



Journal of Biological Sciences

ISSN 1727-3048

science
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Central Nifedipine-induced Alterations in Salivary Flow and Compounds: Role of Nitric Oxide

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Abstract: The aim of this study was to examine the role of nifedipine and Nitric Oxide (NO) on salivary flow and compounds (salivary amylase, saliva total proteins, saliva calcium, sodium and potassium). Male Holtzman rats weighting 200-250 g were anesthetized with zoletil 50 mg kg⁻¹ (tiletamine chloridrate 125.0 mg and zolazepan chloridrate 125.0 mg) into quadriceps muscle and stainless steel cannulas were implanted into their lateral ventricle of the brain (LV). Animals in divided group were injected with nifedipine (50 µg µL⁻¹) alone and in combination with 7-nitroindazol (7-NIT) (40 µg µL⁻¹), neuronal NO Synthase Inhibitor (nNOSI) and Sodium Nitroprussate (SNP) (30 µg µL⁻¹) NO donor agent. As a secretory stimuli, pilocarpine dissolved in isotonic was administered intraperitoneally (ip) at a dosage of 10 mg kg⁻¹ body weight. Saliva was collected for 7 min with four cotton balls weighing approximately 20 mg each, two of which were placed on either side of the oral cavity, with the other two placed under the tongue. Nifedipine treatment induced a reduction in saliva secretion rate and concentration of amylase, total protein and calcium without changes in sodium and potassium concentration in comparison with controls. Co-treatment of animals with nifedipine and SNP retained flow rate and concentration of amylase, total protein and calcium in normal levels. Co-treatment of animals with nifedipine and 7-NIT potentiated the effect of nifedipine on the reduction of saliva secretion and concentrations of amylase, total protein and calcium. Nifedipine (dihydroperidine) calcium-channel blocker widely in use is associated with salivary dysfunction acting in the central nervous system structures. NO might be the mechanism for protective effect against the nifedipine-induced salivary dysfunction, acting in the CNS.

Key words: Water intake, blood pressure, lateral ventricle, nifedipine, nitric oxide, saliva

INTRODUCTION

Nifedipine is widely used as a calcium channel blocker. The frequent side-effect is gingival hyperplasia with reduction in the benefic effect of saliva flow and compounds. The overall quality of life is affected by the symptoms of a permanently dry mouth, inability to enjoy foods and the trouble and expense of frequent dental treatments. Nifedipine-induced gingival hyperplasia is associated with salivary dysfunction (Shourangiz *et al.*, 2005). Activation of cGMP-dependent positive signal-transduction mechanism in salivary glands might be the

mechanism for protective effects of Nitric Oxide (NO) against nifedipine-induced gingival hyperplasia and reduction in the physiological function of saliva (Abdollahi *et al.*, 2003). NO is present in saliva and is a putative neurotransmitter in salivary gland. The role of NO in saliva secretion has been demonstrated. Specific activity of Nitric Oxide Synthase (NOS) was detected in the submandibular gland (Lomniczi *et al.*, 1998; Modin *et al.*, 1994). NO is present in neuronal terminals within the gland and in the ductile system (Bred *et al.*, 1990; Lohinai *et al.*, 1995). Both the histochemical and functional results have suggested

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that Nitric Oxide (NO) plays an excitatory role in the regulation of the parasympathetic nerve inducing salivary secretion in the submandibular gland of rats (Takai *et al.*, 1999). L-NAME, a nitric oxide synthase inhibitor, increased the salivation induced by pilocarpine (Damas, 1994). The alkaloid pilocarpine (pye loe KAR peen) is a useful cholinergic agonist compared with acetylcholine (ACh) and its derivatives but it is far less potent. It is unaffected by acetylcholinesterase. Pilocarpine has been used extensively over the last century as one of the best sialogogues rather than other cholinomimetic agents (Wiserman and Faulds, 1995). Increased generation of NO upon stimulation of cells by muscarinic agonists was detected. A series of muscarinic agonists such as pilocarpine and carbachol stimulated NOS (Wang *et al.*, 1994). Extracellular NO seems to inhibit the ability of the M(2) receptors in decreasing acetylcholine (ACh) release from the parasympathetic nerves. It has been demonstrated *in vitro* that endogenous NO had inhibited the ability of M(2) receptors in decreasing ACh release by using L-NAME and pilocarpine (Golkar *et al.*, 2000). These studies have presented the ability of pilocarpine in affecting the NO activity. The involvement of some areas of the CNS in the control of salivary secretion in rats has been shown by several studies (Renzi *et al.*, 1989).

Muscarinic activation of salivary gland increases the intracellular-free calcium levels that activate NOS leading to the production of NO and then an activation of cGMP that opens the ions channels to initiate the secretory process (Lomicz *et al.*, 1998). NOS activity in the salivary glands is blocked in the absence of calcium or the presence of a calmodulin (Mitsui *et al.*, 1997).

Earlier studies indicate that nifedipine causes significant alterations in the submandibular acinar cells and lowers the flow rate and calcium of submandibular saliva with possible relation to gingival overgrowth (Dehpour *et al.*, 1995). Altered calcium metabolism in gingival cells has been suggested as a mechanism for nifedipine-induced gingival hyperplasia (Pernu *et al.*, 1989; Das and Olsen, 2000). The administration of L-arginine, the precursor of NO, increase salivary flow and calcium concentrations (Abdollahi *et al.*, 2000; Abdollahi and Safarhamidi, 2002). A synergistic action of nifedipine and NOS inhibition has been previously demonstrated when tested in formalin-induced antinociception (Abdollahi *et al.*, 2001).

We have investigated the effect of cholinomimetic agonist pilocarpine injected into LV on salivary secretion of rats with anteroventral third ventricle (AV3V) electrolytic lesion. We have concluded that the central nervous system, particularly the AV3V region, is

important for the effect of pilocarpine on salivary secretion in rats (Renzi *et al.*, 1993). Morphological, morphometric and stereological changes of submandibular glands were observed after the lesion of the ventromedial nucleus of hypothalamus and the lesion of the AV3V region (Renzi *et al.*, 1989, 1990). Central injections of L-NAME and L-arginine interfere with the salivary secretion which implies that they might participate in pilocarpine-induced salivary secretion. The interaction between cholinergic and beta-adrenergic receptors of the CNS for the control of salivary secretion can also be postulated (Saad *et al.*, 2002a, 1999). The septal area plays an important role in the central mechanism that regulates salivary secretion, cardiovascular and electrolyte balance by inhibiting or releasing NO (Saad *et al.*, 2004; Vaucher *et al.*, 1997).

Our earlier results indicated that central mechanisms activated by α_2 -adrenergic and imidazoline receptor agonist blocked pilocarpine-induced salivation in rats. The inhibition of the salivation by activation of septal α_2 -adrenergic and imidazoline receptors may involve at least two distinct efferent mechanisms: (1) activation of inhibitory pathways from the septal area to the salivary glands involving more imidazoline than α_2 -adrenergic receptors; (2) inhibition of septal excitatory mechanisms activated by pilocarpine to induce salivary secretion utilizing more imidazoline than α_2 -adrenergic receptors. These receptors of the septal area have an inhibitory mechanism on salivary secretion (Saad *et al.*, 2004). Taking these findings into consideration, we have examined how the central stimulation or inhibition of NO synthesis might affect nifedipine-induced alteration in saliva flow and in saliva composition. For this, SNP, a NO donating agent and 7-NI, a neuronal NO synthase inhibitor, were used in a rat model nifedipine-induced impairment in saliva.

MATERIALS AND METHODS

Present study was conducted in the Basic Institute of Biosciences-UNITAU, Taubaté, SP, Brazil and in the Department of Biology and Health Science UNIARA Araraquara SP Brazil in the year of 2004/2005.

Experimental protocol: The study of salivary flow started 5 days after the brain surgery. Each animal was submitted to 3 or 4 experimental sessions at 3 day intervals. These parameters were obtained from different experimental sessions and from several groups of animals.

Subjects: The animals were housed in individual metabolic cages. Food (Purina Rat Chow) and tap water

was available *ad libitum*. The temperature was maintained at $22\pm 2^{\circ}\text{C}$. The light cycle was held at 12:12 with lights on 06:00 h. All experiments were conducted during the light period, between 09:00 AM and 03:00 PM. The Medical Ethics Committee of the Universidade Estadual Paulista UNESP approved all protocols in this study.

Cerebral surgery: Male Holtzman rats weighing 250-300 g were anesthetized with zoletil 50 mg kg^{-1} (tiletamine chloridrate 125.0 mg and zolazepan chloridrate 125.0 mg) into quadriceps muscle. The stereotaxic coordinates for the LV were obtained from Paxinos and Watson rat brain atlas (1986). Antero-posterior = 0.92 mm posterior to bregma, Lateral = 1.3 mm lateral to middle sagittal line and vertical = 3.4 mm below the duramater. A stainless steel cannula with 10 and 12 mm long and 0.7 mm OD was implanted into the LV according to the coordinates of Paxinos and Watson atlas rat brain (Paxinos and Watson, 1986). The cannulae were fixed to the skull with the aid of jeweler screws and dental acrylic resin and protected with a stiletto. The experiments begin after 5 days of the brain surgery.

Drug injection: The drugs were injected into the LV by using a Hamilton micro syringe ($5\text{ }\mu\text{L}$) connected by a PE-10 polyethylene tubing (25 cm) to a needle (0.3 mm o. d.), which was introduced into the brain through the cannula previously fixed to the animals' head. The volume of injection was always $0.5\text{ }\mu\text{L}$ injected over a period of 30-60 sec.

- 0.15M NaCl injected into the LV (control)
- Pilocarpine (10 mg kg^{-1} body weight) (Sigma Chemical Company, St. Louis, MO) injected ip
- Nifedipine 1,4-Dihydropyridine ($50\text{ }\mu\text{g}$ $0.5\text{ }\mu\text{L}^{-1}$) Sigma Chemical Company, St. Louis, MO) into LV
- 7-nitroindazol (7-NIT) ($40\text{ }\mu\text{g}$ $0.5\text{ }\mu\text{L}^{-1}$) 7-nitroindazol (Tocris Cookson Inc, Ballwin, MO, USA) injected into the LV
- Sodium Nitroprussade (SNP) ($30\text{ }\mu\text{g}$ $0.5\text{ }\mu\text{L}^{-1}$) (Sigma USA) injected into the LV

Treatment: Salivary flow was stimulated by pilocarpine (10 mg kg^{-1} body weight) injected ip. The animals were anaesthetized with urethane 1.25 g kg^{-1} b.wt. intraperitoneally (i.p.). Saliva was collected with pre weighed small cotton wool balls inserted into the animals' mouth, a technique slightly different from that used by Schallert (1978). Such technique led us to collect the whole saliva. Saliva was collected with four cotton

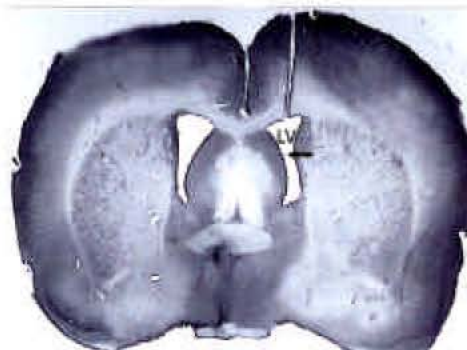


Fig. 1: Photomicrography of a hematoxylin-stained transverse section showing the site of injection into the LV (Arrow)

balls weighing approximately 20 mg each, two of which were placed on either side of the oral cavity, with the other two placed under the tongue. The amount of saliva secreted was measured 5 min before the injection of pilocarpine (baseline salivary secretion) and 5 min after the injection of pilocarpine (stimulated salivary secretion). The nifedipine were injected into the LV 5 min before pilocarpine or were injected without pilocarpine. SNP or 7-NI was injected into LV 5 min before nifedipine.

Biochemical analysis

- The salivary amylase was determined by Carawary's method (Carawary, 1959).
- The total proteins were determined by Bradford's method (Bradford, 1976).
- The calcium was determined by Cresolftalein's method (Connerty and Brigs, 1966).
- The sodium and potassium were determined by photometer of flame (model IL 143 Lexington, Massachussettes USA).

Histology: At the end of the experiments, the rats were anesthetized with ether and given perfuse with saline and buffered formalin. The brains were removed, fixed in 10% formalin, frozen to -25°C and cut into 20-30 μm coronal sections. Only animals in which the injection was placed in the LV (Fig. 1)

Statistical analysis: The results are reported as mean \pm SEM. The ANOVA and Newman-Keuls post-hoc test were used to determine the significance. The values were considered statistically significant with 5% level ($p<0.05$).

RESULTS

Effects on salivary flow: Salivary flow rate induced by pilocarpine-injected ip decreased in rats treated with nifedipine injected into the LV when compared with controls ($p < 0.05$). The decrease in the salivary flow was potentiated by the treatment with SNP and recovered by 7-NI injected prior to nifedipine into the LV ($p < 0.05$) (Fig. 2).

Effects on salivary total protein: Salivary total protein concentration decreased in rats treated intracerebroventricular (icv) with nifedipine (4.7 ± 0.2 vs 6.7 ± 0.3 mg kg⁻¹) compared with controls ($p < 0.05$). Prior treatment with SNP (6.0 ± 0.3 mg kg⁻¹) recovered the effect of nifedipine and 7-NI potentiated the nifedipine reduction effect on total salivary protein (2.4 ± 0.1 mg kg⁻¹) ($p < 0.05$).

Effects on salivary amylase: Salivary amylase was reduced by nifedipine injection into LV compared with control values (620 ± 25 vs 805 ± 40 U m LI⁻¹) ($p < 0.05$). Prior treatment with SNP blocked the inhibitory effect of nifedipine 800 ± 39 U m LI⁻¹) whereas 7-NI potentiated it 430 ± 22 U m LI⁻¹) ($p < 0.05$).

Effects on salivary calcium concentration: Salivary calcium concentration decreased in rats treated with nifedipine into the LV when compared with control values 2.7 ± 3.2 vs 3.2 ± 0.2 mEq L⁻¹) ($p < 0.05$). SNP plus nifedipine recovered the calcium concentration and reached control levels 3.7 ± 0.2 mEq L⁻¹) ($p < 0.05$). 7-NI potentiated the inhibitory effect of nifedipine (2.3 ± 0.1 mEq L⁻¹) ($p < 0.05$).

Effects on salivary sodium concentration: Salivary sodium concentration decreased after the injection of nifedipine into the LV compared with control (4.5 ± 0.2 mEq L⁻¹) vs 6.0 ± 0.3 mEq L⁻¹) ($p < 0.05$). SNP plus nifedipine potentiated the effect of nifedipine on salivary sodium concentration 3.2 ± 0.1 mEq L⁻¹) ($p < 0.05$). 7-NI plus nifedipine produced an increase in salivary sodium concentration 10.0 ± 0.6 mEq L⁻¹) ($p < 0.05$).

Effects on salivary potassium concentration: Salivary potassium concentration increased after the injection of nifedipine into the LV compared with control 10.8 ± 0.5 vs 7.2 ± 0.4 mEq L⁻¹) ($p < 0.05$). 7-NI plus nifedipine decreased salivary potassium concentration (4.2 ± 0.2 mEq L⁻¹) ($p < 0.05$).

Effects on water intake and arterial pressure: The water intake after injection of nifedipine into LV did not

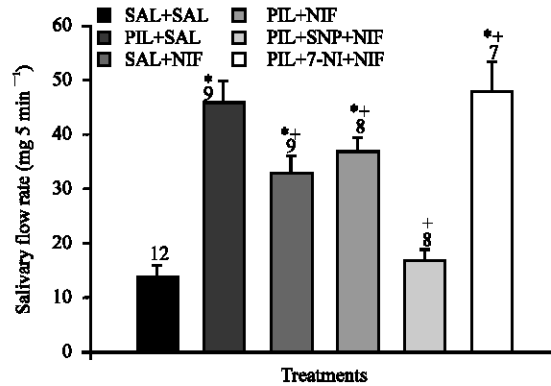


Fig. 2: Effect of pretreatment with saline+pilocarpine (SAL+PIL), saline+nifedipine (SAL+NIF), pilocarpine+sodium-nitroprussade+nifedipine (PIL+SNP+NIF) and pilocarpine+7-nitroindazol+nifedipine (PIL+7-NI+NIF) on saliva flow. Nifedipine, sodium nitroprussade and 7-nitroindazol were injected into lateral ventricle vehicle. Pilocarpine was administered intraperitoneally as secretagogue. The number of animals is represented at the top of each column. Data are reported as mean±SEM. * $p < 0.05$ compared to the saline group; + $p < 0.05$ compared to the PIL+SAL group (Neuman-Keuls post-hoc test)

changed in comparison with the levels of the control animals. Also the arterial pressure did not changed after nifedipine injection into LV. These values were obtained at the same time that the saliva was collect (5 min).

DISCUSSION

The results presented here indicate that nifedipine injected into the LV of the rat brain induces salivary dysfunction. The salivary flow rate decreased, accompanied by a reduction in total protein, salivary amylase and calcium concentration. It has been demonstrated that nifedipine decreased the salivary amylase (Kondo *et al.*, 1984). The salivary sodium concentration decreased by nifedipine, accompanied by an increase in potassium salivary concentration.

Systemic acute administration of nifedipine in rats reduced salivary flow and calcium concentration (Dehpour *et al.*, 1995). Both chronic and acute systemic administrations of nifedipine decreased the salivary flow and calcium concentration while total protein is influenced by chronic administration (Shourangiz *et al.*, 2005). Fluid secretion from salivary glands is stimulated by the cholinergic system which is mediated by an

increase in free cytoplasm calcium concentration (Baum, 1987; Edgar, 1992). The L-type calcium channel complex contains at least four distinct binding sites (Catterall and Striessnig, 1992; Spedding and Paoletti, 1992). These are the dihydropyridine site and isradipine (PN200-110) bind and compete with each other for binding; the phenylalkylamine site at which verapamil and flunarizine bind; and the benzothiazepine site which diltiazem binds and a site to which 1,3-diphosphonates can bind (Rossier *et al.*, 1989).

Pretreating rats with nifedipine, L-type calcium channel blocker which centrally binds to the dihydropyridine site, reduced the salivary flow rate, calcium, total protein and amylase. The present study has shown the protective effect of NO of the Central Nervous System (CNS) from nifedipine-induced salivary alterations. Co administration of SNP exacerbates nifedipine effect and co administration of 7-NI blocked nifedipine effects on salivary flow. The inverse was found in salivary component studies. The presence of NO in many structures of the CNS has been described (Ignarro *et al.*, 1987; Bredt *et al.*, 1990). NO through cGMP-dependent processes, acts as a neurotransmitter in the regulation of blood flow and secretion in salivary glands (Lomniczi *et al.*, 1998; Lohinai *et al.*, 1995; Abdollahi *et al.*, 2000; Abdollahi and Safarhamidi, 2002). NO centrally or systemically plays important role in the regulation of electrolyte excretion (Saad *et al.*, 2002). Nifedipine is proposed to lower NO concentration.

Nifedipine is a potential drug to induce oxidative stress that could result in salivary flow and component dysfunctions. Thus, the release of NO is the other mechanism for protective role of salivary dysfunction. It has been demonstrated that calcium antagonist injected icv attenuated the pressor response to icv ANGII, but not the drinking response. The possibility that calcium antagonists injected icv might not have arrived at the specific area which is important for drinking behavior, cannot be completely ruled out (Kondo *et al.*, 1984). This possibility was confirmed by studies conducted by Zhu and Herbert (1997). Rats pre-treated icv with nifedipine, followed by angiotensin II (ANGII), drank significantly less water than controls. Nifedipine was found to suppress both angiotensin II-induced corticosterona release and *c-fos* expression in the following areas of the CNS Organum Vasculosum of the Lamina Terminalis (OVLT) median preoptic nucleus (MnPO), hypothalamic paraventricular nucleus (PVN) and Supraoptic Nucleus (SON) (Zhu and Herbert, 1997). Our earlier study has shown that the injection of L-NAME into MnPO or SON prior to pilocarpine ip or centrally increase the salivary secretion whereas the SNP decreased it

(Saad *et al.*, 2003, 2004). That the Anterior Ventral Third Ventricle (AV3V) is an important region of the CNS for the effect of pilocarpine on the salivary secretion in rats (Renzi *et al.*, 1993).

Morphological, morphometric and stereological changes of submandibular gland were observed after the lesion of the ventromedial nucleus of hypothalamus and the lesion of AV3V region (Renzi *et al.*, 1989, 1990). Nifedipine injected into the LV decreased the salivary sodium concentration with an increase in salivary potassium concentration. These effects were potentiated by SNP and reduced by 7-NI. Previously we demonstrated that NO in the septal area participated in the regulation of arterial pressure, sodium and water renal excretion and salivary excretion (Saad *et al.*, 2002b). The effect of ip administration of pilocarpine and angiotensin II influences saliva flow, protein and electrolytes, showing that pilocarpine have more potent effect than angiotensin II (Saad *et al.*, 2005). The fact that nifedipine reduced the salivary parameters and *c-fos* expression has other implications. By blocking the calcium channels, water intake and arterial blood pressure might have been reduced, simply as a part of a non-specific, toxic, reaction to a procedure that might have wide-ranging results on neuronal function. In the CNS, Ca²⁺ signaling may have more general effects in neurons responsive to central effects of nifedipine. The systemic administration of this L-type calcium blocked agent may reach some structures of the CNS and cause dysfunctions in salivary flow and components. Summers and colleagues proposed a hypothetical model for AT₁ receptor-mediated effects in neurons directly responsive to ANGII (Summers *et al.*, 1994) which is similar to its peripheral signaling mechanism (Catt *et al.*, 1993). The excitation of neuronal membrane sets up an action potential which results in Ca²⁺ influx at the terminal, which controls the release of neurotransmitters (Kostyuk and Verkhatsky, 1995). Calcium occupies a central role in the nervous tissue function. Studies have demonstrated the presence of specific calcium antagonist binding sites in the brain. The central effects of nifedipine on central pressor and dipsogenic effect of angiotensin II in rats have been demonstrated (Kondo *et al.*, 1984). Nifedipine injected into the LV may reach circumventricular structures and interfere with Ca²⁺ influx in the presynaptic terminals, whereas L-type calcium channels play important roles in the modulating presynaptic neurotransmitters as previously shown (Miller, 1988; Siveramakrishnan and Laurent, 1995).

Nifedipine injected into the LV may have altered Ca²⁺-dependent signaling events in postsynaptic neurons. The results of this study as well as other previous

studies, have demonstrated the permissive action of voltage-sensitive calcium channel on NMDA receptor-mediated Ca^{2+} influx (Burnashev, 1996). Present results provide evidence that calcium signaling plays important roles in the saliva physiological roles. The L-type calcium channel blocked agent widely used in many hypertension stages determines alterations in salivary flow, components and electrolytes. Flow rate and calcium of submandibular saliva were lower than controls. Parotid calcium was decreased after nifedipine injection (Dehpour *et al.*, 1995). AV3V lesion influences pilocarpine-induced pressor response and salivary gland vasodilatation (Takakura *et al.*, 2005).

NO contributes to the control of vascular tone in salivary glands of rats. NO of the many brain structures plays an important protective role in the effect of nifedipine. Nifedipine have not effect on water intake and arterial blood pressure in the same time that the saliva was collected showing that the thirst and blood pressure did not influence the alteration in saliva induced by nifedipine. This effect leads to salivary gland dysfunction induced by nifedipine centrally injected. The protective action of NO can also be postulated. Further experiments need to be done to support this. In conclusion nifedipine injected into the LV might reach areas of the CNS that promote alterations in saliva flow and compounds. NO influences nifedipine effects on saliva.

ACKNOWLEDGMENTS

The authors greatly appreciate the assistance of Luciana Rizuti Saad for preparation of the manuscript and Ana V. Oliveira and Fernando L. Capelli for animal care. Research supported by CNPq (520408/96.9), FAPESP (99/06582-2 and 02/03806-1), FUNDUNESP (01/06756-3), PRONEX and FUNADESP-UNIARA.

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