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UV – VIS Spectral and Morphological Studies on the Effect of Sildenafil Citrate on Testis of Ethanol Fed Albino Mice

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Abstract

Erectile dysfunction (ED), a common disorder among men, is on the surge in recent days. Even though many treatments are available, Sildenafil citrate is proven to be the best oral treatment. Sildenafil is an oral phosphodiesterase inhibitor that enhances erectile function through the same general pathway used by nature. Sildenafil also has been shown to be effective in men with hypertension, diabetes, nonvascular organic etiologies for ED, and psychogenic causes. Sildenafil citrate and ethanol consumption are used in societies worlds wide and have been identified as injurious to human health. The aim of this study was to determine Lipid Peroxidation level by using UV – VIS Spectrophotometric technique and to determine changes in Histoarchitecture of Testis of Albino mice treated combinely with Caverta and Ethanol of using microscopic technique. Healthy male Wistar Albino mice (Mus Musculus), aged about 60 days and weighing about 25-30 gm, were procured from central Animals House. Animals were selected and grouped into seven each group consisting of six animals (Group S_1, S_2, S_3, S_4, S_5, S_6 and S_7). Drug, here, refers to the 50mg tablet of Sildenafil Citrate (CAVERTA Ranbaxy, India) dissolved in an appropriate Volume of Conductivity water to get 20ppm solution. This solution was used as the drug and the animals were fed with this drug @ 1µg/gm body weight. Ethanol, here, represents 18% or 100ppm of Ethanol and the experimental Albino Mice were fed with this Ethanol solution @ 0.01 µg/gm body weight. The animals in the group S_1 were considered as control animals and were fed intragastrically with conductivity water. The animals in the group S_2 and S_3 were fed with a single close of the drug chosen (Caverta) for 15days and 30 days respectively. The groups S_4 and S_5 were treated with a single close of Ethanol for 15 and 30 days respectively and the animals belonging to the last pair of groups (S6 and S7) were treated with a single close of drug and Ethanol combinely for 15 and 30 days respectively. The animals belonging to the groups S_2, S_4 and S_6 were sacrificed on 15^{th} day of initial drug administration while those belonging to S_3, S_5 and S_7 were sacrificed on 30^{th} day of initial drug administration. All these experimental animals were decapitated on the terminal day of the dosage, after four hours of drug administration. The operative procedures were carried under strict aseptic precautions. A vertical ventral midline incision was made in the abdominal wall to collect both the left and right testis samples, after using open Ketlar anaesthesia. These collected testis samples were examined by using UV-VIS Spectral technique and Optical Microscopic technique. If Caverta and Ethanol were fed combinedly, the impact has been noticed to be severe on the level of LPO in tissues of Testis of the experimental animals. There is a heavy surge in the level of LPO and the value was detected to be ≃ 23% and 61% respectively for 15 and 30 days of treatment. The long term treatment Ethanol fed Albino mice with Caverta (30days)

the Testis showed a total distortion in the Histoarchitecture of the seminiferous tubules involving vacuolization of cells, prominent Interstitial Oedema and various congestion. The outcome of the present work indicates an enhanced LPO and drug – induced morphological changes in the tissue of Testis of experimental Albino mice. Therefore, it is concluded that the combined dosage of Caverta and Ethanol produce adverse effects on Testis of Albino mice. In this chapter, it is suggested that the utilization of sophisticated analytical techniques would probably throw limelight on the mechanism of action of these drugs.

Key Words: Erectile Dysfunction, Sildenafil citrate (Caverta), Ethanol, Albino mice, Testis, Lipid and Lipid peroxidation, morphological studies

Introduction

Erectile dysfunction (ED) has been defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual intercourse **(NIH Consensus Development Panel on Impotence, NIH Consensus Conference, 1993).** The Massachusetts Male Aging Study surveyed 1290 primarily white men aged 40 to 70 years and found that ED was present in 52% of this large community sample. **(Feldman H.A et al., 1994).**

Ethanol is a central nervous system depressant and has significant psychoactive effects in sublethal doses; for specifics, see effects of alcohol on the body by dose. Based on its abilities to change the human consciousness, ethanol is considered a psychoactive drug.[David A et al., 2002). Death from ethyl alcohol consumption is possible when blood alcohol level reaches 0.4%. A blood level of 0.5% or more is commonly fatal. Levels of even less than 0.1% can cause intoxication, with unconsciousness often occurring at 0.3–0.4%. **(Hingson R et al., 2003)** The main goal of this study was to investigate the role cadmium in the promotion of Lipid peroxidation in the homogenates of rat testes and the effect of selenium on lipid peroxidation in testes of rats after cadmium injection. Data suggest that lipid peroxidation was associated with cadmium toxicity in testes and that the addition of selenium was found to be effective in attenuation of this effect. **(Shuenn-Jiun Yiin et al., 1999).**

Spermatogonial degeneration can result from exposure to toxic chemicals, heat and radiation, deficiencies of hormones or growth factors and immunodeficiency. **[(Russell LD et al., 1987), (Richburg JH et al., 1996), (Yoshinaga K et al., 1991) and (Miraglia SM et al., 1993)].** Many types of DNA lesions are produced in cells by ionizing radiation and chemicals during cancer therapy **(Somosy Z 2000)**. It has been reported that human spermatozoa are capable of spontaneous LPO and generating reactive oxygen species and that superoxide dismutase present in sperm may play a major role against LPO **(Alvarez JG et al., 1987).**

The recent survey of literature has shown clearly the fact that there is a paucity of research work on the impact of the combined dosage of Sildenafil citrate and Ethanol on the Vital functions of Testis of Albino mice.

Therefore, the Present Investigation has designed with the following aim and objectives. 1) To determine Lipid

Peroxidation level by using UV – VIS Spectrophotometric technique and 2) To determine changes in Histoarchitecture of Testis of Albino mice treated combinedly with Caverta and Ethanol of using microscopic technique.

Materials & Methods:

Animals: Healthy male Wistar Albino mice (Mus Musculus) aged about 60 days and weighing about 25-30 gm, were procured from central Animals House, Rajah Muthaiah Medical College, Annamalai University, Annamalai Nagar, Tamil Nadu. These animals were fed with standard pillet die (Hindustan Lever Ltd., Mumbai, India) and water ad libitum.

Grouping: Animals were selected and grouped into seven each group consisting of six animals (Group S_1, S_2, S_3, S_4, S_5, S_6 and S_7).

Drug Treatment: Drug, here, refers to the 50mg tablet of Sildenafil Citrate (CAVERTA Ranbaxy, India) dissolved in an appropriate Volume of Conductivity water to get 20ppm solution. This solution was used as the drug and the animals were fed with this drug @ 1µg/gm body weight. Ethanol, here, represents 18% or 100ppm of Ethanol and the experimental Albino Mice were fed with this Ethanol solution @ 0.01 µg/gm body weight.

Dosage: The animals in the group S_1 were considered as control animals and were fed intragastrically with conductivity water. The animals in the group S_2 and S_3 were fed with a single close of the drug chosen (Caverta) for 15days and 30 days respectively. The groups S_4 and S_5 were treated with a single close of Ethanol for 15 and 30 days respectively and the animals belonging to the last pair of groups (S6 and S7) were treated with a single close of drug and Ethanol combinedly for 15 and 30 days respectively. The animals belonging to the groups S2, S4 and S6 were sacrificed on 15th day of initial drug administration while those belonging to S3, S5 and S_7 were sacrificed on 30th day of initial drug administration. All these experimental animals were decapitated on the terminal day of the dosage, after four hours of drug administration.

Testis Collection: The operative procedures were carried under strict aseptic precautions. A vertical ventral midline incision was made in the abdominal wall to collect both the left and right testis samples, after using open Ketlar anaesthesia. These collected testis samples were examined by using 1) UV-VIS Spectral technique and 2) Optical Microscopic technique.

U-V Visible Spectral Analysis

Sample Preparation

The testis tissues from both the control and the experimental animals were dissected out and quickly rinsed in 4% of saline. Tissue samples were dried and homogenized using appropriate buffer by Teflon pestle. Using UV-VIS Spectrophotometer the Lipid peroxidation in testis was estimated by measuring Thiobarbituric Acid Reactive Substances (TBARS). TBARS was estimated using the method of **Nichans and Samuelson (1968).**

Results

Statistical Analysis: All the results obtained are expressed as Mean ± SD of six rats in each group Statistical evaluation was done by using Analysis of variance (ANOVA) followed by Duncan's Multiple range Test (DMRT). The statistical significance was at $p<0.05$ level.

Table – 1: Thiobarbituric Acid Reactive Substances (TBARS) in Testis of Albino mice treated combinedly with Sildenafil citrate (Caverta) and Ethanol

Groups	Concentration of TBARS (nmol/g tissue)
S_1	2.27 ± 0.16^{a}
S_2	2.38 ± 0.22^{ab}
S_3	2.58 ± 0.17^{bc}
S_4	2.65 ± 0.20^{c}
S_5	3.10 ± 0.22^{d}
S_6	2.79 ± 0.15^{c}
S_7	3.65 ± 0.26^{e}

S_1 – Control, S_2 – Drug (15 Days), S_3 – Drug (30 Days), S_4 – Ethanol (15 Days), S_5 – Ethanol (30 Days), S_6 – Drug + Ethanol (15 Days), S_7 – Drug + Ethanol (30 Days)

- ✓ Values are mean ± SD of six mice from each group
- ✓ Values not sharing a common superscript letter differ significantly at P<0.05(DMRT) P<0.05(ANOVA)
- ✓ 'Drug', here, denotes Slidenafil citrate (Caverta)

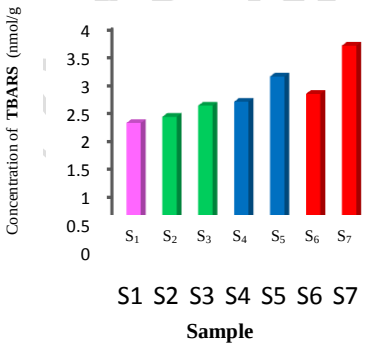

Fig.1 Change in the concentration of Thiobarbituric acid Reactive Substances (TBARS) in Testis of control and experimental Albino mice

S_1 – Control, S_2 – Drug (15 Days) , S_3 – Drug (30 Days), S_4 – Ethanol (15 Days), S_5 – Ethanol (30 Days), S_6 – Drug + Ethanol (15 Days), S_7 – Drug + Ethanol (30 Days)

'Drug', here, denotes Sildenafil citrate (Caverta)

Table - 1 illustrates the results of the level of Lipid Peroxidation in term of TBARS as carried out in the present study while Fig 1 represents the Graphical form of the same. From the table, it is clear that there is a significant increase in the level of Lipid Peroxidation (LPO) when the animals were treated with the Sildenafil citrate (Caverta). The increase has been found to be 5% and 14% for Testis. Samples of Albino Mice treated with Sildenafil citrate for 15 & 30 days respectively.

In the case of Testis samples of the experimental animals fed with a single dosage of Ethanol alone, the percentage of increase in the intensity of Lipid Peroxidation was observed to be ≃ 17 and 37 for 15 and 30 days of continuous dosage respectively.

On the other hand, if Caverta and Ethanol were fed combinedly, the impact has been noticed to be severe on the level of LPO in tissues of Testis of the experimental animals. There is a heavy surge in the level of LPO and the value was detected to be ≃ 23% and 61% respectively for 15 and 30 days of treatment.

Therefore, it is crystal clear, from the present study that the long term combined administration of Sildenafil citrate (Caverta) and Ethanol induced a statistically significant amount of Lipid Peroxidation in Testis of Albino mice.

Morphological Studies

Sample Preparation

The testis tissues collected from both the control and the experimental animals were fixed using Bowin's fluid and then dehydrated using alcohol. After debydratikon they were cleared using XY lene and embedeled in a Paraffin wax. Separate paraffin blocks for each tissue were prepared. Using a Rotary Microtome (WesWoX Company, India), sections of thickness were cut these sections were deparattinised in xylene and the slides were initially stained with haemotoxylin and then with Eosin. After staining, these slides were dehydrated through ascending grades of alcohol, cleared in XYlene and mounted in DPX.

Using the procedures described above, each Testis sample was serially sectioned and a minimum of 100 sections per Testis sample were stained and studied. Morphological studies were made using the stage microscope (NIKON, Japan) available in the histology section of Department of Anatomy, Raja Muthaiah Medical College, Annamalai University, Annamalai Nagar, Tamilnadu.

The Testis Samples of control and experimental animals have been collected and subjected to morphological studies using Light microscopic technique with an intention of studying the drug-induced structural changes.

Fig.1.1 to Fig 1.11 represents the section of testis samples of control and experimental Albino mice. Following observations have been made :

a) In case of S_1 0 hr samples, the testis of Albino mice show normal pattern consisting of interstitial space and Basement membrane. The structure of the testis was intact as shown in.Fig.1.1 and Fig 1.2. b) After fifteen days of continuous dosage of drag (Sildenafil citrate (Caverta) treatment the testis of the experimental Albino mice exhibited mild Interstial Odema and separation of spermatogonia cells from Basement membrane (Fig.1.3). c) After a single dose of Caverta treatment continuously for 30 days (S_3) there is an enhancement in Interstitial Oedema and venous congestion. Separation of spermatogonia cells from basement membrane was more and fat bubbles were also observed (Fig.1.4 and Fig.1.5). d) In case of testis of albino mice s4 samples treated with Ethonal for 15 days continuously 0.01 µg/g body wt. a normal texture of semineferous tubule, Interstial space and mild separation of spermatogonial cells from basement membrane were observed (Fig 1.6)

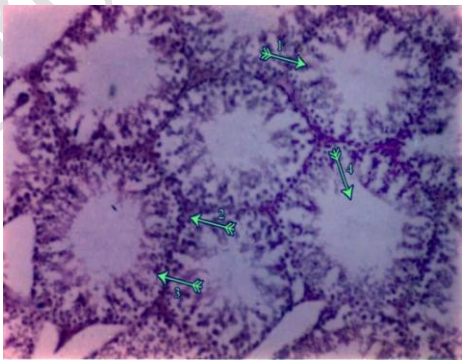

Fig.1.1 Section of Testis of Albino mice [S_1(0 hr.)] showing normal pattern consisting of (1) seminiferous tubule, (2) Interstitial space, (3) basement membrane and (4) Lumen. Haematoxylin – eosin x100.

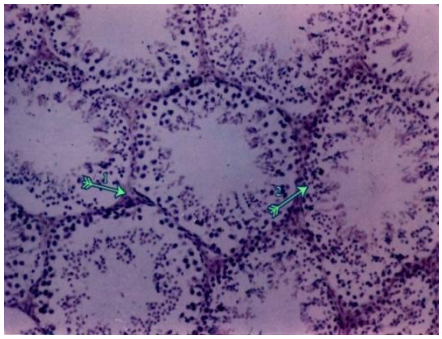

Fig.1.2 Section of Testis of Albino mice [S_1 (0 hr.)] showing normal pattern consisting of (1) Interstitial space and (2) Basement membrane. Haematoxylin – eosin x100.

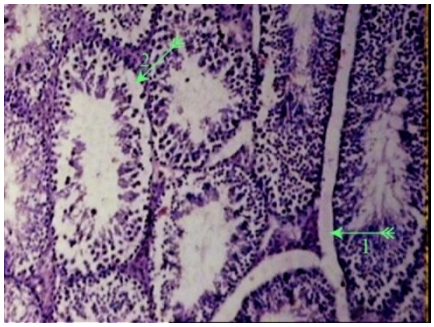

Fig.1.3 Section of Testis of Albino mice [S_2(15 days Caverta treated)] showing (1) Interstitial Oedema and (2) separation of Spermatogonia cells from Basement membrane. Haematoxylin – eosin x40.

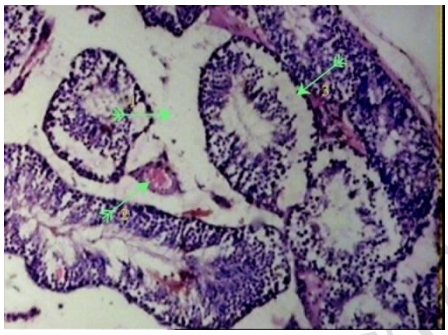

Fig.1.4 Section of Testis of Albino mice [S_3(30 days Caverta treated)] showing (1) marked Interstitial Oedema, (2) Venous congestion and (3) marked separation of Spermatogonia cells from Basement membrane. Haematoxylin – eosin x40.

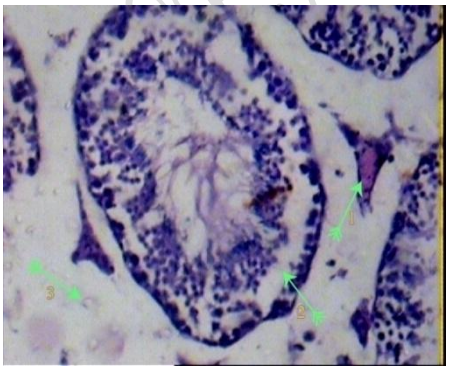

Fig.1.5 Section of Testis of Albino mice [S_3 (30 days Caverta treated)] showing (1) venous congestion and (2) marked separation of Spermatogonia cells from Basement membrane and (3) fat bubble. Haematoxylin – eosin x100.

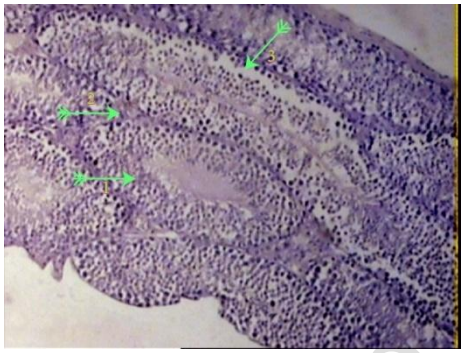

Fig.1.6 Section of Testis of Albino mice [S_4 (15 days Ethanol treated)] showing (1) Seminiferous tubule, (2) Interstitial space and (3) mild separation of spermatogonia cells from Basement membrane. Haematoxylin - eosin x40.

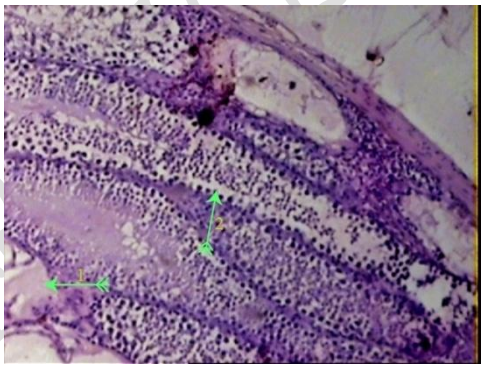

Fig.1.7 Section of Testis of Albino mice [S_5 (30 days Ethanol treated)] showing (1) mild Interstitial Oedema and (2) mild separation of cells from Basement membrane. Haematoxylin – eosin x40.

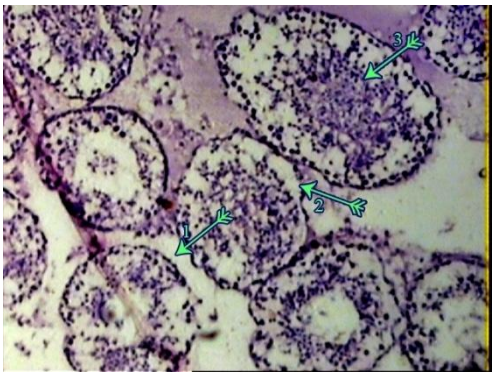

Fig.1.8 Section of Testis of Albino mice [S_6 (15 days combined dosage of Caverta and Ethanol)] showing (1) Interstitial Oedema, (2) Separation of cells from Basement membrane and (3) increased number of cells at the centre of the Seminiferous tubule. Haematoxylin – eosin x40.

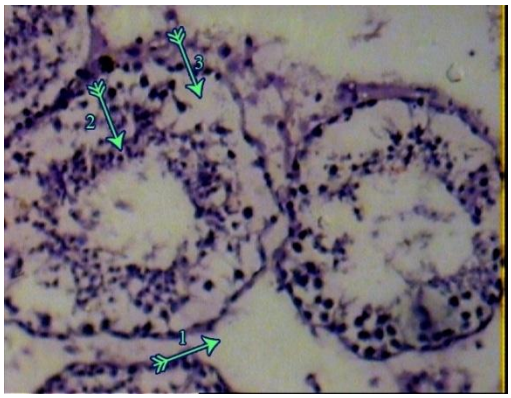

Fig.1.9 Section of Testis of Albino mice [S_6 (15 days combined dosage of Caverta and Ethanol)] showing (1) marked Interstitial Oedema, (2) noceable increase in the cells at the centre of the Seminiferous tubule and (3) marked separation of cells from the Basement membrane. Haematoxylin – eosin x100.

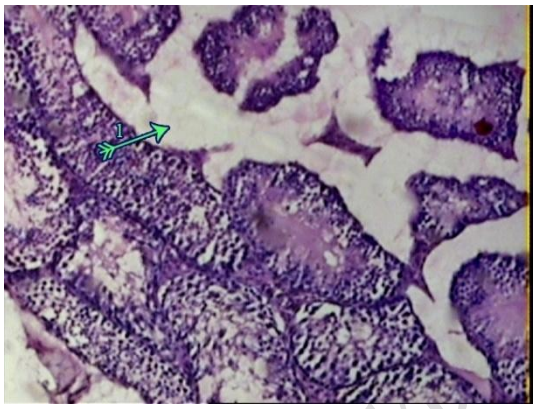

Fig.1.10 Section of Testis of Albino mice [S_7 (30 days combined dosage of Caverta and Ethanol)] showing (1) striking alterations in the Histoarchitecture of the Seminiferous tubule. Haematoxylin – eosin x40.

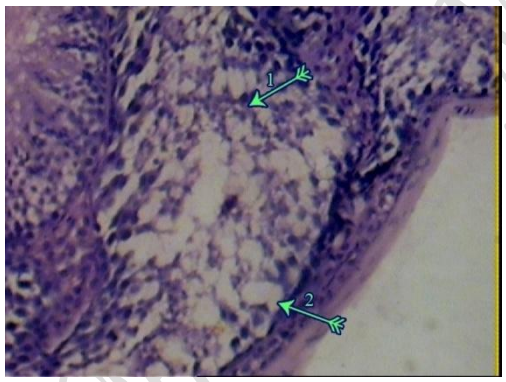

Fig.1.11 Section of Testis of Albino mice [S_7 (30 days combined dosage of Caverta and Ethanol)] showing (1) marked alterations in the Histoarchitecture of the Seminiferous tubule and (2) Vacoulization of cells in the Seminiferous tubule. Haematoxylin – eosin x100.

As the duration of Ethanol treatment gets increased to 30 days (S_5) there was mild interstitial Oedema and mild separation of cells from basement membrane as shown in Fig.1.7. e) In the case of S_6 (15 days) samples, treated with the combined dosage of caveat and Ethanol continuously for 150 days a noticeable increase in the cells at the centre of the seminiferous tubule and enhancement of Interstial Oedema along with district separation of spermatogonia cells from the basement membrane these observations are evident from Fig.1.8 and Fig.1.9. The long term treatment Ethanol fed Albino mice with Caverta (S_7 – 30days) the Testis showed a total distortion in the Histoarchitecture of the seminiferous tubules involving vacuolization of cells, prominent Intersitial Oedema and various congestion (Fig. 1.10 and Fig 1.11)

Discussion: From the results obtained in the present study, it is well understood that combined administration of Sildenfil citrate (Caverta) and Ethanol induced a significant amount of Lipid Peroxidation.

Sildenafil citrate is the agent approved for treatment of Erectile dysfunction in men **Klonner and Jarrow, 1999.** The use of Sildenafil citrate for the treatment of Erectile dysfunction by many patients has been found to result in Cardio Vascular disease due to the properties of this drug **(Gillies et al., 2002). Sivasankaran et al., (2007)** have reported the use of Sildenafil citrate to produce Lipid Peroxidation in Testis of Albino rats. In the present study also similar results indicating and increase in LPO due to the treatment Sildenafil citrate alone have been noticed.

Ethanol abuse is the major cause of health problem and a public health issue. **(Choi et al., 1998)** Ethanol is rich in Calories and devoid of nutrients thus contributing to accumulation of fat in the Testis **(Menden hall et al., 1996). (Day et al., 1993)** It is a powerful inducer of hyper lipidemia both in animals and human **(Hiryama et al., 1998)** and also causes changes in the metabolism of lipoprotein **(Verdy and Gatterian, 1998).**

Testicular development involves a complex combination of cell differentiation, migration, proliferation, and apoptosis, which occurs in a strict temporal order and anatomical pattern **(Capel 2000).**

Numerous studies have indicated that ethanol exposure has profound inhibitory effects on adult testis function in animals and humans. For example, chronic ethanol abuse in males has resulted in decreased testosterone production, reduced sperm output, and testis atrophy **(Van Thiel et al, 1980; Adler, 1992; Villalta et al, 1997).**

There is growing concern that environmental chemicals both natural and man- made, having estrogenic property may be causing a variety of reproductive disorders in wildlife and human population **(Chitra et al., 1999).** The testes of humans and other mammals are highly susceptible to damage produced by genetic disorders, environment or occupational exposure to chemical or other means. Specific causes of testicular damage have been catalogued **(Jadaramkunti and Kaliwal, 2002**

The spermatogenesis is a highly sensitive process toward the physical and chemical agents in which precursor cells form mature haploid spermatozoa within seminiferous tubules. It has been reported that, pesticides with such properties have been shown to cause overproduction of reactive oxygen species (ROS) in both intra and extra cellular spaces, resulting in a decline of sperm count and infertility in wildlife and human **(Gangadharan et al., 2001).**

The testis is sensitive to a variety of stressors, such as hyperthermia, in ammation, radiation and exposure to agents that induce apoptosis of germ cells **[(Lue, Y. H., et al., 1999), (O'Bryan M.K et al., 2000), (Hasegawa M et al., 1997) and (Richburg, J. H. (2000)].** Because oxidative stress in the testis is one of the major factors that induces germ cell apoptosis, Among various phthalate esters, DEHP is one of the most widely studied toxicants in the male reproductive organs. Administration of DEHP reduces the fertility and induces testicular atrophy of laboratory animals **[(Oishi, S. et al., 1979) (Thomas, J. A. et al., 1984) and (Oishi, S. 1986)].** Administration of DEHP induces vacuolation in Sertoli cells **[Richburg, J. H et al., 1996].** Under the present experimental conditions, vacuolation in Sertoli cells of the DEHP-treated rat testis was also observed by electron microscopy.

Histological examinations of the testes in both control and irradiated groups revealed that the diameter of seminiferous tubules, diameter of lumen and seminiferous epithelium height were decreased significantly in all doses of treatment and the decreases were dose-dependent. Irradiated adult testis showed that diameters of testis and lumens decreased and probably this change were resulted from spermatogenic cell loss and tubules disorganization **[Creemers, L.B., et al., 2002].**

Hafeiz et al. (1984) has reported that a single dose of fraction VII from L. quinquestriatus venom given to rats is capable of inducing degenerative changes in seminiferous tubules, resulting in necrozoospermia. action of Nigella sativa seems to be like other several anti-oxidative antidotes (Vitamin E , Ascorbic acid& Melatonin..) which prevent the degeneration of male germ cell by minimizing testicular cytotoxic effect in animal treated with pesticides ,chemicals, mutagens, and metals **[(Foraga 1991), (Kkhan and Sinha 1996), (El –Bahy 1997) and (Hsu et al., 1998)].** In the present study the administration of combined dosage of Sildenfil citrate (Caverta) and Ethanal for long term may induce vacuolization of cells in the seminiferous tubule and drastic alterations in the Histoarchitecture of the seminiferous tubule)

Conclusions: The outcome of the present work indicates an enhanced LPO and drug – induced morphological changes in the tissue of Testis of experimental Albino mice. Therefore, it is concluded that the combined dosage of Caverta and Ethanol produce adverse effects on Testis of Albino mice. In this chapter, it is suggested that the utilization of sophisticated analytical techniques would probably throw limelight on the mechanism of action of these drugs.

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