CHAPTER-IV

EFFECTS OF GLYCOWITHANOLIDES ON

ESTERASE AND AMYLASE ACTIVITY

IN SALIVARY GLANDS OF

D-GALACTOSE STRESSED

MICE
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1. INTRODUCTION

Salivary glands were considered a neglected “appendix” of digestive system. But during the past few decades the number of biologically active polypeptides and/or factors have been isolated from submandibular glands are found important in growth, differentiation, maintenance of homeostasis, regulation and digestion. In all twenty seven biological active polypeptides are synthesized in submandibular GCT (Barka, 1980). The NGF (Nerve Growth Factor) is one of the most extensively studied growth factor of submandibular gland origin. It plays a pivotal role in the development and maintenance of the functional integrity of sympathetic and some sensory neurons. The chemical and biological activities of NGF have been extensively reviewed by several investigators (Levi-montalcini, 1961, 1968, 1972; Bradshaw and Young, 1976; Gospodarowicz and Morgan, 1976; Freed, 1976). Nerve growth factor exists in two forms the 2.5 S NGF and 7 S NGF. The biological activity of 7 S NGF is associated with beta subunit. The gamma subunit is an arginine esteropeptidase which is involved in processing of the synthetic precursor of NGF. In the 7 S NGF complexes the enzyme is minimally active because of the Zn ion which is inhibitory for the enzyme.

Next to NGF, EGF (Epidermal Growth Factor) is the best characterized and most extensively studied factor that occurs in the submandibular gland (Cohen, 1962). EGF is potent mitotic stimulant for variety of cell types; it enhances keratinization and inhibits gastric acid secretion. In crude homogenates of mouse submandibular gland EGF occurs as a high m. wt. (74000) complex, consisting of two moles of EGF and two moles of an EGF–binding proteins. The EGF–binding protein is an arginine esteropeptidase (m. wt. 29300).
Among the submandibular gland factors, involved in maintenance of homeostasis, kallikreins are cerine proteases and specifically a biological active decapetide and plays an important role in local blood flow regulation in salivary glands. Recent structural and immunological data suggest that kallikreins and esteropeptidases derived from a common ancestor molecule. Release of kallikrein like esterase from rat submandibular gland was investigated by Hiroshi, (1984). In submandibular gland the concentration of kallikrein is highest, while that of in sublingual and parotid is negligible. Kallikreins of Submandibular gland occur as multiple isoenzymes (Hopsue-Havu et al, 1967; Brandrzaeg et al, 1976). Immunocytochemical observations established that kallikreins are localized in the GCT and striated duct cells in submandibular gland of rat (Brandrzaeg et al, 1976), cat (Hojima et al, 1977; Maranda et al, 1978) and pig (Dietl et al, 1978). Lauer et al, (1975) suggested that kallikrein is initially localized in the acinar cells and that latter the GCT cells may produce the enzyme.

Apart from these growth factors, presences of several enzymes in salivary glands have been reported by many researchers. Alkaline phosphates activity was observed by (Hill and Bourne, 1954). Thiamine pyrophosphatase in sublingual gland (Kim, 1976), ribonuclease in the end pieces of the mandibular glands of mouse (Mayner and Ackerman, 1962), acid phosphatase (Garett and kid, 1976; Van Lennep et al, 1977 and Pillai and Nadar 1986 - 87) in the secretory cells of salivary glands, esterase-B in GCT of submandibular glands (Torill et al, 1992), non specific esterase in the duct epithelium of mandibular gland (Blood et al, 1977; Tomake and Pillai, 1993), α-amylase in granular ducts of mouse submandibular gland (Smith et al, 1971; Tomake and Pillai, 1996), amylase and trypsin in salivary glands of mice (Pillai et al, 1989).
Esterases are very diverse group of enzymes. The natural substrates of esterases are esters of carboxylic acids. The enzymes acting on simple esters such as methyl butyrate of naphthyl acetate are classified as non-specific esterases. The majority of these esterases have shown non-specific behavior towards various esters and inhibitors. It has been concluded that human tissues possess a heterogeneous mixture of carboxyl esterases with a wide range of properties. Esterase activity can be demonstrated in lysosomes (Pillai et al, 2008). Esterases are proteolytic enzymes. Salivary levels of these enzymes are high. Non-specific esterases have been found to be suitable marker enzyme for certain changes in the physiological states. Esterases may be having the physiological role in the regulatory processes of salivary gland. Arginine-easters complex with m-EGF may involved in development, differentiation and functions of salivary glands in vivo. It also enables the activity of GF in cell culture system, suggesting stimulation of growth in cell culture by salivary esteroproteases (Kenneth, 1976). Kamada et al, (1990) reported esterase-B (Homologous to the RSKG-7 kallikrein gene) is an activator of tissue kallikrein proenzyme.

From above data, it is clear that esterolytic activity along with growth factors synthesis occurs in salivary gland.

Amylases are groups of hydrolases that hydrolyze complex carbohydrate containing α-D-galactose unit linked through carbon 1 and 4 located on adjacent glucose residues. Salivary amylase is α-amylase (Ptyalin) which converts starch into maltose and contributes 40% to 50% to the total protein produced by the glands. Amylase is most abundant protein in salivary gland. Amylase activity has been demonstrated in mammalian parotid (Redman and Sreebny, 1971) in mouse submandibular gland granular duct (Smith et al, 1971; Amerongen et al, 1981). Chretein, (1977) histochemically proved that amylase activity is
located in GCT of submandibular glands. Krause and Mestecky, (1971) demonstrated presence of amylase activity in salivary glands by immunohistochemistry. Daoust, (1965); Smith et al, (1971); Shear, (1972) demonstrated the amylase activity in salivary glands by substrate film technique. Amylase activity has been reported by Pillai et al, (1989) in mice salivary glands. Smith et al, (1971) and Bhoola et al, (1973) showed correlation between development of GCT and amylase activity. Kobayashi et al, (1983) in dog reported that the release of amylase stimulated by pilocarpine or K^+ is dependent on Ca^{++} and that the activation of calmodulin is involved in the process of the release. Increased activity of amylase in submandibular gland was associated with GCT hypertrophy (Raynaud, 1950).

Several studies reported age related changes in amylase activity. Reduction in amylase content in mixed saliva has been reported in elderly persons (Meyer et al, 1937; Meyer and Necheles, 1940; and Chilla et al, 1974). Significant age related decrease in the rate of amylase synthesis in rat parotid was reported by Kim, (1981); Irwin and Mandal, (1984); Kim and Arisumi, (1985). Tomake and Pillai, (1996) reported decrease in amylase activity in salivary glands of castrated adult male mice and it was increased many folds on testosterone propionate injection but not in old mice receiving testosterone. Tomake and Pillai, (1996) in another study reported age related decline in amylase activity in old mice salivary glands. Significant decrease in amylase release from rat parotid gland with age was reported by Mahay et al, (2004).

Amylase is considered to be good indicator of proper functioning of salivary glands (Enberg et al, 2001) as it shows alterations. It is not only important to investigate the effect of aging on the morphology of salivary glands but also to give equal attention on the effects of aging on the physiological processes involved in secretion of salivary amylase.
Amylase activity shows alterations during development, reproductive status, aging and age related disorders like xerostomia, dental caries, burning mouth syndrome, periodontal diseases (Caplan and Hunt, 1996). It is present in GCT cells, where all other factors are synthesized. It can be considered as an indication of proper functioning of salivary gland in digestion, as this is a digestive enzyme. Therefore, for healthy and clean oral physiology, it is pertinent to investigate the effect of aging on amylase activity and also to improve salivary gland physiology and functioning, which get affected during ageing and stress.

2. MATERIAL AND METHODS:

A. MATERIAL:

a. Animal:

Male mice (Mus musculus) were used for the study. They were received Amrut mice feed (Pranav Agro Industries, Pvt. Ltd, Sangli) and water ad libitum. The record of their age and body weight was maintained.

Adult male mice of 5 to 6 month age, weighing 50 to 55 ± 2 gm body weight and old male mice of 16 to 18 month age, weighing 40 to 45 ± 2 gm body weight were used. Both adult and old male mice were divided into main two group viz. protective and curative group. Each group further divided into 4 batches as described in Chapter II.

B. METHODS:

a. Estimation of total proteins from salivary glands (Lowry et al, 1951):

Salivary glands dissected out from the mice were weighted, thawed and homogenized in distilled water (2mg/ml) then centrifuged at 3000 rpm for 10 minutes. The supernatants were used for estimation of proteins. In 0.5 ml sample 3.5 ml distilled water and 5.5 ml Lowry’s C reagent were added and mixed well. After 10 min. 0.5 ml folin-reagent
(diluted 1:1) was added. The optical densities were read after 30 minutes at 600 nm against blank.

b. Estimation of non-specific esterase activity (Bier, 1955):

The homogenates of submandibular and sublingual glands prepared in 0.66 M phosphate buffer pH 7.0 were centrifuged at 5000 rpm at 10\(^\circ\)C for 10 minutes. 1 ml of supernatants added to 5 ml ice cold water and 2 ml phosphate buffer (0.66 M, pH 7.0), all sample tubes were centrifuged at 2000 rpm for 5 minutes and then 2 ml of working substrate solution was added. It was incubated for 20 minutes at 20\(^\circ\)C. Readings were taken at 400 nm against distilled water as blank. The optical densities were converted to µmols of P-nitrophenol from the P-nitrophenol standard curve.

c. Histochemical demonstration of non-specific esterase (Burstone, 1962):

Salivary glands were fixed in 2% cold formol calcium for 24 hours. Fixed frozen cryostat sections kept in incubation medium (prepared as described in Chapter II), washed in cold distilled water, mounted in polyvinyl pyrolidone (PVP) and observed under microscope. Photographs were taken to record observations. Intense red color represented the esterase activity.

d. Kinetic study of esterase:

For determination of Km and Vmax values of esterase, different concentrations of p-nitrophenyl acetate were prepared in phosphate buffer 0.66 M and pH was adjusted to 7.0 pH with the help of 1N HCl. The concentrations of p-nitrophenyl acetate were taken as mentioned in Chapter II. Optical densities at various concentration of substrate were converted to µmole of P-nitrophenol from p-nitrophenol standard graph. The graphs were plotted as enzyme activity Vs substrate concentrations.
e. **Estimation of amylase from salivary glands** (Jayaraman 1988):

Salivary glands were homogenized in 0.1N phosphate buffer pH 6.7 (2mg/ml), centrifuge, 0.5 ml supernatant used for estimation. Reaction mixture contained, 2.5 ml phosphate buffer (0.1N, pH 6.7) + 2.5 ml 0.5% starch solution + 1 ml 1% NaCl mixed well and incubated at 37°C for 10 minutes. Then 1 ml water + 0.5 ml supernatant added and incubated for 15 minutes at 37°C. Then added 0.5ml 2N NaOH and 0.5 ml 1% DNSA, heated in boiling water bath for 5 minutes, cooled at room temperature and readings were taken at 520 nm against blank. Calculations were done as described earlier in chapter II.

f. **Histochemical localization of amylase from salivary glands using starch film method** (Smith and Frommer 1973):

Salivary glands were fixed in 2% formol-calcium for 24 hours at 10°C and fixed frozen cryostat sections of 6 µ thickness were placed on starch substrate film, (preparation of substrate film was described in chapter II) incubated in moist chamber at 37°C for 45 minutes. Slides were fixed in a mixture of methanol, acetic acid and distilled water (5:1:5) for 1 hour, rinsed in running tap water for one minute, stained with Lugol’s iodine solution for 1 minute. Wiped off starch film of unused side of the slide. Unstained areas on starch film were corresponding to the sites of amylase in the tissue sections. Photographs were taken to record the observations.

g. **Kinetic study of amylases:**

For the determination of values of Km and Vmax of amylase the rate of hydrolysis of starch by the enzyme was measured using starch of different concentrations as 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 percent. The pH was adjusted to 6.7; incubation time was 15 minutes and temperature 37°C. The enzyme activity was estimated at each substrate concentration. The amylase activity was calculated for per mg proteins.
from standard graph. The graphs were plotted as enzyme activity Vs substrate concentrations to obtain Km and Vmax.

3. RESULTS:

A. Effects of glycowithanolides (WSG) on total protein content in salivary glands of D-galactose (Dg) stressed mice.

a. Submandibular gland of adult protective group: (Table 3, graph 13).

The total proteins in submandibular glands of control batch was 0.134 ± 0.0054 mg and was decreased to 0.0961 ± 0.0054 mg in Dg-treated batch. In Dg + cent and Dg + WSG it was increased significantly (P<0.001) to 0.146 ± 0.0054 mg and 0.16 ± 0.0070 mg respectively as compared to Dg-treated batch. There was non significant (P>0.1) difference in protein content of Dg + cent and Dg + WSG.

b. Sublingual gland of adult protective group: (Table 3, graph 14).

Protein content of sublingual gland is depicted in table 3 and graph No. 14. Like submandibular glands, significant decrease in proteins was observed in D-galactose treated sublingual gland. In both the antioxidant treatment the protein content in sublingual gland showed significant increase.

c. Submandibular gland of adult curative group: (Table 3, graph 15).

The total proteins in submandibular glands of control batch was 0.13 ± 0.0316 mg and in Dg → saline batch, it was decreased to 0.082 ± 0.0027 mg. This decrease was significant (P<0.001). In Dg → cent and Dg → WSG batches, proteins were increased significantly (P<0.001) to 0.144 ± 0.0054 mg and 0.154 ± 0.0054 mg respectively. There was no significant (P>0.1) difference between Dg → cent and Dg → WSG batches.
d. **Sublingual gland of adult curative group:** (Table 3, graph 16).

In control batch the total proteins was 0.075 ± 0.0015mg and was decreased to 0.056 ± 0.0054 mg in Dg → saline batch. In Dg → cent and Dg → WSG batches there was significant increase in total proteins and was 0.0912 ± 0.0010 mg and 0.104 ± 0.0089 mg respectively. The increase in proteins in Dg → WSG batch was significant (P<0.02) as compared to Dg → cent batch.

e. **Submandibular gland of old protective group:** (Table 4, graph 17).

The total proteins in control batch was 0.116 ± 0.005477 mg and was decreased significantly (P<0.001) to 0.06 ± 0.0073 mg in Dg-treated batch. In Dg + cent and Dg + WSG batch it was increased significantly (P<0.001) to 0.126 ± 0.0026 mg and 0.13 ± 0.0015 mg respectively when compared to Dg-treated batch. There was non significant (P>0.1) difference between Dg + cent and Dg + WSG batches.

f. **Sublingual gland of old protective group:** (Table 4, graph 18).

In control batch the total protein content was 0.0834 ± 0.0016 mg and it was significantly decreased to 0.04 ± 0.0070 mg in Dg-treated batch. The mice received centrophenoxine or WSG along with D-galactose showed significant (P<0.001) increase in total proteins i.e. 0.0858 ± 0.0016 mg and 0.0872 ± 0.0025 mg respectively compared to Dg-treated mice. The protein concentration of Dg + cent and Dg + WSG batches was nearly same.

g. **Submandibular gland of old curative group:** (Table 4, graph 19).

Protein in control batch was 0.114 ± 0.0114 mg and was decreased to 0.0382 ± 0.0086 mg in Dg → saline batch. Mice received centrophenoxine or WSG after Dg treatment showed significant increase in total proteins i.e. 0.124 ± 0.0134 mg and 0.13 ± 0.00158 mg respectively. There was non significant difference between Dg → cent and Dg → WSG batches.
h. **Sublingual gland of old curative group:** (Table 4, graph 20).

The control batch showed 0.081±0.0054mg proteins in its sublingual gland. There was significant decrease in proteins to 0.0344 ± 0.0035 mg in Dg → saline batch. In Dg → cent and Dg → WSG there was significant (P<0.001) increase in total proteins and was 0.082 ± 0.0021 mg and 0.0846 ± 0.0024 mg respectively. When compared Dg → cent and Dg → WSG batches there was non significant (P>0.1) difference in protein contents.

B. **Effects of glycowithanolides on Esterase activity (in µmols p-nitrophenol/mg proteins) in salivary glands of D-galactose stressed male mice.**

a. **Submandibular gland of adult protective group:**

The esterase activity was significantly decreased in submandibular gland of D-galactose treated mice but in D-galactose along with centrophenoxyine or glycowithanolides treatment there was significant increase in esterase activity as compared to Dg and even compared to control (Table 5, graph 21).

b. **Sublingual gland of adult protective group:**

The esterase activity in control batch was 42.3 ± 0.781 µmols. It was significantly (P<0.001) decreased to 33.616 ± 1.6527 µmols in Dg- treated batch. In Dg + cent and Dg + WSG it was significantly (P<0.001) increased to 44.66 ± 0.4277 and 47.8 ± 0.4472 µmols respectively as compared to Dg batch. In Dg + WSG batch there was significant increase (P<0.001) when compared to Dg + cent batch. (Table 5, graph 22).

c. **Submandibular gland of adult curative group:**

In control batch the esterase activity was 57.4 ± 0.5477 µmols and was decreased significantly in Dg → saline treated batch. In Dg → cent and Dg → WSG batches it was significantly increased. The increase in
esterase activity was highly significant in Dg → WSG batch as compared to Dg → cent batch. (Table 5, graph 23).

d. Sublingual gland of adult curative group:

Esterase activity in control batch was 41.2 ± 0.8366 µmols and was significantly decreased to 33.8 ± 1.788 µmols in Dg → saline batch. After receiving centrophenoxine or WSG sublingual gland showed significant increase in esterase activity and was 42.8 ± 0.4472 µmols and 46.6 ± 0.5477 µmols respectively. In WSG treatment the increase was highly significant than Dg → saline, Dg → cent and control batch. (Table 5, graph 24).

e. Submandibular gland of old protective group:

Submandibular gland of control batch contains 49.4 ± 0.5477 µmols esterase and was decreased significantly to 41.8 ± 1.3032 µmols in Dg-treated batch. In Dg + cent and Dg + WSG batches esterase activity was significantly increased to 53.6 ± 0.5377 and 54.6 ± 0.5477 µmols respectively. But there was no significant difference between Dg + cent and Dg + WSG esterase activity. (Table, 6 graph 25).

f. Sublingual gland of old protective group:

In control batch esterase activity was 40.4 ± 0.5477 µmols and was decreased (P<0.001) to 30.8 ± 0.4472 µmols in Dg-treated batch. There was significant increase in Dg + cent and Dg + WSG batches to 42.0 ± 0.7071 and 43.0 ± 1.5811 µmols respectively. No significant difference (P>0.1) between Dg + cent and Dg + WSG batches. (Table 6, graph 26).

g. Submandibular gland of old curative group:

Esterase activity was decreased in Dg → saline batch. It was significantly increased in Dg followed centrophenoxine or WSG treated mice, but no significant difference between Dg → cent and Dg → WSG batches. (Table 6, graph 27).
**h. Sublingual gland of old curative group:**
Batch received saline after 20 days treatment of D-galactose showed significant decrease in esterase activity as compared to control batch. But after receiving centrophenoxyine or glycophanolides, significant increase in esterase activity was noticed. (Table 6, graph 28).

**C. Effects of glycophanolides on histochemical changes in esterase in D-galactose stressed male mice.** (Plates 5, 6, 7, & 8)

**a. Submandibular gland of adult male mice.** (Plate 5, Figs. 1 to 8).

The cross section of submandibular gland, of control batch of adult male mice of protective group showed dark red colored esterase activity in granular convoluted tubules. (Plate 5, Fig.1).

The cross section of submandibular gland of Dg-treated batch of adult protective group showed reduction in esterase positive staining reactivity in GCT. (Plate 5, Fig. 2)

The submandibular gland of mice received centrophenoxyine along with D-galactose showed intense red staining in GCT, indicating increase in esterase activity. (Plate 5, Fig. 3)

Significant increase in staining intensity was also observed in mice received glycophanolides. (Plate 5, Fig. 4)

In curative group intense esterase activity at GCT was also observed in the salivary glands of D-galactose stressed mice after receiving centrophenoxyine and glycophanolides, but not in the GCT of submandibular gland of mice receiving saline. (Plate 5, Figs. 5 to 8)

**b. Sublingual gland of adult male mice.** (Plate 6, Figs. 1 to 8)

The cross section of sublingual gland of control batch of adult protective group showed esterase activity in serous demilune (DM) and
acinar cells (AC) which were stained reddish. Esterase activity was not observed in mucous acini (MA). (Plate 6, Fig. 1).

In D-galactose treated batch of adult protective group intensity of red color was significantly decreased indicating reduction in esterase activity in sublingual gland. (Plate 6, Fig. 2)

Mice received centrophenoxine or WSG along with D-galactose showed significant increase in esterase activity in demilune (DM), acinar cells (AC), excretory duct (ED) and striated duct (SD), as intensity of red color was more as compared to control and Dg batches. (Plate 6, Figs. 3 & 4)

The curative group also showed same pattern of staining in its all batches like that of protective group but staining intensity was slightly less than respective batches of protective group (Plate 6, Figs. 5 to 8).

c. **Submandibular gland of old male mice.** (Plate 7, Figs. 1 to 8)

In submandibular gland of control batch in old protective group showed damaged GCT and acinar cells (AC) and reduced staining intensity, few GCT indicated reduction in esterase activity (plate 7, Fig. 1).

Similar results were also observed in submandibular gland of control batch of old curative group (plate 7, Fig. 5).

The cross section of submandibular gland of Dg-treated batch of old protective group showed reduced staining intensity in GCT, indicating decrease in esterase activity than control batch. (Plate 7, Fig. 2)

In mice receiving centrophenoxine or WSG along with Dg, showed still intense staining in GCT than control. (Plate 7, Figs. 3 & 4)

Batches of curative group showed similar staining pattern but staining intensity was relatively less as compared to batches of protective group (Plate 7, Figs. 6, 7 & 8).

d. **Sublingual gland of old male mice** (Plate 8, Figs. 1 to 8)
The cross section of sublingual gland of control batch of old protective group, showed increased esterase activity in demilune (DM) and acinar cells (AC) (Plate 8, Fig. 1) and was decreased significantly in Dg-treated batch (Plate 8, Fig. 2).

Batch received centrophenoxine along with D-galactose, showed increased intensity of red color in demilune (DM), striated duct (SD), acinar cells (AC) and also WSG receiving batch (Plate 8, Figs. 3 & 4).

Esterase activity was still reduced in control batch of curative group (Plate 8, Fig. 5) than control batch.

The cross section of sublingual gland of Dg → saline batch showed esterase activity in excretory duct (ED) only (Plate 8, Fig. 6).

Sublingual gland of Dg → cent batch showed increased esterase activity but was less than Dg + cent batch (Plate 8, Fig. 7).

The cross section of sublingual gland of Dg → WSG batch showed further increased intensity of red color in demilune (DM) than control, Dg → saline and Dg → cent batches indicated increased esterase activity. (Plate 8, Fig. 8)

**D. Kinetic study of Esterase**

a. **Effect of substrate concentration on Esterase activity in salivary glands**

The enzyme activity assay was determined as per reaction at different substrate concentrations (described in chapter II Material and Methods) under optimum pH and temperature. Plot \(1/v \text{ Vs } 1/s\) was used to determine \(K_m\) and \(V_{max}\). In all samples esterase affinity for the substrate was not changed, it was 1.5 \(\mu\)mols of P-nitrophenyl acetate. Though there was no change in the km, Velocity max for control, centrophenoxine treated and WSG treated salivary glands was increased. There was reduction in Vmax in Dg treated adult and old and naturally old mice.
Km and Vmax of esterase activity from submandibular gland graphically represented in graph numbers 29, 30, 31 and 32. The Km and alterations in Vmax were of same nature, so only graphs describing Km and Vmax of submandibular esterase are included and not of the sublingual gland, but all Km and Vmax values are depicted in table No.7.

E. Effects of glycowithanolides on amylase activity (in µg maltose/mg proteins) in salivary gland of D-galactose stressed male mice.

(Table 8, graphs 33, 34, 35, 36 and Table 9, graphs 37, 38, 39, 40)

a. Submandibular gland of adult male mice of protective group:

Amylase activity in control batch was 4517 ± 64.8652 µg maltose/mg proteins and was significantly decreased to 3550 ± 205.010 µg maltose/mg proteins in Dg-treated batch. In Dg + cent, it was increased significantly than Dg batch and was 5552 ± 552.58. In Dg + WSG batch, it was still increased significantly to 5911 ± 37.8153 µg maltose/mg proteins but no significant difference in Dg + cent of Dg + WSG batches. (Table 8, graph 33).

b. Sublingual gland of adult protective group: (Table 8, graph 34).

There was significant decrease in amylase activity in D-galactose treated batch but increased significantly in both batches received centrophenoxine and WSG along with D-galactose and it was 2515.4 ± 179.4165 and 2625 ± 78.74 µg maltose/mg proteins respectively.

c. Submandibular gland of adult curative group: (Table 8, graph 35).

In control batch amylase activity was 3875 ± 34.2782 µg maltose/mg proteins and it was significantly decreased (P<0.001) to 3095.8 ± 47.1826 µg maltose/mg proteins in Dg → saline batch. In Dg → cent and Dg → WSG batches it was significantly increased to 4017 ± 23.8746 µg maltose/mg proteins and 4727 ± 433.4685 µg maltose/mg
proteins respectively. The increase in amylase activity in Dg → WSG batch was significant when compared to Dg → cent.

d. Sublingual gland of adult curative group :( Table 8, graph 36).
Amylase activity was 1785 ± 22.3606 µg maltose/mg proteins in control batch and was significantly decreased to 1120 ± 27.3861 in Dg → saline treated batch. Mice received centrophenoxine or WSG after D-galactose treatment the amylase was 2045 ± 44.7213 and 2142 ± 27.5227 µg maltose/mg proteins respectively. No significant difference was observed in both Dg → cent and Dg → WSG batches.

e. Submandibular gland of old protective group : ( Table 9, graph 37).
Amylase activity in submandibular gland of control batch was 2974 ± 6.5192 µg maltose/mg proteins and it was significantly decreased to 2166 ± 88.2043 µg maltose/mg proteins in Dg-treated batch. In Dg + cent and Dg + WSG batches, it was significantly increased to 2976 ± 11.9373 and 3024.8 ± 31.5230 µg maltose/mg proteins respectively. The increase in Dg + WSG batch was significant (P<0.05) than Dg + cent batch.

f. Sublingual gland of old protective group : ( Table 9, graph 38).
In sublingual gland of control batch amylase activity was 945 ± 3.7682 µg maltose/mg proteins and it was decreased significantly to 801.2 ± 2.8635 µg maltose/mg proteins in Dg-treated batch. Centrophenoxine or WSG treatment along with D-galactose showed significant increase in amylase activity to 1004 ± 8.07465 and 1022 ± 4.9497 µg maltose/mg proteins respectively. No significant difference between Dg + cent and Dg + WSG batches.

g. Submandibular gland of old curative group: (Table 9, graph 39)
In control batches amylase activity was 2456 ± 16.3401 µg maltose/mg proteins and was significantly decreased to 2074 ± 16.6042
µg maltose/mg proteins in Dg → saline batch. In Dg → cent and Dg → WSG batches, it was significantly increased to 2444.4 ± 17.9397 and 2472.4 ± 14.0107 µg maltose/mg proteins respectively. But no significant difference in Dg → cent and Dg → WSG batches in amylase activity.

**h. Sublingual gland of old curative group:** (Table 9, graph 40).

Mice received saline for 40 days showed 821.6 ± 0.894 µg maltose/mg proteins amylase in its sublingual gland but significantly decreased to 802.2 ± 1.9235 µg maltose/mg proteins in Dg → saline batch. In Dg → cent and Dg → WSG batches, it was significantly increased to 892.6 ± 2.3021 and 916.0 ± 19.8116 µg maltose/mg proteins respectively when compared to Dg → saline batch. In Dg → WSG increase in amylase activity was almost significant (P<0.05) as compared to Dg → cent batch.

**F. Effects of glycowithanolides on Histochemical Changes in amylase activity in D-galactose stressed mice.**

**a. Submandibular glands of adult male mice:** (Plate 9, Figs. 1-8)

Submandibular gland of control batch of protective group showed normal structure of GCT and acinar cells (AC). The amylase activity was observed in the form of white dots in GCT. (Plate 9, Fig. 1)

Dg-treated batch of protective group showed damaged GCT and acinar cells (AC) and very poor amylase activity in GCT in the form white unstained areas. (Plate 9, Fig. 2)

Mice received centrophenoxine along with D-galactose showed increased white dots in GCT indicating increased amylase activity. (Plate 9, Fig. 3)

The cross section of submandibular gland of Dg + WSG batch showed enormous increase in amylase activity in GCT which was observed in the form of many white dots. (Plate 9, Fig. 4)
Control batch of curative group showed normal structure of GCT and acinar cells (AC). White areas corresponded to amylase activity but in this batch amylase activity was slightly less than control of protective group. (Plate 9, Fig. 5)

More blue area and very less white area was observed in submandibular gland of Dg → saline batch, indicating decreased amylase activity than control. (Plate 9, Fig. 6)

In a cross section of submandibular gland of Dg → cent of curative group, showed increased unstained white dots in GCT indicating increased amylase activity. (Plate 9, Fig. 7)

Mice received WSG after D-galactose treatment, showed increased white granular dots in GCT indicated increased amylase activity but less than Dg + WSG. (Plate 9, Fig. 8)

b. Sublingual glands of adult male mice : (Plate 10, Figs. 1-8).

The cross section of sublingual gland of control batch of protective group, showed normal structure of mucous acini (MA), demilune (DM), striated duct (SD). Amylase activity was in mucous acini (MA) indicated in the form of white area. (Plate 10, Fig. 1)

Sublingual gland of Dg-treated batch showed a very poor amylase activity, in mucous acini (MA) than control. (Plate 10, Fig. 2)

In Dg + cent batch of protective group showed increased white area in mucous acini (MA) indicated increased amylase activity. (Plate 10, Fig. 3)

The cross section of sublingual gland of Dg + WSG batch showed increased size of MA and increased amylase activity. (Plate 10, Fig. 4)

Amylase activity was observed in mucous acini (MA) in sublingual gland of control batch of curative group. (Plate 10, Fig. 5).

The cross section of sublingual gland showed decrease in size of mucous acini and also decreased amylase activity. (Plate 10, Fig. 6)
Section of sublingual gland of Dg → cent batch showed increased white granules in mucous acini indicated increased amylase activity. (Plate 10, Fig. 7)

Mice received WSG after D-galactose treatment showed increase in amylase activity in mucous acini. (Plate 10, Fig. 8)

c. Submandibular glands of old male mice : (Plate 11, Figs. 1 - 8).

Submandibular gland of control batch of protective group showed shrunked GCT. White dotted area in GCT showed amylase activity. (Plate 11, Fig. 1)

The cross section of submandibular gland of Dg-treated batch of protective group showed damaged GCT with very few white dots indicating very poor amylase activity as compared to control. (Plate 11, Fig. 2)

Increased white dots in GCT indicated increased amylase activity in submandibular gland of Dg + cent batch. (Plate 11, Fig. 3) WSG treatment along with D-galactose also showed similar results. (Plate 11, Fig. 4)

As compared to batches of protective group, curative group showed decreased amylase activity in its all batches in the same pattern i.e. decreased amylase in Dg → saline but increase in Dg → cent and Dg → WSG batches. (Plate 11, Figs. 5 to 8)

d. Sublingual glands of old male mice : (Plate 12, Figs. 1 - 8).

Plate 12, Fig. 1 is a cross section of sublingual gland of control batch of old protective group showed normal structure with whitish area in mucous acini (MA) represented amylase activity.

The cross section of sublingual gland of Dg-treated batch of old protective group showed decreased amylase activity in GCT as most of the areas remain blue. (Plate 12, Fig. 2)
Treatment of centrophenoxine or glycowithanolides along with D-galactose showed increased white area in mucous acini indicated increased amylase activity. (Plate 12, Figs. 3&4)

No significant change was observed in amylase activity in sublingual gland of curative group than protective group. (Plate 12, Figs. 5 to 8).

G. Kinetic Study of Amylase

• Effect of substrate concentration on amylase activity in salivary Glands:

\( \alpha \)-amylase activity assay was determined as per reaction at different substrate concentrations (described in chapter II Material and Methods) under optimum pH and temperature. Plot \( 1/v \) Vs \( 1/s \) was used to determine \( K_m \) and \( V_{max} \). In all samples amylase affinity for the substrate was not changed, it was 0.4% of starch. There was no change in the \( K_m \) of amylase; Velocity max for control, centrophenoxine and WSG treated salivary gland was increased. There was reduction in Velocity max in Dg-treated adult and old and naturally old mice. \( K_m \) and \( V_{max} \) of amylase activity from submandibular gland graphically represented in graph numbers 41, 42, 43 and 44. The \( K_m \) and alterations in \( V_{max} \) were of same nature, so only graphs describing \( K_m \) and \( V_{max} \) of submandibular amylase are included and not of the sublingual gland, but all \( K_m \) and \( V_{max} \) values are depicted in table No 10.

4. DISCUSSION:

Previous studies have indicated age related changes in the function of salivary glands of human and rodents. The rate of flow is reduced in elderly persons (Meyer and Necheles, 1940; Pedersen et al, 1985; Navazes et al, 1992; Percival et al, 1994; Yeh et al, 1998). About 30% of the elderly persons suffer from oral dryness and related complaints. Therefore it is often assumed that salivary secretion reduces with age.
(Vissink et al, 1997). The proteins are synthesized and secreted by granular convoluted tubular cells and ducts cell including striated duct cells (Cutler et al, 1991; Acini et al, 1999). Several studies reported age related decline in rate of salivary glands protein synthesis and m-RNA (Kuyatt and Baum, 1981; Kim, 1981; Kim and Allen, 1993).

There was significant decrease in total protein content in both salivary glands in aging and stressed mice. But when mice of both the groups were treated with galactose along with plant drug glycowithanolides and synthetic drug centrophenoxine, there was no loss in protein content. Mice receiving drugs after stress also showed enzyme activity similar to control batch. One remarkable observation is that there was no significant difference in the treatment of both the drugs.


The decrease of protein content can be related to destruction of protein synthesizing machinery in salivary glands or due to destruction of salivary glands structure. Significant decline in the synthesis of proteins was observed during old age (Rattan, 1996). The damage of normal structure of salivary glands was described in chapter III (Plates 1 & 2, Figs. 2 & 6 and plate 3 & 4, Figs. 1 & 5). Age related decrease in the synthetic capacity of ribosomes may be due to reduction in the number of active ribosomes (Britton and Sherman, 1975; Kurtz, 1978). Not only damaged gland structure or cellular elements reduce the protein synthesis but also free radical attack on DNA. Damage of DNA could not
transcribe and translate through various RNAs, ultimately reduce the protein synthesis (Browman, 1976). Previous studies have indicated that, age related changes in microsomes, causes cellular changes in protein synthesis due to the reduction in the number of active ribosomes (Buetow and Gandhi, 1973; Britton and Sherman, 1975). According to Kim et al, (1980) age related decline in protein synthesis in parotid gland might be due to reduced responsiveness of salivary glands to cholinergic stimulation. According to Kuyatt and Baum, (1981) low protein content in submandibular gland of aged rats might be due to decrement in biosynthetic process or increased intracellular protein degradation. Lysosomal instability during aging was described by several workers which may lead degradation of protein. The free radicals cause damage to the biomolecules like DNA, RNA, proteins and enzymes (Leibovitz and Siegel, 1980; Akiyama, 1999). D-galactose induces oxidative stress followed by accelerated aging and formation of advanced glycation end product (Song et al, 1999; Deshmukh et al, 2006). Ho et al, (2003) found that D-galactose induced aging effects like increased malondialdehyde and decreased SOD activity with accumulation of AGEs in natural aging. AGEs cross link with proteins and form insoluble aggregate, which can disturb physiological function. Oxidative stress and lipid peroxidation have been observed to increase Reactive Oxygen Species (ROS). Production of ROS is also reported in aging (Pillai et al, 2002; 2003; Gajare et al, 2006; Deshmukh et al, 2006) during D-galactose induced aging. ROS react with H₂O₂ to generate OH⁻ radicals which in turn enhance lipid peroxidation and decreased membrane fluidity.

Venkataraghavan et al, (1980) studied growth promoting effect of Ashwagandha in healthy children of age 8–12 years old and found significant increase in total proteins and body weight. Increase in protein content in salivary glands might be due to regeneration of salivary gland
cells promoted by glycowithanolides (Mote et al, 2009). Rajangam et al, (2009) found increase in serum and tissue protein in W. somnifera roots and leaves extract Alloxan induced diabetic rats. W. somnifera has been reported to produce anabolic effect, enhancing the synthesis of certain modulator proteins in rat liver and increasing the body weight in human (Anbalagan and Sadique, 1981).

Significant decrease in total proteins and m-RNA synthesis during aging was reversed by centrophenoxine treatment in rats (Zs- Nagy, 1989). He also reported that centrophenoxine reversed the age dependent decrease of water solubility of proteins and increase of total protein content in the brain of old rats.

Regenerative changes in salivary gland structure in D-galactose and naturally aged mice received centrophenoxine and glycowithanolides were observed (described in Chapter III). The improvement in salivary structure and GCT was noticed at structural level. Protein content, amylase activity increased in centrophenoxine treatment in galactose receiving and aging mice. These regenerative changes in secretory cells, might regain their secretory ability. So, there was increased total protein concentration in submandibular and sublingual glands of Dg + cent, Dg + WSG, Dg → cent and Dg → WSG batches of this study.

Centrophenoxine and glycowithanolides are powerful antioxidants and free radical scavengers. They might protect the cells from damaging and preserve their secretory ability. From the above discussion it is clear that during stress and after stress, compared to centrophenoxine, WSG is more powerful though there seems to be no significant difference. W. somnifera has been reported to be a potent enhancer of cellular antioxidants and possesses a significant free radical scavenging activity in various disease models (Bhattacharya et al, 1997; 2001; Davis and Kuttan, 2000). Gupta et al, (2003) reported increased
Glutathioneperoxidase activity and inhibition of lipid peroxidation in *W. somnifera* treatment in aging spinal cord of Wister rats it also inhibits protein oxidative modification. Ashwagandha leaf is a potential agent in treating oxidative damage and physiological abnormalities in mouse model of Parkinson’s disease. Treatment of *W. somnifera* extract significantly improved SOD, CAT and malondialdehyde antioxidant levels in brain of mouse model of Parkinson’s disease (Rajasankar *et al*, 2009).

Changes in the lysozymes and their secretion in senescent tissue have been used as biomarker (Bolanowaski *et al*, 1983) and the abnormalities in lysosomal protein degradation pathways are supposed to contribute pathology associated with age. Bogart, (1967) demonstrated age related decrements occurring with several rat submandibular gland hydrolytic enzymes (Succinic dehydrogenase, non specific esterase, cholinesterase and alkaline phosphates). Przybylo *et al*, (2004) found decreased specific activity of lysosomal hydrolases parallel to increasing age of the animals. Earlier also several studies reported age related changes in lysosomal enzymes in various tissues, in retinal pigment epithelium (Gorthy *et al*, 1971; Gorthy, 1978), in cardiac muscle cells (Kurne and Pillai, 1993), in brain (Constantinesue, 1981; Nakamura *et al*, 1989; Ashokan, 1993; Tomake and Pillai, 1995 and Vora, 2005), in salivary glands (Tomake and Pillai, 1993; Yoshimura and Mori, 2005).

Decreased esterase activity in D-galactose and naturally aged mice, can be correlated with significant decrease in total proteins content in submandibular and sublingual glands of D-galactose stressed and naturally aged mice. Activities of all enzymes taking part in the secretory process are decreased with age or it may be due to instability of lysosomes in aging. Through lysosomal membrane there may be leakage of lysosomal enzymes responsible for degradation of cellular proteins.
involved in various metabolic processes and protein synthesis. Tomake and Pillai, (1993) showed increase in cytosolic lysosomal enzyme activity compared to the enzymes from proper lysosomes. The formation of AGEs may be responsible for oxidative burden on lysosomes bringing peroxidation of lysosomal enzyme or formation of cross linkages between enzymes making lysosomal enzymes insufficient in the disposal of cellular garbage in salivary glands. Enzyme activity decreased due to changes in the environment of the cytoplasm, loaded with nonfunctional lysosomes in brain, these are called lipofuscin granules. Increased free radicals damage leading to accumulation of lipofuscin granules and lysosomes get overloaded, so it becomes difficult to degrade wastes (Brunk, 2002). Supplementation and treatment of antioxidants may help in prevention and removal of free radicals and free radical damage, which are the causes of lysosomal insufficiency. Esterase being the lysosomal enzyme increased its activity at biochemical and structural level. Antioxidant ability of W. somnifera and centrophenoxine described by Bhattacharya et al, (2001); Gupta et al, (2003); Harikrishan et al, (2008); Rajasankar et al, (2009); Zs Nagy and Nagy (1980).

This view is supported by our observations in this case increase in lysosomal enzyme activity. Hydrocotyl asiatica is potent antioxidant plant when fed to the old mice it showed increase in the lysosomal enzymes (Pillai et al, 2003). Similar results were obtained in the mice fed with Lactuca sativa, where tremendous increase in the lysosomal enzyme was noticed (Pillai et al, 2002). Vora, (2005) found significant increase in esterase activity in Parsley treated mice. Pillai et al, (2008) showed increased level of esterase enzyme in lysosomal fraction of brain tissue in mice received Brahmi extract and reported that Brahmi extract protect the lysosomal membrane and act as stabilizing agent by decreasing oxidative stress. This shows that the antioxidants like glycowithanolides and
centrophenoxine protect the lysosomes and lysosomal degradation during aging. More effect was noticed in case of glycowithanolides.

Salivary amylase is a major protein in mouse saliva. It is a common secretory enzyme in salivary gland of many mammals. Smith et al, (1971); Chrestein, (1977) histochemically proved that amylase activity is located in GCT of submandibular gland. Previous studies have indicated age related changes in structure and functions of the salivary glands in human and rodents. The rate of salivary flow, enzyme content of saliva and the content of α-amylase reduced in elderly persons (Meyer and Necheles, 1940; Chilla et al, 1974). Kim, (1981); Kim and Arisumi, (1985), reported significant decline in amylase synthesis rate in rat parotid gland with age.

In present study amylase activity was reduced in D-galactose stressed and naturally aged male mice. The reason for this decline in amylase activity can be relate with structural changes and reduction in protein synthesis in salivary gland. Structural damage in salivary glands of D-galactose stressed and naturally aged mice was observed (Described in Chapter III). Reduction in the synthesis of secretory proteins may be due to structural damage. Since the acinar cells are responsible for protein synthesis and release, it was relevant to investigate the effect of aging on amylase release. Meisel et al, (1988) revealed that the basal amylase levels are reduced during aging may possibly be due to age related destruction of acinar cells in the parotid glands. Baum, (1987) reported decrease in synthesis of amylase during old age. Kim and Arisumi, (1985) reported decline in the rate of amylase synthesis due to decline in rate of protein synthesis with age. Kim and Allen, (1993) reported decline in the rate of protein synthesis and their m-RNA in salivary glands of aged animals. Tissues from older animals are less active in protein synthesis due to changes in microsomes (Buetow and Gandhi, 1973;

Zs-Nagy Imer, (1989) reported OH⁻ radical scavenging capacity of centrophenoxine prevents cellular damage. Centrophenoxine might be increasing amylase activity by increasing total protein m-RNA synthesis. Similarly glycowithanolides are also powerful antioxidant and free radical scavenger (Bhattacharya et al, 1997). It prevents cellular damage due to free radicals by increasing concentrations of antioxidant enzymes like SOD, CAT and GPx. Significant improvement in architecture and weight of salivary glands indicated repair process in gland, regaining protein synthesis ability of gland and ultimately increased activity of amylase in salivary gland.