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Evaluating the effect of three newly approved overactive bladder syndrome treating agents on parotid and submandibular salivary glands: Modulation of CXCL10 expression

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ABSTRACT

Background: Despite enormous progresses in understanding pathophysiology of the lower urinary tract, antimuscarinics remain the chief clinically well-established approach for improving symptoms of overactive bladder (OAB). Dry mouth on the other hand remains one of the most untolerated systemic side effects of these drugs that limits their uses and results in high discontinuation rate. Three novel drugs have been recently approved by US Food and Drug Administration for treatment of OAB: trospium, darifenacin, and solifenacin.

Aims: This study has been conducted to provide clear head to head comparative studying of histological and ultrastructural effect of those newly emerging drugs on parotid and submandibular salivary glands and to demonstrate the differential expression of CXCL10 to make a cogent structural and molecular assessment of the relative tolerability of these drugs and the potential mechanisms of occurrence of dry mouth.

Methods: Fifty male *Sprague Dawley* rats were equally divided into five groups: Group I (control), Group II (oxybutynin-treated), Group III (trospium-treated), Group IV (darifenacin-treated) and Group V (solifenacin-treated). Histological and ultrastructural studies were performed on parotid and submandibular glands. Measurement of salivary flow, PCR analysis and immunohistochemical assessment of CXCL10 expression have been carried-out.

Results: Muscarinic receptor antagonists led to various histological, morphometric and ultrastructural changes together with diminished salivary secretion and up-regulation of CXCL10 expression with the mildest alterations observed with solifenacin.

Conclusions: Solifenacin has shown the least adverse effects to salivary glands. CXCL10 is involved in degenerative changes of salivary glands induced by muscarinic antagonists.

1. Introduction

Overactive bladder (OAB) is a highly prevalent complex chronic, distressing condition characterized by symptoms of urgency, frequency with or without reflex urgency-associated urinary incontinence affecting millions of people of all ages and both sexes worldwide (Burrows and Garely, 2002; Rovner et al., 2002). Both voluntary and involuntary detrusor muscle contraction involves stimulation of postganglionic muscarinic receptors (Andersson, 1997), thus, muscarinic receptor antagonists (antimuscarinics) serve as the cornerstone and the first line pharmacotherapy in management of OAB as well as urge urinary incontinence (UUI). Nevertheless, a wealth of clinical evidence supports the view that high incidence of dry mouth remains a common drawback of these drugs making them fall short of any high expectations, limiting their long-term benefits and causing a significant issue regarding low persistence of therapy (Chapple and Abrams, 2005).

One of the most frequently employed agents used for the treatment of UUI and OAB is oxybutynin chloride. The recent approval of a number of new antimuscarinic molecules, namely trospium, darifenacin, and solifenacin, has expanded the therapeutic armamentarium for OAB (Lam and Hilas, 2007). Emerging data from head-to-head clinical trials have revealed differences in the clinical profile of these drugs with greater bladder-to-salivary selectivity ratios for darifenacin (Chapple and Abrams, 2005) and solifenacin (Ikeda et al., 2002) when compared to oxybutynin in animal models.

C-X-C motif chemokine 10 (CXCL10) also known as Interferon gamma-induced protein 10 (IP-10) or small-inducible cytokine B10 is an 8.7 kDa protein encoded by the CXCL10 gene. It has been reported to be significantly elevated in diseases characterized by xerostomia such as Sjögren's syndrome (Ogawa et al., 2005) and was reported to play a

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pivotal role in progression of chronic inflammation (Singh et al., 2007) being an essential T cell-related chemokine (Ogawa et al., 2002). It has also shown up-regulation in the rat submandibular glands following parasympathectomy (Kang et al., 2010). Moreover, inoculation of CXCL10 antagonist markedly reduced salivary parenchymal injury (Hasegawa et al., 2006).

While there is still an indispensible need for antimuscarinics, advances in basic research have not yet offered clear well-established mechanisms for improving symptoms of dry mouth as the most frequently reported reason for discontinuation of antimuscarinic treatment (Benner et al., 2010). In the current study, evaluating CXCL10 expression as a crucial molecule involved in various pathological consequences and in the meantime an important chemokine considerably up-regulated in dry mouth-related conditions might offer a potential line of therapy for achieving better tolerability of these drugs via minimizing their propensity to cause dry mouth.

To the best of our knowledge, no previous study has comprehensively demonstrated the effect of these drugs on the structural, ultrastructural as well as the molecular and immunohistochemical expression of CXCL10 on the parotid and submandibular salivary glands (SMG).

2. Material and methods

2.1. Experimental animals

Fifty adult male albino rats (150–200 g, 8–10 weeks old) of *Sprague Dawley* strain were purchased from the animal care unit of Kasr El-Ainy, faculty of medicine, Cairo University, Egypt. All experimental procedures were carried out according to international guidelines for care and use of laboratory animals published by the U.S. National Institute of Health. Animals had free access to food and water ad libitum. The experiment was conducted in accordance to the guidelines of the ethical committee of laboratory animals at Kasr El-Ainy, Faculty of medicine, Cairo University.

2.2. Experimental design

Animals were randomly divided into 5 groups, 10 rats each. Group I (control, received no treatment), Group II (received i.v oxybutynin hydrochloride 1 mg/kg/day (Oka et al., 2001; Geyer et al., 2008)) (Sigma-Aldrich-O5015), Group III (received i.v trospium chloride 1 mg/kg/day (Geyer et al., 2008)) (Sigma-Aldrich-Y0000429), Group IV (received i.v darifenacin hydrobromide 1 mg/kg/day (Ikeda et al., 2002)) (Sigma-SML1102) and Group V (received i.v solifenacin succinate 1 mg/kg/day (Suzuki et al., 2005; Oger-Roussel et al., 2014)) (Sigma-Aldrich-Y0001763). To avoid any variance, the dose was equalized for all the injected drugs (1 mg/kg/day) and all the injections were performed once daily, in the tail vein at the same time point in the circadian cycle, at 9:00 a.m. All drugs were purchased from Sigma (St. Louis, MO, USA) and were administered for 6 weeks; the estimated time needed for initial management of OAB and for development of dry mouth (Wagg et al., 2016).

2.3. Salivation studies

Rats were anesthetized with pentobarbital (50 mg/kg, i.v.) (Ikeda et al., 2002). Both background saliva and salivary response to pilocarpine hydrochloride (1 mg/kg, subcutaneously) were quantitated in 10-min period by collecting saliva on filter paper 2 h after administration of the drugs using the method described by Oki et al. (2006).

2.4. Histopathological examination

Parotid and SMG from all groups were dissected, weighed and fixed immediately in 10% formol saline. Then the specimens were washed, dehydrated in ascending grades of ethanol, cleared in xylene and impregnated in paraffin. Sections of 5 µm thickness were cut and subjected to hematoxylin and eosin, Masson's trichrome and immunohistochemical staining.

2.5. Immunohistochemistry for CXCL10

The procedure involved the following steps; 4 µm thick paraffin sections were de-waxed, dehydrated, then incubated with10-mM citric acid (pH 6.0) buffer for antigen retrieval. Ten slides have been obtained from each animal in the different groups. Endogenous peroxidase activity was inhibited by peroxidase-blocking solution (3% H_2O_2). Nonspecific binding of antibodies was blocked by incubation with protein block for 1 h (normal horse serum, 10% v/v in phosphate-buffered saline, Novocastra). Sections were incubated with rabbit polyclonal anti-CXCL10 (OABF00732) primary anti-body (Aviva systems biology, San Diego) (1:100, IHC-P, species specificity including rats). After washing in Tris-buffered saline, sections were then incubated with the secondary antibody ImmPRESS™ HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit derived from horse serum (MP-7401, Vector, CA, USA). Paraffin-embedded mouse spleen tissue was used as a positive control. Negative controls were done through omission of the primary antibody in the automated staining protocol. All techniques were done according to the manufacturer's instructions.

2.6. Electron microscopic examination

Parotid and SMG specimens were immersed in 2.5% glutaraldehyde (pH 7.4). Semithin sections were prepared at 0.5 µm thickness and stained with 1% toluidine blue in 1% borax. Ultrathin sections were double stained with 4% uranyl acetate and 0.1% lead citrate, examined and photographed by JEOL JEM 1010 transmission electron microscope (Japan).

2.7. Quantitative analysis of gene expression of CXCL10 by RT PCR

2.7.1. Total RNA extraction

Total RNA was extracted from parotid and SMG homogenate using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instruction. RNA concentrations and purity were measured with an ultraviolet spectrophotometer.

2.7.2. Complementary DNA (cDNA) synthesis

The cDNA was synthesized from 1 µg RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer's protocol (#K1621, Fermentas, Waltham, MA, USA). In brief, 1 µg of total RNA was mixed with 50 µM oligo (dT) 20, 50 ng/µL random primers, and 10 mM dNTP mix in a total volume of 10 µL. The mixture was incubated at 56 °C for 5 min, and then placed on ice for 3 min. The reverse transcriptase master mix containing 2 µL of 10 × RT buffer, 4 µL of 25 mM MgCl2, 2 µL of 0.1 M DTT, and 1 µL of SuperScript® III RT (200 U/µL) was added to the mixture and was incubated at 25 °C for 10 min followed by 50 min at 50 °C.

2.7.3. Real-time quantitative PCR

Real-time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs for CXCL10 were 5-TGTAGCATTCAAAGCATTT GCC-3, antisense 5-CCTCGTCGTACGACCCAAA-3 (Gene bank accession number NM_195227.2) and GADPH sens:5′-CAG GAT GGC GTG AGG GAG AGC-3′,antisense:5′-, 5′-AAG GTG TGA TGG TGG GAA TGG-3′- and were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from the gene bank. Quantitative RT-PCR was performed in a 25-µl reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer