

# PROTECTIVE EFFECT OF GLYCOWITHANOLIDES ON SUBMANDIBULAR GLANDS OF D-GALACTOSE STRESSED MICE

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## ABSTRACT

The study was undertaken to evaluate the effect of glycowithanolides (WSG) on the structure of submandibular gland of aging mice. The aging was induced by 5% D-galactose. Adult male mice (*Mus musculus*) were divided into main two group viz. protective and curative group. Each group further divided into four batches viz. (A) control batch, received 0.5ml 0.9% saline per day, (B) D-galactose stressed batch, received 0.5ml 5% D-galactose per day, (C) centrophenoquine treated batch, received 0.5ml centrophenoquine in 0.9% saline per day (80 mg / kg body weight), and (D) glycowithanolides treated batch, received 0.5 ml WSG per day (20 mg / kg body weight). Histological study of submandibular gland showed loss of structural integrity of acinar cells as well as GCT cells, irregular nuclear distribution, vacuolated cytoplasm in D-galactose stressed mice. Significant structural recovery was observed in antioxidant treatment, increase in size of acinar cells as well as GCT was observed in centrophenoquine and WSG treatment. Structural recovery was more pronounced in WSG treatment than centrophenoquine.

## INTRODUCTION

Salivary glands are not only the organ of digestion and maintenance of oral health, but they synthesize and secrete many enzymes, growth factors and glycoproteins. These products are essential for normal development and maintenance of post mitotic cells in the body. Salivary glands especially submandibular gland is the site, where numbers of polypeptides are synthesized and secreted (Barka, 1980). Structure as well as functioning of salivary glands is altered with decreased salivary flow/output during aging. Several researchers have reported age related changes in morphology (Kim, 1981), in histology (Sashima, 1986; Scott *et al.*, 1986), in ultra structure (Bogart, 1970) and in biochemistry (Kim and Arisumi, 1985; Sashima, 1986) of the salivary glands. There are many evidences of age related changes in structure and histochemical parameters of salivary glands in man and in different animal species (Rybakava, 1982). During aging there is accumulation of free radicals which are often referred as Reactive Oxygen Species (ROS). ROS induce oxidative damage to macromolecules (Harman, 1957). Oxidative stress definitely contribute to tissue injury following various diseases like Parkinson's disease, Alzheimer's disease, cardiovascular disorder etc. and contribute aging process (Sorcha and Thomas, 2009). In early age redox status of the cell is maintained between cell's antioxidant system (including SOD, CAT, and GPx) and ROS production in order to prevent oxidative damage. At various levels cells are equipped with defense system and repair mechanism, so that cells can withstand the stress. Unfortunately these defense mechanisms decline during aging process. Taking into consideration the importance of sali-

vary glands, it is very essential to find out solution to maintain salivary glands structure and physiology. Several efforts are being made to decrease the level of damage to the salivary glands due to the aging stress. Herbs like Brahmi (Pillai *et al.*, 2003), Parsley (Vora *et al.*, 2009) Ashwagandha (Mote *et al.*, 2009) and Lettuce (Patil *et al.*, 2008) are the promising candidates in treatment of oxidative stress. Among these Ashwagandha (*Withania somnifera*) called 'Indian ginseng' is supreme important medicinal herb. In India it is widely used as a home remedy for several diseases. Several national and international researches proved its use in rheumatism, cancer, immune disorders, diabetes (Chopra *et al.*, 1965; Sharma, 2002; Uniyal, 2002; Stan *et al.*, 2008; Rajangam *et al.*, 2009). Among the different constituents from Ashwagandha, glycowithanolides (WSG), isolated by Bhattacharya *et al.*, (1997) and tested using HPTLC are the chemical soul of Ashwagandha and believed to account for its multiple medicinal applications. These are the active principles of *W. somnifera* possesses antioxidant property (Ghosal *et al.*, 1989). Glycowithanolides consists of sitoindoside VII to X and withaferin; induce significant antistress effect (Ghosal *et al.*, 1989). The present study was undertaken to investigate the protective effect of glycowithanolides from *W. somnifera* leaves extract on structure of submandibular glands of D-galactose stressed male mice. Centrophenoquine was used as synthetic antioxidant drug for comparison.

## MATERIALS

### 1. Animals

Male mice (*Mus musculus*) were used as an experimental

animal. Breeding pairs were obtained from Hindustan Antibiotics, Pune. They were housed and maintained in departmental animal house under proper conditions of light, temperature and humidity. The animals were housed in aluminium cages having dimensions of 10"x8"x5" and allowed to live in groups of 3 to 4 per cage. They were supplied with 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 (age of 5 to 6 month) weighing about 50 to 55 ± 2g were divided into two group viz. protective group and curative group. Each group further divided into four bathes as below.

#### Batch A: Control

Male mice were given 0.5mL, 0.9 % saline per day subcutaneously for 20 days and 40 days for protective and curative groups respectively.

#### Batch B: D-galactose stressed

Adult male mice were given D-galactose to accelerate the aging process (Song *et al.*, 1999; Deshmukh *et al.*, 2006). The control batch for protective group received 0.5 mL 5% D-galactose per day subcutaneously for 20 days, while the control batch of curative group received 0.5mL 5% D-galactose per day subcutaneously for 20 days and then received 0.5mL 0.9% saline per day subcutaneously for next 20 days.

Protective batch denoted as Dg-stressed and curative batch denoted as Dg → saline.

#### Batch C: Centrophenoxine treated

Adult male mice were received 0.5mL 5% D-galactose along with Centrophenoxine (80 mg/kg body weight.) per day subcutaneously for 20 days (Patro and Sharma, 1984) for protective group, while curative group received 0.5 mL 5% D-galactose per day for 20 days and then 0.5mL centrophenoxine (80 mg / kg body wt.) per day for further 20 days.

Protective batch is denoted as Dg + cent and curative batch is denoted as Dg → cent.

#### Batch D: Glycowithanolides (WSG) treated

Male mice of protective group were received 0.5mL 5% D-galactose along with WSG (20 mg/kg body weight) per day subcutaneously for 20 days (This dose was selected by Bhattacharya *et al.*, (1997) for *Mus musculus* to study the effect of WSG on the brain). While curative group received 0.5mL 5% D-galactose per day for 20 days and then dose of 0.5mL WSG (20 mg/kg body weight) per day for further 20 days.

Protective batch is denoted as Dg + WSG and curative batch is denoted as Dg → WSG. All drug administrations and sacrifice of the animals were done between 09.00 am and 12.00 am.

## 2. Plant Extract

Fresh Ashwagandha plants (*W. somnifera* Dunal) were obtained from Shri Prasad Nursery, Kognoli, Dist- Belgum (Karantaka State). Fresh green leaves were separated and washed properly with water and rinsed with distilled water. Washed leaves were blotted properly with blotting paper and kept for drying in the shade. Glycowithanolides from these leaves was separated as described by Bhattacharya *et al.*, (1997). An aqueous concentrate of *W. somnifera* leaves was

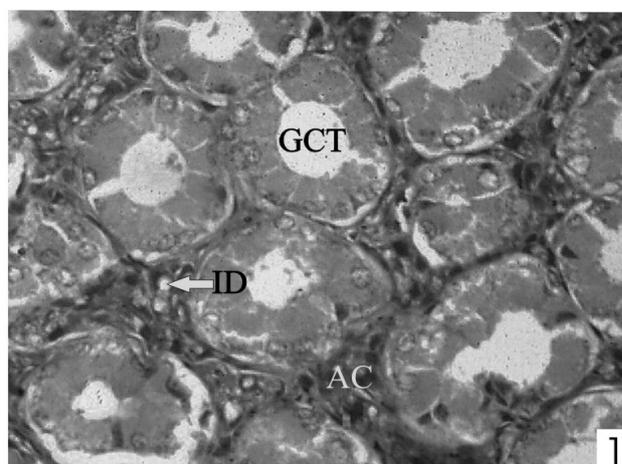
exhaustively extracted with chloroform to remove fatty materials and separates the withanolides. The aqueous solution was then spray dried. After spray drying a thick paste like extract was obtained and stored in glass bottle. It was kept refrigerated (4°C) for further use.

## 3. Histology

For histological study mice from each batch of both protective and curative group were sacrificed, their submandibular glands were dissected out and fixed in 10% formaldehyde for 24 hours, washed, dehydrated through alcoholic grades, cleaned in xylene and embedded in paraffin. The 7 µ paraffin section were subjected to routine Haematoxyline-Eosin technique for histological observations.

## RESULTS

Histological changes in submandibular gland of adult male mice were depicted in Figs. 1 to 8. Fig.1 is a cross section of submandibular gland of control batch of adult protective group showed large number of small secretory acini with darkly stained nuclei. Granular convoluted tubules (GCT) were distinct, stained dark with eosin and have darkly stained nucleus in cells. Intercalated ducts (ID) were also distinct and scattered between the acini.

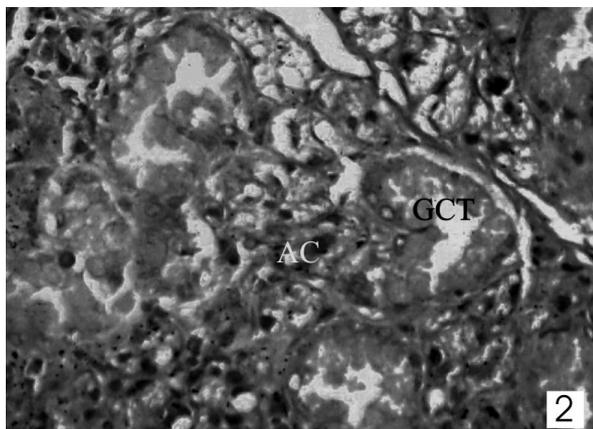


**Figure 1:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of protective group received 0.5 mL saline for 20 days X 450; GCT = Granular convoluted tubule. AC = Acinar Cells. ID = Intercalated Duct.

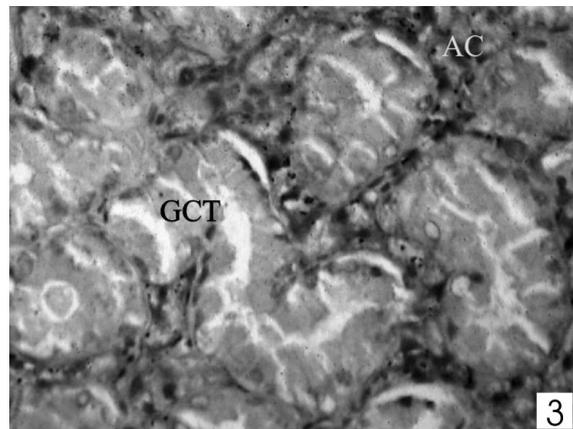
In submandibular gland of Dg-stressed batch there was decrease in structural integrity of acinar cells, nuclear distribution was irregular, the presence of vacuolated cytoplasm and loss of GCT integrity (Fig. 2).

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 treated batch (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 (Fig. 4).

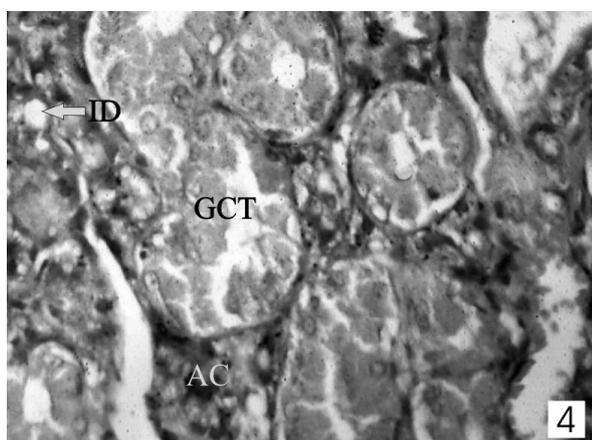
Fig. 5 is a cross section of submandibular gland of control batch of curative group showed similar structure as that of control batch of protective group.



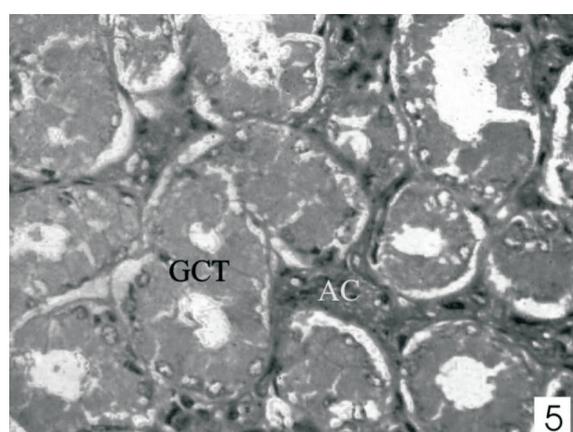
**Figure 2:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of protective group received 0.5 mL D-galactose X 450; GCT = Granular convoluted tubule. AC = Acinar Cells.



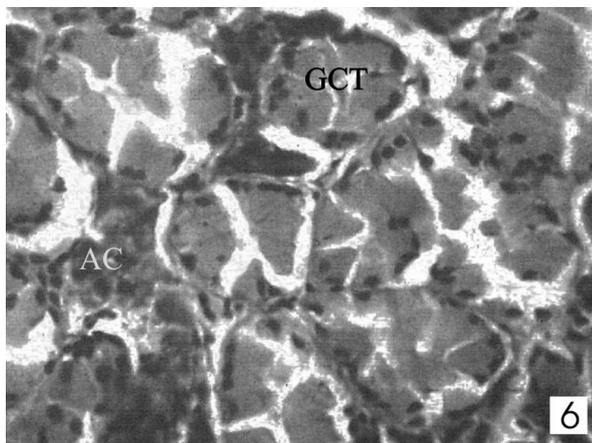
**Figure 3:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of protective group received 0.5 mL D-galactose along with centrophenoxine X 450; GCT = Granular convoluted tubule. AC = Acinar Cells.



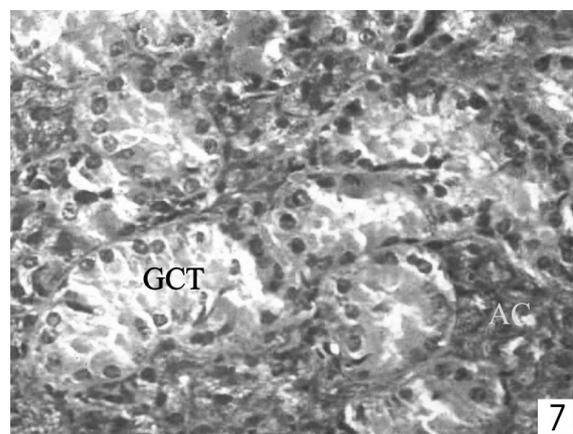
**Figure 4:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of protective group received 0.5 mL D-galactose along with glycowithanolides X 450; GCT = Granular convoluted tubule. AC = Acinar Cells. ID = Intercalated Duct.



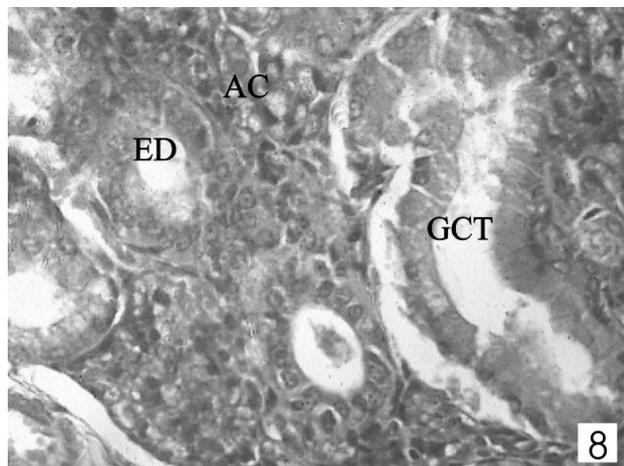
**Figure 5:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of curative group received 0.5 mL saline for 40 days. X 450; GCT = Granular convoluted tubule. AC = Acinar Cells.



**Figure 6:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of curative group received 0.5 mL D-galactose for 20 days, sacrificed 20 days after cessation of D-galactose treatment. X 450; GCT = Granular convoluted tubule. AC = Acinar Cells. ID = Intercalated Duct.



**Figure 7:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of curative group received 0.5 mL D-galactose followed by centrophenoxine for 20 days X 450; GCT = Granular convoluted tubule. AC = Acinar Cells.



**Figure 8:** The sections of submandibular salivary glands of adult male mice stained with Haematoxyline + Eosin. C. S. of submandibular gland of male mouse of curative group received 0.5 mL D-galactose along with glycowithanolides for 20 days. X 450; GCT = Granular convoluted tubule. AC = Acinar Cells. ED = Excretory Duct.

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 (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 (Fig. 7).

Section of submandibular gland of adult Dg → WSG batch of curative group showed well developed acinar cells, GCT were increased in size and regained structural integrity (Fig. 8).

## DISCUSSION

In present study the submandibular glands of D-galactose stressed mice showed loss of their normal architecture, loss of cellular integrity in acinar as well as GCT cells, decrease in the number of secretory units, pyknotic nuclei and vacuolated cytoplasm (Figs. 2 and 6). The changes induced by D-galactose were similar to the natural aging process. D-galactose is aging inducing agent. (Song *et al.*, 1999; Deshmukh *et al.*, 2006). Our observations were supported by previous studies. During normal aging decrease in number of acinar cells (Scott, 1977b; Kim and Allen, 1993), increased amount of connective tissue (Wilde *et al.*, 1986), atrophy of acinar and ductal epithelia (Scott, 1977a; Azevedo *et al.*, 2005) have been observed. The changes in submandibular gland's structure induced by D-galactose might be due to free radicals mediated oxidative stress. D-galactose causes free radical formation, leading to increased Advanced Glycation End products (AGEs) which accelerate natural aging process (Song *et al.*, 1999). The free radicals can cause oxidative tissue damage. (Singh, 1997; Anasari, 1997). The oxidative stress can causes increased lipid peroxidation (Baskaran *et al.*, 1999). 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 submandibular glands within very short time and induce the degenerative changes

in them. Decrease in the proteins content may also reduces the cellular regeneration. Reduction in the total protein content in submandibular glands of D-galactose stressed mice was observed by Mote *et al.*, (2009). 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 submandibular glands of adult male mice treated with centrophenoxine and WSG was observed (Figs. 3 and 7, 4 and 8 respectively). This recovery might be due to antioxidant properties of WSG and centrophenoxine. *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 provides protection to liver from lipid peroxidation due to heavy metal toxicity. Ashwagandha cures all negative conditions associated with aging (kuppurajan *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. The centrophenoxine is also powerful free radicals scavenger (Zs Nagy and Nagy, 1980), prevents cellular damage. The structural recovery in submandibular glands was more pronounced in WSG treatment than centrophenoxine treatment. Thus in present study regeneration in submandibular gland structure might be due to antioxidant property of glycowithanolides of Ashwagandha.

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