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Evaluation of Gonadal Toxicity of Cantharis Q in Male Swiss Albino Mice

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Abstract: The present study was carried out to investigate the toxicity of Cantharis Q treatment in male mice with respect to their gonadal function. The biochemical and histological parameters were considered to evaluate the effect of acute (7 days) and chronic (30 days) treatment and its subsequent autorecovery due to daily oral dosage of 500 µg/kg body weight of Cantharis Q which is used as a mother tincture in homeopathic medicine in its potentized form. It is prepared as a crude alcoholic extract from the blister beetles, *Lytta vesicatoria*, and contains the active component, cantharidin, that is responsible for the vesication property of these beetles when they are crushed. It also has a long history of being an aphrodisiac and there are several reports of its consumption for this purpose. Therefore, the study was aimed at establishing its clinical potential through biochemical markers of testicular function namely acid phosphatase (ACP), alkaline phosphatase (ALP), hyaluronidase (HLA) and cholesterol. The results were supported by the parameters that comprised of organ weight, epididymal sperm count and frequency of all the spermatogenic stages. The study revealed that the chronic treatment showed higher adverse effects on the gonadal cytoarchitecture and function and its subsequent autorecovery was not very promising.

Keywords: Cantharis Q, Testicular toxicity, Spermatogenesis, Epididymal sperm count, Biochemical markers, Testicular histology

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Introduction

Homeopathy is a medical system based on the belief that illness can be cured by taking a minute (dilute or potentized) dose of a substance that, if taken by a healthy person, would produce symptoms like those being treated. It employs a variety of plant, animal, mineral and even toxic substances in very small doses to stimulate the body's natural healing powers and to bring the body back into balance. Sometimes crude extracts (mother tinctures) are also used as medicine, which is termed as non-homeopathic use of the substance. Cantharis Q is one such product obtained from blister beetle, *Lytta vesicatoria* of Fabricius belonging to Family Meloidae that includes over 2500 species worldwide. It is commonly called Spanish fly since it is found frequently in Spain, and it also has an unwarranted reputation as an aphrodisiac

(Honaken, 1993). These beetles have been reported to be consumed by humans for aphrodisiac purposes. However, there have been studies regarding the poisoning effect of this extract (Zhang *et al*., 2018; Polettini *et al*., 1992; Marcovigi *et al*., 1995). This is mainly because Genito – urinary systems come under its sphere of action (Clarke, 2000). After ingestion, it is absorbed from the GI tract and is rapidly excreted by the kidneys. During excretion it irritates the entire urinary tract. The irritation of the urethra increases the blood flow to this region and might result in priapism, a persistent abnormal erection of the penis. It is, therefore, likely that the priapism is the origin of use of Spanish fly as an aphrodisiac (Metcalf and Flint, 1962). These effects are due to a venomous substance that these beetles possess which is diffused throughout their body, especially in their blood, called cantharidin, a potent chemical having caustic or blistering properties when the insects are accidentally crushed or handled roughly (Aiello, 1998). This toxic principle, cantharidin is an odourless, colourless compound that is soluble in various organic solvent but only slightly soluble in water (Davidson *et al*., 1987). The cantharidin content of Spanish fly varies from zero to high values. There are many cases of death and severe injury through its use. It inhibits protein phosphatase 2A, which is important in signal transduction (Honaken, 1993). However, cantharidin has also been used in Chinese herbal medicine as a stimulant called 'Spanish Fly' and is currently being explored for use as an anticancer drug (Karras *et al.,* 1996; Dang and Zhu, 2009). Recent studies have reported the cytotoxic effects of cantharidin on several cancers, such as hepatocellular (Le *et al*., 2016; Zhu *et al.,* 2020), gastric (Sun *et al*., 2017; Song *et al*., 2020), breast (Li *et al*., 2017; Pan *et al*., 2019) and colorectal cancers (Huang *et al*., 2011; Han *et al*., 2014). The present study was carried out to understand the effect of Cantharis Q on the male gonads of mice due to chronic oral intake which could establish its clinical potential. The autorecovery studies were also carried out in order to explore the reversibility of the effect, if any, due to such a treatment. The results were determined for the biochemical as well the histological parameters of testis in order to have comprehensive examination of the toxicity of Cantharis Q using male Swiss albino mice as the model animals. The biochemical parameters included cholesterol content and marker enzymes of testicular toxicity namely acid phosphatase, alkaline phosphatase, and hyaluronidase. The other parameters included observation of changes in organ weight, histo-pathological changes in testis cytoarchitecture, frequency of spermatogenic stages and epididymal sperm count.

Materials and Methods

Experimental animals and their maintenance:

Adult male albino mice of Swiss strain were procured from the animal house of Haffkine Institute, Parel, Mumbai, India. The body weight was about 25 – 30 g. The animals were kept under the standard laboratory conditions as per the CPCSEA guidelines at the Animal Testing Centre of Ruia College, Matunga, Mumbai, Registration Number 315/CPCSEA. They were kept with a 12-h light-dark cycle. Regular mice food and tap water was provided *ad libitum*. They were acclimated for a week.

Dosage preparation and treatment:

Cantharis Q (mother tincture) manufactured by Sarada Homeo Laboratory (SHL), Kolkata, India was used as the study agent. Drug was evaporated at room temperature to concentrate it thereby reducing the amount of alcohol in it. Dry weight of the drug per ml of the concentrate was estimated. The appropriate dose of Cantharis Q (500 µg/kg body weight) was calculated according to the weight of the experimental rats. Experimental animals were orally administered the concentrated drug diluted in 1 ml of distilled water as a single dose per day using GI gavage. Control animals were orally administered proportional quantity of evaporated 90% alcohol (which did not exceed 0.25 ml) in 1 ml of distilled water, as a single dose per day.

Experimental design:

After acclimation, the animals were divided into six groups, Group I as control set for Group II which were experimental mice treated for 7 days, Group III served as control set for Group IV that comprised of experimental animals treated for 30 days. Group V was kept as control set for Group VI that had 30 days treated mice kept for autorecovery period of 30 days (without being dosed). Dosing was done daily throughout the respective durations of treatment periods of 7 and 30 days. Daily record of body weight of the animals, food and water intake was kept during the entire period of study. Sets of control and experimental mice were sacrificed using anaesthetic ether at the end of the respective durations.

Sampling and organ preparation:

After sacrificing the animals at appropriate times, testes were quickly excised from the dissected animals. They were cleaned in cold normal saline, blotted and immediately weighed and were homogenized with chilled distilled water using Remi Tissue Homogenizer and immediately processed for biochemical analysis. Pieces of the excised testis were fixed in aqueous Bouin's fixative and processed for histological studies by standard microtechnique and Hematoxylin-Eosin staining.

Epididymal sperm count:

The sperm count per cauda epididymis was done as per the modified protocol of D'Souza (2003). The cauda epididymis was cut and minced finely in 2 ml Phosphate buffer saline (PBS). This was centrifuged at 3000 rpm for 15 min. After centrifugation, the supernatant was discarded; the pellet was suspended in 5 ml PBS. The mouth of the tube was covered with a stopper and the suspension was mixed thoroughly by inversion. This diluted suspension was loaded on one side of Neubauer's chamber. Sperm heads were counted in the four WBC squares under light microscope. Number of sperms per cauda was calculated as:

$$N/4 \times 5 \times 10^4$$

where, N = total no. sperm heads counted. The sperm count was expressed in million/cauda epididymis.

Frequency of the various stages of spermatogenic cycle:

Tubules were staged according to criteria published by Russell *et al*. (1990). Each sample tubule was staged into the following groups: Stages I-III, IV-VIII, IX-XI, XII. Tubules were staged according to the appearance of round and elongated spermatids as well as the germ cells especially the primary and secondary spermatocytes.

Seminiferous tubule diameter:

The diameter of the seminiferous tubules was measured with the help of micrometer stage of least count 0.01 mm. The ocular micrometer was used to observe the tubules and was accordingly calibrated for different magnifications as per the objective used. One hundred tubules were observed at random and their diameter was accordingly measured at the magnification of 200X.

Chemicals:

Enzyme substrates namely Phenolphthalein glucuronic acid-sodium salt and Hyaluronic acid-sodium salt (from human umbilical cord) were purchased from Sigma Aldrich Chemicals, USA. Other chemicals of AR grade were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. and Qualigens Ltd.

Biochemical estimations:

Testes tissue homogenates were prepared using Remi Tissue Homogenizer. Assays of acid phosphatase (ACP) (King and Jegatheesan, 1959); alkaline phosphatase (ALP) (Kind and King, 1954); hyaluronidase (HLA) (Linker, 1972) were performed. Readings were taken on Systronics UV-Visible Spectrophotometer.

Histopathology:

The fresh testis pieces were fixed in Bouin's fixative for 24 h and then washed with several

changes of 70% alcohol to remove excess of fixative. They were then dehydrated in ethyl alcohol gradients of 90% and absolute alcohol. The dehydrated tissue was cleared in xylene and embedded in paraffin block. Sections of 6 µm thickness were taken on the rotary microtome. Sections were mounted on clean glass slides with a small amount of Mayer's albumin serving as an adhesive. The staining was done with hematoxylin and eosin after rehydration through alcohol grades. Sections were covered with coverslip using DPX as mounting medium. Photographs were taken using Olympus CH20*i* microscope with digital imaging facility.

Statistical analysis:

Results are expressed as Mean ± SD. Statistical analysis was done using Two-way ANOVA followed by Dunnett's post hoc test and values of $P< 0.05$ were considered significant. The statistical analysis was performed using Microsoft Excel2019 Analysis ToolPak.

Results and Discussion

Testicular histology:

The weight of the testis and the diameter of the seminiferous tubules showed significant increase in 7 days treated mice whereas it was significantly decreased after 30 days treatment. The group kept for recovery after chronic treatment had the weight more or less similar to their control counterpart but showed significant increase in the seminiferous tubule diameter (Table 1). Studies with other drug intake have reported similar results such as intake of Busulfan, an anti-cancer chemotherapeutic drug treatment leads to a progressively decrease in the weight of the testes and the epididymal sperm count (Qu *et al*., 2018). Li *et al*. (2010) studied the effects of daily intragastric administration of sasanguasaponin (SGS), a compound from defatted seeds of *Camellia oleifera*, in adult male mice. Abnormal spermatogenesis, decreased sperm count and increase in the number of abnormal spermatozoa was reported in mice treated with SGS at 400 mg/kg dose. Testicular weight and seminiferous tubular area gradually decreased during the treatment of 6 weeks duration. These results suggested that adult exposure to SGS induced spermatogenic apoptosis through increased oxidative stress in male mice. This supports the histological results obtained in the present study where the decreased sperm count and seminiferous tubule diameter significantly decreased after 30 days treatment with Cantharis Q (Table 1, Fig. 1B). Mice from Group II and VI showed increase in both these parameters, however, the histological observations showed abnormal changes in the seminiferous epithelium (Figs. 1A, 1C). Similar results have been obtained in the present study with the oral dosage of 500 µg/kg body weight of Cantharis Q in the male mice. The testicular sections showed degenerative changes in their germinal epithelium associated with exfoliation of germ cells. This was observed in case of 7 days treated mice also, even though they showed increased testicular weight, tubular diameter as well as the epididymal sperm count (Table 1).

Spermatogenic activity:

All the spermatogenic stages showed significant changes in their frequency for the acute as well as chronic treatment (Table 1). This points to the fact that testosterone is crucial in the differentiation of spermatids to mature spermatozoa (Sun *et al*., 1990; McLachlan *et al*., 1994; O'Donnell *et al*. 1994).The significantly decreased frequency of stage XII for both the durations of treatment with Cantharis Q suggested that it affected the normal spermatogenesis process wherein the second meiotic phase was affected, thus hindering the normal sperm production. This could be further asserted as the recovery mice of the chronic treatment showed a significant increase in the frequency of this stage, probably indicating reversal due to stoppage of treatment. However, the histological studies in these mice still showed disorganized germinal epithelium and debris of degenerated spermatozoa in the lumen (Fig. 1C). Thus, it could be stated that the recovery was not very promising in these chronically treated mice.

Table 1: Effect of Cantharis Q treatment on weight of testis, seminiferous tubule diameter epididymal sperm count, and frequency of various spermatogenic stages of male albino mice after 7 and 30 days treatment and recovery group post treatment of 30 days

Parameters assessed	7 days treatment		30 days treatment		Autorecovery of 30 days	
	Control	Treated	Control	Treated	Control	Treated
Testis weight (mg/g b.w.)	3.172 ± 0.0832	4.2 ± 0.123*	3.472 ± 0.228	2.574 ±0.550*	3.42 ±0.303	3.46 ± 0.055
Seminiferous tubule diameter (mm)	0.170 ±0.002	0.186 ± 0.006*	0.187± 0.002	0.1738 ±0.008*	0.193 ±0.007	0.2034 ± 0.002*
Epididymal sperm count (10^5 / cauda)	43.2 ±1.304	62 ± 2*	52 ± 7.349	23.4 ±2.510*	62 ±2.0	70.4 ± 1.673*
Frequency of stages I-III (%)	24.2 ±0.837	24 ± 0.707	16 ± 1	25.4 ±0.894*	33.8 ± 0.837	21.4 ± 0.548*
Frequency of stages IV-VIII (%)	33.6 ±0.548	32.2 ± 0.837*	37.2 ± 0.837	37 ±1.0	35.4 ± 0.548	34.2 ± 0.837*
Frequency of stage IX - XI (%)	34.2 ±0.837	38.6 ± 0.548*	40.4 ± 0.548	31.2 ±0.837*	21.2 ± 0.837	35.4 ± 0.548*
Frequency of stages XII (%)	5.6 ±0.548	3.6 ± 0.548*	5.2 ± 0.837	3.6 ±0.548*	7.6 ± 0.548	10.4 ± 0.548*

The number of animals in each group, n = 5; values are expressed as Mean ± SD. *Differences that are significant between the respective control and experimental groups at P<0.05

The seminiferous tubules of mice treated for 7 and 30 days showed shortened elongated spermatids with lumen devoid of matured spermatozoa (Figs. 1A, B) indicating that the testicular toxicity of germ cells degeneration was accompanied by inhibition of androgen production.

Testicular biochemistry:

ACP is a marker enzyme for androgen activity. ACP has been studied in different reproductive organs of the male rat, in somatic cell lines derived by cloning from both rat and mouse testes, in primary cultures of rat Sertoli cells, and in isolated spermatogenic cells of the mouse (Mann, 1964). According to Mathur and Shrivastava (2006) testicular ACP activity is dependent upon androgen stimuli and is an accurate biochemical marker of specific stages of spermatogenesis. In the present study there has been an insignificant decrease in the activity of ACP for both the durations of treatment as well as the recovery group which indicates disturbance of the normal spermatogenic function in the treated mice.

ALP is known to play an important role in maturation of spermatozoa. Fluctuations in the testicular ALP suggests impairment in sperm production and maturation (Roussel and Stallcup, 1966). There has been a significant decrease in the activity of this enzyme for both the durations as well as the recovery group. Since the enzyme activity is more in the late spermatids (Kornblatt *et al*., 1983) it could be inferred that the degenerated spermatids led to this decreasing level of enzyme, as seen in the histological examination (Fig. 1).

Hyaluronidase activity has been studied during hormone - dependent cell differentiation of the germinal epithelium in the rat testis. Sperm adhesion molecule-1 (SPAM1), which has neutral and acidic (bimodal) activity, is the widely conserved mammalian sperm hyaluronidase and sperm function depends on the concerted activity of both germ cell and 'somatic' hyaluronidases

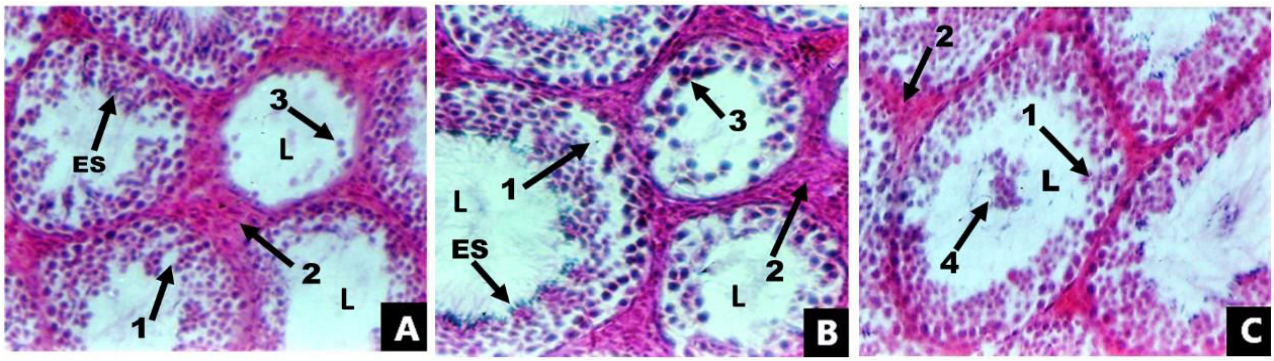

Fig. 1: Photomicrograph of T.S. of testis of treated mice (Magnification: X 400). A - Group II experimental mice after 7 days treatment showing cross sections of seminiferous tubules of reduced diameter; B – Group IV experimental mice after 30 days treatment showing abnormal seminiferous tubules; C – Group VI – kept for autorecovery after 30 days treatment having abnormal seminiferous tubules. Arrow 1 - disorganized seminiferous epithelium with vacuolation; Arrow 2 - thickened intertubular space showing lack of proper interstitial cells; Arrow 3 - sloughed off germinal elements; Arrow 4 - accumulated debris of degenerated spermatozoa; ES- the bundle of degenerating elongated spermatids; L – lumen of seminiferous tubule.

Table 2: Effect on activity of testicular enzymes and biochemical parameters of male albino rats after 7 and 30 days treatment and subsequent 30 days autorecovery period compared with their respective control groups

Parameter assessed /g wet tissue	**7 days treatment**		**30 days treatment**		**Autorecovery of 30 days**	
	Control	**Treated**	**Control**	**Treated**	**Control**	**Treated**
HLA (IU)	0.235 ± 0.003	0.211± 0.026	0.211± 0.016	0.195 ± 0.034	0.218 ± 0.024	0.163 ± 0.022*
ACP (IU)	0.840 ±0.231	0.676 ± 0.013	0.745 ± 0.109	0.716 ± 0.112	0.778 ± 0.022	0.674 ± 0.012
ALP (IU)	1.157 ± 0.226	0.342 ± 0.033*	1.152 ± 0.487	0.515 ± 0.112*	1.172 ± 0.070	0.623 ± 0.104*
Cholesterol (mg)	7.054 ± 1.710	6.932 ± 1.098	6.664 ± 0.332	4.178 ± 0.573*	6.57 ± 0.1	5.34 ± 0.08*

The number of animals in each group, n = 5; values are expressed as Mean ± SD. * Differences that are significant between the respective control and experimental groups at P<0.05

(Martin-Deleon, 2011). A slight, insignificant decrease was reported during both the periods of treatment with dosage of 500 µg/kg which progressively became significant in the recovery mice probably indicates the detrimental effects of spermatozoa maturation as seen in Fig 1C where degenerating spermatozoa as well as decreased spermatozoa were seen in the lumen of the tubules.

Cholesterol is utilized for membrane biogenesis in the gonadal tissue. It forms an important raw material (precursor) in androgen biosynthesis. Decreased level of cholesterol corresponds to decreased androgenesis (Hu *et al*., 2011). A general decrease in lipid fraction is seen when germinal cells are damaged or removed (Oresti *et al*., 2010). In the present study significantly decreased amount of cholesterol for 30 days treatment probably indicates lowered androgen synthesis which can also be confirmed by histological observation of decreased cells of Leydig (Figs. 1A, B,C). This could be because of the lack of stimulus due to low levels of LH (Adamczewska *et al*., 2022). Extract of *Lytta vesicatoria,* the agent that is used in the present treatment study is known to inhibit

steroidogenesis and steroidogenic acute regulatory protein (StAR) expression in cultured rat preovulatory follicles and this StAR synthesis is LH induced. There is a direct relationship between StAR expression and steroidogenesis as proven in the studies of mouse Leydig tumour cells. (Clarke and Stocco, 1995; Yu CC *et al.,* 2001). The depleted level of testicular cholesterol content reported is, therefore, in agreement with the above findings.

Conclusion

The overall analysis of the enzyme biochemistry supported by the histological observations reported in this study has shown a detrimental effect on the gonads of the model animal - male albino mice with the regimen of 500 µg/kg of Cantharis Q for 7 and 30 days duration. The chronically treated mice when kept for recovery period of 30 days continued to show differences in these parameters with respect to their control counterparts, suggesting only a partial recovery.

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