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Pyrimac® (Chlorpyrifos), a commonly used pesticide, alters structural integrity of epididymal epithelium and epididymal sperm function in rats

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Abstract
Chlorpyrifos is a known biological toxicant and is widely used in agriculture. We have studied effects of Chlorpyrifos on epididymal sperm structure and function. Wistar rats (n=12 each group) were given oral doses of 0.6(T1), 0.25(T2), or 0.125(T3) mg/body weight kg Chlorpyrifos or distilled water daily for 14 days. Epididymal morphology and sperm parameters were studied on the day 15th post treatment. Epididymal weight of Chlorpyrifos treated groups increased but total sperm count decreased significantly ($P<0.05$) in the distal regions of the epididymis in comparison to those in the controls. A significant ($P<0.05$) reduction of highly motile sperm number was observed in the distal regions of the epididymis in all the treated groups. Tail abnormalities were significantly increased in treatment groups in comparison to the control. Significantly high pre-implantation loss was observed in females mated with the T3 group males. In the T3 group, distal cauda epididymal epithelium microvilli were completely absent. Present study confirms that epididymal epithelial structure is altered due to low exposure of Chlorpyrifos. Chlorpyrifos exposure interferes with epididymal structure significantly affecting epididymal functions in the rats.

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Introduction

Many chemicals used in industries, agriculture, and domestic purposes and in many other fields are blamed to be a cause for fertility damage (Carlsen *et al.* 1992). In this context, male gamete has been given serious attention since it is subtle and is vulnerable to chemical exposures. It is believed that unintentional exposure to harmful chemicals may alter reproductive capability since intentional effects have been recorded using laboratory animals (Sharp 1994). Although various aspects of pathology of male reproductive system, spermatogenesis, epididymal sperm transfer have been dealt widely, maturation of the epididymal spermatozoa and their effects due to chemical exposure have not been studied thoroughly (Perreault *et al.* 2001). However, sperm motility is an important sperm function since motility is correlated to fertilizing capacity (Moore and Akhondi, 1996). Chlorpyrifos [O,O- diethyl-O (3,5,6- trichloro-2-pyridinyl) Phosphorothioate] is a pesticide widely used in Sri Lanka for agricultural purposes. Chlorpyrifos has anti cholinesterase activity in animal tissues. The pesticide is highly toxic to many animals including Cladoceran (*Daphnia magna*), fish (*Morone saxatilis*) and mammals (Odenkirchen and Eisler 1988).

The present study was designed to investigate effects of Chlorpyrifos on epididymal structure, epididymal sperm parameters and fertilizing ability of sperm in male rats after oral exposure to the pesticide.

Materials and methods

Maintenance of test animals

Wistar rats (age 12-14 weeks) were purchased from Medical Research Institute, Colombo, Sri Lanka. They were acclimated to animal house conditions (temperature 28 $\pm$ 2 ^{0}C; RH 90 $\pm$ 10) for further 3 weeks before experiments were started. Animals were fed with pelleted food (Agro industries, Seeduwa, Sri Lanka) and water freely.

Chemicals

All chemicals were purchased from Sigma Chemical Company Ltd (USA) unless stated otherwise. The test material Pyrimec® (Chlorpyrifos, 400 mg/ml) was purchased from Lankem Ltd, Colombo, Sri Lanka.

Experiment set I: investigation of effective dose level and experimental procedures

In this part of the experiment a preliminary investigation was used for the identification of effective concentration. Previous findings have shown LD_{50} of Chlorpyrifos to rats as 250mg/kg body weight (Odenkirchen and Eisler 1988). Therefore, in the present investigation sub lethal doses were selected.

Experimental design

Male rats were divided into four groups and each group consisted 12 animals. First three groups were treated with different concentrations of test material and a fourth group was treated with distilled water, orally using a feeding tube on every day for 14 days. Hence, treatment group 1 (T1), group 2 (T2), group 3 (T3) were administered 0.6, 0.25, 0.125 Chlorpyrifos mg/kg body weight respectively and the group 4 (T4) 0 Chlorpyrifos mg/kg body weight. At all times the total volume administered was 1.0 ml. On the 15th day all the animals were used for further investigations.

Epididymal, testicular and sperm parameters

Animals were sacrificed and reproductive organs were used for experimental protocols as appropriate. Each epididymis was sectioned into five parts, distal cauda (Dcauda), proximal cauda (Pcauda), distal corpus (Dcorpus), proximal corpus (Pcorpus) and caput. For sperm counting purposes, respective region of the epididymis was minced in a disposable plastic vial (Kestrel freight and customs PVT LTD, Australia) with 2 ml of mammalian saline. Sperm counting and motility assessments were undertaken using Nebuer haemocytometer (Weber Scientific, England). Immotile (sperm that cannot move), twitching (sperm that show tail movements but cannot move), slow moving/slow motile (sperm show slow movements across the microscopic field) and highly motile (sperm can move across the microscopic field faster than motile sperm) sperm were scored. For testicular sperm counts, a known sub sample of testis was taken and weighted. This sample was minced in mammalian saline medium and sperm number was counted using a haemocytometer. In this regard only sperm with normal appearance were taken into consideration.

Sperm abnormalities

Sperm smears were made on glass slides and stained with Geimsa (1:16 in tap water for 30 minutes). Slides were air dried and used to assess abnormalities. Two hundred sperm from each slide were evaluated. The most common abnormalities observed were head, mid piece or tail abnormalities. In a typical rat sperm there is a characteristic sperm head

shape and a characteristic curvature and was obvious in the absence of abnormalities. Mid piece, abnormalities consisted coiled mid piece, enlarged mid piece and mid piece membrane, damages were observed. For tail abnormalities abnormal tail curvatures, tail less sperm, sperm with a short tail and also tail breakage were considered.

Histopathology studies

Epididymis and testes were removed from randomly chosen two animals in each group and fixed in Bouin's fluid. Tissues were kept in the fixative for 7 days and used for histological examinations. Tissues were dehydrated through a graded ethanol series 70 - 100% and cleaned in chloroform. For histopathological analysis 5 µm – 6 µm sections were obtained and double staining was carried out using haemotoxalin and eosin. Epididymal epithelium thickness, height of microvilli and tubule diameter were measured.

Experiment set II: investigation of effective dose on sperm function

From the initial experiment it was evident that the lowest dosage (0.125 Chlorpyrifos mg/kg body weight) used had higher effects on epididymal sperm. Therefore, we carried out further investigations to understand effects of Chlorpyrifos 0.125 mg/kg body weight on sperm fertilizing ability. Selection of animals, animal maintenance conditions and chemicals were similar to experiment set I.

Male rats (15-17 weeks old) were divided into two groups of 10 animals each. The control group (group) received 1 ml of distilled water per day orally. The other group (treatment group) received 1 ml of 0.125 mg/Kg Chlorpyrifos solution orally as mentioned previously. Control and treated animals were dosed for 14 days continuously and used for studies as mentioned previously. However, BWW medium (Biggers *et al.* 1971) was used for sperm counting.

Mating studies

Pro-oestrous female rats (14-16 weeks old) were selected by vaginal smearing. Males were taken on the 12th day of treatment in the control group and in the treatment group. For mating study, males were separately kept in individual cages from 14.00 hours until females were introduced. A pro-estrous female was introduced into each cage housing a male at 18.00 hours. The couple was kept overnight, because usually mating occurs during the midnight. Successful mating was confirmed by the presence of sperm in the vaginal smear in the following morning (07.00 - 08.00 h). If no sperm was present in the vaginal smear in the following morning, male was considered as, unsuccessful in mating. However, all the females were kept and on the day 14 of gestation the female rats were laporatomized. Upon laporatomy number of uterine implants and the number of corpora lutea in the two ovaries and the width of implants were recorded. Females were maintained in the laboratory until parturition. Gestation period, pup weights and pups survival were recorded.

Studying DNA Integrity of epididymal sperm

Preparation of slides - Sperm smears from cauda region were prepared on pre-cleaned glass microscopic slides and air dried for 5 minutes. Acridine orange staining was used to stain the slides. The percentage of sperm with normal DNA was determined by

counting 200 sperm from each slide with 10 x 100 magnification (oil immersion lens) with excitation of 490 nm.

Statistical analysis
All statistical analysis was performed using SPSS statistical package. Body weight, testes weights, epididymal weights, sperm concentrations and sperm motility were analyzed using ANOVA and least significant test. For the other data, log transformations were carried out before using ANOVA and Least Significant test. At all times P<0.05 was considered as significance difference.

Results
Behavior and external features
Following the Chlorpyrifos treatment, rats showed lethargy and long sleeping hours. No significant loss of body weights was recorded during the study period. In addition, treated rats showed transient testicular enlargement for 6-7 days post treatment with testicular fur reduction. Although not measured quantitatively, apparent food intake of the treated rats appeared to be reduced up to 5 - 6 days post treatment. However, after day 5 testicular sacs appeared normal. General behavior of the animals changed to less active during the first 3 days of treatment. None of these observations were seen in control animals.

Total sperm count in various regions of the epididymis
Data for total sperm count is summarized in Figure 1. The study showed reduction of sperm count in treatment groups compared to the control. Reduction was highly significant and clearly seen in the distal regions of the epididymis than in the proximal regions. However, the lowest total sperm count was recorded in the lowest treatment (T3) in the epididymis.

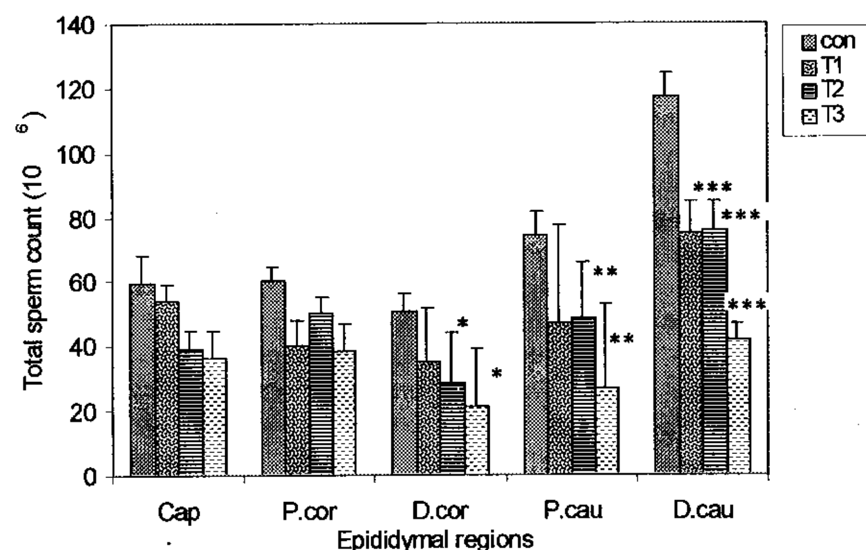

Figure 1. Mean (SEM) percentage of total sperm counts in different regions of the epididymis of the controlled and treated animals (Treatment 1= 0.6 mg/Kg bodyweight, Treatment 2= 0.25 mg/Kg bodyweight, Treatment 3=0.12 mg/Kg bodyweight, *p<0.05, ** p<0.01, ***p<0.001) followed by Student New mann Kuels Test.

Sperm motility parameters
Sperm motility data is summarized in Table 1. All treatment groups showed higher immotile epididymal sperm count compared to the control. Percentage of immotile sperm number increased significantly in Dcorpus (P<0.001), Pcauda (P<0.05) and Dcauda (P<0.001) regions in all the treatments. In the distal regions of the epididymis immotile sperm count was significantly different in all the groups. However, the highest increase in total immotile sperm percentage was evident in the lowest treatment group.

Table 1. Epididymal sperm motility recording for the treatment groups and the control group

Mean (±SEM) immotile sperm percentage					
	Caput	P.corpus	D.corpus	P.cauda	D.cauda
Control	90.85±1.96	79.36±4.66	.71.74±7.19	68.71±8.63	61.81±9.16
T1	94.37±3.33	92.24±2.37	91.03±3.64***	82.73±7.29*	83.07±4.91***
T2	92.47±5.17	85.84±3.32	94.83±2.19***	88.36±4.29*	91.18±2.76***
T3	93.21±1.74	88.59±3.71	95.91±1.50***	98.47±0.88*	94.43±1.35***
Mean (±SEM) slightly motile sperm percentage					
Control	13.14±4.52	16.29±3.46	19.85±6.41	14.99±4.89	21.66±5.61
T1	5.05±3.17	7.57±2.36	7.36±2.15	9.49±4.18*	7.69±1.99***
T2	3.05±1.78	13.25±3.25	4.06±2.07	4.89±2.76*	2.71±1.01***
T3	14.68±8.29	10.75±3.26	4.10±1.50	0.73±0.49*	3.75±1.37***
Mean (±SEM) twitching sperm percentage					
Control	0.53±0.53	3.31±1.46	1.37±0.87	2.93±1.42	2.93±1.13
T1	0.44±0.44	0	1.61±1.61	1.89±1.18	2.50±1.04
T2	1.1±1.1	1.34±1.26	0.75±1.07	2.17±0.86	2.32±0.98
T3	0	0.81±0.62	0	0.40±0.40	0.91±0.47
Mean (±SEM) slow moving sperm percentage					
Control	0.00	0.92±0.41	3.58±1.29	5.05±1.76	5.31±2.18
T1	0.15±0.15	0.19±0.19	0.00*	3.23±2.19	2.50±1.04
T2	1.10±1.10	0.26±0.17	0.5±0.5*	1.87±1.46	2.32±0.98
T3	0.00	0.25±0.25	0.00*	0.00	0.91±0.47
Mean (±SEM) fast moving sperm percentage					
Control	0.00	0.17±0.17	3.44±1.30	6.05±1.53	9.33±1.86
T1	0.00	0.00	0.00**	2.87±2.25	2.86±1.58**
T2	1.28±1.28	0.11±0.11	0.25±0.25**	2.23±1.84	2.28±1.06**
T3	0.00	0.00	0.30±0.30**	0.00	1.89±0.86**

Slightly motile sperm number in the epididymis always showed a reduction compared to the control. However, significant reduction in the percentage of slightly motile sperm number was recorded in Pcauda and Dcauda. The results did not show significant difference in twitching sperm number in any part of the epididymis at any dose level compared to the control although generally total twitching sperm number was low compared to the control. Slightly motile sperm count showed a significant reduction at all three doses levels of Chlorpyrifos in Dcorpus region (P<0.01) to distal cauda region than the control.

Highly motile sperm number gradually increased from caput to cauda in the control (Figure 2). However, in all three-treatment groups, percentage of highly motile sperm count showed significant reduction. In the proximal regions of the epididymis sperm motility was negligible compared to the control group data. However, a highly motile sperm population was observed in the distal regions of the epididymis. But this was significantly reduced compared to the control data at least in Dcorpus and Dcauda regions (P<0.05). Therefore, in general motile sperm count decreased in all treatment groups but was more prominent and significantly different mostly in distal regions of the epididymis.

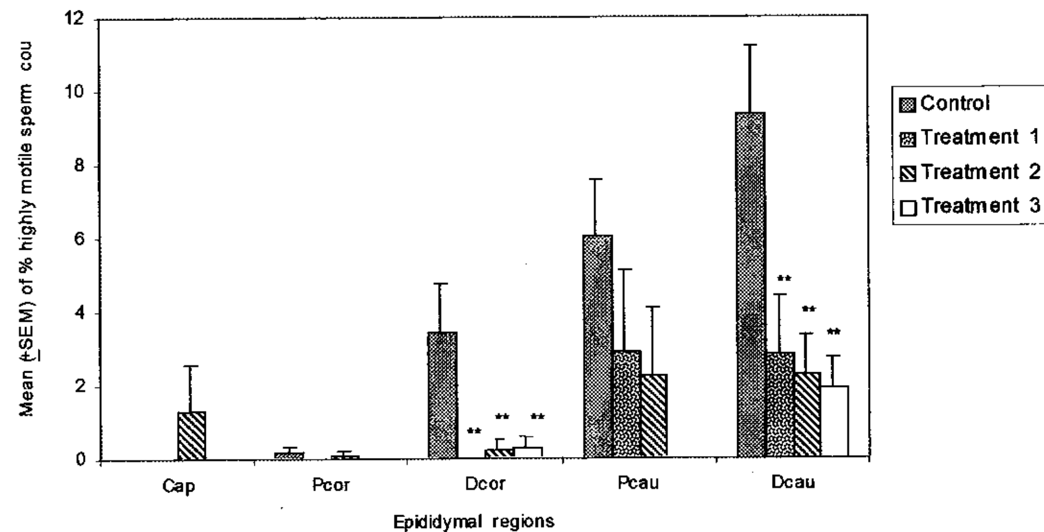

Figure 2: Mean (SEM) percentage of sperm motility patters in different regions of the epididymis of the control and treated animals (Treatment 1= 0.6 mg/Kg bodyweight, Treatment 2= 0.25 mg/Kg bodyweight, Treatment 3=0.12 mg/Kg bodyweight; P values, Compared to control data *p<0.05, ** p<0.01, ***p<0.001) followed by the student Newmann Keules test.

Cap: caput, Pcor: proximal corpus, Dcor: distal corpus, Pcau: proximal cauda, Dcau: distal cauda

Epididymal and testicular morphology

Percentage ratio of epididymal weight: body weight increased in all treated groups but significantly different from the control only in T3 (0.1172 g ±0.01 in control and 0.2214 g ±0.11 in T3 P<0.05). Epididymal weights in all treatment groups significantly

(P<0.001) increased compared to the control group (T1:0.4965 g±0.02, T2: 0.5994 g ±0.02, T3: 0.6133 g ±0.04 and in the control group 0.3524 g ±0.01). see Table 2.

Table 2 Mean (±SEM) of percentage testicular weight and epididymal weight

	% Testicular weight	**% Epididymal weight**
Control	0.3524±0.01	0.1172±0.01
T1	0.4965±0.02***	0.1708±0.01
T2	0.5994±0.02***	0.1717±0.01
T3	0.6133±0.04***	0.2214±0.11*

*P< 0.05, ***P< 0.001. Percentages were calculated as Testicular weight/ Body weight X 100
Other classification similar to Figure 1

Sperm morphology (Sperm abnormalities)

Sperm abnormalities significantly increased in all the treatment groups. In the lowest treated group abnormalities were at least doubled than compared to the control data. In the caput region head abnormalities of the control group was 10.88 % ±1.68 but in the T3 group increased three fold to 34.54 % ±7.93. Head abnormalities were common to all treated groups and in all regions of the epididymis. Significant increase of tail abnormalities in caput (P<0.05), Dcorpus (P<0.05), Pcauda (P<0.05) and Dcauda (P<0.05) regions were observed. Tail abnormalities (%) were mostly confined to the Dcauda region of the epididymis and significantly increased in all treatment groups (Control 16.48, T1 25.60, T2 22.80, T3 43.00) (Table 3).

Table 3 Total abnormal sperm numbers (±SEM) x 106 of the treatment groups and control group in different regions of the epididymis

	Caput	**Pcorpus**	**Dcorpus**	**Pcauda**	**Dcauda**
Control	22.62±1.87	23.02±2.85	19.77±3.04	19.99±2.17	16.48±2.13
T1	35.21±4.24***	30.69±3.08***	23.90±2.84	20.41±2.40	25.60±3.54**
T2	36.30±4.49***	35.43±3.24***	26.08±2.34**	23.31±4.84	21.31±2.59**
T3	57.42±7.93***	54.45±7.87***	49.96±9.61**	45.86±9.33**	43.00±9.32**

**P< 0.01, ***P< 0.001; other classification similar to Figure 1

Histopathology of the epididymal epithelium

In treated rats epididymal tubule diameter did not show any significant difference compared to the control. However, thickness of the epididymal epithelium and height of microvilli of the epithelial cells showed a significant difference than the control. Thickness of the epithelium significantly (P<0.001) decreased in all treatment groups. Usually a healthy epididymal epithelium consists of principal cells (70% – 90%) with microvilli. Thus, normal conditions were observed in control group. Height of the microvilli in control group was 2.26 μm but in T1 treatment microvilli height significantly (P<0.001) reduced with mean to 0.196 μm. At the lowest treatment T3, microvilli of the epithelial cells were completely absent (Figure 3). Therefore, results confirmed that the reduction of microvilli was dose dependent.

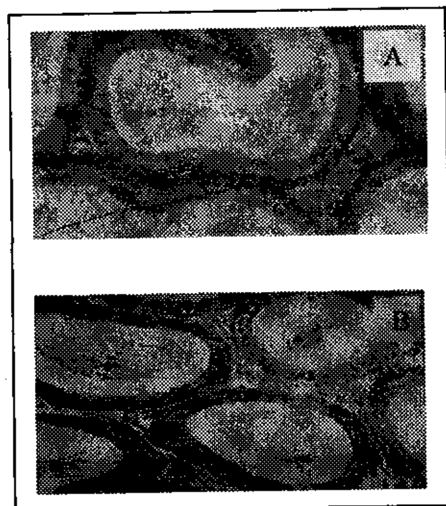

Figure 3. Photomicrographs showing detail histology of cauda epididymis from treatment (0.12 mg/kg dose) group and control group. A: cauda from a control animal showing intact appearance of the histology. B: cauda from a treatment animal showing disappearance of microvilli on the epithelium

Implantation loss and DNA integrity

Females mated with T3 treated males showed a significant pre-implantation loss than the control group (Mean 41.62±11.35 in control compared to the value of T3 73.87±8.67). No significant difference observed in caput and cauda DNA integrity of T3 males (However, 9.67±2.77 caput sperm showed red to yellow colour sperm heads compared to control data 5.73±2.08). In T3 cauda sperm showed mean 4.70±1.00 yellow to red colour sperm compared to control data 4.10±1.71. Therefore, apart from pre implantation loss there was no possible indication of implantation loss thereafter.

Discussion

According to the data gathered in the present study, it is clear that there is no any significant reduction of body weight of rats during the study period. This shows that the exposure to the pesticide has not affected their general body weight patterns. However, observations made during the study have shown enlargement of testicular sacs. This might have occurred due to swelling of the testes rather than the scrotum. It is worth investigating this phenomenon although further analyses on this aspect were not carried in the present study.

Our study also revealed that the total epididymal sperm count was affected in treatment groups. Reduction was mainly observed in the proximal and distal cauda epididymal regions of the epididymis. Reason for the total sperm count decreased was not clear from this study. However, in comparison with the data gathered for histopathological study it is clear that reduction of epithelium microvilli had a potentiality to reduce some basic functions of the epididymis thus possible to accept as a valid reason for the reduction of total sperm count. However, epididymal weight showed an increase. Decrease in total sperm count and increase in epididymal weight may not relate to each other. Results of the present study shows epididymal weight increase were mainly confined to the

proximal regions of the epididymis, but not in the distal regions of the epididymis. Several workers have observed toxicological effects on spermatogenesis after long term treatments (Luiz *et al.* 2000). However, the present findings suggest that possible pathological changes in the testis may have been the cause for weight changes in the proximal parts of the epididymis.

The importance of rete testis fluid and factors that is carried with the fluid has been given much attention by several workers (Hess *et al.* 2000). Those studies have clearly shown the relevance between the testis and the epididymis is extremely important. Nevertheless, it is understood that several necessary factors are transported to the testis via initial segment of the epididymis. During transit through the proximal epididymis (caput to proximal cauda region) sperm change from being functionally immature cells incapable of fertilizing ova, to cells with a potential for fertilizing ova which can develop normally and result in offspring (Amann *et al.* 1982). It is evident that acquisition of the fertilizing ability of sperm is associated, at least partly, with the acquisition of motility, minor morphological changes, and shifts in metabolic characteristics and alterations in cellular membrane (Amann *et al.* 1982). Epididymal secretions interact with sperm within the lumen and are associated with the modifications that lead to maturation and survivability. However, the mechanisms by which these processes take place have not been explained satisfactorily. Both in vivo and in vitro experiments have shown that the epididymal epithelium is in intimate contact with sperm within the epididymal lumen. According to the present findings, we can clearly say that fluid that carried to the proximal parts of the epididymis through the initial segment may have had some effects on functional ability of the proximal parts of the epididymis. Therefore, synthesis ability of the proximal epididymal epithelium and maturational events of caput and corpus region might have in jeopardy. Maturational events ultimately lead to the induction of sperm progressive motility in the distal parts of the epididymis (Cooper *et al.* 1989).

Results clearly show a decrease of the number of highly motile sperm and a increase in the incidences of sperm head abnormalities in the proximal regions of the epididymis. Reduction in sperm motility, especially progressive motility might have had a relationship with the loss of microvilli on epididymal epithelium. In the epithelium the most important cell type, the principal cell, is also mainly responsible for presence of microvilli and synthesis and secretary activity. Therefore, with the evidence it is clear to believe that destruction or alteration of structure and function of epithelium of the distal epididymis might have lead to the decrease of sperm motility.

On the other hand, epididymis is also responsible for maturation of sperm tail structures such as flagellum. Our data showed a higher percentage (but non significant) of treated sperm heads that consist of damaged DNA. This phenomenon showed changed sperm head nuclear integrity from treated epididymis. Although during the transit the gross morphology of sperm does not change significantly during maturation (Yanagimachi 1988), many structures undergo stabilization such as chromatin, and dense fibers of the mid piece and tail components (Calvin and Bedford 1971). Oxidation of thiol groups in sperm tail on sperm motility has been established (Yeung *et al.* 1994)). Change in thiol-disulphide bonds might have affected change in sperm motility. Therefore, further investigations might be necessary to find out the cause for sperm motility changes.

In the present study we observed at least 10 % of all observed sperm in any region of the epididymis of the treated group were decapitated sperm. Similar observations have been recorded in previous findings in the epididymal sperm (Rao and Chinoy 1983). Sperm abnormalities in the epididymis were related to epididymal functional capability mainly because epididymal functions are hormone dependent (Goyal *et al.* 2001). Both estrogen and progesterone hormones are considered important for better functioning of the epididymis (Goyal *et al.* 2001) shows low level of testosterone as the only or even a major factor in inducing the epididymal and sperm disorders, yet they have not seen functional or structural effects. In a separate study, rats treated with 10 mg/ kg ethinyl estradiol for 3 and 5 days had an undetectable level of testosterone but sperm concentration in distal parts of the epididymis was not affected (Kaneto *et al.* 1999). Testosterone replacements could only enhance such effects to some extent (Meistrich *et al.* 1975). However, some functional restoration was possible with testosterone replacements (Goyal *et al.* 1998).

Although pre implantation loss was evident no obvious post implantation loss was occurred. When compared with sperm motility data it is clear that reduction in sperm motility might have contributed to the reduction in implantation loss. Functional ability of sperm is extremely important for a successful fertilization. For a successful fertilization tail movements of the sperm and integrity of sperm head are extremely important, because fertilization is a highly specific and an extremely subtle process. Increase of head abnormalities indicates the possible cause for the reduced fertility of sperm. Plsamamembrane of the sperm is involved with fertilization process, mainly during the hyperactivation process, acrosome reaction and binding to the egg plasma memabrne (Ian and Chi 1999). Abnormalities in the tail structure could contribute to impaired function and thus can change fertilization potential. Because disulphide bonds are very important in epididymal maturation of spermatozoa. These factors collectively may possibly relate to reduce pre-implantation loss. On the other hand, possible loss of cauda epididymal functions can lead to loss of successful storage. However, it is necessary reiterate that in the present study indications of DNA damages to the sperm head were not observed.

From the present study it is clear that oral exposure of Chlorpyrifos (0.125 mg/kg) has reduced the total number of sperm in proximal and distal cauda and has decreased sperm motility which affecting pre-implantation. Reduction or alteration of microvilli patterns of cauda epididymis may also help to find out any mechanism/s involved in the above processes.

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