



## Original Research Article

# Protective effects of glycowithanolides on antioxidative enzymes in testes and accessory reproductive organs of D-galactose induced stressed mice

N.H.Shaikh, S.R.Desai and M.V.Walvekar\*

Department of Zoology, Shivaji University, Kolhapur, 416001 (MS), India

\*Corresponding author

## ABSTRACT

### Keywords

*Withania somnifera*;  
testes and  
accessory  
reproductive  
organs;  
antioxidative  
enzymes.

Antioxidants are compound that protect cell against the damaging effects of reactive oxygen species. Glycowithanolides are the most active and functional constituents in *Withania somnifera* (Ashwagandha). The aim of this study was to investigate the effect of glycowithanolides on antioxidative enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in testes and accessory reproductive organs in mice during aging. For the present investigation mice (*Mus musculus*) were divided into four groups viz, control (Group I), D-galactose treated (Group II), protective (Group III) and curative (Group IV). The effect of glycowithanolides (20 mg/kg body weight) was evaluated against aging. The activity of antioxidative enzymes in the testes and accessory reproductive organs was recorded during aging. There was decrease in all the three antioxidative enzymes in D-galactose treated (Group II) group as compared to control (Group I). After the co-treatment with glycowithanolides (WSG) significant increase in level of antioxidative enzymes in testes and accessory reproductive organs in protective (Group III) and curative groups (Group IV) was observed. Thus it proves that glycowithanolides found to be effective antioxidative agent which reverses D-galactose induced oxidative damage.

## Introduction

The term antioxidants, also called antioxygen in the 19<sup>th</sup> and early 20<sup>th</sup> century, has been referred specifically to a chemical that prevented the consumption of molecular oxygen. Antioxidants are the subject of extensive research as they protect cell against the damaging effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, peroxy radicals, hydroxyl radicals and

peroxynitrite. An imbalance between antioxidants and ROS results in oxidative stress (OS), leading to cellular damage (Sikka *et al.*, 1996). However, high levels of ROS production leads to peroxidation of the sperm acrosomal membrane and diminished acrosin activity (Zalata *et al.*, 2004) and impaired sperm - oocyte fusion (Aitken *et al.*, 1989). Secondly, ROS directly damage the sperm DNA,

compromising the paternal genomic contribution to the embryo. Protective enzymatic and non enzymatic antioxidant defense mechanisms reduce oxidative stress by degrading ROS. The enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), which causes reduction of hydrogen peroxide to water and alcohol.

Superoxide dismutase (SOD) is metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide (Malstrom *et al.*, 1975). SOD is considered the first line antioxidant defense system against cellular oxidants while CAT complements the SOD antioxidant defense process by converting any remaining oxidant species to non reactive H<sub>2</sub>O (Chelikani *et al.*, 2004). Glutathione (GSH) is not only a co-factor for GPx but can also react as a direct scavenger of ROS (Calvin *et al.*, 1981).

Although the use of medicinal plants or their active principle in the preventions or the treatment of chronic diseases is based on the experience of traditional system of medicine from different ethnic societies, their use in modern medicine suffer from lack of scientific evidence (Buhler and Miranda, 2000). Only very few medicinal plants have been attracted the interest of scientists and one such plants is *Withania somnifera* Lin. The active principle of *W.somnifera* is glycowithanolides (WSG), consisting sitoindosides VII to X and withaferin (Bhattacharya *et al.*, 1996). They have been shown to induce significant antistress, immunomodulatory (Ghoshal *et al.*, 1989.) and cognition facilitating effects. *W.somnifera* has been documented in ancient Indian ayurveda and unani system of medicine, for its capability to improve endurance against

stress, general resistance against infections, retardation of the aging process and improvement of male sexual health in disorder such as psychogenic impotence and unexplained infertility (Ahmed *et al.*, 2010). The aim of the present study was to throw a light on protective effects of glycowithanolides on antioxidative enzymes in testes and accessory reproductive organs in D-galactose induced stressed 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 extracted from leaves of *Withania somnifera* plant as described by Bhattacharya *et al.*, (1996). Fresh leaves of *Withania somnifera* were collected, separated, washed with distilled water, blotted properly and kept in shade for drying. Dried leaves were crushed, powdered and sieved. Then soaked in chloroform for 72 hrs to remove fatty material and separate the withanolides, the solution was filtered and chloroform evaporated by evaporator, thick paste was obtained. It was stored in glass bottle at 4<sup>0</sup>C and used as active ingredient for dose preparation.

### Animals

Swiss albino male mice (*Mus-musculus*) were used as an experimental animal. They were reared and bred in departmental animal house (CPCSEA/233) under proper

conditions of light, temperature and humidity. They were supplied with Amrut mice feed (Pranav Agro industries, Pvt. Ltd Sangali.) and water *ad libitum*. All animals were treated in accordance with the (CPCSEA), New Delhi, India. Adult male mice of six month age weighing about 50 to 55 gm were divided into following four groups.

- 1) **Control group (Group I)** - Mice were injected with 0.5 ml sterile water subcutaneously for 20 days.
- 2) **D-galactose treated group (Group II)** -Mice were injected with 0.5 ml of 5% D-galactose subcutaneously for 20 days to accelerate aging (*Song et al.*, 1999).
- 3) **Protective group (Group III)** - Mice were injected with 0.5 ml of 5% D-galactose subcutaneously along with WSG at the dose of 20 mg/kg body wt/ day for 20 days (*Bhattacharya et al.*, 1996).
- 4) **Curative group (Group IV)**- Mice were injected with 0.5 ml of 5% D-galactose per day for 20 days and then dose of WSG (20 mg/kg body wt ) per day for further 20 days.

After the completion of dose mice were killed between 8.00 am to 10.00 am by cervical dislocation. The testes and accessory reproductive glands was removed, blotted and weighed and was proceed for following estimations.

- i) Estimation of SOD was done by Beaucham and Fridovich (1971) method.
- ii) Estimation of CAT was done by Luck (1974) method.
- iii) Estimation of GPx was done by Beers and Sizer (1952) method.

## Statistical analysis

All values are expressed as mean  $\pm$  S.D. The statistical analysis was performed using students 't' test. A value of  $P < 0.001$  was considered statistically highly significant.

## Results and Discussion

In control group (Group I), SOD activity was maximum in epididymis as compared to testes and seminal vesicle. CAT and GPx activity was maximum in testes in control mice (Group I) as compared to epididymis and seminal vesicle. The activity of all studied enzymes in the testes, seminal vesicle and epididymis was decreased in mice with D-galactose induced aging group (Group II) as compared to control (Group I) and decrease was significant ( $P < 0.01$ ); while there was increase in activity of the antioxidative enzymes in tissue studied from protective group (Group III) and curative group (Group IV) mice as compared to aging induced mice (Group II) and significance was  $P < 0.01$ . In *Withania somnifera* treated groups significant increase was observed in curative groups as compared to protective group (Table 1, 2 and 3).

Free radical oxidative stress has been implicated in pathogenesis of variety of diseases resulting usually from defective natural antioxidant defense. De Brain and collaborators, (2002) suggested that age related decline in fertility is influenced by stress. There are several reports linking male infertility to stress. D-galactose is aging inducing agent that causes free radical formation, leading to increased advanced glycation end products (AGEs) which accelerates the natural aging process (*Song et al.*, 1999).

**Table.1** Protective effect of glycowithanolides on superoxide dismutase (SOD) activity in testes and accessory reproductive organs of D-galactose induced stressed mice. (Enzyme activity expressed in unit/mg protein/hr). Values are mean  $\pm$  S.D. (Numbers in parenthesis denotes number of animals).

Sr. No.	Group (n=5)	SOD Activity in Testes	Statistical Significance	SOD Activity in Epididymis	Statistical Significance	SOD Activity in Seminal vesicle	Statistical Significance
1	Control	30.9588 $\pm$ 0.0233	1:2, P<0.01	37.38 $\pm$ 0.0743	1:2, P<0.01	34.8162 $\pm$ 0.0729	1:2, P<0.01
2	D-Galactose	20.7988 $\pm$ 0.0259		28.6575 $\pm$ 0.5261		24.6375 $\pm$ 0.0369	
3	Protective	22.18 $\pm$ 0.0644		31.0575 $\pm$ 0.1226		25.9463 $\pm$ 0.0434	
4	Curative	29.64 $\pm$ 0.0784	2:3, P<0.01	36.165 $\pm$ 0.01116	2:3, P<0.01	29.1975 $\pm$ 0.1361	2:3, P<0.01
			2:4, P<0.01		2:4, P<0.01		2:4, P<0.01
			3:4, P<0.01		3:4, P<0.01		3:4, P<0.01

P<0.01= significant.

**Table.2** Protective effect of glycowithanolides on catalase (CAT) activity in testes and accessory reproductive organs of D-galactose induced stressed mice. (Enzyme activity expressed in unit/mg protein). Values are mean  $\pm$  S.D. (Numbers in parenthesis denotes number of animals)

Sr. No.	Group (n=5)	CAT Activity in Testes	Statistical Significance	CAT Activity in Epididymis	Statistical Significance	CAT Activity in Seminal vesicle	Statistical Significance
1	Control	1.9916 $\pm$ 0.0065	1:2, P<0.01	1.1936 $\pm$ 0.003 8	1:2, P<0.01	0.9791 $\pm$ 0.0048	1:2, P<0.01
2	D-Galactose	0.7735 $\pm$ 0.0060		0.5033 $\pm$ 0.003 2		0.5683 $\pm$ 0.0028	
3	Protective	1.1373 $\pm$ 0.0048		0.627 $\pm$ 0.0057		0.6871 $\pm$ 0.0050	
4	Curative	1.6648 $\pm$ 0.0036	2:3, P<0.01	0.9186 $\pm$ 0.005 1	2:3, P<0.01	0.7152 $\pm$ 0.0017	2:3, P<0.01
			2:4, P<0.01		2:4, P<0.01		2:4, P<0.01
			3:4, P<0.01		3:4, P<0.01		3:4, P<0.01

P<0.01= significant.

**Table.3** Protective effect of glycowithanolides on glutathione peroxidase (GPx) activity in testes and accessory reproductive organs of D-galactose induced stressed mice (Enzyme activity expressed in unit/mg protein). Values are mean + S.D (Numbers in Parenthesis denotes number of animals)

Sr. No.	Group (n=5)	GPx Activity in Testes	Statistical Significance	GPx Activity in Epididymis	Statistical Significance	GPx Activity in Seminal vesicle	Statistical Significance
1	Control	1.6657±0.0043		0.7341±0.0054		0.8396±0.0072	
2	D-Galactose	0.6418±0.0052	1:2, P<0.01	0.3617±0.0058	1:2, P<0.01	0.4336±0.0040	1:2, P<0.01
3	Protective	0.8366±0.0037	2:3, P<0.01	0.4237±0.0060	2:3, P<0.01	0.5152±0.0089	2:3, P<0.01
4	Curative	1.5132±0.0057	2:4, P<0.01 3:4, P<0.01	0.6161±0.0059	2:4, P<0.01 3:4, P<0.01	0.6493±0.0075	2:4, P<0.01 3:4, P<0.01

P<0.01= significant.

In the present investigation antioxidative enzymes such as SOD, CAT and GPx significantly decreased in D-galactose treated group (Group II) as compared to control group (Group I). Eskiocak *et al.*, (2005, 2006) showed that psychological stress reduces the sperm quality by increasing seminal plasma ROS generation and by reducing antioxidant protection. Animals studies using Brown Norway rat, an established model of male reproductive aging, confirm that sperm from older animals produce more free radicals than from young animals and have reduced enzymatic antioxidants activity, resulting infertility( Zubkova *et al.*, 2005; Weir and Robaire, 2007). It is well recognized that oxidative stress (OS) is one of the major causes of sperm DNA damage (Aitken *et*

*al.*, 1988). The present study showed increase in antioxidative enzymes in testes, epididymis and seminal vesicle, activity in both protective (Group III) and curative group (Group IV) as compared to D-galactose treated group (Group II).

Abdel – Magied and colleagues, (2001) demonstrated that administration of aqueous extract of *W. somnifera* is able to decrease the serum level of FSH and to increase the LH level in rat. Ahmed *et al.*, (2010) observed that administration of *W. somnifera* also significantly increased serum testosterone and LH levels, and also reduced the levels of FSH in men. On the other hand, it was observed that *W.somnifera* inhibit lipid peroxidation (LPO) and reduce oxidative stress in mice

and rat. Bhattacharya *et al.*, (1996) reported that WSG exert significant antioxidant effects on various areas of rat brain by increasing antioxidants enzymes like SOD,CAT and GPx .

The active principle of *W. somnifera*, sitoindosides VII to X and withaferin (glycowithanolides) have been shown to reactivate the major free radical scavenging enzymes.

In conclusion the above mentioned observations are suggesting that increasing age might be responsible for the induction of oxidative stress (OS) in healthy subjects and may increases male infertility, decreases sperm egg interaction and reduces *in vivo* fertility by altering the levels of SOD, CAT and GPx. To counteract these changes *W. somnifera* leaves extracts treatment after the stress will be definitely beneficial and might reduces infertility.

## References

- Abdel – Magied, E.M., Abdel –Rehman, H.A. and Harraz, F.M. 2001. The effect of aqueous extract of *Cynomorium coccineum* and *Withania somnifera* on testicular development in immature wistar rats. *J. Ethnopharmacol.* 75: 1-4.
- Ahmed, M.K., Mahdi, A.A., Shukla, K.K., Islam, N., Rajender, S. and Madhukar, D. 2010. *Withania somnifera* improves semen quality by regulating reproductive hormone level and oxidative stress in seminal plasma of infertile males. *Fertil Steril.* 94 (3): 989-996.
- Aitken, R.J. and Clarkson, J. S. 1988. Significance of reactive oxygen species and antioxidants in defining efficacy of sperm preparation techniques. *J Androl.* 9:367.
- Aitken, R.J., Clarkson, J.S. and Fishel, S. 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol Reprod.* 41: 183-197.
- Beauchamp, C. and Fridovich, I. 1971. Superoxide dismutase: improved assay and assay applicable to acrylamide gels. *Anal Biochem.* 44: 276-278.
- Beers, R. and Sizer, I. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem.* 195: 133-134.
- Bhattacharya S. K., Kalkunte S. S. and Ghoshal. S. 1996. Antioxidant activity of glycowithanolides from *Withania somnifera*. *Indian journal of experimental Biology.* 35: 236-239.
- Buhler, D.R. and Miranda, C. 2000. Antioxidant activities of flavonoides. Oregon State University: USA.
- Calvin, H., Cooper, G., and Wallace, E. 1981. Evidence that selenium in rat sperm is associated with a cysteine rich structural protein of the mitochondrial capsule. *Gamete Res.* 4: 139-45.
- Chelikani, P., Fita, I. and Loewen P. C. 2004. Diversity of structures and properties among catalases. *Cell. Mol. Life Sci.* 61 (2): 192-208.
- De brain, J.P., Dorland. M. and Spek, E.R. 2002. Ultrastructure of the resting ovarian follicle pool in healthy young women. *Biol Reprod.* 66 (4): 1151-66.
- Eskiocak, S., Gozen, A, S., Yaper, S. B., Tavas, F., Kilic, A. S. and Eskiocak, M. 2005. Glutathione and free sulphhydryl content of seminal plasma in healthy medical students during and after exam stress. *Hum Reprod.* 20: 2595-2600.
- Eskiocak, S., Gozen, A.S., Tavas, F., Kilic, A. S., Taskiran, A., Eskiocak,

- M. and Gulen, S. 2006. Effect of psychological stress on the L-arginine-nitric oxide pathway and semen quality. *Braz J Med Biol Res.* 39: 581-588.
- Ghosal, S., Kaur, R., Shrivastava, R. S., Bhattacharya, S. K., Upadhyay, S. N., Jaiswal, A. K., and Chattopadhyay, U. 1989. Immunomodulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. *Phytother Res.* 3: 201.
- Luck, H. 1974. Catalase. In "Methods in enzymatic Analysis" 2, edited by Gergmeyer. Academic press, New York. 885-894.
- Malstrom, B., Andreasson, L. and Reinhammer, B. 1975. In *The enzymes*. Edi. Boyer, P. XIII. Academic press, New York. 533.
- Sikka, S. C. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Frontier in Bioscience.* 1: 78-86.
- Song, X., Bao, M., Li, D. and Li, Y.M. 1999. Advanced glycation in D-galactose induced mouse aging model. *Mech Aging Dev.* 108: 239-251.
- Weir, C.P. and Robair, B. 2007. Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oxygen species production during aging in the Brown Norway rat. *J Androl.* 28: 229-240.
- Zalata, A. A., Ahmed, A.H., Allmaneni, S.S., Comhaier, F.H. and Agarwal, A. 2004. Relationship between acrosine activity of human spermatozoa and oxidative stress. *Asian J Androl.* 6:313-318.
- Zubkova, E.V., Wade, M. and Robaire, B. 2005. Changes in spermatozoa chromatin packaging and susceptibility to oxidative challenge during aging. *Fertil Steril.* 84(suppl 2): 1991-1998.