of the immunoreaction (percentage area) was assessed in 30 fields at ×400 magnification.

The Statistical Package for the Social Sciences (SPSS) version 16 was utilized to analyze the behavioral, biochemical, and image analysis data. Parametric data for different groups were compared using ANOVA (F-test), followed by a Bonferroni post hoc test to analyze each pair of groups, thereby avoiding a multiple-comparison effect. Statistical significance was considered to be present at $P < 0.05$.

## Results

### Effect of CUMS on body weight

There was a significant increase ($P < 0.001$) in the body weight of mice at the end of the experiment in comparison to that before the experiment in all groups except the control. A significant increase in body weight was also observed in mice exposed to CUMS along with the different treatments, in comparison with the untreated mice. On the other hand, the mice treated with musk after exposure to CUMS had a significantly lower ($P = 0.01$) body weight than CUMS-exposed, untreated mice (Table 1).

### Effect of CUMS on serum corticosterone

It was found that CUMS significantly increased ($P < 0.001$) the level of serum corticosterone in comparison with unexposed mice, while treatment with FLU ($P < 0.001$) or musk ($P < 0.001$) significantly reduced it relative to the untreated mice (Table 1).

### Effect of CUMS on behavior

The CUMS-exposed mice exhibited a significant decrease ($P < 0.001$) in the time spent in the open arms of the EPM when compared with the control mice. Administration of FLU or musk resulted in reduction of anxiety-like behavior, as evidenced by an increase in the time spent in the open arms, in comparison to CUMS-exposed mice ($P = 0.001, P < 0.001$) (Fig. 1).

Table 1: Effect of FLU and musk on body weight and serum corticosterone

| Variables                  | Groups $(n = 10$ each) | Control          | CUMS             | CUMS+FLU          | CUMS+M            |
|----------------------------|------------------------|------------------|------------------|-------------------|-------------------|
| Weight before (gm)         |                        | 32.09 ± 3.2      | 32.67 ± 1.8      | 30.29 ± 1.04      | 32.54 ± 1.7       |
|                           | $P = 0.99$             | $P = 0.76$       | $P = 0.21$       | $P = 0.99$        |                   |
| Weight after (gm)          | $P^2 = 0.06$           | 34.5 ± 1.99      | 43.31 ± 3.99     | 41.55 ± 0.87      | 39.38 ± 1.51      |
|                           | $P < 0.001$            | $P < 0.001$      | $P = 0.18$       | $P < 0.001$       |                   |
|                           | $P ^1 < 0.001$         | $P ^1 < 0.001$   | $P < 0.001$      |                   |                   |
| Serum corticosterone (ng/mL)| $P = 0.001$           | 21.03 ± 2.1      | 130.97 ± 8.8     | 56.89 ± 4.8       | 41.25 ± 4.2       |
|                           | $P < 0.001$            | $P < 0.001$      | $P < 0.001$      | $P < 0.001$       |                   |
|                           | $P ^1 < 0.001$         | $P ^1 < 0.001$   | $P < 0.001$      | $P < 0.001$       |                   |

Data are presented as mean ± SD. $P$: significant difference compared with the control group. $P^1$: significance difference compared with the CUMS group. $P^#$: significance difference compared with measurements taken before starting the experiment. Significance was considered at $P < 0.05$. 

For photography, an Olympus BX-51 (Tokyo, Japan) Microscope with an attached digital camera was used. Pro Plus image analysis software version 6.0 was used to assess immunoreactivity of ASMA and BDNF. The extension of the immunoreaction (percentage area) was assessed in 30 fields at ×400 magnification.

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Results

Effect of CUMS on body weight

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Exposure to CUMS increased the amount of spontaneous locomotor activity during the OFT as the mice moved ($P < 0.001$) and performed rearing ($P < 0.001$) significantly more often than the control mice. Exposure to FLU or musk after CUMS significantly reduced ($P < 0.001$) these activities in comparison to the untreated group (Fig. 1).
Effect of CUMS on the histological structure and immunohistochemical reactivity of the salivary gland

The SMG of control mice was formed of intact seromucous acini and numerous well developed large granular convoluted ducts (GC) that possessed a strongly acidophilic cytoplasm filled with secretory granules, as well as other ductal components. Exposure to CUMS resulted in congestion of the blood vessels and vacuolation of some seromucous acinar cells while the GC appeared intact. Treatment with FLU or musk slightly decreased the CUMS-induced vacuolation of acinar cells and blood vessel congestion (Fig. 2).

Observation of the MECs surrounding the glandular acini and ducts revealed that SMGs in the control group had strongly positive immunoreactivity for ASMA, indicating that the MECs were intact. On the other hand, exposure to CUMS resulted in significant reduction \((P < 0.001)\) of ASMA expression, indicating that the structure of the MECs had been affected. Inhalation of musk after exposure to CUMS significantly increased \((P < 0.001)\) the expression of ASMA in both salivary acini and ducts in comparison to the CUMS group, whereas FLU elicited no such effect (Figs. 3, 4A).

BDNF was expressed mainly in the GC ducts of the SMGs, and the expression was significantly increased \((P < 0.001)\) after exposure to CUMS in comparison with the unexposed mice. Treatment with FLU or musk significantly increased the expression of BDNF \((P = 0.03, P = 0.01)\) in comparison with untreated mice (Figs. 3, 4B).

The parotid gland in control mice was formed of...
closely packed, intact pure serous acini and striated ducts in addition to the other ductal components. After exposure to CUMS, the serous acini appeared smaller in size and some of them were atrophic. Some of the acinar cells were vacuolated. These changes were also observed in the glands of mice treated with FLU or musk, but to a lesser degree (Fig. 5).

A significant \( P < 0.001 \) decrease of ASMA immunoreactivity was observed in the parotid gland after exposure to CUMS. Although treatment with FLU increased the expression of ASMA, this increase was not statistically significant, whereas treatment with musk led to a significant \( P < 0.001 \) increase of ASMA expression (Figs. 4A, 5).

Immunoreactivity for BDNF was observed in striated ducts of the parotid glands. Semi-qualitative assessment showed that BDNF expression in the parotid gland was significantly increased \( P < 0.001 \) following exposure to CUMS when compared to that in control mice. Administration of FLU or musk significantly increased the expression \( P < 0.04, P = 0.02 \) relative to that in untreated mice (Figs. 4B, 5).

**Discussion**

In the present study, we observed that exposure of mice to CUMS led to development of a depressed state characterized by increased spontaneous locomotor activity during the OFT, and this was confirmed by the EPM test. This depressed state was associated with an increased corticosterone level. These findings were consistent with those of Mizuki et al. (20) and Liu (21). Inhalation of musk exerted an anti-depressive-like effect, in accord with previous studies (11). FLU, a selective serotonin reuptake blocker antidepressant used for pharmacological validation in the present study, ameliorated the behavioral and biochemical changes induced by CUMS. Mice exposed to CUMS exhibited a significant increase in body weight, in concordance with the finding of Savignac et al. (22). This gain in body weight might have been attributable to the increase observed in the level of corticosterone after exposure to stress. On the other hand, some studies have reported weight loss in rats after exposure to chronic stress (23,24).
This study showed that exposure to CUMS induced acinar cell vacuolation along with a reduction in the size of some acini of the SMG and parotid gland. As MECs are not easily identified in routinely stained sections, their structural integrity was assessed using an antibody against ASMA, a specific immunohistochemical marker. This revealed that exposure to CUMS affected the structural integrity of MECs around both acini and ducts, which may have affected their contractility and subsequently reduced their saliva section. Pellegrini et al. observed similar histopathological changes in the submaxillary glands of male albino rats exposed to chronic stress (25). Taher reported similar changes in the SMGs of rats after administration of hydrocortisone for 2 weeks (26). This suggests that the increased corticosterone level evident in this study after exposure to CUMS was related to the histopathological changes observed in the salivary glands. We noticed that the duct system of both the SMG and parotid gland was affected after exposure to CUMS, similar to the observations reported by Saruta et al. in the rat SMG after chronic restraint stress (5).

In the present study, exposure to CUMS significantly increased the immunoeexpression of BDNF in the submandibular and parotid glands, supported many previous studies that had demonstrated increased salivary gland expression of BDNF in response to different types of stress. Irie et al. reported a significant increase of BDNF expression in the rat SMG after exposure to occlusal disharmony-induced chronic stress for 8 weeks (27). Not only chronic stress, but also the acute stress has been shown to increase the expression of BDNF in salivary glands (7,8). On the other hand, many studies have demonstrated decreases in the BDNF content of the rat hippocampus following chronic stress (28). In more recent studies, the levels of both BDNF mRNA and protein were down-regulated in the mouse hippocampus after exposure to CUMS (11,29). Thus the changes in BDNF levels induced by stress appear to be tissue-specific, as reported by Saruta et al. (5).

Reduced hippocampal expression of BDNF resulting from chronic stress has been attributed to corticosterone secretion (27). On the other hand, Saruta et al. have reported that during repeated restraint, the elevated levels of circulating glucocorticoids did not reduce salivary gland production of BDNF (5). This is supported by the findings of the present study, as salivary BDNF was shown to increase whereas the serum corticosterone level increased. It is worth mentioning that although the gene and protein expression levels of BDNF are elevated in the salivary glands during chronic stress, the SMG is not the only contributor to the elevated plasma BDNF level during stress, as BDNF is expressed and secreted by many other organs, including the heart, lung, liver, pancreas, spleen and vascular endothelial cells (30-32).
In this study, treatment of mice exposed to CUMS in combination with FLU or musk showed a significant increase of BDNF expression in the SMGs and parotid glands relative to untreated mice. In previous studies, FLU was found to increase the level of BDNF in stressed mice following exposure to CUMS (28,29). Saruta et al. have stated that secretion of BDNF may represent a protective mechanism that plays important roles in homeostasis under stress conditions (5). In a more recent study by Ayuob et al., musk was shown to increase both gene and protein expression in the hippocampus of mice exposed to chronic stress (11).

The protective role of musk on salivary glands might be attributable to its ability to reduce stress-induced elevation of the corticosterone level. Another explanation for the protective effect of musk may be its high content of steroids, which accounted for up to 14% of the musk examined in this study, similar to levels previously reported (33). The major steroid in musk is muscone, which is reported to have estrogenic activity (34), and sex steroid receptors including those for androgen, progesterone and estrogen receptor β are expressed in the salivary glands (35). However, this issue will require further study.

In conclusion, the present study has revealed that musk improves chronic stress-induced depression in mice, and also ameliorates stress-induced structural changes in the submandibular and parotid glands. This effect might be mediated through up-regulation of BDNF secretion by the glands. The present findings suggest that further studies to test the effectiveness of musk for relieving stress-associated salivary changes in humans would be justified.

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Conflict of interest
The authors have no conflict of interest to declare.

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