

Effect of Lithium on Secretory Factors and Growth Stimulation by Bombesin in Rat Pancreas and Salivary Glands

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Abstract

Lithium, a drug of choice in bipolar affective disorders, also affects the metabolism and cell proliferation in a diverse array of organisms. In this study, we investigated the effect of lithium on bombesin-mediated function in excretion and growth of the pancreas and the salivary glands. The weight, protein content, amylase concentration and salivary flow rate of the pancreas, parotid and the submandibular glands were determined in male Wistar rats after consumption of either water or lithium chloride (600 mg/l) for 14 days and each group received s.c. injection of either saline or bombesin (10 µg/kg) during the last 4 days of experiment. Our results revealed that administration of bombesin in lithium-treated group not only suppressed pancreas and parotid weight augmentation due to bombesin, but also significantly decreased pancreas growth. Chronic lithium consumption significantly inhibited the protein content augmentation due to bombesin in the salivary glands. Getting bombesin, as well as saline in lithium-treated group, resulted in notable decrease in salivary protein content. Protein content of pancreatic gland increased considerably in the bombesin-injected groups either treated with saline or lithium. In conclusion, the stimulatory effect of bombesin on the growth and protein content of the pancreas and the salivary gland was inhibited by lithium. Lithium seems to be a potent inhibitor of growth factors induced by bombesin probably through inhibiting phosphatidylinositol 4,5-bisphosphate hydrolysis.

Key Words: bombesin, lithium, parotid gland, submandibular gland, pancreas

Introduction

Lithium (Li⁺) has been used for 50 years as a mood stabilizer for the treatment of manic depression (33, 38). It has been shown that lithium affects metabolism, neuronal communication and it can inhibit proliferation of some cells in a diverse array of organisms

(16, 36, 45). Although the mechanism of lithium actions is not yet resolved, increasing evidence confirms that it exerts its therapeutic effects by interfering with signal transduction through G-protein-coupled pathways (21) or by direct inhibition of specific targets in the signaling systems including inositol 1-phosphatase (7, 34) and glycogen synthase kinase-3 (27). Recently,

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Received: November 4, 2008; Revised: April 6, 2009; Accepted: May 12, 2009.

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lithium has also been reported to stimulate extracellular signal-regulated kinase pathway (14). There is evidence that Li^+ inhibits the inositolphosphatase hydrolyzing inositol 1-phosphate and thereby blocking the recycling of inositol (7, 34, 48).

Bombesin (BBN), an amphibian skin equivalent of gastrin-releasing polypeptide (GRP), is a regulatory peptide which elicits various physiological effects in mammals such as inhibition of feeding, smooth muscle contraction, exocrine and endocrine secretion, thermoregulation, blood pressure and sucrose regulations and cell growth (1, 4, 24, 31, 35, 49, 50). BBN is well known for its ability to promote the release of gastroenteropancreatic hormones such as gastrin and to potentiate glucose-stimulated insulin release from pancreatic islets of Langerhans (15, 28, 32). Moreover, BBN acts as a growth factor for Swiss 3T3 fibroblasts (42) and pancreatic and gastrin cells (23, 26). Besides, GRP receptors are over-expressed in several types of human cancer cells including breast, prostate, small cell lung and pancreatic cancers (10, 19, 30). Several studies have shown that BBN induces inositol 1,4,5-trisphosphate formation through rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG) in some cell lines (29, 37, 40, 47, 48, 51). The previous studies showed that in the presence of lithium, the BBN-induced elevation of inositol phosphates is decreased compared to controls in some cell lines (48).

The likely mechanism of lithium action is interruption of PIP_2 hydrolysis through inhibiting the recycling of inositol substrate which causes depletion of the second-messenger source. On the other hand, bombesin induces PIP_2 hydrolysis and generates secondary messengers. Therefore, it seems that lithium administration can modify the stimulatory effects of bombesin on the growth of target tissues and cells through interaction with PIP_2 pathway (6, 46). As organogenesis of epithelial systems, histological and functional properties are similar in different epithelial organs such as pancreas and salivary gland (18, 22). In this study, we investigated the effect of BBN and lithium on rat pancreatic, parotid and submandibular glands to determine if there were possible similarities or differences in their excretory function and proliferation. We also assessed the effect of BBN in the presence of lithium to see whether it could prevent the effect of BBN.

Materials and Methods

Animals and Chemicals

Adult male albino Wistar rats were obtained from the Pasteur institute of Tehran. The animals were handled in accordance with the criteria outlined

in the Guide for the Care and Use of Laboratory Animals (NIH US publication 86-23 revised 1985). Rats were housed 1-2 per cage, with controlled condition ($22 \pm 1^\circ\text{C}$ and a humidity of 65-70%) on a 12-hour light-dark cycle and were allowed free access to a standard pellet chow and tap water *ad libitum*.

All drugs were freshly prepared in physiological saline solution. Drugs were purchased from commercial sources unless otherwise indicated. BBN (fragment 8-14) and lithium chloride were purchased from Sigma (St. Louis, MO, USA).

In this study, we assessed the growth and excretory function of rat pancreatic, parotid and submandibular glands, in addition of liver, duodenum and stomach weight changes in response to BBN and lithium and their combination.

Pancreatic, Parotid and Submandibular Glands Weight and Assessment of Protein Content

Male Wistar rats (170-180 g) were randomly divided in two experiment groups. The weight and protein content of the pancreatic glands were assessed in the first group as opposed to the second group in which the parotid and submandibular glands pancreatic glands weight and protein content were evaluated.

The treatment groups, each consisted of 8-10 rats, consumed either water or lithium chloride (600 mg/l) added to tap water, for 14 consecutive days. For the last 4 days of experiment, each group received s.c. injection of either 1 ml 0.9% saline or bombesin (10 $\mu\text{g}/\text{kg}$) three times a day. At the end of the experiment, the rats were lightly anesthetized with diethyl ether after an overnight fast and then sacrificed. In the first group, the pancreatic glands (rat pancreas is a rather diffuse organ divided into three parts *i.e.* the biliary, duodenal and gastrosplenic portions), liver, duodenum and stomach were rapidly excised, stripped of connective tissues, and weighed as well as the parotid and submandibular glands in the second group. The results are represented as normalized gland weight (= gland's weight / total body weight).

Besides, a portion of the pancreas and salivary glands (approximately 200 mg) was separated and homogenized in 10 ml of saline phosphate buffer (0.05 molar, pH 6.9) using the Omni-Mixer 17106 homogenizer. Protein content of whole homogenates was determined by the method of Folin-Lowry (8) with bovine serum albumin as a standard.

Assessment of Secretory Factors

Male Wistar rats (200-250 g) were divided in treatment groups each consisting of 8-10 rats, and were treated for 14 days in the same way as mentioned above. At the end of 14 days, animals were kept

fasted for one night and the next morning, the secretions of pancreas, parotid and submandibular were collected. In the first group, sampling of pancreatic secretions of anesthetized animals (pentobarbital, 50 mg/kg) was designed particularly to inhibit integration of digestive secretions with pancreatic discharge. In other words, the first cannula was entered through the distal stomach into the duodenum and tied closely in order not to let saliva and gastric secretions enter the duodenum. After exposing and tying the biliary duct, a second cannula was inserted through the end of duodenum. The parotid and submandibular ducts in the second group of anesthetized animals were exposed and cannulated.

All samples were collected using polyethylene cannulas (0.28 mm internal diameter) during carbachol infusion (100 µg/kg) for 30 min.

To measure the amylase concentration of pancreatic and parotid secretions, an EnzChek® Amylase Fluorometric (Green) Assay Kit, (Invitrogen) was used.

Submandibular Flow Rate Measurement

In this experiment, volume of secreted saliva was measured according to animal weights. Flow rate was calculated in ml/min/gram of body weight and ml/min/gram of submandibular gland weight. The flow rate measurement in ml/min/gland's weight is much more accurate than the measurement in ml/min/body weight because the variations in the animal body weight are not included in the former measurement. Therefore, the results emphasize on flow rate measurements according to calculation in ml/min/gland's weight.

Measurement of Lithium Plasma Concentration

In all rats receiving lithium chloride, 2-5 ml of heart blood was sampled after collecting the saliva. After centrifugation, serum lithium concentration was measured in all samples by using atomic-absorption spectrophotometry (44).

Histological Studies

Pancreas, parotid and submandibular tissues of control and treatment groups were fixed in 10% formalin, processed in a tissue processor for light microscopy evaluation. Paraffin-embedded blocks were prepared and sectioned in 5 µm thickness; the sections were stained by hematoxylin and eosin.

Statistical Analysis

All data are presented as means ± S.E.M.

Statistical analysis was performed by the SPSS software version 11.5 (SPSS Inc. Chicago, IL, USA). We used Wilcoxon ranks test to compare means among groups. *P* value < 0.05 was considered as statistically significant.

Results

Results from the growth and secretion of pancreatic, parotid and submandibular glands after administration of BBN, in lithium- or water-consumed groups and their control group are shown as follows:

Alterations in Pancreas Gland Growth and Secretions

Administration of BBN (10 µg/ml) in water-fed rats increased pancreas weight significantly (*P* < 0.01), whereas in lithium-treated groups, administration of either BBN or saline decreased pancreas weight substantially (*P* < 0.01) (Table 1).

Protein content of pancreas gland (mg/gland) increased considerably in the water- and lithium-treated groups which were administered BBN compared with the control group (*P* < 0.01). Anyhow, there was a notable decrease in the protein content of the lithium-treated group which were administered BBN compared to the water-fed group. On the other hand, no significant change was observed in the pancreatic protein content of the lithium-treated group (Fig. 1A).

Even though administration of BBN or saline to lithium-treated groups caused a notable decrease in pancreatic protein secretion (*P* < 0.01), BBN administration did not amplify this parameter significantly (Fig. 1B).

Receiving BBN in the water-fed group increased amylase activity in a significant manner (*P* < 0.01), but in lithium-treated group, the amylase activity significantly decreased after the administration of BBN in comparison with the water-fed animals (*P* < 0.05). Lithium treatment did not show any considerable effect on amylase activity (Fig. 1C).

Histopathologic studies of pancreas glands showed normal appearance in both the endocrine islets and exocrine acini. No edema, atrophy, leukocytic infiltration and fibrotic changes were found in the treatment groups (Fig. 2, A-D).

Alterations in Parotid Gland Growth and Secretions

Administration of BBN in the water-fed rats increased parotid weight significantly (*P* < 0.01), whereas lithium treatment did not result in notable changes in the parotid weight. Nevertheless, lithium treatment significantly inhibited the augmentative effect of BBN on parotid weight (Table 1).

Table 1. Weight (mg) and normalized weight (gland's weight/total body weight) of the pancreas, parotid and the submandibular gland

	Control	Water + BBN	Lithium + Saline	Lithium + BBN
Pancreas gland				
Weight (mg)	1035 ± 13	1315 ± 11**	794 ± 9**	941 ± 10 ⁺⁺
Normalized Weight	0.534 ± 0.009	0.666 ± 0.005**	0.434 ± 0.012**	0.494 ± 0.004 ⁺⁺
Parotid gland				
Weight (mg)	230 ± 6	280 ± 8**	203 ± 6*	214 ± 5 ⁺
Normalized Weight	0.118 ± 0.004	0.141 ± 0.004**	0.111 ± 0.004	0.112 ± 0.002 ⁺⁺
Submandibular gland				
Weight (mg)	431 ± 23	430 ± 21	372 ± 7*	380 ± 4
Normalized Weight	0.222 ± 0.011	0.217 ± 0.010	0.201 ± 0.006	0.199 ± 0.001

Data are presented as the mean of at least 8 independent determinations ± S.E.M. * $P < 0.05$ and ** $P < 0.01$ in comparison with the control group; ⁺ $P < 0.05$ and ⁺⁺ $P < 0.01$ in comparison with the BBN-treated group.

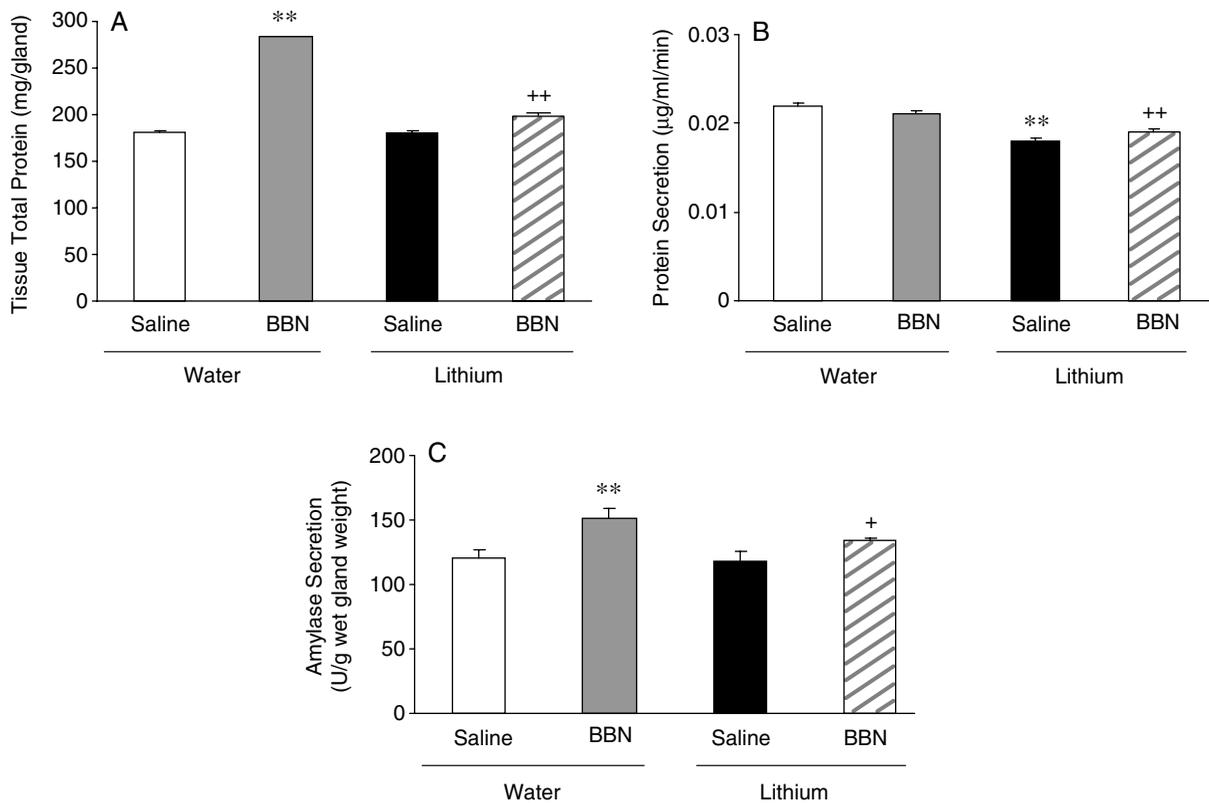


Fig. 1. Representative pancreas gland growth and excretory parameters: (A) gland total protein (mg/gland), (B) gland protein secretion rate ($\mu\text{g/ml/min}$), (C) amylase (U/g wet gland weight). Data are presented as the mean of at least 8 independent determinations ± S.E.M. ** $P < 0.01$ in comparison with the control group; ⁺ $P < 0.05$ and ⁺⁺ $P < 0.01$ in comparison with the BBN-injected group.

Protein content of the gland in the BBN-treated group increased considerably compared with the control group, while lithium did not cause a substantial change. However, injection of BBN in the lithium-treated group, when compared with the water-fed group, resulted in a notable decrease ($P < 0.01$) in the protein content of the parotid (Fig. 3A).

Lithium treatment in saline- and BBN-injected rats resulted in a substantial decrease in parotid protein secretion, but BBN administration in the water-fed group did not significantly amplify this parameter (Fig. 3B). In all of the experimental groups, amylase secretion changed in a significant manner (Fig. 3C).

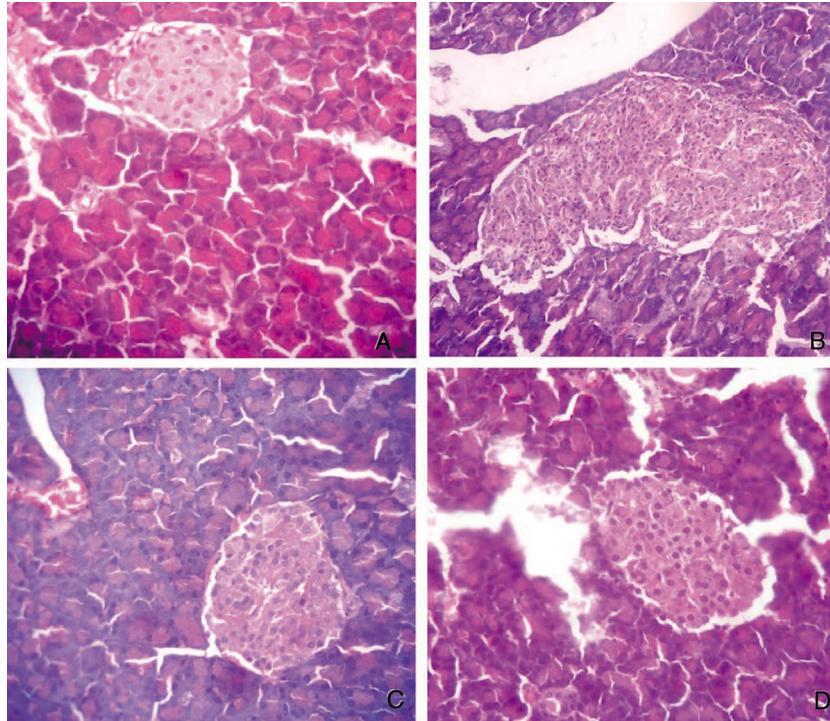


Fig. 2. Representative histopathologic appearance of the pancreas gland from saline-treated group (A), BBN-treated group (B), lithium-treated group (C), and lithium + BBN-treated group (D). Exocrine acini and endocrine islets show normal appearance in all treated groups (Hematoxylin and eosin staining. Original magnification: A and C-D, $\times 400$; B, $\times 100$).

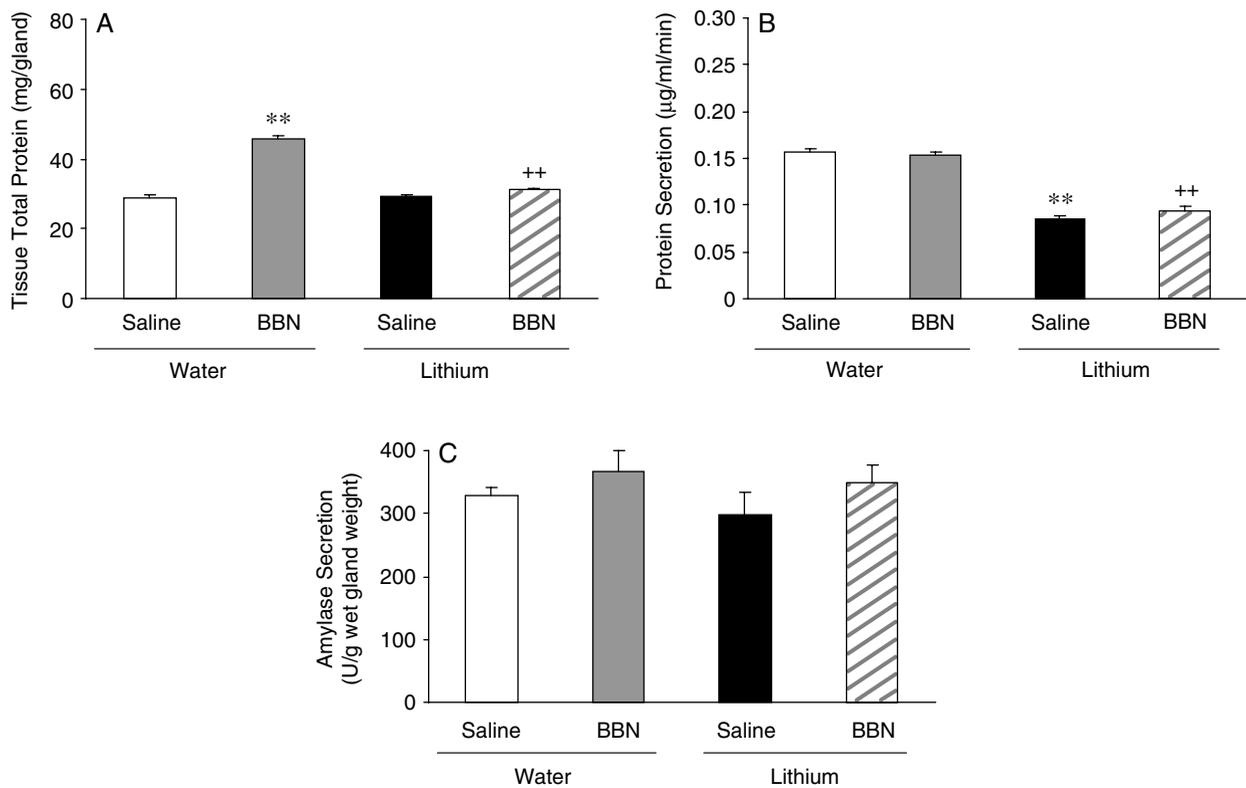


Fig. 3. Representative parotid gland growth and excretory parameters: (A) gland total protein (mg/gland), (B) gland protein secretion rate ($\mu\text{g}/\text{ml}/\text{min}$), (C) amylase (U/g wet gland weight). Data are presented as mean of at least 8 independent determinations \pm S.E.M. $**P < 0.01$ in comparison with the control group; $++P < 0.01$ in comparison with the BBN-injected group.

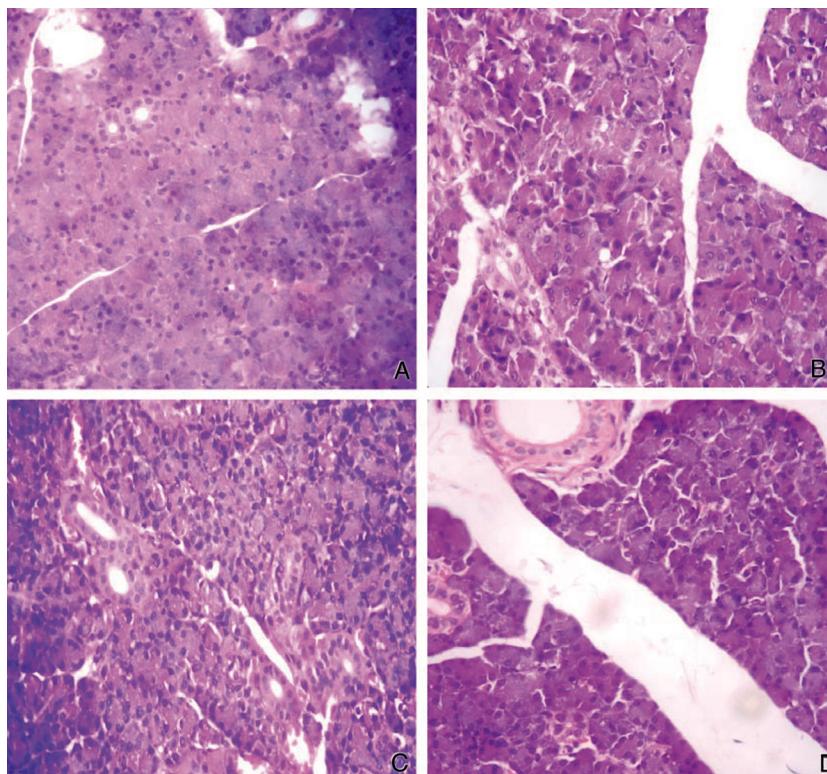


Fig. 4. Representative histopathologic appearance of the parotid gland from saline-treated group (A), BBN-treated group (B), lithium-treated group (C), and lithium + BBN-treated group (D). Serous acini and ducts are seen in the images (A-D). No histopathologic changes are noticeable in the treated groups (Hematoxylin and eosin staining. Original magnification: A-D, $\times 400$).

A histopathologic study of the parotid glands, parenchymal zones (acini and ducts) and the connective tissue revealed normal appearance in all the treatment groups. Mucous acini and a few mixed acini were seen in some samples. No edema, atrophy, leukocytic infiltration, fibrotic changes were found (Fig. 4, A-D).

Alterations in the Growth and Secretions of the Submandibular Gland

Submandibular gland weight did not change significantly in any experimental group. BBN augmented tissue total protein content in the water-fed group ($P < 0.01$), but the augmentation was completely reversed in the lithium-treated rats (Table 1).

Protein content of the submandibular gland increased significantly in the BBN-treated group in comparison with the saline group ($P < 0.01$). Lithium consumption did not change the protein content of the gland, either in the saline- or the BBN-injected groups (Fig. 5A).

Lithium significantly reduced total secreted protein from the submandibular gland ($P < 0.01$) whereas BBN increased protein secretion significantly ($P < 0.01$). However, lithium treatment in the BBN-injected group significantly decreased ($P < 0.05$) the

protein content compared with the BBN-injected water-fed group (Fig. 5B).

Flow rate decreased significantly in the lithium-treated group in comparison with the water-fed one ($P < 0.05$). The same results were observed when lithium and BBN were administered together. BBN did not significantly change the flow rate in both water-fed and lithium-treated groups (Fig. 5C).

Acinar cells, both mucous and serous cells, were normal in histopathologic studies. Granules of serous cells were normal and no vacuolization was noticed. Ductal structures were normal and connective tissue showed normal appearance. Edema, atrophy, leukocytic infiltration, fibrotic changes were found in all groups (Fig. 6, A-D).

Alterations in Stomach, Liver, and Duodenum Weight

Administration of BBN, lithium and their co-administration did not significantly change stomach, liver and duodenum normalized weight (data not shown).

Lithium Plasma Concentration

Lithium plasma concentrations in animals consuming lithium chloride and in those taking lithium

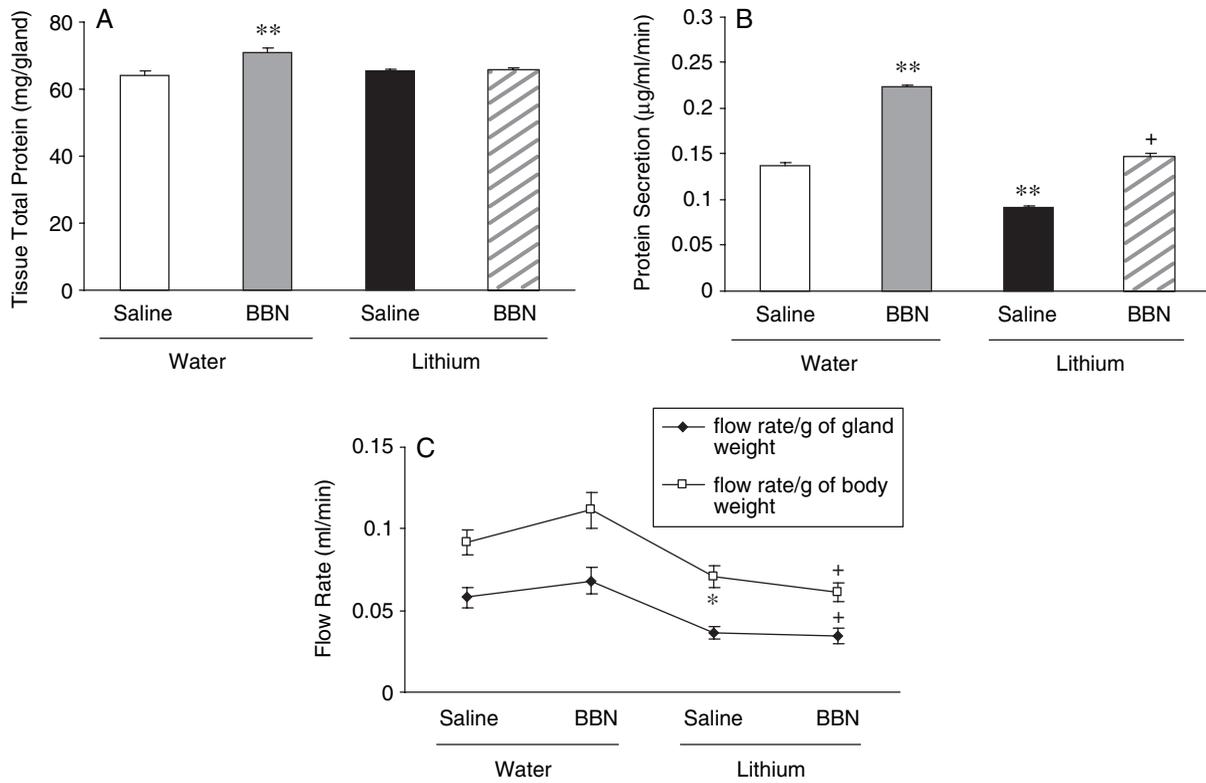


Fig. 5. Representative submandibular gland growth and excretory parameters: (A) gland total protein (mg/gland), (B) gland protein secretion rate ($\mu\text{g/ml/min}$), (C) flow rate (ml/min per gland weight and body weight). Data are presented as mean of at least 8 independent determinations \pm S.E.M. * $P < 0.05$ and ** $P < 0.01$ in comparison with the control group; + $P < 0.05$ in comparison with the BBN-injected group.

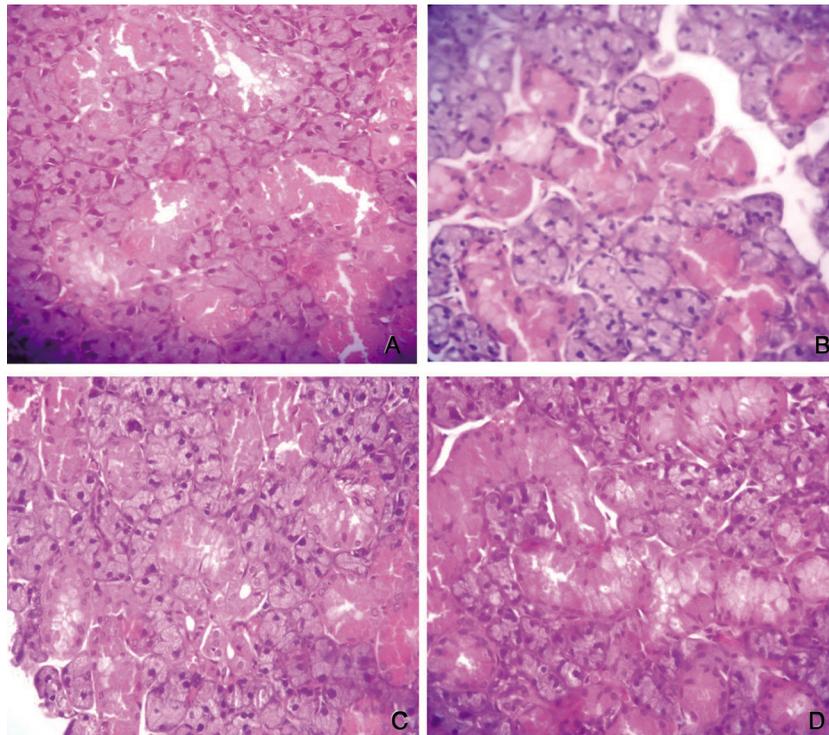


Fig. 6. Representative histopathologic appearance of the submandibular gland from saline-treated group (A), BBN-treated group (B), lithium-treated group (C), and lithium + BBN-treated group (D). Mixed acini (serous and mucous) and intercalated ducts are seen. All treated groups reveal normal histology (Hematoxylin and eosin staining. Original magnification: A-D, $\times 400$).

chloride followed by BBN injection were 0.321 ± 0.005 and 0.314 ± 0.006 mM, respectively. There were no statistically significant differences between these two groups.

Discussion

Li^+ has been reported to affect growth and proliferation in a wide variety of cells. Although growth is inhibited in some cells, such as murine neural and embryonal cells, lithium shows mitogenic effects on human lymphocytes, human myeloid progenitor cells, human bone marrow cultures and murine pluripotent progenitor cells (16, 45). Considering the controversies on the effects of Li^+ on growth and proliferation, we investigated the effects of Li^+ on the known properties of BBN on growth and secretion of salivary glands and pancreas.

The results of this study revealed that BBN caused a significant increase in the weight of the pancreas and parotid. Simultaneous administration of lithium and bombesin not only suppressed pancreas and parotid weight due to BBN, but also significantly decreased pancreatic growth. Lithium administration reduced pancreas weight by itself, but it did not change parotid weight. Therefore, this effect can be assumed as to be lithium-BBN mechanism interaction only in the case of parotid gland but not the pancreas. Histological study did not show noticeable microscopic changes in any treated groups. In addition, BBN administration in water-fed or lithium-treated rats increased protein content of the pancreas in a significant manner, but the water-fed group receiving BBN showed a notable increase compared to the lithium-treated group. Lithium consumption did not considerably change the protein content of the pancreas. Therefore, it seems that the loss of the pancreatic weight was not due to protein reduction.

The achieved results from this survey pointed out that BBN administration significantly increased the protein content of the parotid and the submandibular glands, whereas lithium treatment inhibited the stimulating effect of BBN on the protein content of both glands.

Although BBN has been discovered for about 40 years, there is still controversy on its mechanism of action on different tissues, particularly on the pancreas. Ashton and his colleagues have shown the existence of BBN receptors in the pancreas in 1990 (2). At the same time, Scarpignato *et al.* reported that the weight and content of the pancreas increased due to BBN (43). On the one hand, a group of scientists assert that BBN induces growth and secretion of the pancreas *via* releasing gastrointestinal hormones such as gastrin and CCK (12, 50). On the other hand, a few scientists believe that BBN exerts its

stimulatory effects directly through binding to BBN receptors in the pancreas (43). The role of neuropeptides such as VIP, somatostatin and substance P has been studied by the other researchers (17). These neuropeptides can also play a critical role in the growth and secretion of salivary glands (20).

BBN induces hydrolysis of PIP_2 and generates secondary messengers IP_3 and DAG (25, 48). Researchers have shown that some particular growth factors perform their effect on the stimulation of cellular growth through phosphoinositide hydrolysis. Besides, it was remarked in 1984 that these two secondary messengers might co-operate in the regulation of some ionic processes that are responsible for the initiation of cellular growth (5). Growth factors not only enhance Ca^{2+} levels but also act as inducers for Na^+-H^+ carrier exchangers (41, 48). It is generally assumed that IP_3 causes the movement of intracellular Ca^{2+} , while DAG might stimulate antiport of Na^+-H^+ and protein kinase C (5). In fact, metabolites of phosphoinositide can function as mediators for activities of growth factors.

Lithium is one of the agents which selectively interferes with the phosphoinositide cycle. It leads to increases in inositol monophosphate through inhibiting the enzyme inositol monophosphatase and finally leading to the halt of the recurrent cycle of PIP_2 synthesis (5, 48). Lithium suppresses this enzyme in extracellular concentration of more than 0.1 mM. Such a concentration is tenfold lower than the concentration necessary for the suppression of adenylyl cyclase (13). The plasma concentration of lithium in our experiments was around 0.3 mM. Since, in this concentration, lithium is able to decrease the PIP_2 level, the inhibitory effects of lithium on BBN-induced growth parameters can be interpreted as its effect on the two mentioned secondary messengers.

Regarding to salivary protein secretion, there are two main mechanisms involved: 1) activation of adenylyl cyclase which causes an increase in the cAMP level and finally results in protein secretion in the saliva (9); 2) activation of phospholipase C which hydrolyzes PIP_2 to generate IP_3 and DAG. Augmentation in saliva volume is due to IP_3 production (3), whereas DAG can cause protein secretion (39). In this study, BBN significantly increased protein secretion of submandibular saliva while lithium administration not only reduced salivary protein content but also suppressed the stimulating effects of BBN on saliva protein of the submandibular gland. It seems that BBN can increase protein secretion *via* PIP_2 hydrolysis. But as lithium administration significantly reduced protein secretion by itself, this effect, therefore, can not be interpreted as the reducing effect of lithium on the PIP_2 level. Instead, it can be concluded that lithium and BBN act through an independent mechanism

on salivary protein secretion.

In accordance with other reports (11), the secretion of salivary protein from the parotid gland did not change due to BBN, whereas lithium decreased it. It seems that higher concentrations of BBN are necessary to execute its effect on salivary protein secretion in the parotid. Moreover, BBN could not affect the total secreted protein from the pancreas. It appears that subcutaneous injections do not provide adequate plasma levels to influence the acinar cells.

Flow rate in the submandibular gland did not change noticeably due to BBN. In contrast, lithium could reduce submandibular flow rate significantly that is explainable by the blockage of recycling of PIP₂ synthesis.

In the parotid gland, BBN and lithium, either administered alone or together, did not induce significant reduction in the amylase level. As amylase secretion is accompanied by increases in the cAMP level (11), it can be concluded that neither BBN nor lithium changes the level of cAMP.

Amylase secretion from the pancreas amplified significantly in the BBN-treated set in comparison with the control set. This amplification could be due to IP₃ formation and subsequent increases in cytoplasmic Ca²⁺ concentration. Besides, lithium treatment did not affect the amylase concentration (U/ml) of the pancreas though it can be concluded that higher concentrations of lithium are necessary to inhibit amylase secretion. In addition, amylase discharge may depend on cAMP. In other words, this concentration of lithium could not have affected the cAMP level. In the pancreas, lithium induced a significant reduction in amylase secretion increased by BBN. It seems that lithium has reduced pancreas amylase secretion through a decrease of the PIP₂ level which subsequently results in the decline of the IP₃ and DAG levels.

In conclusion, the stimulatory effects of BBN on growth and protein content of the pancreas and the salivary glands were confirmed in this study. On the other hand, lithium was determined to be a potent inhibitor of growth parameters induced by BBN probably through inhibition of PIP₂ hydrolysis. Nonetheless, it seems that BBN has an indispensable role, even any, in the activation of the adenylyl cyclase pathway. Therefore, more experiments are necessary to clarify the root of BBN and lithium action on isolated tissue and the role of the PIP₂ pathway in the growth of the pancreas and the salivary glands.

Acknowledgments

We would like to thank Dr. Reza Rahimian and Ms. Maryam Rahimi-Balaei for their kind help in the preparation of the present article. We also express

our gratitude to the reviewers for their nice and considerable comments on this study.

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