Research Article

Glycowithanolides Enhances Lactate Dehydrogenase Activity in Reproductive Organs of mice during Oxidative Stress.

Shaikh Nilofar[1], Walvekar Madhuri[2*], Desai Sneha[3]

[1]Assistant professor, Department of Zoology, Dattajirao Kadam Arts, Science and Commerce College, Ichalkaranji, Maharashtra, India.

[2] Assistant professor, Department of Zoology, Shivaji University, Kolhapur, Maharashtra, India.

[3]Research student, Department of Zoology, Shivaji University, Kolhapur, Maharashtra, India.

***Corresponding author's E-mail:** madhuri_walvekar@rediffmail.com

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ABSTRACT

Aging is inevitable process, characterized by diminishing functional capacity of an organism so it falls to cope up with stress. Varieties of antioxidants are suggested by researchers to reduce adverse effects of aging. The present study was undertaken to assess the effect of glycowithanolides, extracted from *Withania somnifera* leaves on protein contents and LDH activity. For this purpose, adult male mice (*Mus musculus*) of six month old, weighing 50 to 55 gm were divided into following four groups viz. control group (I), D-galactose treated group (II), protective group (III) and curative group (IV). The lactate dehydrogenase (LDH) activity as well as protein content was significantly reduced in testes epididymis and seminal vesicle in D-galactose treated group (II). The protein content was significantly increased in testes, epididymis and seminal vesicle in protective as well as in curative group. In protective group LDH activity was again significantly increased in testes and epididymis except seminal vesicle but in curative group it was significantly increased. A specific LDH –X isoenzyme, a marker enzyme of active spermatogenesis was seen after electrophoresis in control group but it disappeared in D-galactose stressed mice. However, it reappeared in both protective and curative group. Further, it was more prominent in curative group than protective group. Thus increase in LDH activity and protein content indicates antioxidative role of glycowithanolides during oxidative stress.

Keywords: Oxidative stress**,** D-galactose, Glycowithanolides (WSG).

INTRODUCTION

Lactate dehydrogenase is a hydrogen transfer enzyme that catalyses the oxidation of L- lactate to pyruvate with nicotinamide- adenine dinucleotide $(NAD)^+$ as a hydrogen acceptor, the final step in the metabolic chain of anaerobic glycolysis. The enzyme is composed of four peptide chains of two types M and H, each under separate genetic control. Heart (H) subunit or muscles (M) subunit are so named because of their predominance in the respective tissues. The subunit compositions of the five isoenzymes in order of decreasing anodal mobility in an alkaline medium are: LDH-1 (HHHH; H_4); LDH-2 (HHHM; H_3M); LDH-3 (HHMM; H_2M_2); LDH- 4 (HMMM; HM_3); and LDH-5 (MMMM; M_4)[1-2]. LDH –X is an isoenzyme of the LDH that is present only in mature sperm[3-4]. It is not found in the seminal fluid or serum. Appearance of seminal fluid in seminal plasma has been postulated to be a sign of leakage from spermatozoa or from their precursor cells [5-6]. It is one of the best characterized germ cell specific isozyme[7] that plays an important role in the process of spermatogenesis and has been shown to be vital for sperm survival and motility. The studies on rats have shown gradual decrease in the LDH activity during aging, more specifically M4 isozyme[8]. The several studies have shown a major consequence of oxidative stress is damage to nucleic acid bases, lipids, and proteins. Alterations in the rate and extent of protein synthesis, accuracy, post translational modifications and turnover are among the main molecular characteristics of aging. A decline in the cellular capacity through proteasomal and lysosomal pathways to recognize and preferentially degrade damaged proteins leads to the accumulation of abnormal proteins during aging[9-10].

Most plants have protective biochemical functions of naturally occurring antioxidants in the cells. Many secondary compounds and enzymes of higher plants have been demonstrated with *in vitro* experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. In view of this and the present understanding about ROS –induced multiple diseases, we have selected one such important popular ayurvedic herb, *Withania somnifera* commonly known as ashwagandha for examination of its antioxidative status. This herb is found in all over world. Both aqueous as well as alcoholic extracts of the plant (root as well as leaves) were found to possess strong antibacterial activity[11]. Glycowithanolides, the major active constituent has been isolated from *Withania somnifera*[12]. In present contribution attempts have been made to study the effects of *Withania somnifera* on LDH activity and protein contents of testes and accessory reproductive organs of stressed male mice.

MATERIALS AND METHODS

Plant material

The plant was identified by taxonomist from Botany Department, Shivaji University, Kolhapur. Fresh leaves of *Withania somnifera* were collected from Town hall garden Kolhapur.

Plant extraction

Glycowithanolides were extracted from leaves of *Withania somnifera* plant as described by[12]Fresh leaves of *Withania somnifera* were separated, washed with distilled water, blotted properly and kept for shade drying. Dried leaves were crushed, powdered, sieved and then soaked in chloroform for 72 hrs to remove fatty material and to separate withanolides. The solution was filtered and chloroform was evaporated by evaporator and thick paste was obtained. With the help of TLC and GCMS the active principal glycowithanolides was confirmed. It was stored in glass bottle at 4^0C and used as active ingredient for dose preparation.

Animals

Swiss albino male mice *Mus musculus* (Linn) of age six months, weighing 50 to 55 gm were used for present investigation. The animals were maintained in departmental animal house (1825/PO/EReBi/S/15/ CPCSEA) under proper condition. Experimental work was conducted in accordance with the guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC). Mice were divided into following four groups of ten mice each.

I) Control group – Mice were injected subcutaneously with 0.5 ml distilled water/day/animal for 20 days.

II) D-galactose treated group- Mice were injected subcutaneously with 5% D-galactose 0.5 ml/day/animal for 20 days[13-14].

III) Protective group-Mice were injected subcutaneously with 0.5 ml of 5% D-galactose/day along with glycowithanolides 20mg/kg body weight for 20 days[15].

IV) Curative group- Mice were injected with 0.5 ml of 5% D-galactose for 20 days, and then glycowithanolides was injected 20mg/kg body weight for next 20 days.

Methods

The animals from respective groups were killed by cervical dislocation after 24 hours of completion of treatment testes and accessory reproductive organs were dissected out, blotted homogenized in cold water with the help of Teflon pestle and mortar. The concentration of homogenate was 1 mg/ml for LDH estimation and 25 mg/ml for electrophoresis. The homogenate was centrifuged at 10 °C at 1000 rpm for 10 minutes and the supernatants were used for estimation of lactate dehydrogenase[16]. Biochemically the enzyme was estimated by Sevela and Tovorek's method[17] using sodium lactate as substrate and NAD as coenzyme. Electrophoresis of LDH was carried out using polyacryl amide gel electrophoresis Estimation of protein was done by using Bovine serum as standard[18].

RESULTS

Table 1: Effect of glycowithanolides on lactate dehydrogenase (LDH) activity in testes, epididymis and seminal vesicle of D- galactose induced aged mice (Enzyme activity /mg protein). Values are mean ± S.D. (Numbers in parenthesis denotes number of animals).

LDH Activity in relative organs (Enzyme activity /mg protein)							
S.N.	**Treatment (8)**	**Testes**	**Statistical significance**	**Epididymis**	**Statistical significance**	**Seminal vesicle**	**Statistical significance**
I	Control	210.558± 3.5401	1:2, P < 0.01	389.798 ± 6.2365	1:2, P < 0.01	358.577 ± 9.2807	1:2, P < 0.01
II	D-galactose	166.11 ± 2.2571	2:3, P < 0.01	227.23 ± 4.9271	2:3, P < 0.01	284.686 ± 9.5136	2:3, Non significant
III	Protective	186.622 ±3.5075	2:4, P < 0.01	251.308 ± 14.4238	2:4, P < 0.01	286.241 ± 4.0731	2:4, P < 0.01
IV	Curative	194.563 ± 2.8466	3:4, P < 0.01	296.872 ± 6.2326	3:4, P < 0.01	323.352 ± 4.6747	3:4, P < 0.01

P<0.01= significant, P >0.5 = Non significant

Table 2: Effect of glycowithanolides on protein content (µg/ mg tissue) in testes, epididymis and seminal vesicleof D-galactose induced aged mice. Values are mean ± S.D. (Numbers in Parenthesis denotes number of animals).

Protein content in relative organs (µg/ mg tissue)							
Sr.No.	**Treatment (8)**	**Testes**	**Statistical significance**	**Epididymis**	**Statistical significance**	**Seminal vesicle**	**Statistical significance**
I	Control	750 ± 37.796	1:2, P < 0.01	506.25 ± 32.043	1:2, P < 0.01	406.25 ±32.043	1:2, P < 0.01
II	D-galactose	437.5 ± 23.145	2:3, P < 0.01	181.25 ± 25.877	2:3, P < 0.01	231.25 ± 37.201	2:3, P < 0.01
III	Protective	525 ± 37.796	2:4, P < 0.01	293.75 ± 49.551	2:4, P < 0.01	306.25 ± 17.677	2:4, P < 0.01
IV	Curative	600 ± 46.291	3:4, P < 0.01	418.75 ± 25.877	3:4, P < 0.01	337.5 ± 23.145	3:4, P < 0.01

P<0.01= significant, P >0.5 = Non significant

Table 1 shows the LDH activity from testes, epididymis and seminal vesicle, it was decreased in D-galactose treated aging induced mice as compared to control group and decrease was significant (P< 0.01); while there was significant increase in LDH activity in testes and epididymis from protective group and curative group mice as compared to D-galactose treated aging induced mice.In case of seminal vesicle from protective group LDH activity was slightly increased but increase was non significant (P- Non significant). The enzyme activity was significantly increased in curative group as compared to D-galactose treated aging induced mice (P < 0.01). The better results were observed in curative groups than protective groups.

Figure 1: Lane 1: Sample of testes from control group mice, Lane 2: Sample of testes from D-galactose treated group, Lane 3: Sample of testes from protective group, Lane 4: Sample of testes from curative group.

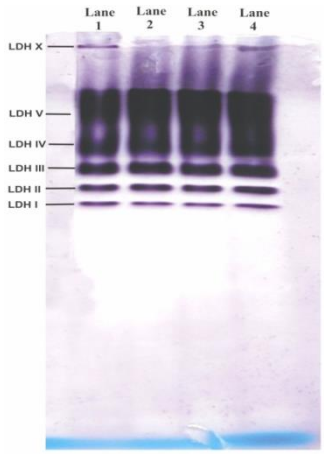

Figure 2: Lane 1: Sample of epididymis from control group mice, Lane 2: Sample of epididymis from D-galactose treated group, Lane 3: Sample of epididymis from protective group, Lane 4: Sample of epididymis from curative group.

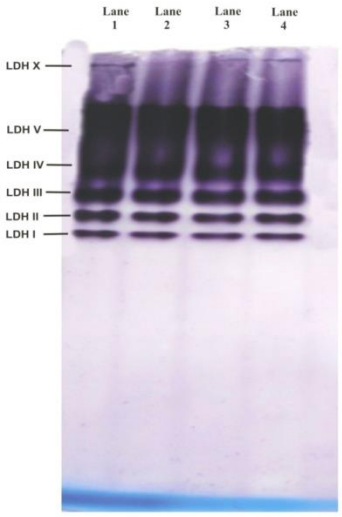

Figure 3: Lane 1: Sample of seminal vesicle from control group mice, Lane 2: Sample of seminal vesicle from D-galactose treated group, Lane 3: Sample of seminal vesicle from protective group, Lane 4: Sample of seminal vesicle from curative group.

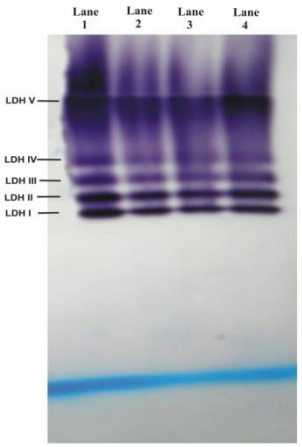

Table 2 shows the protein content in testes, epididymis and seminal vesicle, it was reduced significantly in aging accelerated group as compared to control (P < 0.01); but it was significantly increased in protective and curative group as compared to aging accelerated group (P<0.01). The increase was more in curative groups as compared to protective group.

The LDH isoenzyme was clearly separated into six bands i.e. LDH I, LDH II, LDH III, LDH IV, LDH V, and LDH X in testes and epididymis while in seminal vesicle, it was separated into five bands i.e. LDH I, LDH II, LDH III, LDH IV, LDH V.In D-galactose treated mice LDH X was completely disappeared in testes and epididymis while it was again reappeared in protective and curative group.The staining intensity of all LDH bands which was decreased in D-galactose treated group was again increased in both protective and curative groups in all three organs.

DISCUSSION

Recent advances in the understanding of male infertility has implicated oxidative stress to be a major causative factor [19-20].In the present investigation D-galactose was used to induce oxidative stress. A number of reports have suggested that the type of accelerated aging model induced by D-gal is associated with oxidative stress since some antioxidant enzyme activities were decreased in D-gal treated animals [21-24].Oxidative stress (OS) arises when excess free radicals overwhelm the antioxidant defence of the male reproductive tract[25-26]damaging cells, tissues and organs[27-28].LDH-X comprises 90 % of the total LDH-X activity of the spermatozoa. It plays an essential role in the metabolism of spermatozoa and is involved in the processes specific for these cells that generate energy for their survival, differentiation and motility [29]. There is a link between LDH-X and spermatogenesis since prepubertal males are lacking in this enzyme and amount of LDH-X increases with testis maturity [30]. LDH-X is a testis specific enzyme and has been used as a chemical marker for the germ cell status in somniferous epithelium [31]. It has also been suggested to be of diagnostic value in case of testicular toxicity [32]. In the present study, the decrease in the LDH-X level in D- galactose induced aged mice was

observed. This apparently is a reflection of a decrease in the number of mature spermatocytes which otherwise have high level of LDH-X. However, previous studies have indicated that toxic conditions affected the activity of LDH-X in the testis [32]. Inverse relation has been found between LDH/LDH-X activities and ROS production [33]. Increase in LDH X activity in protective and curative group clearly indicates the antioxidative role of glycowithanolides by removing free radicals. In conclusion, the oxidative stress generated due to the D-galactose may be responsible for affecting directly the process of spermatogenesis and in turn reflecting the reduced LDH-X level in the testesand epididymis while glycowithanolides by antioxidative role reduced the oxidative stress and in turn increased the LDH-X Level in testes and epididymis.

After D-galactose treatment about 50% decrease in protein content from seminal vesicle and testes was observed as compared to control group. In case of epididymis this decrease was much larger as compared to control group. This decrease in total protein content was may be due to reduction in sperm number. In the rat, observations made for specific proteins from the epididymis[34-36] seminal vesicle[37-38] and prostate[39-41], showed marked changes as a function of age. The protein content in testes, epididymis and seminal vesicle was also increased in both protective group and curative group indicating increase in number of sperm content. Better results in curative group than protective group indicates though D-galactose increased the oxidative stress, the treatment of glycowithanolides reduced the oxidative stress.

CONCLUSION

Glycowithanolides administration contributed to normalize LDH X activity in the oxidative stress during aging in reproductive organs that may suggest its remarkable antioxidative role. In addition such study may explore the importance of studying the efficacy of plant product to reduced infertility caused by oxidative stress.

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