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Mechanism of Paraquat Action Shows Interference in Spermatogenesis and Epididymal Maturation of Sperm in Mice

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Abstract

Studies have shown mammalian spermatogenesis and sperm maturation processes are in danger from environmental pollutants but detailed mechanisms of such actions are rare. Paraquat is a broad-spectrum weedicide, potent free radical former and readily soluble in water. The present study aimed to investigate mechanism(s) of toxic effects of Paraquat on mice epididymal sperm. Mice (5 groups, n=10) were exposed with four concentrations i.e. 8(T1), 4(T2), 2(T3), 1(T4) mg/kg bodyweight of Paraquat and distilled water alone to control group, by way of inter-dermal exposure to scrotal sac for a week on the day other day and sacrificed on the day 14th. Testicular and epididymal sperm parameters were measured. DNA integrity of epididymal sperm and abnormalities were assessed using acridine orange staining. There was a marked increase in epididymal weight among treatment groups and significant ($P<0.05$) in T2. Testicular weights did not show changes indicating reduced effects on testicular endocrine milieu. However, testicular sperm count differed significantly ($P<0.05$) for treated groups T1, T2 and T3. Percentage fast moving, slow moving, twitching and immotile sperm in the epididymis were significantly reduced in all treated groups. DNA integrity test and abnormalities revealed marked damages to epididymal sperm. From the present study it is not exactly clear whether reduction in sperm number and sperm count were due to free radical formation by Paraquat. But present findings suggest whether sperm membrane damage *in vivo* make sperm storage vulnerable in mammalian epididymis.

Keywords: *Paraquat, Spermatogenesis, Epididymial Sperm Maturation, Sperm Abnormalities, Mice*

1. Introduction

Environmentally available chemicals may alter sperm production, Sperm maturation and sperm function in the epididymis. Paraquat is one of the most widely used herbicides, and probably the market leader having the highest share for the distribution. Paraquat is sold in about 130 countries for variety of pest control. It has wide spectrum weedicide properties and hence popular among farmers (Odenkirchen & Eisler, 1988). Toxicity of Paraquat and its distribution over the body once absorbed has been given serious attention. Lungs selectively accumulate paraquat and therefore contain higher concentrations than other tissues. Liver damage occurs and renal failure may follow as the kidneys remove absorbed paraquat. At spray strength paraquat is of relatively low acute toxicity. It is classified as a solid and with an acute oral LD50 for rats of 157 mg/kg (WHO, 1996).

Spermatogenesis involves rapid divisions of germinal epithelium cells until they differentiate into sperm. The process involves structural and functional changes to the sperm. The major change spermatozoa undergo is the reduction of DNA material from diploid to haploid. It is a specific requirement for successful fertilisation and result in a viable offspring. The process of spermatogenesis includes a series of mitotic and meiotic divisions. Since spermatogenesis includes nuclear reshuffling it is a delicate process and highly sensitive to toxic damages. Attention has been given to study how environmental toxicants can alter various steps of spermatogenesis process in mammals (Robaire et al, 2006).

The effects of harmful chemical exposure on the male reproductive system have been demonstrated using laboratory animals (Miller et al, 1983; Chapin et al, 1984; Linder et al, 1986; Klinefelter et al, 1997 and Veeramachaneni et al, 2001) and there have been several reports that human semen quality may be in decline. Deterioration in sperm function might be a reflection of increased exposure of the human population to synthetic chemicals although this argument remains to be proven (Creasy et al, 1985; 1990 and Carlsen et al, 1992). Among the various effects to the testes include, decrease in the weight of the testes (Linder et al, 1986 and Foster et al, 1983), flaccid appearance (Miller et al, 1983), reduction in size (Doe et al, 1983) and gross testicular pathology (Lim & Miller, 1997 and Linder et al, 1988a). With regard to gross morphology of the testis, apart from size and weight, it is very difficult for any one to measure the severity of the effect unless work on histopathological, biochemical and molecular changes are involved. Further investingations of sperm maturation are therefore important to assess the threat to human fertility by environmental toxicants. Greater understanding of sperm development may lead also to new methods of contraception in the male and better diagnosis and treatment of infertility. Progress in all these areas depends on a full understanding of the underlying mechanisms of spermatogenesis and the gradual maturation processes in the excurrent duct, which lead to complete fertilizing capacity of sperm (Moore et al, 1998).

Effects of Paraquat, however, on sperm maturation process are not represented well in the available literature. We thought to study the effects of Paraquat on sperm maturation in epididymis in detail. Therefore, we used extremely small doses close to the effective site. Interdermal exposure was made to the scrotal sac and the effects were studied. Aim of the present study was to investigate effects of low concentrations of Paraquat exposure on testicular and epididymal sperm.

2. Materials and Methods

2.1. Test Animals

Mice (age 13-14 weeks, weight 45 ± 5 g) were purchased from Medical Research Institute, Colombo, Sri Lanka. They were acclimatized in animal house conditions (Temperature 28 $\pm$ 2 ^{o}C; RH 90 $\pm$ 10) for further 3 weeks before experiments were started. Animals were fed with pelted food (Agro industries, Seeduwa, Sri Lanka) and water freely. Animals were grouped into 5 groups consisting of 10 animals in each group.

2.2. Chemicals

All chemicals were purchased from Sigma Chemical Company Ltd. (USA) unless stated otherwise.

2.3. Exposure

Four groups of animals (n=10 each) were named as T1, T2, T3 and T4 for the treatment groups and C for the Control Group. Required concentrations of Paraquat were prepared in distilled water and final dosing volume was 100 µl in each case. Dosing was done by way of an inter-dermal exposure to the scrotal sac. Care was taken to insert the needle in between both testicle capsules. Animals were exposed with four concentration of Paraquat, i.e. Treatment 1 (T1) 8 mg/kg body weight, Treatment 2 (T2) 4 mg/kg body weight, Treatment 3 (T3) 2 mg/kg body weight and Treatment 4 (T4) 1 mg/kg body weight for a week on the day other day. Control group (C) was given vehicle alone i.e. distilled water only.

2.4. Behavioural Changes

Animals were observed daily (09.00-11.00 hrs) for any behavioural changes throughout the experiment. Data recorded as either normal or abnormal behaviour. In addition any external changes to major organs such as eyes, ears and testicular sac were also checked daily until termination of the experiment.

2.5. Sacrifice of Animals and Collection of Sperm for Counting

Animals were sacrificed on the day 14 after initial exposure. Testes and epididymides were surgically removed. Tissue was cleaned thoroughly to remove any bloodstains. Both left and right testes were weighted using an electronic balance. Epididymides were isolated and cleaned. Regions of the epididymis were separated according to classification (Figure 1). Separated regions were put into glass vials (10 ml) previously incubated at 37 ^{o}C with 2 ml of BWW solution containing BSA (Bovine Serum Albumin). Tissue minced using a fine pair of scissors and an aliquot of sperm preparation was allowed to pass through a Nebula haemocytometer and numbers of sperm were counted.

2.6. Assessment of Epididymal Sperm Motility

A drop of sperm suspension was introduced on the haemocytometer and sperm count and sperm motility were recorded. Both counting and motility assessment were finished within first 5 minutes. Motility was recorded after categorizing 4 main different patterns of sperm motility i.e. i) non motile- sperm which do not show any motility at all; ii), twitching- sperm that show tail beats and any other movement but that cannot move at all; iii), slightly motile (motile but not fast)- sperm that can move but do not move very fast; iv), highly motile- sperm that show high motility, these sperm usually could cross the filed of view very quickly.

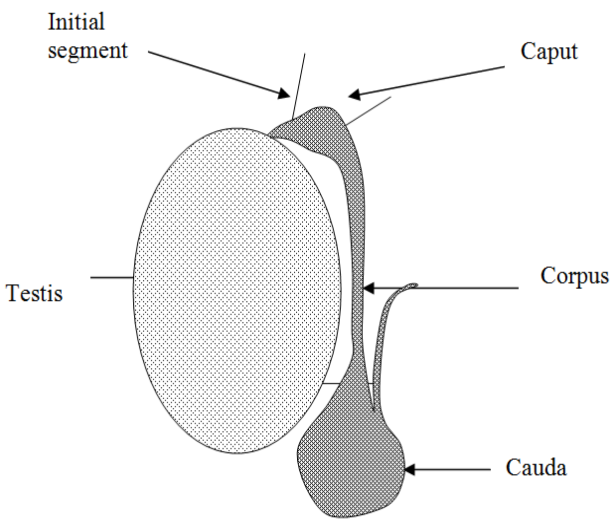

Figure 1. Diagrammatic Presentation of the Epididymal Regions Defined and Used in the Present Study

2.7. Morphological Changes to the Sperm

Sperm smear prepared on glass slides were used to investigate morphological changes to the sperm. A drop of sperm was placed on a clean glass slide and a smear was made. Observations were done using a stereo microscope (Olympus, Japan). Usually morphological changes were clear under the power x400. Morphologically normal sperm were distinguished with their characteristic typical sperm appearance. However any sperm that showed following morphological changes were counted as abnormal morphology i) Sperm head with abnormal shape or size ii) Sperm without sperm heads iii) Sperm with morphologically abnormal tails, such as coiled tails, iv) any other abnormality.

2.8. DNA Integrity Assay

Fresh sperm samples were used to prepare smears. Usually at least 3 sperm smears were prepared from each individual animal using a similar procedure for sperm morphology. Once the smear is dried method described elsewhere was used to analyze DNA integrity using acridine orange test (Peiris, 1998). Briefly, Sperm smears were done using fresh sperm, air dried. Slides were fixed in 3:1 methanol: Glacial acetic acid for 4 hours. Slides stained with acridine orange-citric acid buffer (16:1, 0.1 M Citric acid: 0.3 M $Na_2HPO_4.7H_2O$, pH 2.5, 1% acridine orange 10 ml) for approximately 5 minutes. Slides washed with PBS and checked for yellow to red or green appearance under fluorescence microscope. Results were interpreted as percentage yellow-red heads.

2.9. Statistical Evaluations

Statistical evaluations were carried out using SPSS for Windows. Data were used either as raw data or transformed data. Percentage values were transformed to log values. ANOVA was used to assess all the data mentioned in this work. However, individual comparisons were made using Tukey's test. At all levels <0.05% was taken as significant level. LD50 values calculated using probit analysis method.

3. Results

During the experiment no gross pathological effects were seen externally or internally in the scrotum, testis or epididymis of all mice used. Calculation of the LD50 value of Paraquat for 7 days scrotal inter-dermal exposure was 6.7 mg/kg. Therefore the exposure used in the present investigation was around 50% lethal concentrateon. There was a change in epididymal weight among treatment groups than the control but significantly increased ($P<0.05$) only in T2. Values were 0.043±0.002 in T1, 0.050±0.003 in T2, 0.045±0.002 in T3, 0.042±0.003 in T4 and 0.037±0.002 in Control Group.

Testicular weights of the treated groups were not significantly different from the control group indicating reduced effects on testicular endocrine milieu. However, testicular sperm count differed significantly ($P<0.05$) from the control for treated groups 1, 2 and 3 [(6839 ± 138) $x10^8$ in control, (3225 ± 1390) $x10^8$ in T1, (777 ± 370) $x10^8$ in T3, (1481 ± 892) $x10^8$ in T4].

Length of caput corpus and cauda epididymis did not show significant changes. Similarly epididymal weight was non-significant indicating no apparent alterations to these systems due to toxicant exposure. However, functional aspect of the epididymis showed clear significant changes compared to the control. Total epididymal sperm count was significantly different from the control (Figure 2). There was a marked difference in the T2 although the results were not statistically significant. Percentage fast moving sperm in the epididymis was significantly reduced in all treated groups. This reduction was commonly found in slow moving and twitching sperm. Reduction in percentage immotile sperm needs to be compared with total epididymal sperm count. Because there was a significant reduction in epididymal total sperm count.

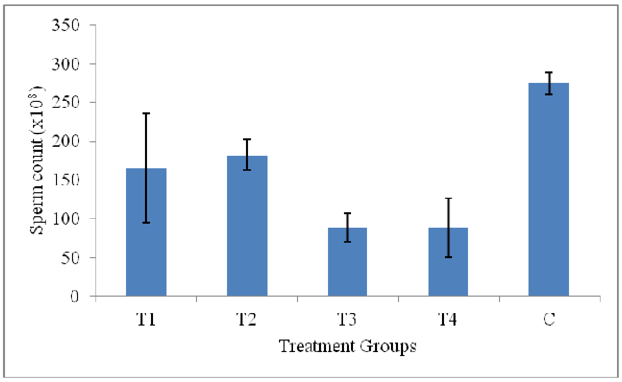

Figure 2. Total Epididymal Sperm Count in Treatment Groups Exposed to Paraquat

When taken alone caput region did not show significant motility changes compared to control. However, percentage-twitching sperm in the caput decreased significantly for Treated Groups 1, 3 and 4. Fast moving sperm behave differently. In the corpus region, there was a slight reduction in fast moving sperm compared to control but percentage twitching and immotile sperm showed a numerical increase. Reasons were not quite evident. Cauda fast moving sperm reduced significantly for all the treated groups (Figure 3).

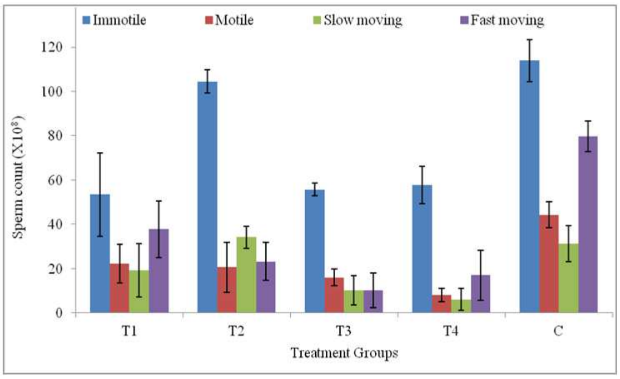

Figure 3. Epididymal Sperm Motility in Treated Groups Exposed to Paraquat

Morphologically sperm from treated groups showed the abnormal sperm heads. This percentage was higher in all treated groups compared to the control group. In addition, percentage of sperm without tails were also higher in treatment Group 4 (1 mg/kg), compared to all the other groups including the Control Group. Abnormal sperm number increase was highest in treated group exposed to the lowest concentration. Head abnormalities were prominent than tail abnormalities in caput, corpus and cauda. However there was a considerable number of tail abnormalities compared to the control. The values are given in the Table 1.

According to acridine orange test, yellow to red appearances in sperms were increased in all treated groups. Results of the caput region in T2, T3 and T4 were significantly different ($P<0.05$) compared to the control (20% in the control while 44%, 57% and 41% in T2, T3 and T4 respectively). Sperm in the corpus region showed the similar trend. Moreover, Cauda sperm showed the highest increase in yellow to red heads ($P<0.001$).

4. Discussion

Present investigation was carried out with a novel technique of toxicant exposure to mice. The exposure method allowed immediate internal exposure of Paraquat to the vicinity of reproductive systems. Since we have used extremely low concentrations, it is suggestive to use this type of a method to investigate mechanism of toxicant exposure. However, our exposure in this experiment was around LD50 value for Paraquat exposure of this method. Therefore it is clear that the method used will help in understanding of the toxicant effect on target tissue. Nevertheless, in earlier experiments it has been shown that LD50 values of Paraquat is 151 mg/kg for rat (Odenkirchen & Eisler, 1988).

Paraquat concentration used in the investigations did not show possible change in testicular weight. This is clear to show the effects were not on the hormonal control of the testes. This also depicted in epididymal weights and length changes. These data suggest that it is possible to exclude any possible explanation due to change of testosterone production or synthesis. However, the marked reduction in testicular sperm count in Treated Groups 1, 3 and 4 against the control group may provide a better understanding.

Reduction in epididymal sperm motility due to Paraquat exposure was significant in all treated groups. This was supported by reduction in total sperm count as well. It is well known that sperm is highly susceptible to reactive oxygen species (Aitken et al, 1994). Because reactive oxygen species will harm lipid layers of sperm thus sperm membrane is in danger. As Paraquat produces free radicals (Samayawardhena et al, 1995), which can damage sperm membranes. If similar mechanism has happened in the present investigation it might have been a probable case for reduction in sperm motility. On the other hand during the epididymal maturation sperm membrane is highly reviewed (Cooper, 1986). The necessity of such development is clear. Epididymis mainly makes final preparation of sperm for a successful fertilization. Whether Paraquat could change fertilization ability need to be properly investigated. However we might have not addressed the entire hypothesis in this investigation, though it is probable to think that Paraquat toxicity might have occurred mainly through free radical formation.

Table 1. Percentage Sperm Abnormalities in Different Regions of the Epididymis in Mice Exposed to Paraquat

Region	Type	Percentage Abnormalities (±SD)				
		T1	T2	T3	T4	C
Caput	Head abnormalities	30.22±1.13	26.13±0.99	26.32±1.52	40.72±5.15	4.89±0.96
	Head less	18.94±3.3	14.93±1.24	23.12±4.13	11.45±2.19	7.54±0.62
	Tail abnormalities	17.67±0.5	8.92±0.63	12.18±1.29	6.31±0.46	10.77±3.13
	Tail less	1.51±0.51	1.42±0.6	0.17±0.17	2.77±0.38	1.47±0.83
	Normal	31.64±2.9	48.55±2.12	38.8±3.87	43.07±3.12	74.29±3.59
Corpus	Head abnormalities	29.09±2.78	26.94±1.02	28.57±2.2	30.1±4.2	2.93±0.83
	Head less	18.27±1.85	12.26±1.22	22.49±4.35	15.62±3.08	5.75±0.65
	Tail abnormalities	14.21±0.94	12.98±2.19	11.12±1.07	8.08±0.69	7.2±1.38
	Tail less	0.75±0.75	1.04±0.65	2.43±1.27	2.58±0.26	1.02±1.02
	Normal	37.41±3.81	46.76±2.32	35.59±4.26	43.54±2.89	80.56±3.74
Cauda	Head abnormalities	20.67±2.4	26.64±1.28	27.98±2.29	33.4±3.34	3.97±0.63
	Head less	19.38±5.6	12.13±1.0	23.55±3.48	15.89±2.39	8.83±1.61
	Tail abnormalities	10.53±1.69	12.09±1.24	10.26±1.27	7.7±0.9	9.31±1.16
	Tail less	6.21±2.44	3.43±1.03	1.86±0.79	2.69±0.28	0.82±0.41
	Normal	43.18±4.83	46.09±1.36	38.53±3.0	40.29±3.75	77.03±3.46

In the present study we found that all doses except the dose 2 showed main differences in sperm count, mortality and abnormalities compared to the control. The fact was not clear why Group 5 (Control) and T2 (2 mg/ml) were in a sub set. Variations in just one group has been recorded earlier when hamsters were treated with alpha chlorohydrins results of 66 mg/ kg dosages were highly variable but not the results of the doses treated with 33 mg, 49 mg and 83 mg (Slot et al, 1995). The fact seems that we cannot find a proper answer for such variations with limited results. In relation to alpha chlorphydrin change in sperm motility has been explained with alteration in sperm transport within female reproductive tract (Slot et al, 1995). Changes in sperm motility are usually considered as a mechanism to alter fertilizing capacity. Because testicular dysfunction, hormonal dependence and alterations in sperm transport are not considered alternative mechanism to explain changes in sperm fertility (Lubicz-Nawrocki & Chang, 1974a and b).

In our study hormonal dysfunction was less likely to be considered as a proposed mechanism of action because we did not observe a significant change in testicular weight, epididymal weight or epididymal length. Probably our data suggests less involvement with hormonal milieu Altered sperm motility can also be associated with sperm metabolism. If metabolism mechanisms are blocked and alter-ed this may have a great impact on sperm motility, such as in fertilization process sperm could show altered abilities in capacitation and acrosome reaction (Dravland & Meizel, 1981). Therefore, perhaps the observed motility reduction in the present study may have far reaching effects that we did not see.

There are significant correlations in vivo fertilizing ability and both uterine and epididymal sperm motility end points when assessed using CASA (Moore & Akhondi, 1996). It is known that velocity end points are better than percentage motility or percentage progressive motility with sperm fertilizing ability. However motile sperm percentage is frequently used end point in toxicological studies.

In the epididymis biochemical mechanisms underlying sperm maturation is largely undefined. Because it is known that during the epididymal passage intrasperm concentrations of cAMP, pH and Ca changes are observed (Goltz et al, 1988). Although development of sperm motility in the epididymis is associated with intrasperm cAMP levels in many species, it is not considered true for all mammalian species (Bedford & Hoskins, 1990). However, calcium and pH act as factors that may modify the events occur by cAMP in the motility enhancement. Changes in Calcium concentration, pH above and below optimum ranges can stimulate or inhibit flagella activity (Goltz et al, 1988 and Lindemann & Kanous, 1989).

Serine/theonine specific proteins are known to be involved in wide range of cellular function. In addition, catalytic protein phosphates are also known to be involved in sperm motility (Vijayarajan et al, 1996).

Functions of epididymal epithelium for sperm maturation while in the epididymal transit cannot be excluded as well. It is evident that the acquisition of the fertilizing ability of sperm is associated, at least partly, with the acquisition of motility, minor morphological changes, shifts in metabolic characteristics and alterations in cellular membranes (Amann, 1982). Epididymal secretions interact with sperm within the lumen and are associated with the modifications that lead to maturation and survivability. Although some proteins originate from rete testis fluid, many proteins present in the epididymal luminal fluid are produced by the epididymal epithelium (Hoskins & Hall, 1975). Epididymal specific proteins have been recorded in many species including bull, boar and rat (Baker & Amman, 1971; Lavon & Boursnell, 1971; Koskimis & Kormano, 1975 and Brooks, 1981). Generally, the caput is the most active secretory region of the epididymis although specific proteins are secreted throughout the duct. Specific epididymal proteins may be tightly bound to the sperm during transit along the lumen. This has been proved experimentally with the requirement of high salt concentrations for the extraction of these proteins (Kohane et al, 1980b). Many secretions are glycoproteins which vary between the regions (Bostwick et al, 1980). Glycoproteins are known to be a major component in the surface protein composition of sperm. Therefore, alterations of such events in the epididymis can also have an influence on loss of sperm maturation or on loss of sperm motility. But such hypothesis need to be further evaluated in the present study.

Many studies have shown that sperm maturation and storage in the epididymis is dependent on androgens directly from the rete testis and from the systemic circulation (Cooper, 1986). *In vitro* studies have identified androgen receptors (ARs) in all regions of the mammalian epididymis. Their distribution in the epididymis and their concentration within the epithelium differs along the duct. For instance, in the rabbit there is a greater concentration of ARs in the caput epididymis; surprisingly in the cauda region the majority of the receptors were for oestrogen (Toney & Danzo, 1988). Oestrogen receptors have also been observed in the ductuli efferentes in the goat (Chan et al, 1995; Goyal et al, 1997). These receptors are important providing necessary receptor mediated supportive functions in the epididymal environment. For instance damage to the epithelium cells due to free radical formation or even depletion of $NADPH_2$ could be attributed to epithelium function thus sperm might not receive necessary attention. However, whether the assumption was true in the present case was not evident but can only suggest.

Androgens are concentrated in the caput region in cytosol and nuclei of principal cells. 5α-dihydrotestosterone is known to be secreted by the epididymis itself (Brooks & Higgins, 1980). Androgen dependent secretory proteins mediated through ARs must have a functional significance since the lack or reduction of androgen dependent proteins leads to a decrease in the acquisition of fertilising ability of epididymal sperm. The chemical composition of epididymal luminal plasma is profoundly influenced by circulating androgens. In the presence of androgen, a stable milieu is maintained in the lumen of the duct (Jones R, Glover, 1973; Orgebin-Crist et al, 1976 and Courty et al, 1987). Sperm are bathed in a fluid rich in dihydrotestosterone, testosterone, 3α-androstanediol and 3β-androstanediol although a direct affect of androgens on epididymal sperm has not been reported. Dihydrotestosterone concentration is maximal in the corpus epididymis. In addition to androgen, progesterone has been reported to be present in the bull caput and corpus epididymis (Ganjam & Amann, 1976). Since the testis is the major source of androgen to the epididymis any possible impact on the testis could change the balance between androgen secretion and proteins mainly dependent on androgens.

With some epididymal proteins their association with sperm does not happen at site of secretion, but appears later (Vernon et al, 1982). The binding of epididymal proteins to surface of sperm may require other factors (such as enzymes) that are present in the distal regions of epididymal tract. Such studies using labelled epididymal tissue or epididymal sperm *in vitro* have been reported for many species including rat, mouse, hamster, rabbit, ram, human (Olson & Hamilton, 1978; Gabel et al, 1979; Oliphant & Singham, 1979; Voglmayer et al, 1980; Olson & Danzo, 1981; Brooks & Tiver, 1983; Cornwall et al, 1990; Vreeburg et al, 1992; Akhondi, 1996 and Moore & Akhondi, 1996). Additionally, several other studies have used the technique of *in vivo* injection of ^{35}S Methionine into rat and mouse followed by total detection of epididymal protein secretions in epididymal fluid or on sperm (Cornwall et al, 1990; Vreeburg et al, 1990 and 1992). These evidences suggest possible role of androgens and the importance of epididymal synthesized and secreted proteins in sperm maturation process. In the present findings we have shown epididymal sperm motility reduces significantly and the effects are more confined to lower doses. Yet possible hormonal interference cannot be used in support to explain data due to no weight change in testis or epididymis after the treatments.

Androgens, such as testosterone are known to regulate many secretory proteins of the epididymis (Orgebin-Crist et al, 1976). The influence of androgens probably occurs by interacting with androgen receptors present at varying concentrations in the epididymal epithelium. Such an interaction leads to promotion of transcription and translation of mRNA that codes for specifically regulated proteins and resulting in change in synthesis of proteins. The secretory activity of hamster epididymis has been reported previously (Cuasnicu et al, 1984 and Robitalle et al, 1991). Although it is possible to speculate no hormonal interference, we cannot exclude the fact that molecular level interference of Paraquat on epididymal epithelium functioning can be considered important in sperm maturation. Therefore, the recorded results in this study probably could support possible interference of Paraquat on epididyml epithelium function and thus effects seen in sperm are secondary. To conclude such hypothesis further experimentation is necessary.

5. Conclusion

Although the mechanism, by which these abnormalities occur in the epididymis, is unclear, we can suggest that alteration in sperm structure in the epididymis can greatly damage sperm function thereafter. Whether this destruction process has an involvement with epididymal epithelium are being studied at the moment. But we know that Paraquat can generate free radicals within the biological system. These free radicals are highly vulnerable to membrane structures including sperm membrane. Per oxidation and damages to the membrane will alter all the functions of the membrane creating a hostile situation to sperm function in the epididymis.

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