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Effect of Methylmethane sulphonate and Alpha chlorohydrin on reproductive organs of wild Indian house rat (*Rattus rattus*)

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KEYWORDS

Alpha chlorohydrins; anti-fertility agents; Methylmethane sulphonate; *Rattus rattus;* testis.

A B S T R A C T

The present investigation was conducted to reveal the effect of Methylmethane sulphonate (MMS) with Alpha chlorohydrin (ALPCH) on the testis of wild Indian house rat (*Rattus rattus*). The combination of MMS with ALPCH (1mg/100g body weight each/day at 1:1 ratio) was administered orally with prepared food for three days. 24 male rats were segregated into two groups in the experiment-I. Group-A rats were served as the control. In the experiment-II, the treated males with normal females were paired with 24 adult proestrous female rats for seven days and fertility was tested. The testis of the experimental groups showed histological changes including severe damage within the seminiferous tubules. Cholesterol, alkaline and acid phosphatases were changed significantly in the testis. The accumulations of lipid, alkaline and acid phosphatases were changed in the testicular tissues. There was an enhanced anti-fertility effect and a lower number of implantation sites in the treated rats. Our results validate combination of Methylmethane sulphonate with Alpha chlorohydrins for very short day's treatment (3 days) acts as a successful anti-fertility agent and prevent spermatogenesis and the methods to control the rodent pest may be followed as an integrated pest management programme.

Introduction

Rodents constitute the largest order of existing mammals. In West Bengal, a large amount of crops is destroying by wild Indian rat. These rodents cause damage to the standing crops due to their burrowing, cutting and hoarding activities, to food in storage, in poultry farms and to other commodities (Prakash and Ghosh, 1992).

Naturally on an emergency basis the rodent populations should be controlled in a judicious way. Many synthetic chemicals, compounds, physical agents have been used to control them.

Methylmethane sulphonate (MMS) produce characteristic anti-fertility effects in rodents. MMS is the DNA damaging agents (Gallego *et al*., 2001). MMS, a potent mutagen in mouse spermatogonia, and of the two post meiotic germ cell mutagens by a single intra-peritoneal injection was reported by Liegibel and Schmezer (1997).

The anti-fertility agent alpha chlorohydrin (ALPCH) has been shown to act post-testicular to cause a reversible loss of fertility in several species in mammals (Jelks and Miller, 2001). ALPCH does not impair mating behaviour, but low doses cause temporary infertility in rats (Li *et al.,* 2010). The present study was undertaken to determine the effect of Methylmethane sulphonate with Alpha chlorohydrins (1mg/100g body weight each, 1:1 combination) and it may be included integrated pest management programme as a controlling agents in controlling the rodent pests.

Materials and Methods

Adult wild male and female Indian rats (*Rattus rattus*) were trapped from various localities of the District Purba Medinipur and Howrah, W.B., India (9.75 meters above sea level at latitude 22°57'N-21°36'N and longitude 88°12'E-86°33'E). After capture, the rats were maintained in individual metallic cages and kept under ambient temperature conditions (12 hours of light, maximum temperature 25°C, minimum temperature 12°C, humidity 74 ± 1%) in the laboratory. Rats were fed a specially prepared diet (50 g/d/rat) and given water ad- libitum prior to treatment. Twenty four male and twenty four female rats of almost equal body weight (95 ± 5 g) were included. Rats were allocated into four separate groups. The combination of MMS with ALPCH (1mg/100g body weight each/day at 1:1 ratio) was administered orally with prepared food for three days. The study was conducted on obtaining permission from the Ethical Committee, Department of Zoology, Bajkul Milani Mahavidyalaya, Bajkul-721655, Purba Medinipur, and West Bengal, India.

Experiment-I

After a period of three days, Group A: Six male *R. Rattus* was weighed and sacrificed. Group B: Six male *R. Rattus* was treated with association of Methylmethane sulphonate and Alpha chlorohydrin (1mg/100g body weight each, 1:1 ratio) and sacrificed.

Experiment – II

To test the effects of Methylmethane sulphonate and Alpha chlorohydrins on the fertility of male rats, adult proestrus female rats of almost equal body weight were paired with treated male rats for seven days after the last oral treatment and fertility was tested. Mating was confirmed by the presence of vaginal spermatozoa and/or a copulatory plug. In the absence of these criteria, daily smearing of the females was performed to check for pseudopregnancy. Fertility of the males was assessed by the number of implantation sites in the females.

Light microscopic procedure

At the conclusion of the experiments, all male rats were weighed and killed by cervical dislocation between 9:00 and 10:00 AM. The right testes were

immediately removed surgically, freed from adherent tissues, weighed, fixed in Bouin's solution, and embedded in paraffin for histological study. These tissues were processed, cut into 6-um-thick sections, and stained with hematoxylin-eosin. For quantitative estimation of the germ cell populations, well-stained tubular sections of the testis of six animals were considered. Cells were counted at a magnification of 1000× (oil-immersion objective 100× and ocular 10×) from 200 different seminiferous tubules that had been randomly selected and counted from each rat. Areas of 100 seminiferous tubules from each group were traced with the camera lucida unit. The seminiferous tubular areas of the testis were measured with the help of an Allbritt disk planimeter with a zero setting device, and the area was magnified with a light microscope of low power (10×10) magnification

Biochemical procedure

Testicular tissues were processed for biochemical estimation of total cholesterol (Zarrow *et al*., 1964), ascorbic acid (Walter and Schutt, 1974), acid and alkaline phosphatases (Nino and Prasad, 1980).

Histochemical changes of the Testicular tissue

Histochemical studies were carried out on frozen sections for lipids (Sudan III & IV) (Kay and Whitehead, 1941), acid phosphatase (Bitensky, 1963) and alkaline phosphatase (Butcher and Chayan, 1966).

Statistical Analysis

For statistical analysis, the data were analyzed with Student's t test (Fisher, 1963).

Result and Discussion

Body and Testis Weight

The body weights of the treated rats were reduced at all fixation intervals compared with control, although these differences were not statistically significant. However, the weight of the testes decreased in the treated animals compared with the controls, and this decrease was statistically significant ($P < 0.001$; Table 1). However, we did not monitor food intake in this study, which was a limitation in our study design.

Histological Changes of the Testicular Tissue

The testes of the control rats were normal in shape and size. The histological sections showed numerous seminiferous tubules, the areas of which were wide with wide lumens containing abundant spermatocytes, spermatids, and spermatozoa. The basement membrane of each seminiferous tubule was very thin (Fig 1). The number of seminiferous tubular areas decreased appreciably after treatment (Fig 2). The nuclear diameters of Leydig cells were reduced considerably after treatment ($P < 0.001$; Table 3). The percentages of Sertoli cells and primary spermatocytes increased considerably and the primary spermatocytes were compact and reduced in size with flattened nuclei compared with the control groups. The percentages of spermatids and spermatozoa were significantly decreased ($P < 0.001$; Table 2).

Biochemical changes of the Testicular tissue

The different biochemical components (cholesterol, alkaline phosphatase, acid

phosphatase and ascorbic acid) in the post treated testis showed significant variations (Table 4).Testicular cholesterol and ascorbic acid levels were significantly increased at the treated rats ($P < 0.05$). But cholesterol and ascorbic acid levels in the testis were decreased ($P < 0.001$).

Histochemical procedure

Sudanophilic lipids gradually increased in the interstitium of the testis in the treated group (Table 5) and restricted to the interstitium. The distributions of alkaline and acid phosphatases within the testicular sections at treated groups were decreased. Alkaline phosphatase activity was localized within the basement membrane. The intensity of reactions of acid phosphatases was found to be very low at seminiferous region.

Effects on Mating and Fertility

The administration of association of Methylmethane sulphonate and Alpha chlorohydrin enhanced the anti-fertility effect because a lower number of implantation sites were found in post-treated rats. However, in the post-treated males were significantly infertile ($p < 0.001$; Table 6).

In the present study, association of Methylmethane sulphonate (MMS) and Alpha chlorohydrin (ALPCH) produced a marked decrease in fertility of male wild Indian house rat. Spermatogenesis is a complex cyto-morphological event controlled by various sets of genes and their products. The expressions of the genes follow a cascade pattern, and the final shape and size of spermatozoa is determined at a very late phase of spermatogenesis. ALPCH inhibits the fertilizing capacity of insufficient spermatozoa, causing an anti-fertility effect in the rams (Elizabeth *et al*., 1976). Shigenari *et al.,(*2000) used MMS on male rat and concluded that a treatment period of 2 weeks (40mg/kg) is sufficient to allow evaluation of toxic effects on reproductive organs. ALPCH interferes with the modification of surface proteins of the immature spermatozoa in the epididymis (Jones, 1989).

Interactions between Sertoli cells, Leydig cells, and germ cells are thought to be essential for spermatogenesis. In the study association of MMS and ALPCH we also observed that the weight of the testes, seminiferous tubular areas, and the nuclear diameters of Ledig and Sertoli cells all decreased considerably after treatment. Spermatogonia are highly polarized Sertoli cells that act as nursery units for developing sperm (Sawada and Esaki., 2003). The Sertoli cells are largely responsible for orchestrating germ cells through sequential phases of mitosis, meiosis, and differentiation. In the present study, Sertoli cells were shown to be increased in number at most of the fixation intervals, but the mechanism for this could not be identified.

The basement membrane plays an important role in maintaining the structural and functional integrity of tissues. Rats post-treated for longer periods of time showed increased thickness of basement membranes with fibrous connective tissue and increased percentages of spermatogonial and Sertoli cells. Other types of germ cells, such as spermatids, spermatozoa, and primary and secondary spermatocytes, decreased significantly. The nuclei showed more prominent clumps of chromatin and their interstitial spaces were considerably degenerated. It might be that MMS and ALPCH arrests spermatogonial

Table.1 Changes in the body, testis, epididymis and vasdeferens (mg/100g body weight) after combination of Methylmethane sulphonate with Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) in the male *Rattus rattus*.

Groups (n= 6)	**Body wt. (g) (Mean ± SE)**	**Relative Testis wt. (mg/100g body wt.) (Mean ± SE)**	**Relative Epididymis wt. (mg/100g body wt.) (Mean ± SE)**	**Relative Vasdiferens wt. (mg/100g body wt.) (Mean ± SE)**
	Final	**Relative**	**Relative**	**Relative**
Group A (Control)	117.83±5.72	1748.17±96.72	562.17±12.07	77.17±3.90
Group B (Treatment)	113.67±4.81 (NS)	999.50±23.25* P<0.001	493.67±10.63* P<0.01	59.33±2.35* P<0.01

Student's 't' test ; NS: Non significant & *: Significant

Table.2 Percentage of different germ cell types due to combination of Methylmethane sulphonate and Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) in the adult male *Rattus rattus*.

Groups (n=6)	Spermatogonia (%) (Mean ± SE)	Primary spermatocyte (%) (Mean ± SE)	Secondary spermatocyte (%) (Mean ± SE)	Spermatid (%) (Mean ± SE)	Spermatozoa (%) (Mean ± SE)	Sertoli cell (%) (Mean ± SE)
Group A (Control)	6.67±0.29	13.04±0.57	20.78±1.10	24.87±1.09	33.36±1.35	1.28±0.09
Group B (Treatment)	52.56±4.31* P<0.001	35.13±2.11* P<0.001	8.06±0.89* P<0.001	00.00±0.00* P<0.001	00.00±0.00* P<0.001	4.25±0.53* P<0.001

Student's 't' test ; NS: Non significant & *: Significant

Table.3 Effects of combination of Methylmethane sulphonate and Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) on the seminiferous tubular area, nuclear diameter of the Sertoli and Leydig cells of the adult male *R. rattus*.

Groups (n=6)	Seminiferous tubular area (cm^2) (Mean ± SE)	Nuclear diameter of Sertoli cell (µm) (Mean ± SE)	Nuclear diameter of Leydig cell (µm) (Mean ± SE)
Group A (Control)	23.69±2.06	2.58±0.34	2.44±0.05
Group B (Treatment)	12.85±1.20* P<0.01	2.39±0.38 NS	2.13±0.03* P<0.001

Student's 't' test ; NS: Non significant & *: Significant

Table.4 Effect of combination of Methylmethane sulphonate and Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) on the biochemical components within the testicular tissues of the adult male *R. rattus*.

Groups (n=6)	Cholesterol (mg/100 mg tissues) (Mean ± SE)	Alkaline phosphatase (µg/100 mg tissues) (Mean ± SE)	Acid phosphatase (µg/100 mg tissues) (Mean ± SE)	Ascorbic acid (mg/100 mg tissues) (Mean ± SE)
Group A (Control)	0.644±0.054	2.53±0.028	0.663±0.050	0.022±0.003
Group B (Treatment)	0.848±0.043* P<0.05	0.091±0.012* P<0.001	0.399±0.037* P<0.01	0.034±0.003* P<0.05

Student's 't' test ; NS: Non significant & *: Significant

Table.5 Effect of combination of Methylmethane sulphonate with Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) on the histochemical components within the testicular tissues of the adult male *R. rattus*.

Groups (n=6)	Regions	Lipids	Alkaline phosphatase	Acid phosphatase
Group A (Control)	i. Capsule (C) ii. Basement membrane (B) iii. Seminiferous tubules (ST) iv. Interstitium (I)	- - ++ -	+ +++ + ++	+ ++ + ++
Group B (Treatment)	i. Capsule (C) ii. Basement membrane (B) iii. Seminiferous tubules (ST) iv. Interstitium (I)	- - - +++	- ++ + -	+ ± + -

- : Negative ; ± :: Insignificant; + :: Just positive; ++ :: Moderately positive; +++ :: Highly positive.

Table.6 Effect of combination of Methylmethane sulphonate with Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) on body weight and genital organ weight of adult female *R. rattus*.

Groups (n=6)	Body weight (gm) (Mean ± SE)	Relative weight of Ovaries (mg/100g body wt) (Mean ± SE)	Relative weight of uterus (mg/100g body wt.) (Mean ± SE)
Group C (Control)	119.67±2.79	25.50±1.93	380.17±8.46
Group D (Treatment)	114.83±1.87 N S	19.00±1.34* P<0.05	314.00±6.85* P<0.001

Student's 't' test ; NS: Non significant & *: Significant

Table.7 Effect of combination of Methylmethane sulphonate with Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) on the biochemical components within the ovaries tissues of the adult female *R. rattus*

Groups (n=6)	Cholesterol (mg/100 mg tissues) (Mean ± SE)	Alkaline phosphatase (µg/100 mg tissues) (Mean ± SE)	Acid phosphatase (µg/100 mg tissues) (Mean ± SE)	Ascorbic acid (mg/100 mg tissues) (Mean ± SE)
Group C (Control)	5.73±0.23	1.09±0.06	0.213±0.02	0.435±0.02
Group D (Treatment)	8.98±0.27* P<0.001	0.29±0.04* P<0.001	0.087±0.01* P<0.001	0.822±0.05* P<0.001

Student's 't' test ; NS: Non significant & *: Significant

Table.8 Percentage of fertility after 3 days treatment of combination of Methylmethane sulphonate and Alpha chlorohydrin treatment (each 1mg/100g body weight, 1:1 ratio) in the adult female *R. rattus*.

Groups (n=6)	Fertility test
Group A + Group C	97.33±0.67
Group A + Group D	1.17±0.31* P<0.001
Group B + Group C	1.17±0.17* P<0.001
Group C + Group D	00.00±0.00* P<0.001

Student's 't' test; NS: Non significant & *: Significant

Fig.1 T.S. of testis of control *Rattus rattus* where prominent seminiferous tubular area was found. Fig.2. T.S. of testis of treated *Rattus rattus* where only spermatogonial cell was found. Fig.3. High intensity of Alkaline Phosphatase was found in the basement area in the control. Fig.4. Moderate intensity of Alkaline Phosphatase was found in the Basement area in the treated rats. Fig.5. Moderate intensity of Acid Phosphatase was found in the Basement area and interstitum area in the control. Fig.6. Low intensity of Acid Phosphatase was found in the seminiferous tubular area in the treated group.

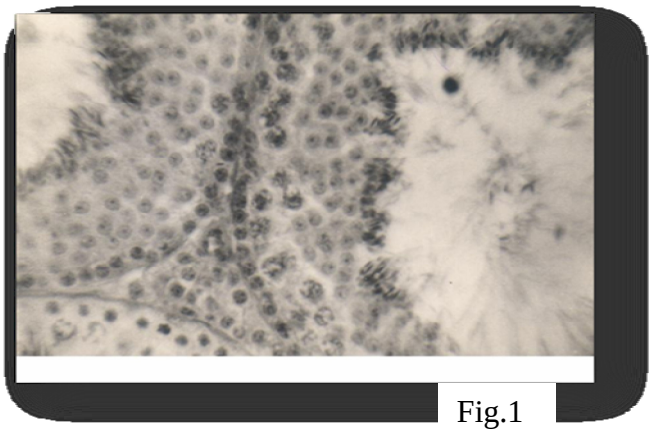

Fig.1

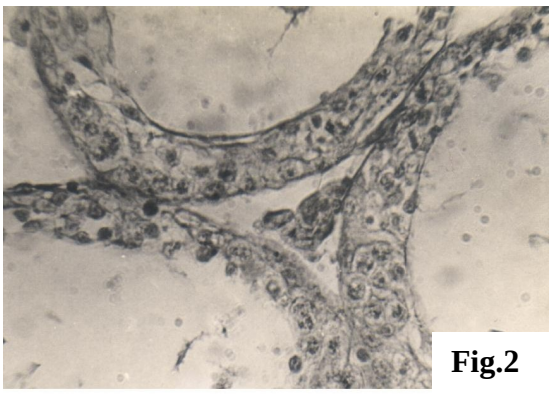

Fig.2

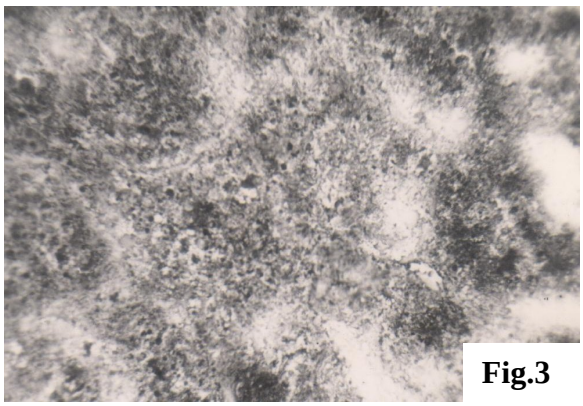

Fig.3

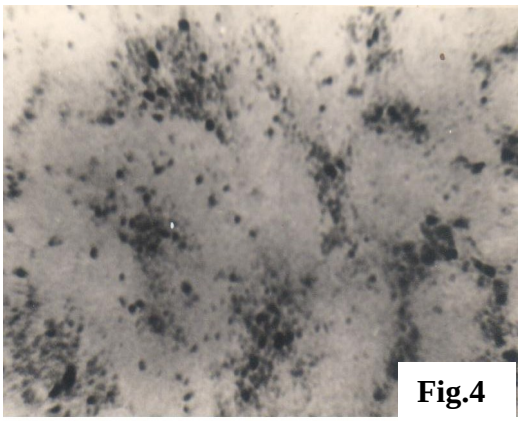

Fig.4

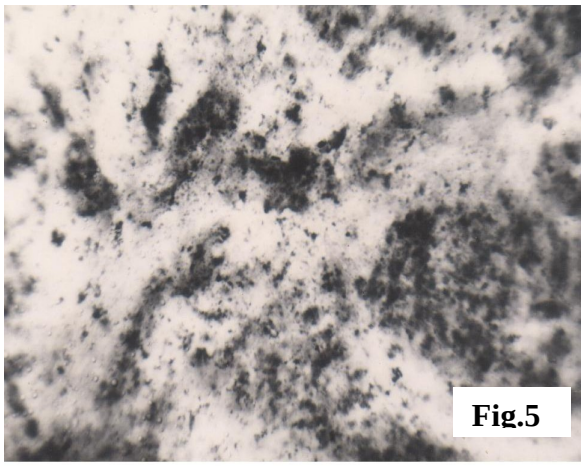

Fig.5

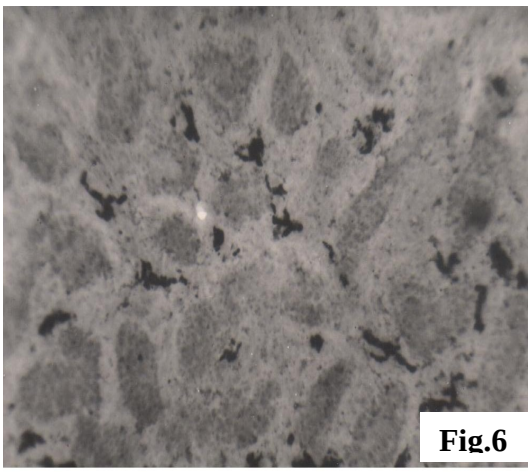

Fig.6

division and maturation, which affect spermatogenesis and other sperm parameters. It might also injure or disrupt the function of Sertoli cells and effectively reduce their supportive roles, which results in decreases in the percentages of spermatids and spermatozoa via apoptosis.
Role of ascorbic acid in the process of spermatogenesis is well-known (Shimizu, 1970). Noach and Van Rees (1958) also suggested that ascorbic acid may either be specifically involved in the production of corticoids or in the formation of steroid hormones from their precursors. In the present study, the high concentration of testicular ascorbate during the treated group may exert a negative influence on steroid biosynthesis. It is also possible that stoppage of steroid biosynthesis results in cholesterol accumulation in testis tissues. Further, ascorbic acid is known as a catalyst for both lipid peroxidation and alteration of unsaturated fatty acid composition (Shimzu, 1970). Higher levels of ascorbic acid during the treated group may be blocked in the process of steroidogenesis.

Alkaline phosphatase is required for the synthesis of glycogen, which in turn appears to participate in the metabolic process of spermatogenesis (Sohval, 1958). It has been reported that the enzymes (acid and alkaline phosphatases) are mainly associated with growth, differentiation, and maturation of the spermatogenic elements of mammalian testis (Singer *et al.*, 1980). These enzymes are also involved in removing cytoplasmic droplets of acrosomes in rat (Terner *et al*., 1975). In the present study, the low level of acid and alkaline phosphatase enzymes in the testis indicates are not associated the active participation of these enzymes in the process of spermatogenesis at the treated groups. Cholesterol is the precursor of androgens (Lofts and Murton, 1973).

Cholesterol transport within the mitochondria has emerged as the key control point for steroidogenesis (Colin, 2002). Low level of cholesterol and a very faint reaction of lipids are found in the seminiferous tubules in the treated group which prevent steroidogenesis within the testicular tissue.

Finally in summary of the results of this study demonstrate that association of MMS and ALPCH produced a marked decrease in fertility in a very short term feeding. Biochemical and histochemical data indicated that association of both fertility agents prevent spermatogenesis in various ways. So, association of Methylmethane sulphonate and Alpha chlorohydrin is an effective contraceptive regimen to control wild rodent pest populations of male wild Indian house rat.

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