CHAPTER-III

EFFECTS OF

GLYCOWITHANOLIDES

ON SALIVARY GLAND’S STRUCTURE

OF D-GALACTOSE STRESSED MICE
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1. INTRODUCTION:

Extrinsic salivary glands located external to the oral cavity and occur bilaterally are named as submandibular glands, sublingual glands and parotid glands, open into the mouth cavity by their long excretory ducts. The submandibular glands consist of parenchymal component which includes seromucous acini, serous granular convoluted tubules and duct system while the stromal component includes fibrous connective tissue, blood vessels, lymph vessels and nerve fibers. The sublingual glands consist of mucous acini, capped with serous demilune and duct system.

Several studies have reported age related changes in histology of salivary glands. They are described in chapter I.

2. MATERIAL AND METHODS:-

A. MATERIAL:

a. ANIMAL:

Male mice (Mus musculus) were used for the study. The breeding pairs were obtained from Hindustan Antibiotics Pune, and were reared in air-conditioned departmental animal house. 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 two main groups viz. protective and curative group. Each group further divided into 4 batches as below

• Batch A: Control

Both adult and old male mice were received 0.5 ml 0.9% saline per day subcutaneously for 20 days in case of protective group and 40 days to curative group.
• Batch B: D-galactose stressed

Adult and old male mice were received 0.5ml, 5% D-galactose per day subcutaneously for 20 days for protective group (Dg-treated), while curative group received 0.5ml, 5% D-galactose per day subcutaneously for 20 days and then followed by 0.5ml, 0.9% saline per day for next 20 days subcutaneously (Dg → saline).

• Batch C: Centrophenoxine treated

Both adult and old male mice were received 0.5 ml 5% D-galactose along with centrophenoxine (80 mg/kg body weight) per day subcutaneously for 20 days for protective group (Dg + cent), while curative group received 0.5 ml 5% D-galactose per day for 20 days and then followed by 0.5 ml centrophenoxine (80 mg / kg body wt.) per day for further 20 days subcutaneously (Dg → cent).

• Batch D: Glycowithanolides treated

Protective group of both adult and old male mice were received 0.5 ml 5% D-galactose along with WSG (20 mg/kg body weight) per day for 20 days (Dg + WSG). Curative groups received 0.5 ml 5% D-galactose per day for 20 days and then followed by dose of 0.5 ml WSG (20 mg/kg body weight) per day for further 20 days subcutaneously (Dg → WSG).

All operations were carried out between 09.00 am and 12.00 am.

B. METHODS:

a. Determination of Body Weight and Glands Weight of Animals

i. Determination of body weight of mice in gm.

Animals were weighed before starting of drug treatment and after completion of drug treatment (at the time of sacrifice) and record of their body weight was maintained.

ii. Determination of glands weight of mice in mg.

After completion of drug treatment animals were sacrificed, submandibular and sublingual glands of both sides were removed and wet
weight of these glands was taken immediately and record of their weight was maintained. Then glands were used for further studies.

b. **Histology by Haematoxylene–Eosin (H/E) technique:**

Age related changes in salivary glands were studied by histological method. Histological studies were made by taking 10% formalin fixed 7 µ sections of salivary glands, stained with routine Haematoxylene–Eosin (H/E) technique. Details of technique were described in the chapter II.

3. **RESULTS:**

   A. **Changes in body weight and glands weight of mice:**

   a. **Changes in body weight of adult male mice of protective group:**

   Illustrated in Table 1 Graph 1.

   The body weight of control male mice was 55 ± 1.5811 gm which was reduced to 41.4 ± 1.1401 gm in D-galactose received mice and this reduction in the weight was significant (P<0.001). The body weight of mice received D-galactose along with centrophenoxine was increased to 65.8 ± 0.8366 gm and this increase was highly significant (P<0.001) as compared to D-galactose treated mice.

   The body weight of mice received WSG along with D-galactose was increased to 67.6 ± 0.5477gm. The increase was highly significant (P<0.001) as compared to D-galactose stressed mice but non-significant (P>0.1) as compared to centrophenoxine treated mice.

   b. **Changes in glands weight of adult male mice of protective group:**

   Given in Table 1, graph 2 & 3

   i. **Weight of submandibular glands (Table 1, graph 2):**

   The weight of submandibular gland of control batch was 162.4 ± 2.073 mg and was reduced significantly (P<0.001) to 130.6 ± 1.5165 mg in Dg-treated batch. In Dg + cent batch the weight of submandibular glands was increased to 176 ± 2.9154 mg and this increase was highly significant (P<0.001) as compared to Dg-treated batch. In Dg + WSG
batch the weight of submandibular gland was 182.4 ± 2.0736 mg. Increase in weight was highly significant (P<0.001) as compared to Dg-treated batch but non-significant (P>0.1) as compared to Dg + cent batch.

ii. Weight of sublingual glands (Table 1, graph 3):

The weight of sublingual glands of control batch was 25.2 ± 1.0954 mg and decreased to 13.8 ± 0.8366 mg in Dg-treated batch, decrease was highly significant (P<0.001). In Dg + cent batch weight of sublingual gland was significantly (P<0.001) increased to 28.4 ± 2.6076 mg as compared to Dg-treated batch. While in Dg + WSG batch it was increased to 37.6 ± 2.6076 mg. The increase was highly significant (P<0.001) as compared to Dg-treated batch but almost significant (P<0.05) as compared to Dg + cent receiving mice.

c. Changes in the body weight of adult curative group:

Illustrated in Table 1, graph 4

The body weight of control batch was 52.6 ± 0.8944 gm and was decreased to 44.6 ± 0.5477 gm in Dg → saline batch the difference was highly significant (P<0.001) as compared to control batch. The body weight of Dg → cent batch was 53.6 ± 1.1401 gm and was significantly increased (P<0.001) compared to Dg → saline batch. In Dg → WSG batch body weight was 56.8 ± 2.1679 gm and was highly significant (P<0.001) to Dg → saline batch but non significant (P>0.1) to Dg → cent batch.

d. Changes in glands weight of adult curative group.

Given in Table 1, graph 5&6

i. Weight of submandibular glands (Table 1, graph 5):

The weight of submandibular glands of control batch was 160.2 ± 1.6431 mg and was decreased significantly to 125.2 ± 1.6431 mg in Dg → saline batch. In Dg → cent batch the weight of submandibular gland was increased to 174.4 ± 2.880 mg and increase was highly significant to
Dg → saline batch. In Dg → WSG batch submandibular gland weight was significantly increased (P<0.001) to 180.2 ± 1.6431 mg as compared to Dg → saline batch but non significant (P>0.1) to Dg → cent batch.

ii. Weight of sublingual glands (Table 1, graph 6):

The weight of sublingual glands in control batch was 25.6 ± 1.1401 mg decreased to 14.8 ± 0.8366 mg in Dg → saline batch (P<0.001). In Dg → cent batch sublingual gland weight was increased to 26.6 ± 1.5165 mg as compared to Dg → saline batch (P<0.001). In Dg → WSG batch increase was 30.4 ± 1.1401 mg and nearly doubled compared to Dg receiving batch.

e. Changes in body weight of old male mice of protective group:

Depicted in Table 2, graph 7

The body weight of the control old male mice was 47.6 ± 0.5477 gm and was significantly (P<0.01) reduced to 44.0 ± 1.5811 gm in Dg-treated batch. It was significantly increased (P<0.001) in Dg + cent batch to 56.8 ± 0.8366 gm and 57.4 ± 0.5477 gm in Dg + WSG batch as compared to Dg-treated batch, but increase was non significant when Dg + cent and Dg + WSG batches were compared.

f. Changes in glands weight of old protective group:

(Table 2, graph 8 & 9)

i. Submandibular glands weight (Table 2, graph 8):

The weight of the submandibular gland was 129.2 ± 1.6431 mg of control but it was reduced to 97.8 ± 1.3068 mg in Dg-treated batch. It was increased significantly (P<0.001) 156.4 ± 3.7815 mg in Dg + cent batch and 164.8 ± 1.3038 mg in Dg + WSG batch as compared to Dg-treated batch but non significant difference in the gland weight of Dg + cent and Dg + WSG batches.
ii. **Sublingual glands weight** (Table 2, graph 9):

The weight of sublingual glands of control batch was $19.6 \pm 1.1401$ mg and was decreased to $11.8 \pm 0.8366$ mg in Dg-treated batch while in Dg → cent and Dg → WSG batches, there was significant (P<0.001) increase in sublingual weight i.e. $23.0 \pm 1.5811$ mg and $26.8 \pm 1.6431$ mg respectively and there was non significant difference in weight of sublingual glands from Dg → cent and Dg → WSG batches.

g. **Changes in body weight of old curative group:**

(Table 2, graph 10)

In curative group the body weight of control batch was $44.8 \pm 0.4472$ gm, and reduced to $42.8 \pm 1.3038$ gm in Dg → saline batch, while in Dg → cent it was increased significantly (P<0.02) to $48.2 \pm 1.1510$ gm. There was significant (P<0.001) increase in body weight to $50.3 \pm 1.2041$ gm in Dg → WSG batch as compared to Dg → saline, while non significant when compared to Dg → cent treated mice.

h. **Changes in glands weight of old curative group:**

(Table 2, graph 11 & 12)

i. **Submandibular glands weight** (Table 2, graph 11):

The weight of submandibular glands of control was $128.8 \pm 1.4832$ mg and was decreased to $100.6 \pm 1.8165$ mg in Dg → saline batch and it was further increased to $153.6 \pm 3.6469$ mg in Dg → cent batch and $159.8 \pm 1.7888$ mg in Dg → WSG batch respectively. There was non significant (P>0.1) difference between Dg → cent and Dg → WSG batches.

ii. **Sublingual gland weight** (Table 2, graph 12):

Same trend was also observed in sublingual gland weight.

B. **Changes in histological structure of salivary glands:**

Histological alterations in salivary glands of adult and old male mice were depicted in Plate 1 to 4.
a. Changes in submandibular gland of adult protective and curative groups stained with H/E technique (Plate 1, Figs. 1 to 8).

Plate 1 Fig. 1 is a cross section of submandibular gland of control batch of adult protective group showed large number of small secretary acini with dark blue stained nuclei. Granular convoluted tubules (GCT) were distinct, stained dark with eosin and have dark blue nucleus in cell. Intercalated ducts (ID) were also distinct, scattered between the acini.

In submandibular gland of Dg-treated batch there was decrease in structural integrity of acinar cells, nuclear distribution was irregular, the presence of vacuolated cytoplasm and loss of GCT integrity (Plate 1 Fig. 2).

There was increase in number and size of acinar cells, large, distinct and darkly stained nuclei were seen. The GCT remained intact and enlarged in size in centrophenoxine batch (Plate 1 Fig. 3).

Structure of submandibular gland of adult Dg + WSG batch showed structural integrity and increased size of both acini as well as GCT as compared to control (Plate 1 Fig. 4).

Plate 1 Fig. 5 is a cross section of submandibular gland of adult control batch of curative group showed similar structure as that of control batch of protective group.

Cross section of submandibular gland of adult curative Dg → saline batch showed destruction of acini and GCT. Pyknotic nuclei were observed. There was a large gap between acini and GCT indicating reduction in the diameter of GCT (Plate 1 Fig. 6).

Cross section submandibular gland of adult Dg → cent batch showed increased number and size of acinar cells and increased size of GCT, cells of GCT not retained structural integrity as compared to that of Dg + cent batch (Plate 1 Fig. 7).
Section of submandibular gland of adult Dg → WSG batch of curative group showed well developed acinar cells and GCT were increased in size, regained structural integrity (Plate 1 Fig. 8).

b. Changes in sublingual glands of adult protective and curative groups (Plate 2 Figs. 1 to 8):

Section of sublingual gland of control batch of protective and curative groups of adult mice showed normal structure, consisted of mucus acini (MA), Demilune (DM), excretory duct (ED), striated duct (SD) and intercalated duct (ID). Mucus acini did not stain with eosin but demilune stained dark due to their serous nature (Plate 2 Fig. 1 and Fig. 5).

Cross section of sublingual gland of adult D-galactose batch showed pyknotic nuclei in striated duct and demilune, atrophy of mucus acini was seen, increase in ductal elements was observed (Plate 2, Fig. 2).

Plate 2, Fig. 3 is cross section of sublingual gland of adult Dg + cent batch of protective group showed structural recovery of mucus acini and striated duct to some extent but not in demilune.

Cross section of sublingual gland of adult Dg + WSG batch of protective group showed spectacular structural recovery in mucus acini, demilune and striated duct. There was increased size of nuclei (Plate2Fig.4)

Sublingual gland of adult Dg → saline batch of curative group showed destruction of mucus acini, demilune were not distinct and pyknotic nuclei in striated duct, demilune and excretory duct were observed (Plate 2 Fig. 6).

The cross section of sublingual gland of adult Dg → cent batch of curative group showed recovery in mucus acini structure but demilune were not distinct, pyknotic nuclei were observed in excretory duct and in demilune (Plate 2, Fig. 7).
While the cross section of adult Dg → WSG batch of curative group showed significant structural recovery in mucus acini, distinct demilune and nuclei were observed (Plate 2, Fig. 8).

c. Changes in submandibular glands of old protective group and curative group (Plate 3, Figs. 1 to 8):

Plate 3, Fig. 1 & 5 are cross sections of submandibular gland of control batches of old protective and curative groups respectively showed decreased volume of acini, atrophy and shrinkage of acini, destruction of cells of GCT.

Cross section of submandibular gland of old D-galactose batch of protective group showing reduction in size of GCT, more destruction of GCT cells as compared to control batch, acini showed pyknotic nuclei (Plate 3, Fig. 2).

Plate 3, Fig. 3 is cross section of submandibular gland of old Dg + cent batch of protective group showed increased size and number of the acini but acini were vacuolated, structure of the GCT was improved than the control

Plate 3, Fig. 4 is cross section of submandibular gland of old Dg + WSG batch of protective group showed increase in size of most of the acini, their number was also increased; GCT structure was well improved than control.

Submandibular gland of old Dg → saline batch of curative group showed destruction of nuclei, acini and increased fibrosis in stromal components. These changes were slightly improved as compared to Dg-treated batch of protective group (Plate 3, Fig. 6).

Submandibular gland of old Dg → cent batch of curative group showed increase in number of acini, GCT structure was improved than the control batch (Plate 3, Fig. 7).
Plate 3, Fig. 8 is cross section of submandibular gland of old Dg → WSG batch of curative group showed increased size of acinar cells as well as GCT.

d. Changes in sublingual glands of old protective and curative groups (Plate 4, Figs. 1 to 8):

The gland of old control batch of protective group showed shrinkage of mucus acini, no change was observed in demilune (Plate 4, Fig. 1).

Plate 4, Fig. 2 is cross section of sublingual gland of old Dg-treated batch of protective group showed loss of structural integrity of mucus acini. Demilune cells were reduced.

Sublingual gland of old Dg + cent batch of protective group retained their structural integrity (Plate 4, Fig. 3).

Dg + WSG batch showed increase in size and number of mucus acini and demilune cells, they became prominent and distinct than control group; nuclei were enlarged and stained more darkly. Eosin staining was intense in excretory duct and serous cells (Plate 4, Fig. 4).

Old control batch of curative group showing normal structure of demilune, mucus acini, striated duct and intercalated duct but number of mucus acini seem to be reduced as compare to control batch. Eosin reactivity was also decreased (Plate 4, Fig. 5).

Remarkable reduction in the size of acini and duct but staining reactivity for nuclei and cytoplasm was observed in the sublingual gland of old mice receiving D-galactose (Plate 4, Fig. 6).

Both the batches of old curative groups, D-galactose treated mice receiving centrophenoxine or WSG showed some alterations in the structure and staining reactions of acini and duct compared to only D-galactose receiving mice. Structural integrity and staining reactivity were similar to control group (Plate 4, Fig. 7 & 8).
4. DISCUSSION:

In the present investigation there was decrease in the body weight and glands weight in D-galactose stressed adult as well as in old male mice. When weight of normal old mice was compared with adult control, the loss in the weight was observed. Also loss in body weight as well as gland weight was remarkable in old mice receiving D-galactose. The reduction in body weight was reported by Bandopadhyay et al, (2003) in rat feeding with 50% galactose diet and Sonavane, (2007) in D-galactose treated mice. The reduction in body weight and glands weight in D-galactose stressed adult and old mice might be due to cellular damage by D-galactose mediated free radicals formation in adult as well as in old male mice. The free radicals are capable of damaging the biomolecules (Leibovitz and Seigel, 1980, Akiyama 1999). Imbalance between free radicals formation and free radicals scavenging capacity of cell resulting into cellular damage and ultimately facilitates the more production of free radicals causes more damage. These changes are most common with advancing age (Harman 1992). The sugars are capable of reacting with proteins, DNA and lipids without any enzymatic intervention, called advanced glycation. The complexes of sugars with macromolecules initiate a chain reaction form Advanced Glycation End products (AGEs). Accumulations of irreversible complexes of AGEs in the cells provoke, increase in the formation of free radicals (Munch et al 1996). D-galactose is a reducing sugar and shown to induce advanced glycation in the cell (Song et al 1999), which ultimately produce free radicals and damage salivary glands might be responsible for reduction in gland weight and body weight. Due to damage of functional GCT of salivary glands of D-galactose stressed and naturally aged mice (Plate 1, Figs. 2&6, Plate 3&4, Figs. 1&5) the production of EGF in salivary gland might be reduced and resulting into reduction of growth hormone secretion (Tustsumi 1987).
EGF plays an important role in maintaining tissue morphology, physiology, cytoprotection and renewal of epithelial healing in rats and mice (Acosta and Lizama 1998). The loss in body weight in salivary adenoectomised mice was reported by Okamoto and Oka, (1984); Tsutsumi and Oka, (1987); Bodare and Pillai (2007).

Thus in present study reduction in body weight and glands weight in D-galactose induced and naturally aged male mice might be due to free radicals mediated salivary glands damage. There was significant increase in the body weight and salivary glands weight, in both adult as well as in old mice treated with centrophenoxine and WSG. The increase in body weight was observed in *W. somnifera* powder receiving children (Venkatraghavan *et al*, 1980); rat (Sharma *et al*, 1986) and mice (Kumar and Kalonia, 2007). The increase in body weight and glands weight was also observed in centrophenoxine receiving mice. The centrophenoxine is a powerful free radical scavenger (Zs-Nagy and Nagy, 1980) and prevent free radical formation due to oxidative stress during aging (Anwer *et al*, 2008). Rajangam *et al*, (2009) observed recovery of body weight by *W. somnifera* treatment in alloxan induced diabetes rats. Thus *W. somnifera* is also responsible in the recovery of body weight loss during D-galactose stress and natural aging stress. The recovery in WSG treatment was more significant than centrophenoxine. This showed that the free radical scavenging activity of WSG was like centrophenoxine or more than that of centrophenoxine. Submandibular gland and sublingual glands of naturally aged and D-galactose stress adult as well as old mice showed loss of their normal architecture, loss of cellular integrity as well as loss in number of secretory units. During normal aging decrease in number of acinar cells (Scott *et al* 1986; Dong, 1989; Kim and Allen, 1993), increased amount of connective tissue (Wilde *et al*, 1986), atrophy of acinar and ductal epithelia (Scott, 1977a,b; Azevedo *et al*, 2005) were
observed by several workers. The loss of glandular structure during aging must be due to free radical reaction. Here the structural damage in salivary glands might be due to free radicals induced by D-galactose. D-galactose is aging inducing agent (Song et al, 1999; Deshmukh et al, 2006) causes free radical formation, leading to increased AGEs which accelerate natural aging process. The changes induced by D-galactose are similar to natural aging process. The free radical formation when exceeds the cell’s natural antioxidant quota, it results into oxidative stress. The given doses of D-galactose caused a significant oxidative damage to the salivary glands within very short time and induce the degenerative changes in them. In present study there was reduction in total proteins content in D-galactose stressed and old mice (Chapter IV Results, Table No. 3 & 4) which might reduce the cellular regeneration. Senescence accelerated decrease in IGF-1 protein may result into lower levels of cellular regeneration, proliferation and wound healing in aged oral tissue (Kobayashi et al, 2004). The spectacular improvement in architecture of salivary glands of adult as well as old male mice treated with WSG and centrophenoxine was observed. In old mice, there was structural recovery after WSG and centrophenoxine treatment though it was not up to the level of adult mice. This recovery was due to antioxidant properties of WSG and centrophenoxine. Many studies also showed free radical scavenging activity of flavonoids and phytophenolic compounds. *W. somnifera* tends to reverse the changes in lipid peroxidation and damage to cells (Dhuley, 2000). Bhattacharya et al (2000) reported that WSG of Ashwagandha provide protection to liver from lipid peroxidation due to heavy metal toxicity. Ashwagandha cure all negative conditions associated with aging (Kuppuragan et al, 1980) by its antioxidant properties. Bhattacharya et al, (1997) reported that WSG exerts significant antioxidant effects on various areas of rat brain by increasing
antioxidant enzymes like Super Oxide Dismutase (SOD), Catalase (CAT) and Glutathion Peroxidase (GPx). Davis and Kuttan, (2000) in histopathological analysis of urinary bladder reported that cyclophosphamide induced necrotic damage of urinary bladder was cured by *W. somnifera* treatment to its normal architecture. Palaniyandi *et al*, (2006) reported that *W. somnifera* along with paclitaxel inhibit free radicals mediated cellular damage by benzo (a) pyrene induced experimental lung cancer in male Swiss albino mice.