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The Effect of Salbutamol on the Parotid Salivary Gland of Albino Rats (Immunohistochemical Study)

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Abstract: Salbutamol is a short-acting selective β2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease. The β-adrenoceptors are also present in the secretory system and have a strong impact on salivary composition. This study was done to evaluate the effect of the selective β2-adrenergic agonist salbutamol on the parotid salivary glands regarding cellular proliferation and apoptosis during long term treatment and after stoppage by immunohistochemical methods. One hundred male Albino rats were divided into control and study groups. The study group received daily injections of 4 mg kg^{-1} salbutamol (Ventolin, GSK) while the control group received equal amounts of saline. Rats were sacrificed at periods of 2, 7, 14 and 25 days of treatment and then 1 week after cessation of treatment. At each period the parotid glands were dissected and processed for H&E stain and immunohistochemical staining with ki-67 and p53 antibodies. After 2 days of treatment marked hypertrophy of the acini was noticed as well as marked increase in the number of proliferating cells as denoted by ki-67 antibody. Ducts and some endothelial cells showed increased proliferative activity. The proliferative activity decreased dramatically after that but did not go back to the normal level of the control. Apoptotic activity denoted by the p53 apoptotic marker showed an increase at 7 days post treatment.

Key words: Salbutamol, Parotid gland, ki-67, p53, Egypt

INTRODUCTION

Asthma is a chronic inflammatory condition of the airways characterized by hyper-responsiveness and episodic reversible symptoms of airflow obstruction. The prevalence of asthma has been increasing since the 1980s across all ages, gender and racial groups and is higher among children than adults (Steinbacher and Glick, 2001).

Short Acting Beta2-Adrenoceptor agonists (SABA) such as salbutamol (albuterol USAN) are the first line treatment for asthma symptoms. Older, less selective adrenergic agonists such as inhaled epinephrine have similar efficacy to SABAs (Fanta, 2009). They are however not recommended due to concerns regarding excessive cardiac stimulation (Cates *et al*., 2009). β-adrenoceptors are also present in the secretory system and have a strong impact on salivary composition (Baum, 1987).

Matsuura and Suzuki detected hypertrophic enlargement and increased DNA synthesis in mouse submandibular gland after stimulation of β-adrenergic receptors by Isoproterenol (IPR) (Matsuura and Suzuki, 1997). Isoproterenol was also found to cause changes in salivary composition where Isoproterenol was shown to stimulate the secretion of α-amylase enzyme from the parotid glands (Kim *et al*., 1982) as well as induction of synthesis of a series of Proline-Rich Proteins (PRPs) and glycoproteins (Wells and Humphreys-Beher, 1985).

The effects of non selective β-adrenergic agonists like isoproterenol have been studied extensively in rodents while the effects of the selective β1 and β2 agonists have been much less studied. The only found study that detected the histological changes of salivary glands after salbutamol treatment was that made by Esfandiari *et al*. (2008). The study found increase in parotid gland weight and acinar hypertrophy after trearment for 15 days (Esfandiari *et al*., 2008). No studies could be found detecting the proliferative effect of the drug on the gland and the role of apoptosis in the process. Also, no studies continued the treatment for long periods of time. So, this study was conducted to study the proliferation and apoptosis process in the gland immunohistochemically for long periods during treatment and after stoppage.

MATERIALS AND METHODS

One hundred male white albino rats weighing 250-300 g were kept under standard housing conditions. The animals were acclimatized before starting the study.

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They were randomized into two groups and kept under the same nutritional and environmental conditions. All experimental procedures were taken under a protocol approved by the Ethical Committee of Faculty of Dentistry, Mansoura University, Egypt. The first group was considered as the control one, consisted of twenty five rats and given saline solution. While the second group was considered as the study one, consisted of seventy five rats and received salbutamol treatment.

Drug administration: The saline and salbutamol were administered through intraperitoneal route. The rats of the study group were given salbutamol in a dose of 4 mg kg^{-1} body weight (Ventolin, GlaxoSmithKlein, Brentford, United Kingdom) daily (Esfandiari *et al*., 2008), meanwhile the rats of the control group were given equivalent volumes of sterile saline. The treatment periods were continued for 25 days (Ryberg and Johansson, 1995).

Five rats of the control and fifteen of the salbutamol treated group were randomly chosen and sacrificed using thiopental at periods of 2, 7, 14 and 25 days. About 1 week after the end of the treatment period, the remaining animals were sacrificed. The parotid glands were excised and fixed in 10% formal saline and prepared for stain.

H&E stain

Immunohistochemical staining with ki-67 and p53 antibodies: After fixation, the tissues were stained with H&E to obtain conventional histologic sections and sections were stained immunohistochemically using monoclonal antibodies against ki-67 and p53. It was considered to be positive strictly nuclear reactions for Ki-67 and p53.

Digital image analysis of immunostained tissue sections was performed by calculating the percentage of positive cells out of the total number of cells per field regardless intensity. Ki-67 and p53 labeling indices were statistically analyzed using Statistical Package for Social Sciences (SPSS, Version 13.0, Chicago, IL, USA). The data were parametric by using Kolmogrov-Smirnov test. The data were expressed as Mean±SD. For comparison between two groups unpaired t-test was used. For different treatment periods, comparisons were carried out by Analysis of Variance (ANOVA) with the Least Significance (LSD) Post hoc analysis for inter group comparison. Pearson correlation was used to assess relations between variables. Significance was considered when $p<0.05$.

RESULTS

Haematoxylin and eosin stain

Control group: The study of the control parotid salivary gland consisted of the well known lobular structure with serous acini and ducts. The serous acini were separated by connective tissue septa. The acini appeared regular and rounded in shape with pyramidal cells surrounding a narrow central lumen with basally spherical nuclei. The duct system appeared intervening between the acini (Fig. 1A).

Salbutamol treated group: At 2 days of treatment the serous acini became markedly hypertrophic and packed together. Some nuclei showed signs of mitosis. Duct outlines appeared normal (Fig. 1B). At 7 days of treatment the acini were hypertrophic with less connective tissue spaces. The acinar outlines were irregular. The nuclei were enlarged and irregular in shape and size with clumped chromatin (Fig. 1C). At 14 days, the acini were also hypertrophic with irregular outline. Some vacuoles appeared in the tissues. Some cells showed multipolar mitosis and some nuclei were hyperchromatic (Fig. 1D).

At 25 days the acini appeared enlarged. The connective tissue septa shrunk in size. Some cells still showed mitosis. The duct structures appeared normal. Vacuolation can be seen in some fields (Fig. 1E). Finally, at 32 days (1 week after stoppage of the treatment), the size of the acini decreased but they were still irregular in outlines. The connective tissue septa started to widen. The nuclei were still irregular in shape and size (Fig. 1F).

Immunohistochemical results

Ki-67 (control group): The normal parotid gland showed only a few cells with positive nuclear reaction (Fig. 2A).

Salbutamol treated group: At 2 days many nuclei of the acinar cells showed positive staining. Some duct cells and endothelial cells showed positive reaction as well (Fig. 2B). At 7 days some acinar cells and occasional duct and endothelial cells showed positive reaction (Fig. 2C). At 14 (Fig. 2D) and 25 days (Fig. 2E) less number of acinar cells showed positive staining and at 32 days few cells showed positive reaction (Fig. 2F).

p53 (control group): The normal parotid gland showed negative reaction with p53.

Salbutamol treated group: At 2 and 7 days, no cells showed positive reaction. At 14 and 25 days, small numbers of positive acinar cells could be detected while at 32 days many acinar cells showed positive reaction. No positive duct or endothelial cells could be detected Fig. 3A-F.

Statistical analysis: Digital image analysis and statistical results for ki-67 immunostaining revealed a significant

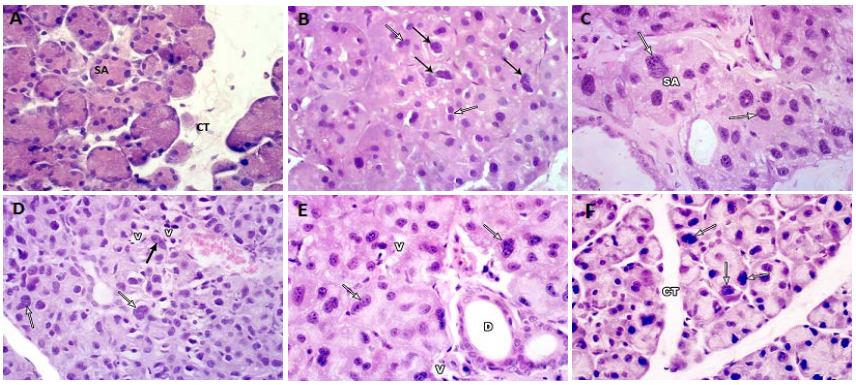

Fig. 1: A) Photomicrographs of the parotid gland sections of the control group showing arrangement of serous Secretory Acinar cells (SA) with large basally located nuclei and narrow lumen. The connective tissue septa are wide and filled with Connective Tissue fibers (CT); B) The salbutamol treated group after 2 days showing hypertrophic packed secretory acini. Some nuclei are enlarged and irregular (black arrows). Some cells showed signs of mitosis (white arrows); C) After 7 days of treatment the acini were hypertrophic and irregular (SA). Nuclei are enlarged with irregular shape. Chromatin condensation can be seen in some nuclei (arrows); D) After 14 days some cells show normal bipolar mitosis (black arrow) and others show multipolar mitosis (white arrows). Vacuoles (v) in the tissue can be seen. Ducts appear normal E) After 25 days irregular acini can be seen with some nuclei showing condensed chromatin (arrows). Vaculation (v) appears in the tissue. The Ducts (D) are normal; F) After 32 days the secretory acini show wide Connective Tissue septa (CT). The nuclei are still enlarged and irregular in shape and size (arrows) (H&E stain 400x)

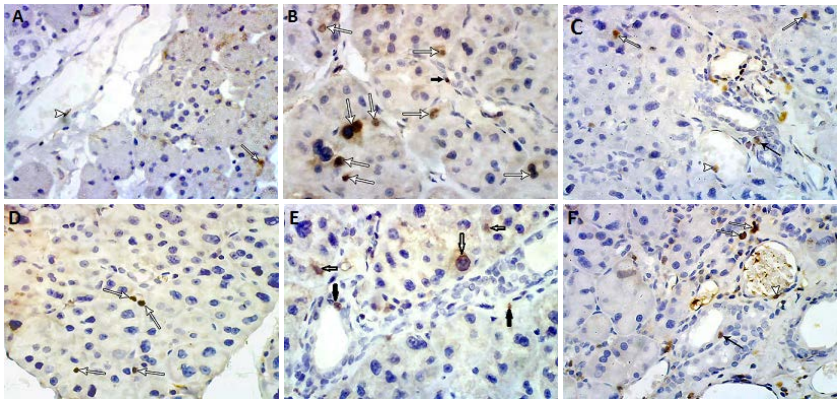

Fig. 2: A) Photomicrographs of the parotid gland sections of the control group showing positive immunoreactivity a few numbers of acinar nuclei (white arrows) and endothelial cells (arrow head); B) The salbutamol treated group at 2 days showing many positive acinar cells; C) At 7 days showing some positive acinar cells (white arrows), duct cells (black arrow) and endothelial cells (arrow heads); D) At 14 days showing some positive acinar cells (arrows); E) at 25 days showing positive acinar cells (white arrows) and duct cells (black arrows); F) At 32 days showing positive duct cells (black arrows), endothelial cells (arrow heads) and some acinar cells (white arrows) (Immunoperoxidase staining with the anti ki-67, DAB chromagen 400x)

difference between the control group and all the treated groups at 2, 7, 14, 25 and 32 days of treatment ($p<0.05$) (Table 1). ANOVA revealed significant difference between the treated group at 2 days and the other treated groups at 7, 14, 25 and 32 days ($p<0.05$).

Digital image analysis and statistical results for p53 immunostaining revealed no significant difference between the control group and the 2 and 7 days treated groups ($p<0.05$) whereas a significant difference was shown between the control and the 14, 25 and 32 days

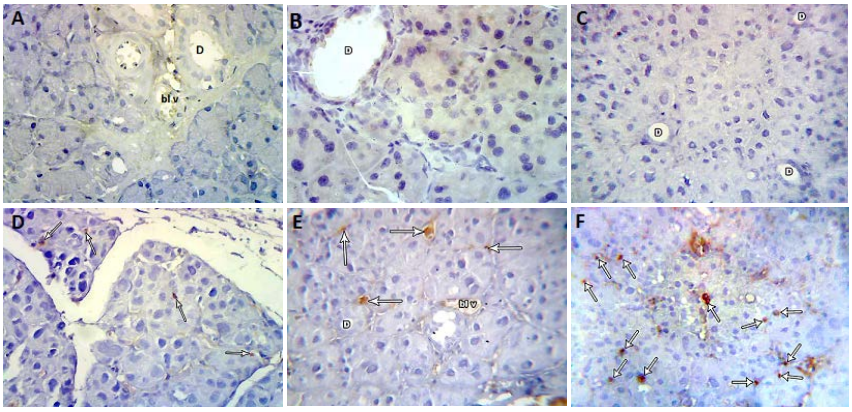

Fig. 3: A) Photomicrographs of the parotid gland sections of the control group showing negative immunoreactivity in acini, Ducts (D) and blood vessels (bl v); B, C) the salbutamol treated group at 2 and 7 days showing negative reaction in Ducts (D) and acinar cells; D) At 14 days few positive cells can be seen (arrows); E) At 25 days some positive acinar cells could be detected (arrows) but Duct (D) and endothelial cells (bl v) were negative; F) At 32 days many acinar cells showed positive reaction (Immunoperoxidase staining with the anti p53, DAB chromagen 400x)

Table 1: Comparison between control and salbutamol treated group on day 2, 7, 14, 25 and 32 according to ki67 immunostaining results

Statistical analysis	Control	Salbutamol treated	P
2 days	1.26±0.27	9.8±2.200	<0.05*
7 days	1.3±0.230	5.05±0.70	<0.05*
14 days	1.23±0.16	3.1±0.500	<0.05*
25 days	1.29±0.20	2.81±1.12	<0.05*
32 days	1.21±0.21	2.09±0.30	>0.05*

Mean±SD represents the ±values

Table 2: Comparison between control and salbutamol treated group on day 2, 7, 14, 25 and 32 according to p53 immunostaining results

Statistical analysis	Control	Salbutamol treated	P
2 day	0	0	-
7 days	0	0	-
14 days	0	1.65±0.3	<0.05*
25 days	0	2.1±0.54	<0.05*
32 days	0	6.07±1.2	<0.05*

All results are expressed as mean±SD standard deviation; Non significant: at p>0.05; Significant (*): at p<0.05; P: significance between control group and salbutamol treated groups on day 2, 7, 14, 25 and 32

study groups (p>0.05) (Table 2). ANOVA showed no significant difference between the 14 days treated group and the 25 days group (p<0.05) although it was significant between the same group and the 32 days group (p>0.05). There was also a significant difference between the 25 and the 32 days groups (p>0.05) where the mean of positive apoptotic cells was highest after stoppage of the treatment at 32 days.

DISCUSSION

Many studies have been conducted on the effects of the non selective β-agonist isoproterenol on different salivary glands. Enlargement of the parotid and submandibular glands caused by chronic administration of isoproterenol is believed to involve both hypertrophy which reflects the excessive activation of protein synthesis, enlargement of acinar cells as well as increase in the wet weight and size of the gland and hyperplasia associated with cell proliferation (Cohen *et al*., 1995; Matsuura and Suzuki, 1997; Melvin *et al*., 2001). Also, there is general agreement that hyperplasia and hypertrophy contribute to gland enlargement both in human and rat sialosis following the administration of isoproterenol (Chisholm and AdI, 1995).

Some studies were conducted on the selective β1 agonists and proved the same results (Bloom *et al*., 1983; Ekstrom and Malmberg, 1994; Schneyer, 1986) but much less studies were conducted on the β2 agonists and they were contradicting (Klein, 1984; Abe *et al*., 1985).

The results of the present study showed acinar hypertrophy and mitotic figures at 2 days of treatment. This is in agreement with Matsuura and Suzuki (1997) who found that the acinar cells increased in size and mitotic figures were often seen in mice submandibular glands after 2 days of treatment with isoproterenol. Similarly, Onofre *et al*. (1997) detected the 1st 3 days to be the period of greatest growth in the acini of rat parotid glands treated daily with isoproterenol for 2 weeks. Cell volume increased in percentage terms, only during the 1st 3 days but continued to grow up to the fifth, stabilizing thereafter. A significant number of mitotic figures were observed from 3-5 days of treatment and a significant increase in the number of bipolar and multipolar mitoses was also observed at the same periods. Also, sharp

increase in mitotic rate was observed by Schneyer *et al.* (1967) (Robinovitch *et al.*, 1977) in rat parotid 2-3 days after IPR was administered twice daily but mitotic activity dropped to zero after the 4th day.

In the study, the parotid gland ducts did not show changes in their general outline which is similar to the results obtained by Katsumata *et al.* (2009) where microscopic examination revealed no definite alteration in the morphology in other component cells other than the acinar cells of rat parotid gland during the 5 days of treatment.

At 7 days of treatment in the present study, the acini were hypertrophic and irregular with less connective tissue spaces. The nuclei were enlarged and irregular in shape and size with clumped chromatin denoting active mitosis. This is in accordance with Onofre *et al.* (1997) who found that after 7 days of isoproterenol treatment, the glandular mass of the rat parotid gland increased with total acinar volume exhibited maximum growth during the period from 0-7 days. During this period both nuclear volume and cytoplasmic volume increased.

Hand and Ho (1985) found that mitotic figures in the acinar cells and some intercalated duct cells were most numerous after six daily injections of IPR. On the contrary, in a study by Novi and Baserga (1971), the number of mitoses in mice submandibular and parotid glands progressively decreased from the second day so that in the parotid, mitotic activity dropped to zero by 6 days.

At 14 days of treatment in this study, the acini were still hypertrophic with irregular outline, the connective tissue spaces were decreased and the duct outlines remained unchanged. These results are similar to the results of the study made by Esfandiari *et al.* (2008) who detected hypertrophy and decreased space between the serous secretory units of rat parotid glands after 15 days of treatment with salbutamol. No morphological changes were noticed in ductal cells after treatment. No histological studies were found on any of the β-adrenergic (β1, β2 or non selective) agonists for periods >15 days.

At 32 days (7 days after stoppage of the treatment), some mitotic figures could still be detected and the acinar cells were still enlarged with granular irregular nuclei. This agrees with the studies by Chisholm and AdI (1995) and Chisholm *et al.* (1995) who observed enlargement of the acini of rat submandibular and parotid glands with enlarged granular nuclei and nucleoli at the 2nd day after cessation of the 9 days daily treatment period. Mitotic figures were readily identified at an early stage in treated glands. Mitotic counts in serous acinar cells reached a sudden peak by day 1 after withdrawal of isoprenaline.

In the present study, the results of the ki-67 immunostaining in the control group showed a few positive cells. At 2 days, the ki-67 immunostaining showed positive reaction in many nuclei of the acinar cells. Some duct cells and endothelial cells showed positive reaction as well. No immunohistochemical studies concerning the effects of the selective β2 agonist salbutamol could be found. Only studies on the non selective β agonist isoproterenol were found. Similar to the present study, Katsumata *et al.* (2009) found that acinar cells of isoproterenol treated rat parotid glands, showed the highest proliferation activity on day 3 of IPR treatment as denoted by ki-67 immunohistochemical labeling. Intercalated duct cells also showed an increase in labeling index during days 2-4 of IPR treatment.

In the present study at 7 days, the anti ki-67 immunostaining showed less number of acinar cells and occasional duct and endothelial cells with positive reaction as compared with the 2 days group. Radley (1968) found that the peak value of DNA amount measured in submandibular glands of isoproterenol treated rats was shown after 7 injections. Similarly, Hand and Ho (1985) detected that mitotic endothelial cells were found and were most numerous after from 4-6 days of treatment but were still significantly increased in number after ten IPR injections. In contrast to this in the study by Matsuura and Suzuki (1997), the numbers of DNA-synthesizing cells after 5 or 7 days of IPR treatment were found to be the same as in intact or saline-injected control mice.

In the present study at 14 and 25 days of treatment some acinar cells were positive as detected by ki-67 but less than the earlier periods. Similar to these results, Novi and Baserga (1971) found that the total amount of DNA in the submandibular and parotid glands continued to increase steadily for at least 10 days after isoproterenol treatment was begun. After the eleventh day till the thirteenth, both the total amount of DNA/gland and the specific activity of parotid DNA remained constant, indicating that after the eleventh day, no further DNA synthesis occurred.

After stoppage of the treatment at 32 days in this study, few acinar cells and also some duct and endothelial cells still showed positive reaction. This agrees with Ochiai *et al.* (2002) who found marked decrease in the number of BrdU (5-Bromo-2-deoxyUridine) positive cells from the 3rd day of administration of IPR and similar low levels after stoppage of 10 days treatment period.

In the present study, the results of the p53 antibody in the control group showed no positive reaction for the antibody. This agrees with a study by Moubarak (2008) where the acinar cells of rat parotid gland of control normal group showed negative p53 immunoreactivity as

normal p53 is virtually undetectable by antibody-mediated staining because of its rapid degradation (Harris, 1996).

No study detecting the apoptotic process during and after salbutamol treatment on the salivary glands could be found but only studies on the non selective agonist Isoproterenol. At 2 and 7 days in the present study, no cells showed positive reaction. At 14 and 25 days few acinar cells showed reaction. At 32 days many acinar cells were positive indicating increased apoptotic activity after stoppage of the treatment to balance the increased proliferative response observed in the early periods of the treatment.

Similarly, in the study by Ochiai *et al.* (2002) by the 3rd day after stoppage of IPR administration, the number of TUNEL (Terminal Deoxyribonucleotidyl Transferase (TDT) mediated-DeoxyUridine-Triphosphate (dUTP) biotin Nick End Labeling) positive cells had dramatically increased. TUNEL positive cells, vacuoles and swollen structures were recognized in acinar areas, located not only between acinar cells but also in their cytoplasm. Few TUNEL positive cells were recognized in the duct areas but in difference with the present study, the total number of TUNEL positive cells decreased rapidly by day 5 and 7 post IPR administration but the treatment period was 10 days whereas the treatment in the present study continued for 25 days.

CONCLUSION

Based on the employed methodology and the obtained results, it may be concluded that treatment with salbutamol drug causes hypertrophy and hyperplasia of the parotid glands especially at the early periods of treatment. The hyperplasia includes acinar, duct as well as some endothelial cells and this hyperplasia is not maintained by prolonged treatment periods. The hyperplastic changes are balanced by apoptosis after cessation of the treatment.

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