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CAN *OCIMUM BASILICUM* (BASIL) RELIEF THE IMPACT OF CHRONIC UNPREDICTABLE MILD STRESS ON THE MICE SALIVARY GLANDS?

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Abstract

Background: Salivary glands are affected in acute and chronic stressful conditions. *Ocimum basilicum* (OB), basil, possesses anxiolytic and antidepressant like effect so this study aimed to evaluate the effect of the chronic unpredictable mild stress (CUMS) on the structure of salivary glands of mice and the efficacy of OB in relieving this effect.
Material and Methods: Forty male mice were distributed equally into four groups; the control, CUMS (exposed to the CUMS for 4 weeks), CUMS+Fluoxetine (treated with Fluoxetine after exposure to CUMS), CUMS+OB (treated with OB after exposure to CUMS). Treatments continued for 2 weeks. Behavioral changes and serum corticosterone level were assessed at the end of the experiment. Submandibular and parotid glands were histopathologically examined and stained with anti-alpha smooth muscle actin (ASMA) and anti-brain derived neurotropic factor (BDNF) antibodies.
Results: A depressive behavior was observed in mice exposed to the CUMS. Serum corticosterone level significantly increased in these mice compared to the control (130.9±8.8 versus 21.03±2.1, $p<0.001$). Structural affection of salivary acinar and myoepithelial cells of stressed mice was evident. BDNF expression in the salivary ductal system significantly ($p<0.001$) increased in the stressed mice and treatment with FLU or OB significantly increased it more ($p=0.03$, $p=0.04$) when compared to the untreated mice.
Conclusion: *Ocimum basilicum* improved chronic stress-induced depressive status and structural changes in the salivary glands. This effect might be mediated through up-regulating salivary BDNF secretion by the glands so it is the time to test OB effectiveness in relieving stress-associated salivary changes on human being.

Keywords: Stress; Depression-ASMA-BDNF; Submandibular; Parotid; *Ocimum basilicum*; Fluoxetine

Introduction

Salivary glands have crucial role in oral and general health as they influence the other body organs including the respiratory tract. In addition to the saliva, which is involved in food digestion, mastication, and antimicrobial activity, salivary glands secret a group of biologically active substances and growth factors like the epidermal growth factor (EGF), the nerve growth factor (NGF) secreted by the submandibular glands (SMG) (Tsukinoki et al., 2007) as well as brain-derived neurotrophic factor (BDNF) and neurotrophins (NTs) (Lewin and Barde 1996). Saliva also contains proteins that can be informative when detecting diseases related to oral health, in particular oral cancer, Sjögren's syndrome, and diabetes (Ambatipudi et al., 2012 and Bencharit et al., 2013).

Exposure to stress results in different physiological and pathological changes in the body and triggers adaptive reactions to cope with it. If the body fail to adapt to stress many psychological disorders such as anxiety or depression may developed (Ravindran et al., 2005). Among the organs that are affected upon exposure to either acute and chronic stress are

the salivary glands. It was reported that both rat and human SMG secrete BDNF, a neurotrophin that is heavily implicated in stress mechanisms (Saruta et al., 2012). The rat SMG was confirmed to be the major source of plasma BDNF during acute immobilization stress (Saruta et al., 2014). Increased levels of plasma BDNF is considered a neuroprotective response in conditions of acute immobilization stress and may play important role in homeostasis during exposure to stress (Saruta et al., 2010). BDNF expression was up-regulated in the SMG after chronic restraint stress. The exact mechanism by which BDNF affects the salivary glands during stressful conditions is still unknown (Saruta et al., 2014).

Chronic unpredictable mild stress (CUMS) is a valid animal model of depression with proved predictive, face and construct validity (Duman, 2010). This model was showed to induce pathophysiological alterations relevant to depression, such as activation of the hypothalamic–pituitary–adrenal (HPA) axis and decreased levels of BDNF and both could be reversed by antidepressant drugs (Kuipers et al., 2013). So CUMS was chosen in this study to study the effect of depression on the salivary glands.

Ocimum basilicum (Family Lamiaceae) (OB), also known as sweet basil, is a common popular annual herb. It originated in Asia. It is fairly common in the savannah regions of Senegal, Guinea, Cape Verde Islands, Mali, Sierra Leone, Liberia, Nigeria, Central African Republic as well as America (Paton 1992). It is widely used in perfumes and foods (Khan and Abourashed 2010). It has been traditionally used in treating many neurological disorders as anxiety, headache, migraine, nerve pains. It has been used as carminative and antispasmodic (Bora et al., 2011). OB is used in the east of Morocco by hyperlipidemic subjects as an alternative therapeutic to treat hyperlipidemia (Paton 1992). This study aimed to evaluate the effect of the chronic unpredictable mild stress on the structure of salivary glands of mice and the efficacy of OB in relieving this effect.

Materials and Methods

Drugs

OB was obtained from the local gardens at south Jeddah, Saudi Arabia and it was confirmed by specialist in Botany from the Faculty of Science, King Abdulaziz University (KAU). The essential oils of OB was extracted as described by Ismail (2006) and its constituents were identified by using gas chromatography coupled with mass spectrometry. Gas chromatography analysis was done using (GC-MS; Agilent, Columbia, USA) with column: DB-5ms (30 m x 0.25 mm x 0.25 µm). Temperature was programmed at 5℃/min from 50℃ to 250℃. Relative percentage amounts were calculated from peak total area by apparatus software. The compounds were identified by comparing their retention times and mass spectra with those obtained from the MS library. OB essential oils was diluted before use by Propylene glycol (5%; Sigma, St. Louis, MO, USA) as described by Chioca et al., 2013.

Fluoxetine, a common antidepressant of the selective serotonin reuptake blocker category, was used in this study for pharmacological validation. It was obtained from Dar Al Dawa Pharmaceuticals Co., Ltd., Jordan. It was dissolved in 0.03% sodium carboxymethyl cellulose (CMC-Na) and was administered through the intragastric gavage with a dose of 20 mg/kg (Li et al., 2014). 5% Amyl acetate was administered to the positive control group because it was reported to be an odorous substance with no effect on anxiety (Pavesi et al., 2011). It was obtained from Sigma (St. Louis, MO, USA).

Experimental procedure:

This study was ethically approved by the biomedical research ethics committee at the Faculty of Medicine, KAU, Jeddah, Saudi Arabia with the reference number 48-16. Dealing with animals was according to the guidelines set by the Animal Care and Use Committee at the King Fahed Medical Research Center (KFMRC), KAU, Jeddah Saudi Arabia. Forty male Swiss albino mice were purchased from animal unit at the KFMRC and left to acclimatize for one week to the behavior laboratory condition (22 ± 3°C and relative humidity of 44%-55% with a 12 h dark/light cycle). The weight of the mice were recorded at the start and at the end of the experiment. The mice were divided into 4 groups; the control group (n=10) was left without exposure to any stress or treatment and the other three groups (n=10 each) were exposed to the CUMS for 4 weeks as described by Doro et al., (2014). During this period, mice were exposed to different types of social stress like placing them in cages soiled by other mice or placing them in cages with wet sawdust, reversing the light/dark cycle, tilting cages at 30°, restraining the mice and placing them in an empty cage with 1 cm of water on the bottom. Mice were exposed to one stressor/day at different times during the day. After exposure to the CUMS for 4 weeks, the mice in the 3 groups; CUMS, CUMS+FLU and CUMS+OB were treated with 5% amyl acetate, FLU and OB respectively for another 2 weeks. Administration of amyl acetate and OB was by inhalation for 15 minutes/day in a special odor-isolated inhalation chamber according to Chioca et al. (2013).

At the end of the experiment, the behavior changes evoked by exposure to CUMS were evaluate using elevated plus maze (EPM) and Open field test (OFT). The EPM was performed as described by Carobrez and Bertoglio, 2005. The numbers of closed arms entries in 6 min as well as the time spent by each mouse in the open arm of the EPM were recorded and expressed in seconds. The OFT was performed as described by Mineur et al. (2006). The number of mouse rearing in

25 min was registered manually and the distance traveled by the mouse during these 25 min was also measured through video tracking system (Columbus Instruments, OHIO 43204, USA).

The day after performing the behavior tests, the mice were anaesthetized, in the morning, using light ether anesthesia, blood samples were obtained from retro-orbital venous plexus, centrifuged for 10 min (2200 g, 4uC) and kept at the refrigerator till the measurement of serum corticosterone levels using RIA (ELISA kits, ALPCO Diagnostics, Orangeburg, NY).

After obtaining the blood samples, the mice were decapitated and the SMG and parotid glands were dissected out and fixed in 10% neutral buffered formalin, dehydrated, cleared and embedded in paraffin. Sectioning using microtome at 4 microns thickness were obtained and stained with haematoxylin and eosin (H&E) according to Bancroft and Gamble 2008 for routine histopathological examination. Another two set of the 4 microns-thick paraffin sections were processed for immunohistochemical examination using streptavidine–biotin–peroxidase technique according to Gu and Herrera 2010. Anti-alpha Smooth muscle actin (ASMA) antibody (Dako Cytomation, Heverlee, Belgium, with dilution of 1/1000), was used as a marker for identification of myoepithelial cells (MECs) (Takahashi et al., 2004). Anti-BDNF antibody (Santa Cruz Biotechnology, Texas, USA with dilutions of 1/400) was used to assess the immunohistochemical expression of this neurotrophin.

During light microscopic examination and photographing an Olympus Microscope BX-51 supplied with a digital camera was utilized. Pro Plus Image analysis software version 6.0 was used to assess immunoexpression of different antibodies used. Extension of the reaction (area percentage) was assessed in 30 field at magnification X 400.

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

Results

Composition of OB essential oils:

Analysis of OB essential oils components showed that it included linalool (about 36%), 1,8-cineole (11.2%), cadinol (10.3%), ocimene (3.7%) camphor (2.3%), and other constituents like caryophyllene, humulene**.** See Table (1).

Table 1: Chemical composition of essential oil of *Ocimum basilicum* (OB) obtained by GC-MS

Compound	Retention time (min)	Percentage
Linalool L	14.699	35.945
1,8-Cineole	13.594	11.228
Alpha-Cadinol	24.297	10.395
Farnesyl acetate	38.401	10.178
E,E-alpha-Farnesene	20.226	4.851
Trans-beta-Ocimene	13.774	3.724
Gamma cadinene	22.022	3.101
(-)-Camphor	15.539	2.331
Borneol	15.932	1.96
Alpha-Copaene	23.882	1.75
Fenchyl acetate	16.591	1.316
L-alpha-bornyl acetate	17.569	1.225
Cis-beta-Terpineol	14.231	1.19
Alpha-Guaiene	20.321	1.162
Alpha-Humulene	20.864	0.931
p-Menth-1-en-8-ol	15.879	0.756
Alpha-bisabolol	24.956	0.721
Trans-epoxy-ocimene	15.305	0.63
(-)- Cadina-1,3,5-triene	22.171	0.588
Trans-Caryophyllene	25.296	0.574
Trans-gamma.-bisabolene	21.958	0.504
Alpha-Thujone	14.561	0.462
Calarene	19.333	0.301
Non-identified compounds	-	4.177

Effect on body weight

A significant ($p<0.001$) increase was observed in body weight of all mice in the different groups at the end of the experiment in comparison to their weight at the start of the experiment except the control group. In addition, there was a significant ($p<0.001$) increase in the body weight of mice exposed to the CUMS when compared to the control. On the other hand, the mice treated with OB after exposure to CUMS have significantly lower ($p=0.002$) body weight when compared to the untreated mice (Table 2).

Effect on serum corticosterone

The CUMS procedure significantly ($p<0.001$) increased the serum corticosterone level when compared to the unexposed mice while treatment with FLU ($p<0.001$) or OB ($p<0.001$) significantly reduced it compared to the untreated mice (Table 2).

Table 2: Effect of FLU, *Ocimum basilicum* on body weight and serum corticosterone

Parameter	**Control (n=10)**	**CUMS (n=10)**	**CUMS+Flu (n=10)**	**CUMS+OB (n=10)**
Weight before (gm)	32.09±3.2	32.67±1.8 p=0.99	30.29±1.04 p=0.76 p1=0.21	31.72±2.6 p=0.99 p1=0.99
Weight after (gm)	34.5±1.99 $p^{\#}$ =0.06	43.31±3.99 p<0.001 $p^{\#}$ <0.001	41.55±0.87 p<0.001 p1=0.18 $p^{\#}$ <0.001	37.26±3.17 p=0.031 p1=0.002 $p^{\#}$<0.001
Serum corticosterone (ng/mL)	21.03±2.1	130.97±8.8 p<0.001	56.89±4.8 p<0.001 p1<0.001	45.5 ±6.2 p<0.001 p1<0.001

Data are expressed as mean±SD; p: significance versus control group; p1: significance versus CUMS group
$P^{\#}$: significance of after versus before measurements; Significance is considered at $p<0.05$

Effect on the behavior

The time spent by mice of the CUMS group in the open arms of the EPM test decreased significant compared to the control ($p < 0.001$) while administration of FLU or OB increased it significantly compared to the untreated group ($p = 0.001$ and < 0.001, respectively) (Fig. 1). In addition, the number of closed arm entries during the EPM test was increased significantly ($p < 0.001$) in the CUMS group compared to the control group. FLU or OB reduced the number of entries significantly ($p < 0.001$ and < 0.001, respectively) compared to the untreated group (Fig. 1).

The spontaneous locomotor activity, observed during the OFT, of the mice exposed to the CUMS increased. These mice travelled significantly ($p<0.001$) longer and performed significantly ($p<0.001$) more rearing compared to the control mice. Treatment with FLU or OB significantly ($p<0.001$) reduced these activities in comparison to the untreated group (Fig. 1).

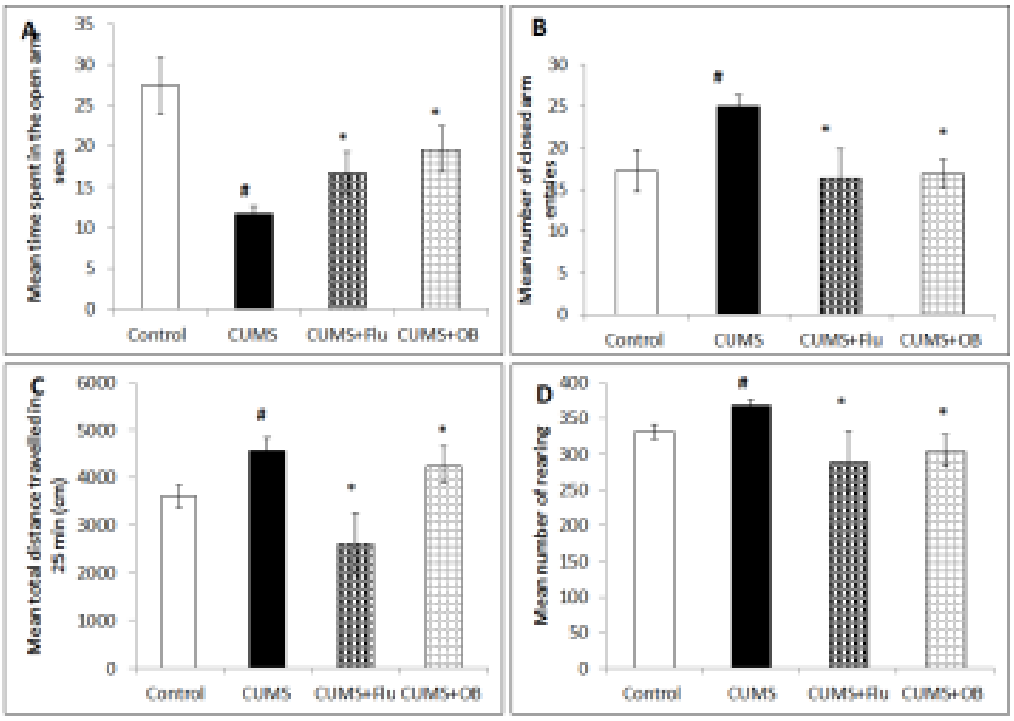

Figure (1)

Figure 1: Elevated plus maze test (A, B) and Open field test (C, D) of the control, chronic unpredictable mild stress (CUMS), fluoxetine-treated (CUMS+FLU) and OB-treated (CUMS+OB) groups (n=10 each). Data was shown as mean±SD. # indicates significance compared to the control group, * indicates significance compared to the CUMS group. (CUMS; chronic unpredictable mild stress, FLU; fluoxetine, OB; *Ocimum basilicum*)

Effect on the histological structure of the salivary gland
The submandibular gland

Submandibular gland of the control mice had intact serous acini, granular convoluted tubules (GCT) with strong acidophilic cytoplasm and secretory granules in addition to the other parts of the duct system. On the other hand, the SMG of the mice exposed to the CUMS showed some congested blood vessels, some vacuolated serous acinar cells and some acini were reduced in size while the GCT appear intact. Treating mice with FLU or OB slightly decreased the acinar cells vacuolation and the blood vessel congestion (Fig. 2).

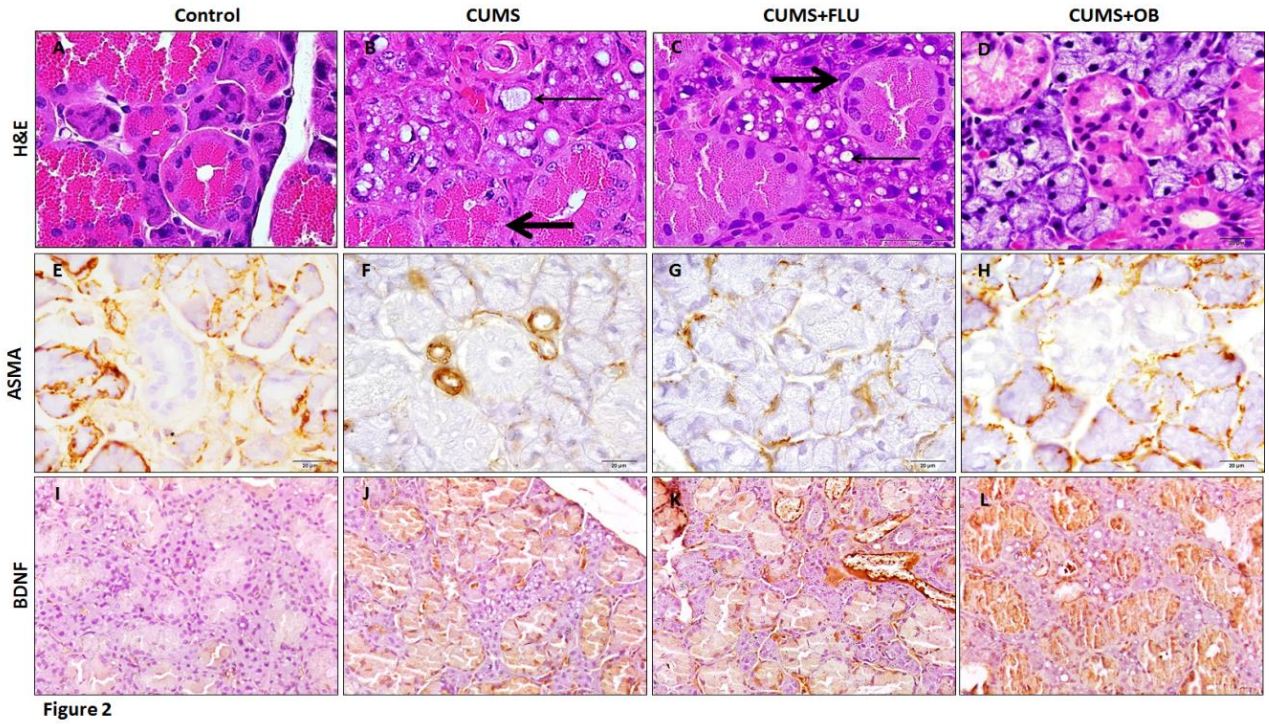

Figure 2: Sections in SMG show serous acini (SA) and numerous large granular convoluted tubules (GCT) filled with acidophilic granules. Note the congestion of the blood vessel (star), vacuolation of SA (thin arrow), while the GCT (thick arrow) appear intact in the SMG of the CUMS(C, D) and CUMS+FLU (E, F) groups CUMS+M (G, H) group (H&EX 200, 600). (SMG; submandibular glands, CUMS; chronic unpredictable mild stress, FLU; fluoxetine, OB; *Ocimum basilicum*).

When it came to the structure of the MECs surround the glandular acini and ducts, it was found that the control SMG showed strong positive ASMA immunoexpression around acini and ducts indicating intact MECs. On the other hand, exposure to the CUMS induced a significant ($p<0.001$) decrease in ASMA expression indicating structural affection of the MECs. Treating stressed mice with OB significantly ($p=0.01$) increased ASMA expression in both acini and ducts compared to the untreated mice while treatment with FLU failed to increase it (Fig. 2, 3).

When the BDNF expression in the salivary glands was assessed it was observed that it significantly ($p<0.001$) increased after exposure to CUMS when compared to the control mice. Treating mice with FLU or OB significantly increased it more ($p=0.03$, $p=0.04$) (Fig. 2, 3).

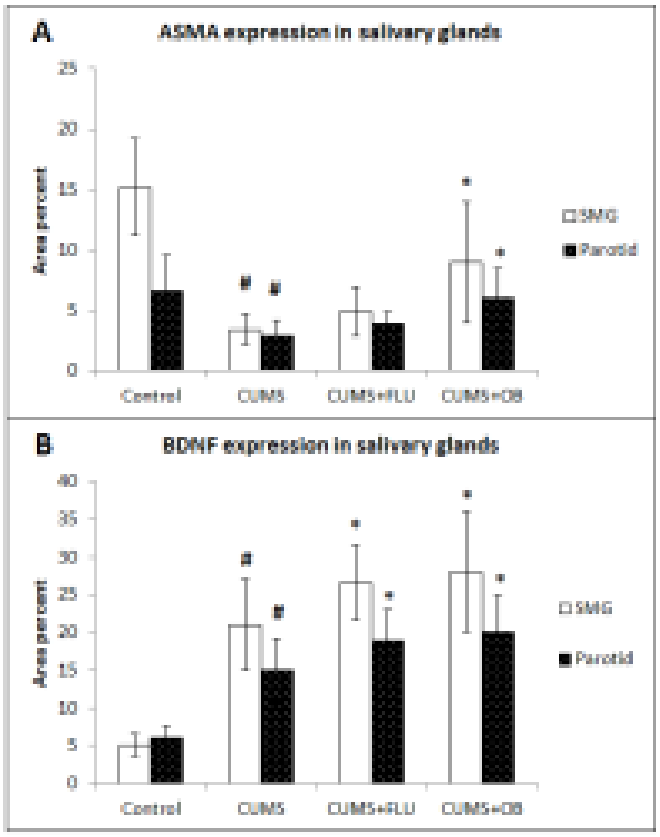

Figure (3)

Figure 3: Immunoexpression of ASMA (A) and BDNF (B) in SMG and parotid of the control, chronic unpredictable mild stress (CUMS), fluoxetine-treated (CUMS+FLU) and OB-treated (CUMS+OB) groups (n=10 each). # indicates significance compared to the control group, * indicates significance compared to the CUMS group. (CUMS; chronic unpredictable mild stress, FLU; fluoxetine, OB; *Ocimum basilicum*; ASMA; apha smooth muscle actin, BDNF; Brain derived neurotropic factor, SMG; Submandibular gland)

The parotid gland

The control parotid gland was formed of intact closely packed serous acini and duct system. Parotid gland of stressed mice showed smaller serous acini with some of them appeared atrophied and others had vacuolated cells. These changes were less frequently observed in the glands of mice treated with FLU or OB (Fig. 4).

It was noticed that there was a significant ($p<0.001$) decrease in ASMA immnuoexpression in parotid gland of mice exposed to CUMS. Although treatment with FLU increased ASMA expression but this increase was statistically insignificant while OB significantly ($p=0.003$) increased it (Fig 3, 4).

Immunoexpression of BDNF in the parotid gland was significantly ($p<0.001$) increased following exposure to CUMS when compared to the control mice. Treating stressed mice with FLU or OB significantly increase it ($p=0.04$, $p=0.03$) more (Fig. 3, 4).

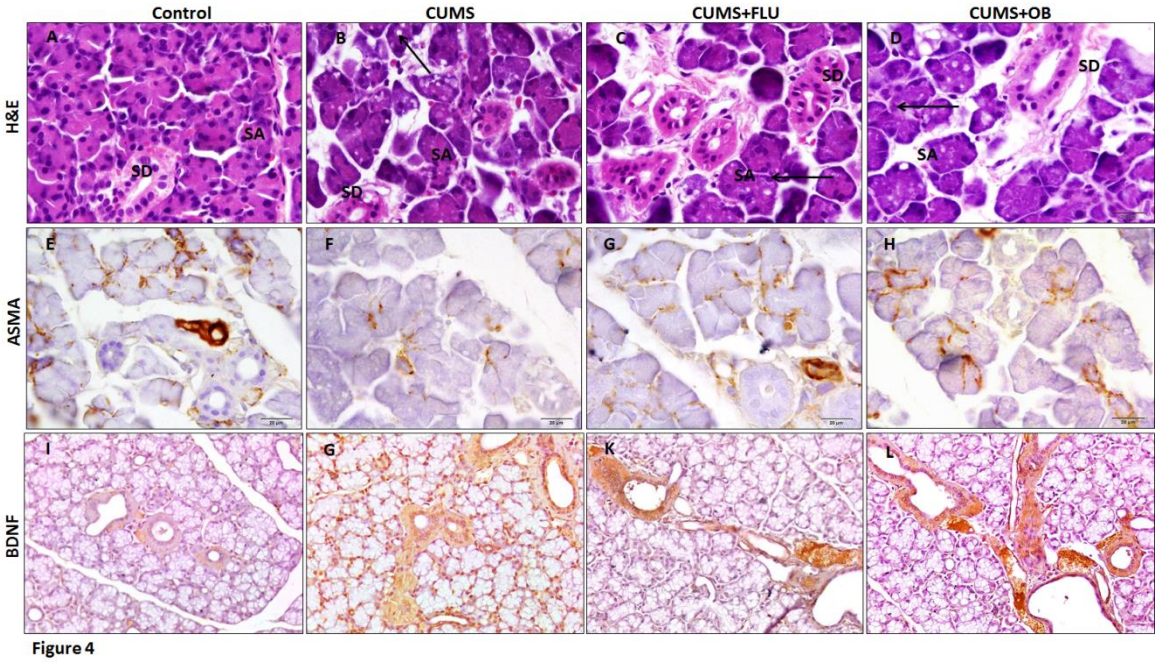

Figure 4: Sections in parotid gland show serous acini (SA) and intralobular ducts (SD). Note the reduction in the size of the SA with some of them appear atrophied and vacuolation of some cells lining these acini (thin arrow) of the glands of the CUMS (B) and CUMS+FLU (C) groups compared to the CUMS+OB (D) group (H&EX 400). Immunoexpression of ASMA (E-H) and BDNF (I-L) of the parotid glands of control, CUMS, CUMS+FLU and CUMS+OB (immunostaining E-HX 400 and I-L X 200). (CUMS; chronic unpredictable mild stress, FLU; fluoxetine, OB; *Ocimum basilicum*; ASMA; alpha smooth muscle actin, BDNF; Brain derived neurotropic factor).

Discussion

Exposing mice to CUMS for 4 weeks, in this study, resulted in a depressive status evidenced by the prolonged time spent in the open arms during the EPM test and reduction in open field activity during the OFT which indicated depression. This depressive status was accompanied by elevated corticosterone level. These findings were consistent with those of Mizuki et al., 2014 and Liu et al., 2014. Fluoxetine ameliorated the CUMS-induced behavioral changes and reduced elevated corticosterone level. The body weight of mice exposed to the CUMS significantly increased compared to the control. Although this finding was supported by the study of Patterson et al., 2013, other studies reported weight loss in rats after exposure to chronic stress (Bekris et al., 2005, Lucca et al., 2008).

In this study, after exposure to CUMS the salivary glands showed reduction in the size of the SMG and parotid gland acini together with vacuolation of some acinar cells. The structural integrity of the MECs around the acini and ducts was affected as evidenced with ASMA immunohistochemistry. This might resulted in affection of the MECs contractility with subsequent reduction in salivary secretion which was reported in stressful conditions (Castle and Castle, 1998). Similar histopathological changes were observed by Pellegrini et al., 1998 in the submaxillary salivary glands of male albino rats exposed to chronic stress. Taher in 2008 also reported similar changes in the SMG of rats after administration of hydrocortisone for two weeks. This might reveal that increased corticosterone level, evident in this study after exposure to CUMS, was behind the histopathological changes observed in the salivary glands after exposure to chronic stress. It was noticed that the duct system of both SMG and parotid gland was not affected after exposure to CUMS, in this study. This was in concordance with what was reported by Saruta et al. (2010) regarding the SMG of rat exposed to chronic restraint stress.

The significant increase in immunoexpression of BDNF in the duct system of the SMG and parotid glands of stressed mice was among the findings elicited in this study and was supported by the findings of many researchers. Irie et al., 2011 reported a significant increase in BDNF expression levels in rat SMG after exposure to occlusal disharmony-induced chronic stress for 8 weeks. Not only the chronic stress, but also the acute stress resulted in an increase in the salivary glands expression of BDNF (Tsukinoki et al. 2006 and Saruta et al., 2014). It seems that changes in BDNF levels induced by stress are tissue specific, as reported by Saruta et al. (2010). Decreased BDNF content in the rat hippocampus following chronic stress has been previously reported (Zheng et al. 2006). In more recent studies both BDNF mRNA and protein levels were down-regulated in mice hippocampus after exposure to the CUMS (Filho et al., 2015, Ayuob et al. 2016). The reduction in hippocampal BDNF expression in chronic stressful conditions was attributed to corticosterone

secretion (Zheng et al. 2006). On the other hand, Tsukinoki et al. 2007 reported that corticosterone is not likely to regulate BDNF expression in salivary gland. In addition Saruta et al. (2010) confirmed that during repeated restraint, the elevated circulating glucocorticoids did not reduce the salivary glands production of BDNF. This is supportive to the findings of this study as salivary BDNF increased although serum corticosterone level increased. It is worth to mention that although the gene and protein expression of BDNF were elevated in the salivary glands during chronic stress, the SMG was not the only contributor to the elevated plasma BDNF level during stress as BDNF is expressed and secreted by many other organs, including heart, lung, liver, pancreas, spleen and vascular endothelial cells (Hanyu et al. 2003 and Schuhmann et al. 2005).
In this study, treating stressed mice with FLU or OB significantly increased the expression of BDNF in the SMG and parotid glands compared to the untreated mice. In previous researches FLU increased BDNF levels in stressed mice following exposure to the CUMS (Filho et al., 2015). Saruta et al. (2010) reported that secretion of BDNF is a protective mechanism as it plays important roles in homeostasis under stress conditions. In the present study, inhalation of OB alleviate the depressive status induced after exposure to the CUMS. This could be attributed to its essential oils 1,8-cineole, linalool, caryophyllene, humulene and camphor, which have been reported to exhibit anxiolytic and sedative effects (Satou et al. 2014). Bora et al. 2011 attributed the anxiolytic effect of OB to its neuroprotective affect exerted by its phenolic, flavonoid and tannin contents, which are scavengers of reactive oxygen species. OB also reduced, in this study, the corticosterone level as well as the pathological changes induced by stress on the salivary glands. Although no previous studies described the effect of OB on the cortisol level, the compounds isolated from an extract of holy basil (*Ocimum sanctum*), another species of the genus Ocimum, were reported to normalize the plasma cortisol levels (Gupta, 2007). It seems that the improved histopathological changes observed in salivary glands of stressed mice treated by OB was attributed to its ability to reduce the corticosterone level in the serum.

Conclusion

The study showed that OB improved the depressive status and the structural changes in the salivary glands induced by exposure to chronic stress. This effect might be mediated through up-regulating the BDNF secreted by the glands. So it is the time to test the effectiveness of OB on reliving stress-associated salivary changes on human being.

Conflict of interest: The authors have no conflict of interest.

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