EFFECT OF GLYCOWITHANOLIDES ON SIALIC ACID CONTENT IN SALIVARY GLANDS OF D-GALACTOSE INDUCED AGING MICE

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ABSTRACT:
The study was undertaken to find out effect of glycowithanolides (WSG) extracted from Withania somnifera leaves on sialic acid content in submandibular and sublingual salivary glands of D-galactose induced aging mice. The adult male mice (Mus musculus) were grouped into protective and curative group. Each group further divided into four batches 1) Control batch of protective and curative group received 0.5 ml 0.9% saline per day for 20 days and 40 days respectively. Mice from 2nd, 3rd and 4th batches of protective group received D-galactose, D-galactose+ centrophenoxine and D-galactose + WSG for 20 days respectively. Mice from 2nd, 3rd and 4th batches of curative group received D-galactose for 20 days followed by saline, centrophenoxine and WSG alone for further 20 days respectively. In D-galactose stressed mice sialic acid content was increased significantly in both the submandibular and sublingual salivary glands compared to control. In centrophenoxine and WSG treatment receiving mice there was significant reduction in sialic acid content as compared stressed mice. During aging stress, induced by D-galactose alteration in sialic acid of salivary glands took place. These alterations were prevented by centrophenoxine and WSG.

Key Words: Glycowithanolides, salivary glands, sialic acid, D-galactose, aging, centrophenoxine.

INTRODUCTION:
Salivary glands are involved in secretion of polypeptides, glycoproteins, growth factors and esteropeptidases which bring growth, regulation and differentiations. Salivary secretion consists of approximately 99% water, variety of electrolytes and proteins [1]. Among salivary proteins up to 26% are the glycoproteins [2]. Numerous histochemical studies were made on glycoproteins in the secretory cells of salivary glands [3, 4]. Salivary glands are rich in sulfated glycoproteins [5, 6]; which includes hexoses, fucose and sialic acids. The presence of sialic acid, hexoses, hexosamine and fucose in human salivary
EFFECT OF GLYCOWITHANOLIDES ON SIALIC ACID CONTENT IN SALIVARY GLANDS

R. N. MOTE, et al.

Gland has been reported by Nisizawa and Pigman [7]. High content of sialic acid is one of the characteristic features of the salivary gland glycoproteins [8, 9]. The structure, occurrence and general functions of sialic acids have been extensively reviewed [10, 11]. The negative charges contributed by sialic acid on cells surface and glycoproteins cause cell to cell repulsion, functional stability and survival of glycoproteins. It also plays an essential role in the lubrication and protection in the digestive tract [12]. Salivary secretion provides an excellent example of age related vulnerability with multiple ramification of clinical significance. Therefore study of salivary secretion is an important issue for the age that affects physiology of various organs. Several studies reported age related changes in glycoproteins. Bruzymski, [13] reported glycoprotein bound hexose protein nitrogen ratio was significantly decreased in submandibular gland of aging guinea pig. Reduction in glycoprotein sialic acid in submandibular glands of old rats was described by Mysliwaski and Zurawska Czupa, [14]. Reduction in N-linked glycosylation was also reported in aged rats by Kousvelari et al, [15]. To overcome age related changes in salivary glands it is essential to supply antioxidants. Many plants from Ayurveda such as Brahmi, Shatavari, Ashwagandha etc. possess antioxidant properties [16].

The major bioactive chemical principle of Withania somnifera is glycowithanolides [17] and has beneficial effects in treatment of stress [18]. Therefore it was felt to find out its effects on sialic acid content of D-galactose stressed submandibular and sublingual salivary glands of mice. Centrophenoxine is efficient OH− radical scavenger [19] and is able to prolong the life spine [20] so it was used as synthetic antioxidant for comparison with WSG.

MATERIALS AND METHOD:

a. Animals:
Adult (5 to 6 months old weighing 50 to 55 ± 2 gm body wt.) male mice (Mus musculus) were selected for the study. They were supplied with Amrut mice feed (Pranav Agro Industries Pvt. Ltd. Sangli) and water ad libitum. They were divided into two group viz. protective group and curative group. Each group further divided into 4 batches.

Batch 1 – Control:
Control batch received 0.5 ml 0.9% saline/day for 20 days and 40 days for protective and curative groups respectively.

Batch 2 – D-galactose (Dg) stressed:
Mice received 0.5 ml 5% D-galactose (prepared in 0.9% saline) per day for 20 days [21] for protective group. Curative group received D-galactose for 20 days and then saline for further 20 days subcutaneously. Protective batch denoted as Dg-treated and curative batch as Dg → saline. 

Batch 3 – Centrophenoxine treated:
Mice of protective group received 0.5 ml 5% D-galactose along with centrophenoxine—a synthetic antioxidant per day (80 mg/kg body wt.; [22]) for 20 days. Curative group received 0.5 ml 5% D-galactose for 20 days and then centrophenoxine for further 20 days, sacrificed on 41th day. Protective batch denoted as Dg + cent and curative batch denoted as Dg → cent.

Batch 4 – Glycowithanolides (WSG) treated:
Mice received 0.5 ml 5% D-galactose along with WSG (20 mg/kg body wt.) [17]/day for 20 days in protective group and denoted as Dg + WSG. In curative group mice received 0.5 ml 5% D-galactose for 20 days and then followed by 0.5 ml WSG alone per day for 20 days. Protective batch denoted as Dg + WSG and curative batch denoted as Dg → WSG.

All treatments were given at 9.00 am. After completion of treatments animals were sacrificed by cervical dislocation between 9.00 am to 12.00 pm in noon. Submandibular and sublingual glands from one side were pulled, weighed, homogenized and centrifuged at 5000 rpm for 10 minutes at 10°C temperature to prepare sample and used for estimation of proteins and sialic acids. Submandibular and sublingual from other side were used for histochemical demonstration of sialic. The protein contents in both the salivary glands were estimated by Lowry et al. [23] and sialic acid by Warren (1959) method. For histochemical studies 2% formal calcium fixed 7 µ thick salivary gland’s sections were subjected to PAS-Sodium borohydride staining as described by Culling et al. [24].

Preparation of plant extract:-
Glycowithanolides extracted from fresh green leaves of Withania somnifera. Fresh leaves were shade dried, crushed and chloroform extract was prepared as described by Bhattacharya et al. [17]. The aqueous concentrate of Withania somnifera leaves was exhaustively extracted with chloroform to remove fatty material and free withanolides. The aqueous solution was then spray dried contained
sitoindosides VII – X and withaferin collectively referred as glycowithanolides (WSG). The later was determined with the help of HPTLC [17]. Glycowithanolides was freely soluble in water and saline. Plant extract was dissolved in sterile water and was given to the experimental mice subcutaneously (20 mg/kg body wt.)

RESULTS:
A. Biochemical changes:

1. In submandibular glands
   Table 1 and graphs, 1 and 2 showed the sialic acid content of submandibular glands after respective treatments. The sialic acid content in D-galactose treated batches of both protective and curative groups showed significant increase as compared to control batch (p<0.001). There was significant decrease in sialic acid content in submandibular gland of centrophenoxine and glycowithanolides (WSG) treatments (p<0.001) in both protective and curative group. No significant difference (p>0.1) was observed between centrophenoxine and WSG treatment in protective group but in curative group the difference was significant (p<0.02).

2. In sublingual glands (Table -1, Graphs 3 and 4)
   The sialic acid content of control batch was 0.00037 ± 8.3666 µg and 0.000338 ± 4.4721 µg per mg proteins for protective and curative groups respectively. In D-galactose stressed batch it was increased significantly to 0.00046 ± 0.00001 µg and 0.000504 ± 5.4772 µg per mg proteins for protective and curative groups respectively. Significant decrease in sialic acid was observed in both the antioxidant treatments. Significant decrease was observed in WSG treatment as compared to centrophenoxine.

B. Histochemical alterations:
1. In submandibular glands (plate 1 fig. 1 to 8)
   Control of protective and curative groups showed light staining reaction in acinar cells and GCT cells of submandibular glands with PAS-sodium borohydride staining. (Plate -1 Figs. 1 and 5). In D-galactose stressed submandibular glands of protective and curative groups, there was destruction of gland’s architecture but some of the acinar cells and GCT cells showed dark staining reaction with PAS-sodium borohydride indicating increased sialomucins. (Plate -1 Figs. 2 and 6).
Centrophenoxine and WSG treated submandibular gland showed remarkable decrease in staining intensity for sialic acid. Staining reaction was completely abolished in GCT during stress and after stress also due to antioxidant treatment (Plate -1 figs. 3 and 7 & 4 and 8 respectively).

2. In sublingual glands (plate 2 fig. 1 to 8)

The control batch showed weak staining reaction with sodium borohydride in membrane of mucous acini and in demilunes (fig.1). Slight increase in staining reaction was observed in control batch of curative group (fig. 5). The remarkable increase in staining intensity was observed in membrane of mucous acini represented by dark magenta pink color. Demilunes also stained darkly (Plate -2 figs. 2 and 6). In protective and curative groups received centrophenoxine light staining intensity was observed in mucous acini, demilunes didn’t show any staining reaction (figs. 3 and 7). There was remarkable decrease in sialomucins in WSG treatment (figs. 4 and 8). The staining intensity in acini was lighter than centrophenoxine.

DISCUSSION:

Sialic acid is present in several tissues. The major sialic acid is N-acetyl neuraminic acid is increasing directly towards the O-acetylated species. O-acetylated sialic acid plays fundamental role in the development of organism, in the regulation of the immunity system, in cancer processes and many other biological as well as pathological events [11]. Histochemically it has been observed that these glycoproteins are present in Granular Convoluted Tubule (GCT) and mucous acini of submandibular and sublingual glands respectively. The present study shows alterations in sialic acid glycoproteins in the submandibular and sublingual salivary glands of D-galactose stressed mice. Changes observed in salivary glands in D-galactose stressed mice are similar to that of aged mice [24, 15, 25]. Changes observed in D-galactose stress are similar to that of normal aging stress in other organs also including brain [26, 21,] heart [27], prostate gland [28]. These changes such as increase in lipid peroxidation in brain [29, 30] in mitochondrial fraction of brain [31], alterations in lysosomal enzymes like acid phosphatase [27, 30]. Although any cellular structure subjected to free radical induced damage, the
EFFECT OF GLYCOWITHANOLIDES ON SIALIC ACID CONTENT IN SALIVARY GLANDS

R. N. MOTE, et al.

Impairment of mitochondrial and lysosomal integrity is the key factor for understanding the age related degeneration [32]. Non-functional lysosomes are responsible for progressive accumulation of lipofuscin granules, with a progressive accumulation of functionally inactive material [33].

Salivary gland sialoglycoproteins and also O-acetylated sialic acid are increased in stressed condition. Increase in sialic acid content in D-galactose stressed mice might be due to accumulation of glycoproteins in acini. During old age there occur alterations in β-adrenergic simple transduction pathways which results in to inhibition of protein secretion [34, 35]. In aging and stressed salivary glands there is reduction in the ductal volume [36]. In stressed condition there is decrease in glycoproteins, on the other hand O-linked sialoglycoproteins are increased. There is also close relationship between O-sialoglycoprotein and pathological condition [37, 38]. Glycoprotein concentration in old age increases in most of the old age related disease like Xerostomia, Sjogren’s syndrome. The increase in intracellular ionic strength causes considerable condensation of the intracellular colloid leading to gradual increase in intracellular dry mass [39]. Accumulation of neutral and acid glycoproteins in acinar cells of submandibular glands of rat during aging was reported by Emmanouil – Nikoloussi et al [40]. Increased accumulation of glycoproteins content may also be due to increase in lipid peroxidation. Gokmen et al [41] have suggested that either the shedding or secretion of sialic acid from cell to cell membrane surface may be partly responsible for increased sialic acid concentration. Increased sialylation of serum proteins may increase sialic acid [42] or reduction in desialylation of plasma glycoproteins increases sialic acid [43]. Increase in O-acetylated sialic acid in D-galactose stressed mice was reported by Kalmade et al [28] in prostate gland, Sonavane [44] in salivary glands and Tomake [45] in naturally old male rat’s salivary glands.

Increase in sialic acid in adult mice was prevented by antioxidant treatment during stress or after stress. Centrophenoxine is a powerful chemical antioxidant [46] and possess OH scavenging capacity [19, 20] which helps to prevent cellular damage and there by reduces lipid peroxidation. Glycowithanolides (WSG) is a powerful natural antioxidant [47] and active principle of Withania somnifera increases cell’s
antioxidant enzymes like SOD, CAT AND GPx level by inhibiting lipid peroxidation [17, 48]

Decrease in sialic acid content was also reported by treatment of antioxidants like *Petroselinum crispum* in salivary glands [44] and *Bacopa monniera* in prostate gland [28] of D-galactose stressed mice. In present work when antistress effect of both the synthetic and natural antioxidant is studied, glycowithanolides is more effective than centrophenoxine in reducing increase in sialic acid and O-acetylated sialic acid in salivary glands of D-galactose stressed mice.

REFERENCES:
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heart during aging. Ph. D. thesis submitted to Shivaji University, Kolhapur, (MS), India.


PLATE 1
PLATE 1
Caption to figures 1 to 8

This plate shows sections of submandibular salivary glands of adult male mice stained with Thionin – Schiff – sodium - borohydride technique.

Fig. 1:- C. S. of submandibular gland of male mouse of protective group received 0.5 ml saline for 20 days X 450.

Fig. 2:- C. S. of submandibular gland of male mouse of protective group received 0.5 ml D-galactose X 450.

Fig. 3:- C. S. of submandibular gland of male mouse of protective group received 0.5 ml D-galactose along with centrophenoxine X 450.

Fig. 4:- C. S. of submandibular gland of male mouse of protective group received 0.5 ml D-galactose along with glycowithanolides X 450.

Fig. 5:- C. S. of submandibular gland of male mouse of curative group received 0.5 ml saline for 40 days. X 450.

Fig. 6:- 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.

Fig. 7:- 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.

Fig. 8:- 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.

Abbreviations:
GCT = Granular convoluted tubule.
AC = Acinar Cells.
GC = GCT cells
SD = Striated Duct.
EFFECT OF GLYCOWITHANOLIDES ON SIALIC ACID CONTENT IN SALIVARY GLANDS

PLATE 2
PLATE 2
Caption to figures 1 to 8
This plate shows sections of sublingual salivary glands of adult male mice stained with Thionin – Schiff – sodium-borohydride technique.

Fig. 1: C. S. of sublingual gland of male mouse of protective group received 0.5 ml saline for 20 days. X 450.

Fig. 2: C. S. of sublingual gland of male mouse of protective group received 0.5 ml D-galactose. X 450.

Fig. 3: C. S. of sublingual gland of male mouse of protective group received 0.5 ml D-galactose along with centrophenoxine. X 450.

Fig. 4: C. S. of sublingual gland of male mouse of protective group received 0.5 ml D-galactose along with glycowithanolides. X 450.

Fig. 5: C. S. of sublingual gland of male mouse of curative group received 0.5 ml saline for 40 days. X 450.

Fig. 6: C. S. of sublingual 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.

Fig. 7: C. S. of sublingual gland of male mouse of curative group received 0.5 ml D-galactose followed by centrophenoxine for 20 days. X 450.

Fig. 8: C. S. of sublingual gland of male mouse of curative group received 0.5 ml D-galactose along with glycowithanolides for 20 days. X 450.

Abbreviations:
MA = Mucous acini
ED = Excretory Duct.
Table 1:-
Effect of Glycowithanolides (WSG) on sialic acid content (in µg/mg protein) in salivary glands of D-galactose stressed adult male mice (5 to 6 months old).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Batches</th>
<th>Submandibular gland</th>
<th>Sublingual gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg sialic acid</td>
<td>t-value</td>
<td>p-value</td>
</tr>
<tr>
<td>I</td>
<td>Protective Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control (5)</td>
<td>0.00015 ± 5.4772</td>
<td>1:2= 7.3638</td>
</tr>
<tr>
<td>2</td>
<td>Dg treated (5)</td>
<td>0.00024 ± 1.9493</td>
<td>2:3= 5.2041</td>
</tr>
<tr>
<td>3</td>
<td>Dg + Cent (5)</td>
<td>0.00011 ± 8.9442</td>
<td>3:4= 1.1710</td>
</tr>
<tr>
<td>4</td>
<td>Dg + WSG (5)</td>
<td>0.00011 ± 5.4772</td>
<td>2:4= 5.2463</td>
</tr>
<tr>
<td>II</td>
<td>Curative Group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control (5)</td>
<td>0.00018 ± 0.00001</td>
<td>1:2= 9.0033</td>
</tr>
<tr>
<td>2</td>
<td>Dg → Saline (5)</td>
<td>0.000296 ± 5.4772</td>
<td>2:3= 17.5260</td>
</tr>
<tr>
<td>3</td>
<td>Dg → Cent (5)</td>
<td>0.000158 ± 8.3666</td>
<td>3:4= 3.0151</td>
</tr>
<tr>
<td>4</td>
<td>Dg → WSG (5)</td>
<td>0.000118 ± 1.7889</td>
<td>2:4= 16.8194</td>
</tr>
</tbody>
</table>

Values in the parenthesis denote the number of mice
p>0.1 = non significant
p<0.001 = highly significant
P< 0.01 = significant
p< 0.02 = significant
p<0.05 = almost significant

Values are Mean ± S.D.
EFFECT OF GLYCOWITHANOLIDES ON SIALIC ACID CONTENT IN SALIVARY GLANDS

Graph 1: Effect of Glycowithanolides on sialic acid content (in µg/mg protein) in submandibular glands of adult protective groups

Graph 2: Effect of Glycowithanolides on sialic acid content (in µg/mg protein) in submandibular glands of adult curative groups

Graph 3: Effect of Glycowithanolides on sialic acid content (in µg/mg protein) in sublingual glands of adult protective groups

Graph 4: Effect of Glycowithanolides on sialic acid content (in µg/mg protein) in sublingual glands of adult curative groups