Low Levels of Pesticides Disrupt the Pituitary-Gonadal Hormone and Reproductive Functions in Male Rodent Model.

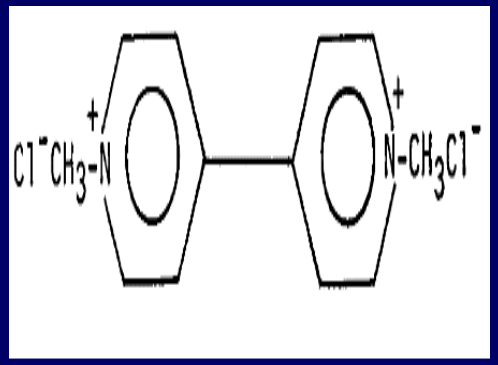
Professor Urban John
Faculty of Medicine & Health Sciences
Universiti Malaysia Sabah, Sabah
Malaysia


SEKOLAH PERUBATAN
UNIVERSITI MALAYSIA SABAH

Organochlorines and organophosphates

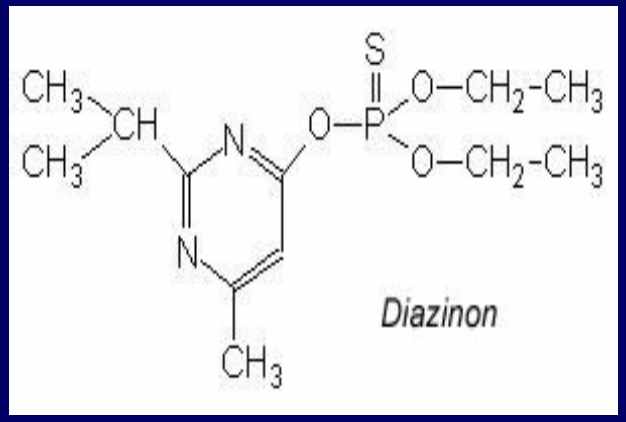
- Pesticides are organochlorine/phosphate compounds.
- Paraquat - organochlorine – herbicide. - ***N,N*-dimethyl-4,4'-bipyridinium dichloride**; LD50 values of 110 to 150 mg/kg in rats.
- Trade names: Crisquat, Cyclone, Dextrone, Dexuron, Gramoxone Extra, Herbaxone, Ortho Weed and Spot Killer, and Sweep.
- Diazinon – organophosphate – insecticide.-. ***O,O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate**, LD50 values of 300 to 850 mg/kg in rats


Paraquat


Cl⁻ CH3-N⁺
N⁺-CH3 Cl⁻


Paraquat threat
Estate workers employed as sprayers have high degree of exposure to the chemical.
A National Poison Centre study in 2002 found that plantation workers used backpack pesticide sprayers for an average of 262 days a year without protective clothing.
Study showed that from 1987 to 1997, 27% of the fatal cases of pesticide poisoning in Malaysia were paraquat related.
The greatest risk to workers is during mixing and loading of spray equipment, where contact with the concentrated chemical occurs.
Prolonged contact with diluted paraquat spray could also be fatal.
Star GRAPHICS © 2006


ORTHO
Diazinon Ultra
Insect Spray
WATER BASED CONCENTRATE
Multi-Purpose Insect Killer
CAUTION


CH3 CH CH3
N
N
O—P(=S)(O—CH2-CH3)(O—CH2-CH3)
CH3
Diazinon


MAXIDE
READY-TO-USE
Diazinon
Insect Killer
Granules
QUICK KILL FORMULA
KILLS 30+ INSECTS

Mechanism of action

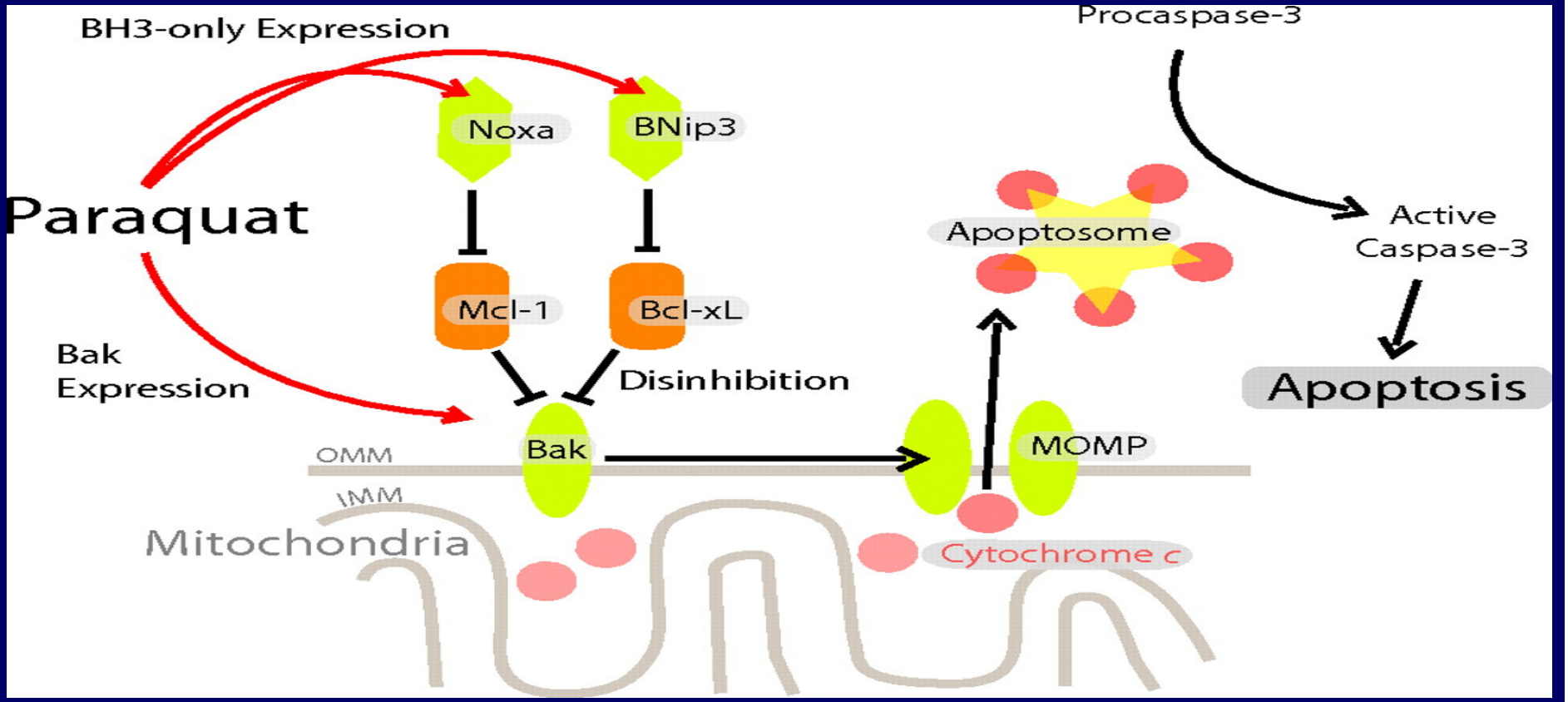
- Paraquat - a non-selective redox cycling agent used in weed control.

- Herbicidal properties - enhanced by the addition of a single electron to the parent cation - free radicals.

  Highly toxic - *in vitro* and *in vivo* systems

Paraquat - Neurotoxicity

- Paraquat causes selective degeneration of dopaminergic neurons in substanitia nigra pars compacta reproducing pathological feature of Parkinson's



Mechanism of action

- Diazinon: A potent neurotoxin.
- Inhibit/permanently suppresses the activity of acetylcholinesterase.
- The mechanism behind this action involves the agent's phosphorus atom binding to the enzyme site.
- Role of acetylcholinesterase is to degrade the neurotransmitter acetylcholine
- Excessive amount is left to concentrate in the synaptic cleft where it can no longer reach neurotransmitter receptors.

Exposure

- Routes of exposures - Oral, dermal or respiratory routes.

Adverse toxicities

Pararquat:

Mutagenic agent- gene mutations

- Increased frequencies of sister-chromatid exchanges, chromosome aberrations
- Increased sperm abnormalities

Adverse toxicities

Diazinon:

Hematological effects

Mutagenic agent- gene mutations

- Hepatotoxicity, neurotoxicity
- Increased sperm abnormalities

Objectives

Effects of 'Low doses':

* Pituitary – gonadal functions – Hormonal role : Does it affect the synthesis of
  FSH, LH, Prolactin and testosterone

* Testicular marker enzyme levels?

* Sperm toxic effects & causes for sperm toxicity?

* Correlation of Hormones, free radicals with testicular & sperm parameters.

Animal Ethics

- University Ethical Clearance was received.

Further information could be retrieved from Universiti Malaya

Paraquat

Control (n=6)	Duration(n=6)	Dose
7 days 14 days 28 days	7 days 14 days 28 days	6mg/kg BW
7 days 14 days 28 days	7 days 14 days 28 days	15mg/kg BW
7 days 14 days 28 days	7 days 14 days 28 days	30mg/kg BW

Diazinon

Control (n=6)	Duration(n=6)	Dose
7 days 14 days 28 days	7 days 14 days 28 days	6mg/kg BW
7 days 14 days 28 days	7 days 14 days 28 days	15mg/kg BW
7 days 14 days 28 days	7 days 14 days 28 days	30mg/kg BW

Methodology

Control

Free access to pellet and water *ad libitum*

Male rats
Sprague-Dawley

Treated group

6, 15, 30 mg/kg
Paraquat or Diazinon

7,14, 28D

Anesthesized

Cervical dislocation

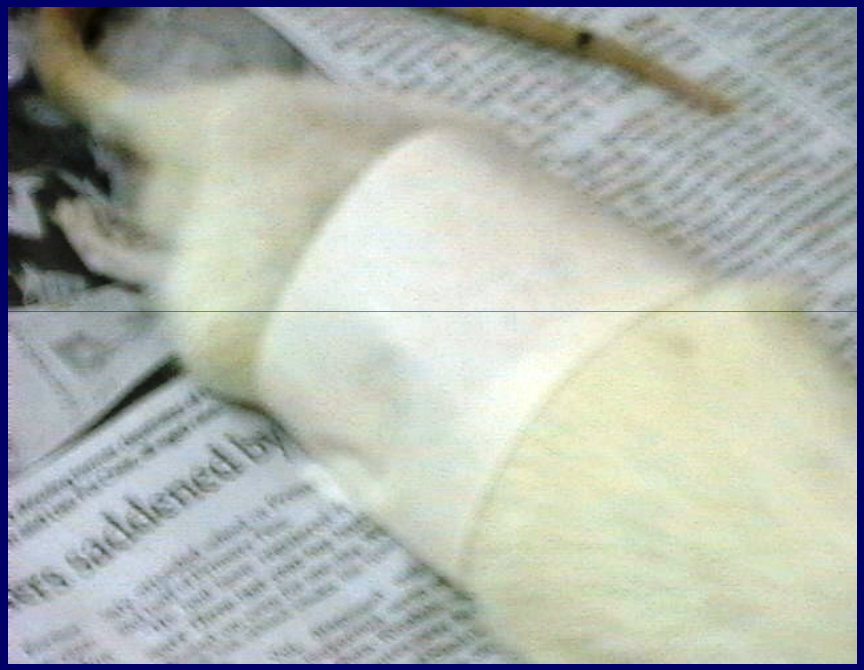
Intracardiac blood sample

Laparatomy

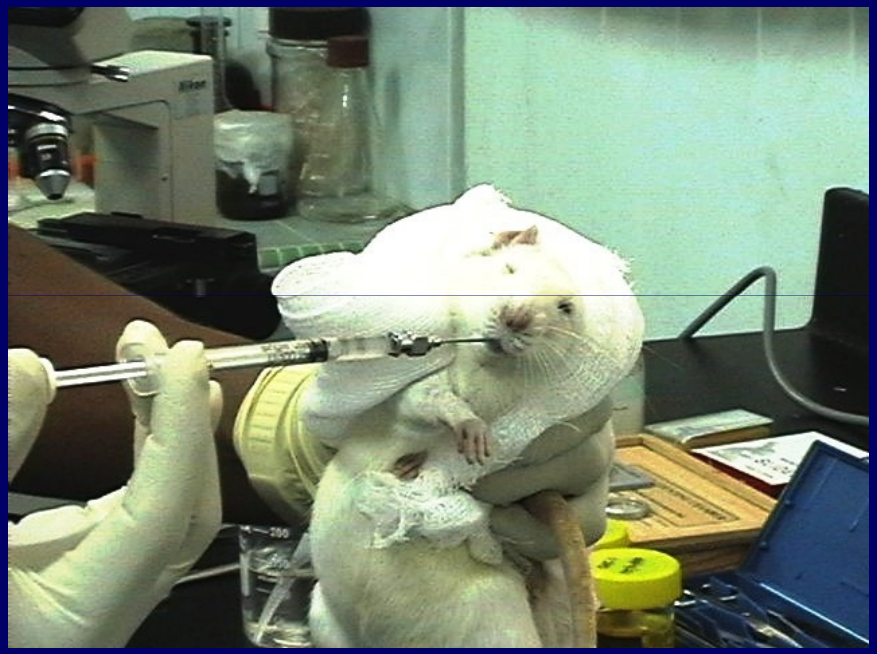
Testis & epididymes

Biochemical and histopathological

Route of exposure



Dermal



Oral

Hormone Assays

- Testosterone assay: (**ImmunoradiometricAssay:** IRMA-nmol/L) The procedure follows the basic principle of radioimmunoassay where there was a competition between a radioactive and a non-radioactive antigen for a fixed number of antibodybinding sites (Yallow & Bearson 1971). The amount of [I-125]-labelled testosterone bound to the antibody was inversely proportional to the concentration of unlabeled testosterone present.
- Follicle-Stimulating Hormone (FSH-IRMA- μ IU/L)
- Luteinizing Hormone (LH-IRMA- μ IU/mL)
- Prolactin IRMA(μIU/ml)

Testicular Marker Enzyme Assays

- **Acid Phosphatase (**IU/L) (Pesee 1987)

- **Lactate dehydrogenase(LDH)**(IU/ml) (Appleby & Morton 1989)

[Reading: The spectrophotometer (Beckmann Coulter Co. USA) was adjusted at 420 nm at 25°C].

Estimation of free radicals

- The LPO assay (Buege and Aust, 2003)
- The LPO level in testis post-mitochondrial supernatant (PMS) was determined by measuring the rate of thiobarbituric acid reactive substance production (MDA equivalents).

Spermatogram

- Epididymal Sperm Count: (Vega et al. 1988).
- Sperm Morphology Assay: (Wyrobek & Bruce 1975; Wyrobek et al. 1983; Narayana & D'Souza 2002).
- Histopathology Evaluation of the Testis: (Culling et al. 1983; D'Souza & Narayana 1993)

Statistical Analysis

- SPSS (version 17.0, 2008) software.

- **One Way ANOVA** (dose-response) and **Two Way ANOVA** (time-response)
  followed by Bonferroni's post – hoc test.

- Results were shown as mean ± standard error mean

- Results were considered significant if $P<0.05$

Results: Paraquat: Testosterone levels

Dose/Duration	7 days	14 days	28 days
Control	29.72 ± 1.88	30.54 ± 1.74	30.21 ± 1.86
PQ – 6 mg/kg DZ-6mg/kg	25.44* ± 1.22 28.44 ± 0.82	29.82 ± 2.02 29.12 ± 1.42	30.52 ± 0.96 29.12 ± 0.74
PQ – 15 mg/kg DZ-15mg/kg	20.12* ± 1.02 24.32* ± 0.92	23.22* ± 1.42 24.42* ± 1.02	28.98 ± 1.09 29.94 ± 1.02
PQ – 30 mg/kg DZ-30mg/kg	18.36* ± 1.64 21.34* ± 1.04	20.48* ± 1.12 25.18* ± 1.08	26.38* ± 2.12 29.42 ± 1.18

FSH

Dose/Duration	7 days	14 days	28 days
Control	5.61 ± 0.32	5.66 ± 0.22	5.63 ± 0.28
PQ – 6 mg/kg DZ – 6 mg/kg	5.34 ± 0.12 5.85 ± 0.12	5.29 ± 0.24 5.46 ± 0.12	5.38 ± 0.16 5.29 ± 0.24
PQ – 15 mg/kg DZ – 6 mg/kg	5.20* ± 0.18 5.42 ± 0.12	5.38 ± 0.22 5.38 ± 0.14	5.34 ± 0.18 5.62 ± 0.12
PQ – 30 mg/kg DZ – 6 mg/kg	4.56* ± 0.16 5.18* ± 0.28	4.95* ± 0.19 5.53 ± 0.04	5.09* ± 0.42 5.42 ± 0.14

LH

Dose/Duration	7 days	14 days	28 days
Control	2.64 ± 0.16	2.58 ± 0.15	2.60 ± 0.18
PQ – 6 mg/kg DZ – 6 mg/kg	2.56 ± 0.10 2.66 ± 0.12	2.64 ± 0.12 2.68 ± 0.12	2.64 ± 0.08 2.75 ± 0.02
PQ – 15 mg/kg DZ – 15 mg/kg	2.62 ± 0.08 2.46 ± 0.14	2.62 ± 0.17 2.64 ± 0.62	2.67 ± 0.09 2.61 ± 0.12
PQ – 30 mg/kg DZ – 30 mg/kg	2.48* ± 0.13 2.46* ± 0.42	2.48* ± 0.09 2.54 ± 0.12	2.66 ± 0.20 2.65 ± 0.10

Prolactin

Dose/Duration	7 days	14 days	28 days
Control	15.58 ± 3.90	15.88 ± 1.76	15.34 ± 3.11
PQ – 6 mg/kg DZ – 6 mg/kg	16.08 ± 2.72 15.28 ± 1.62	12.38 ± 1.48 14.13 ± 1.12	14.92 ± 3.78 15.34 ± 2.43
PQ – 15 mg/kg DZ – 15 mg/kg	15.77 ± 1.08 16.38 ± 2.32	10.88* ± 3.44 12.67 ± 0.88	14.57 ± 2.79 16.22 ± 1.88
PQ – 30 mg/kg DZ – 30 mg/kg	14.88 ± 2.82 15.28 ± 1.72	10.02* ± 5.62 12.46 ± 1.12	10.56* ± 2.38 12492* ± 2.08

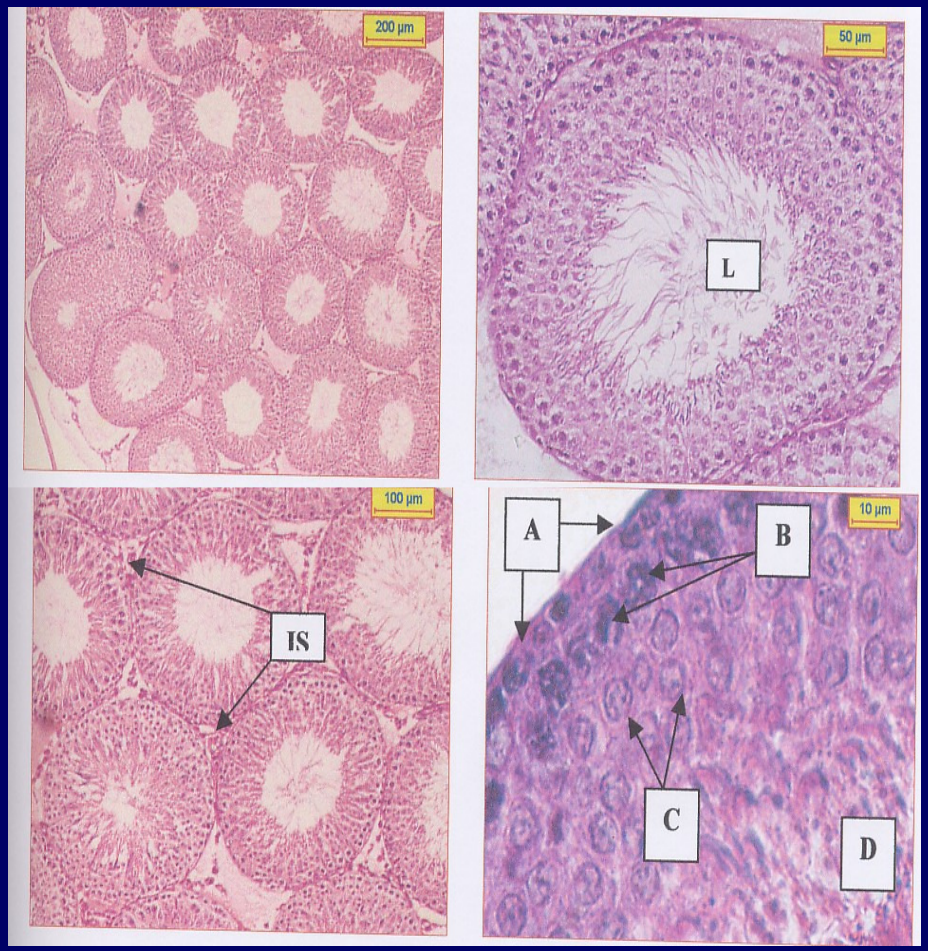
Acid phosphatase

Dose/Duration	7 days	14 days	28 days
Control	11.73 ± 0.68	12.48 ± 0.69	12.55 ± 0.60
PQ – 6 mg/kg	11.86 ± 1.15	12.94 ± 0.87	13.72 ± 1.18
PQ – 15 mg/kg	13.16 ± 1.30	13.01 ± 1.34	13.18 ± 1.02
PQ – 30 mg/kg	13.09 ± 1.04	13.05 ± 0.78	13.19 ± 1.58

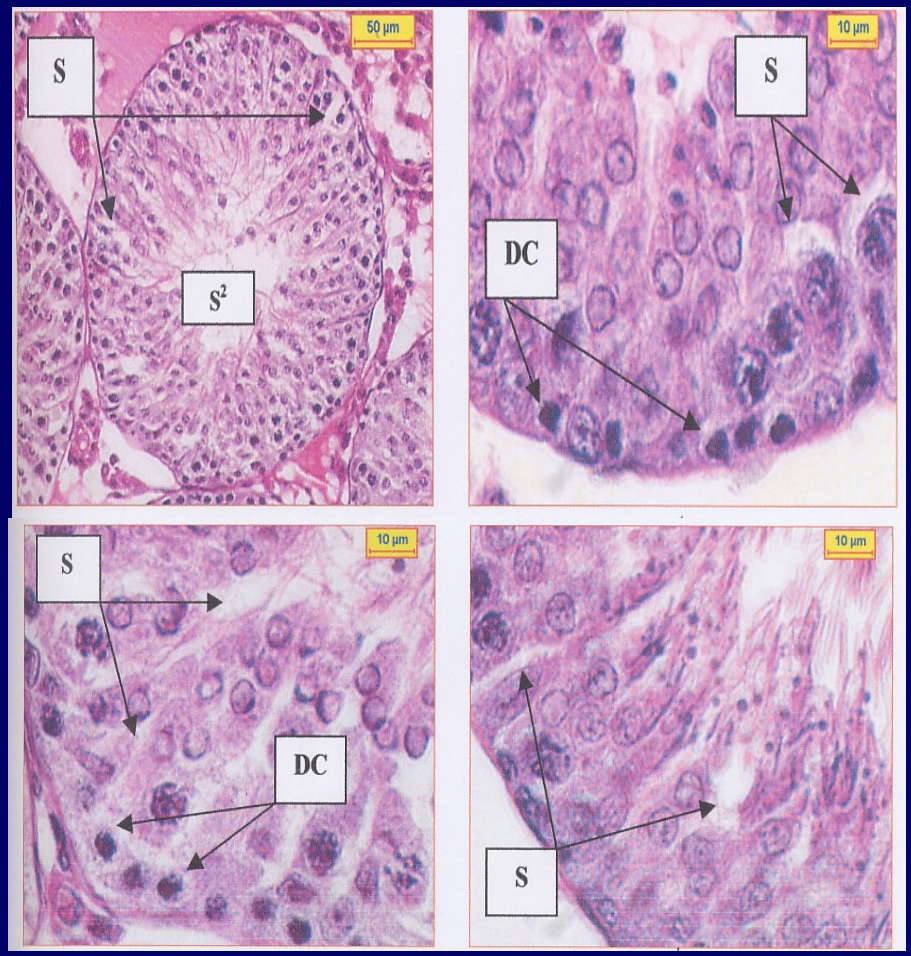
LDH

Dose/Duration	7 days	14 days	28 days
Control	6.94 ± 1.91	7.98 ± 1.71	8.32 ± 1.14
PQ – 6 mg/kg	7.33 ± 0.66	7.23 ± 0.93	7.72 ± 0.79
PQ – 15 mg/kg	10.62* ± 0.86	7.74 ± 0.74	8.24 ± 1.05
PQ – 30 mg/kg	15.98* ± 2.66	11.83* ± 3.38	9.91 ± 0.78

Histopathological changes: Testis

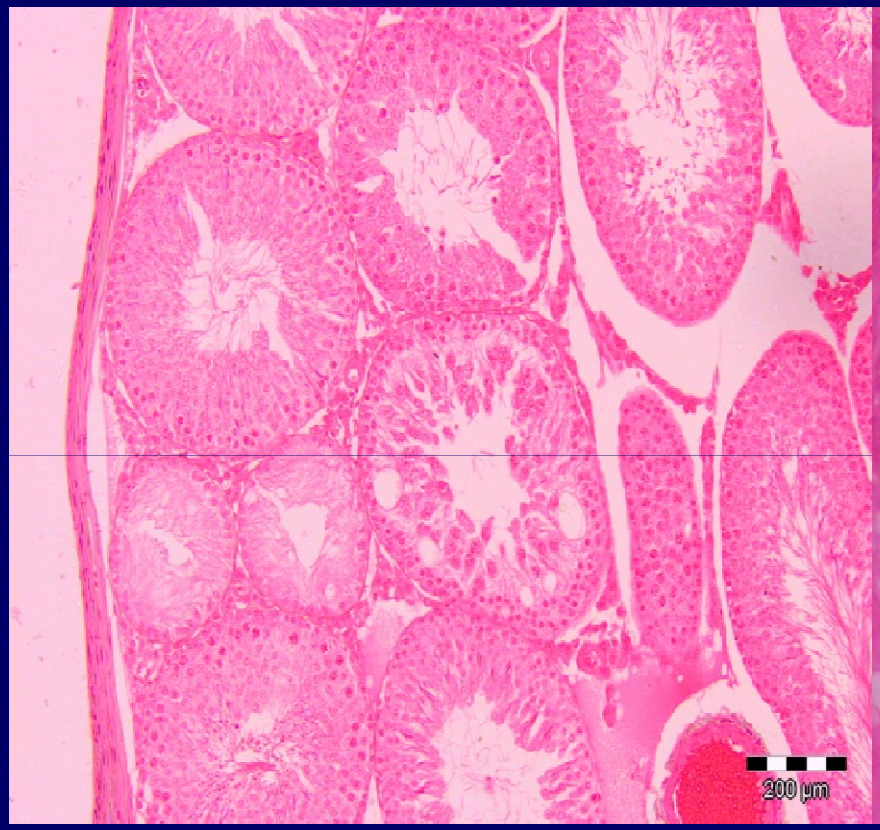


Control

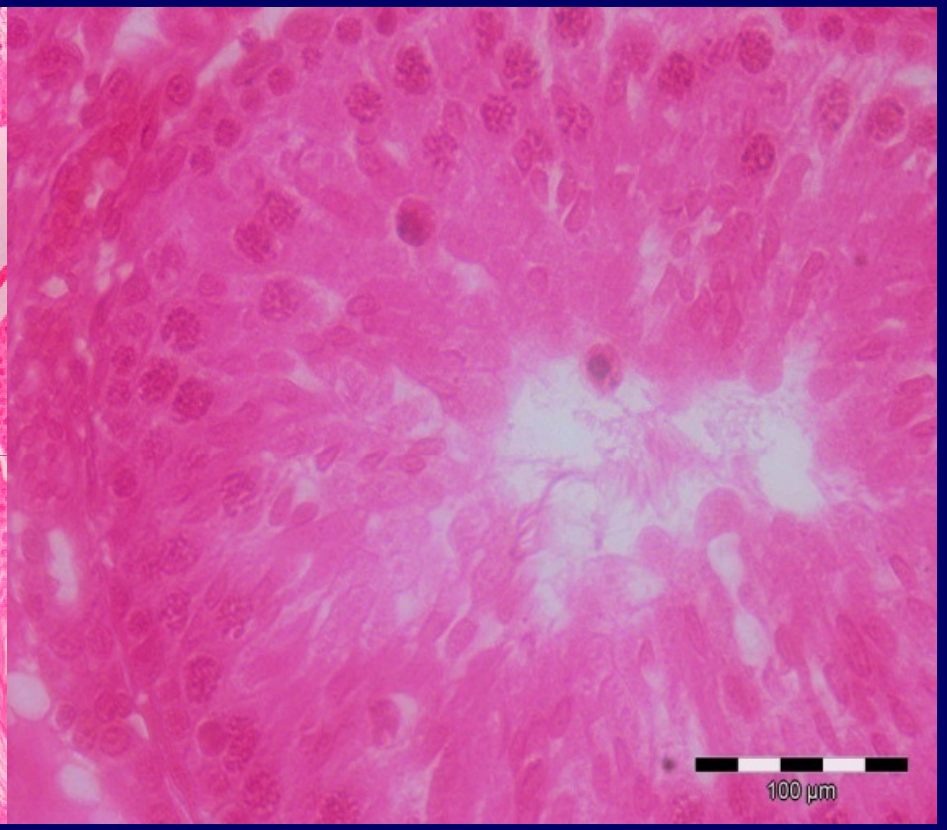


Paraquat -30mg/kg, 7D

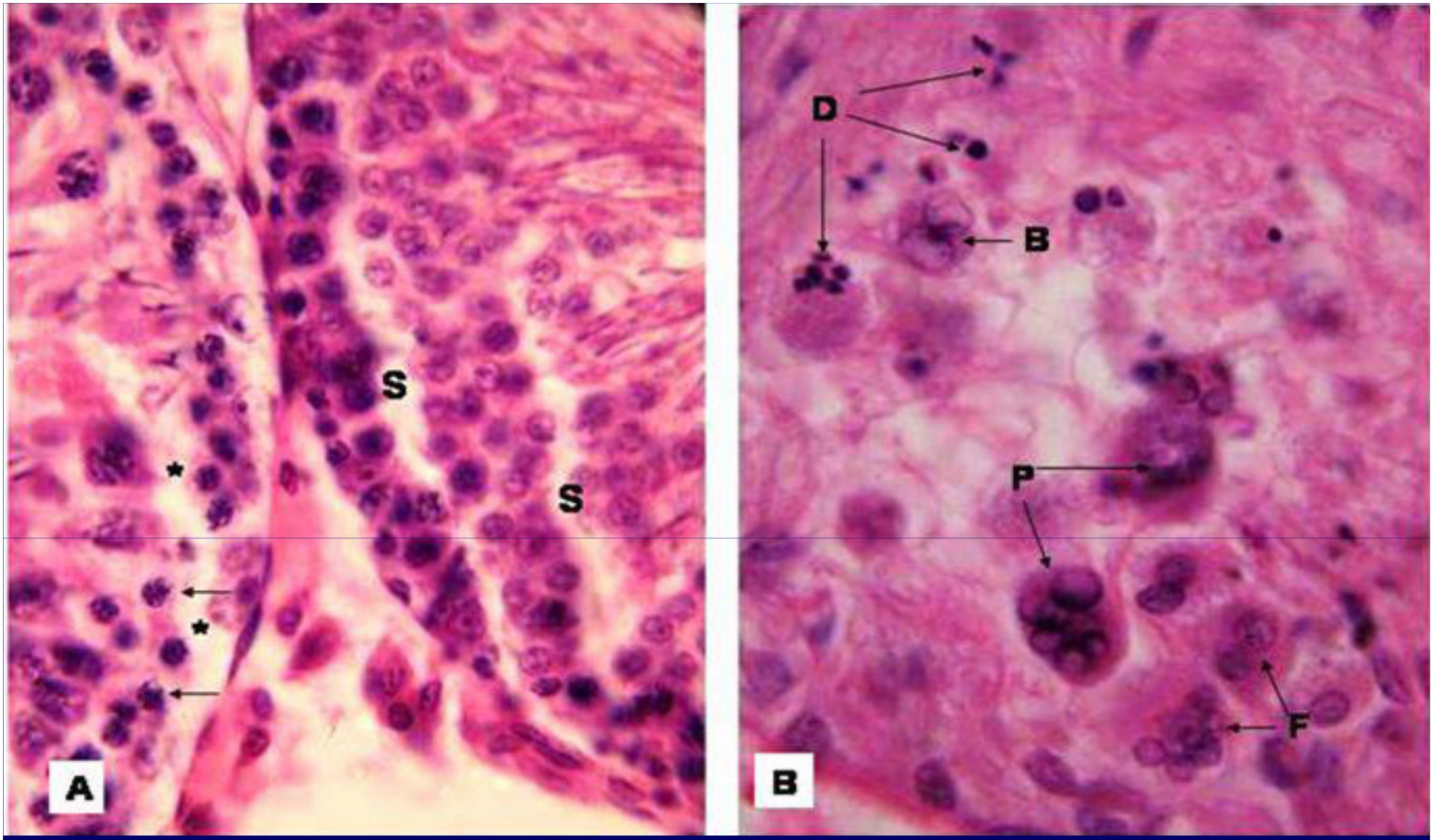
Histopathological changes: Testis



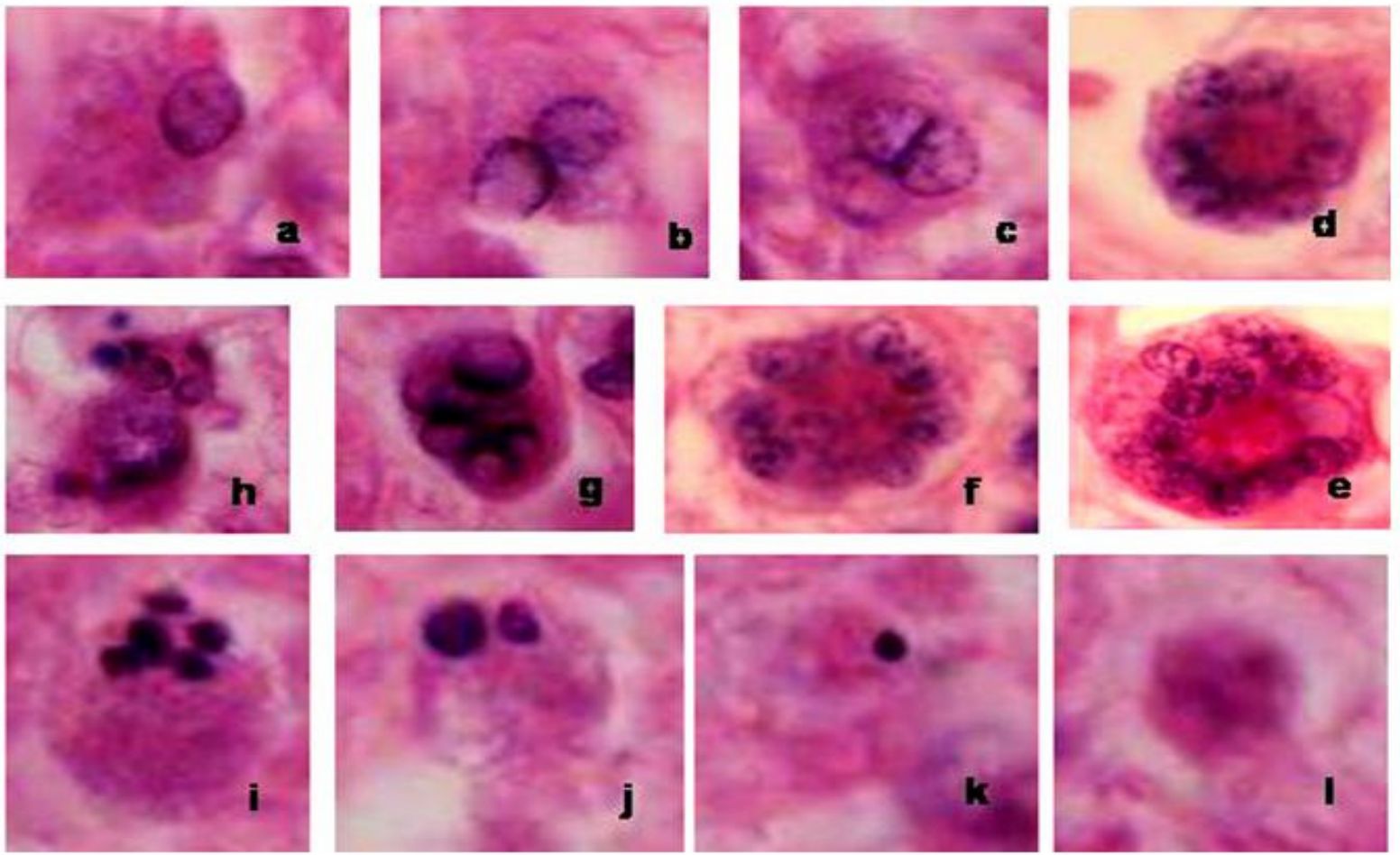
DZ 15 mg/kg, 7D
Sloughing of seminiferous tubule which appears as vacuole-like structure



DZ 30mg/kg, 28D
Abnormal migration of secondary spermatocytes to the lumen

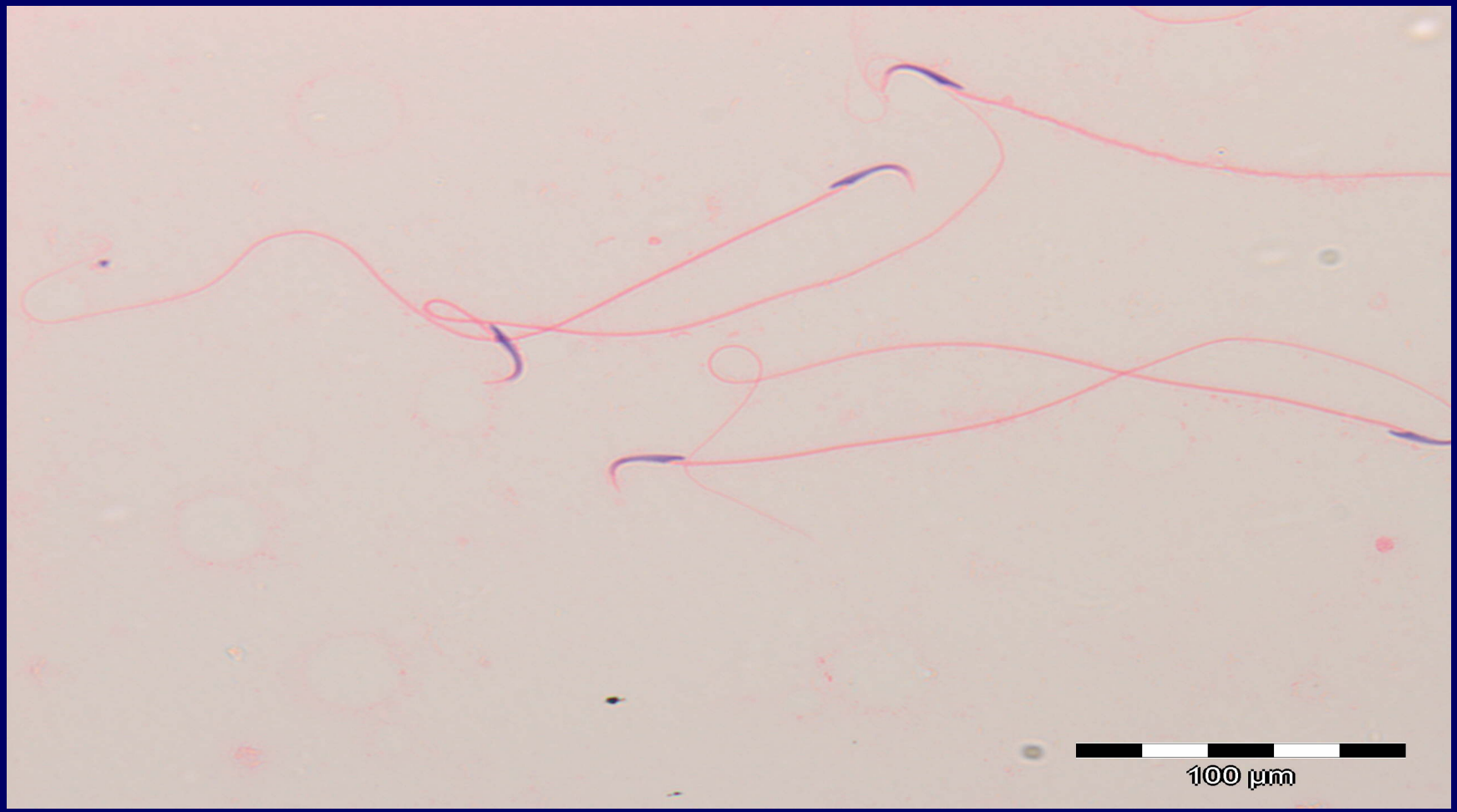


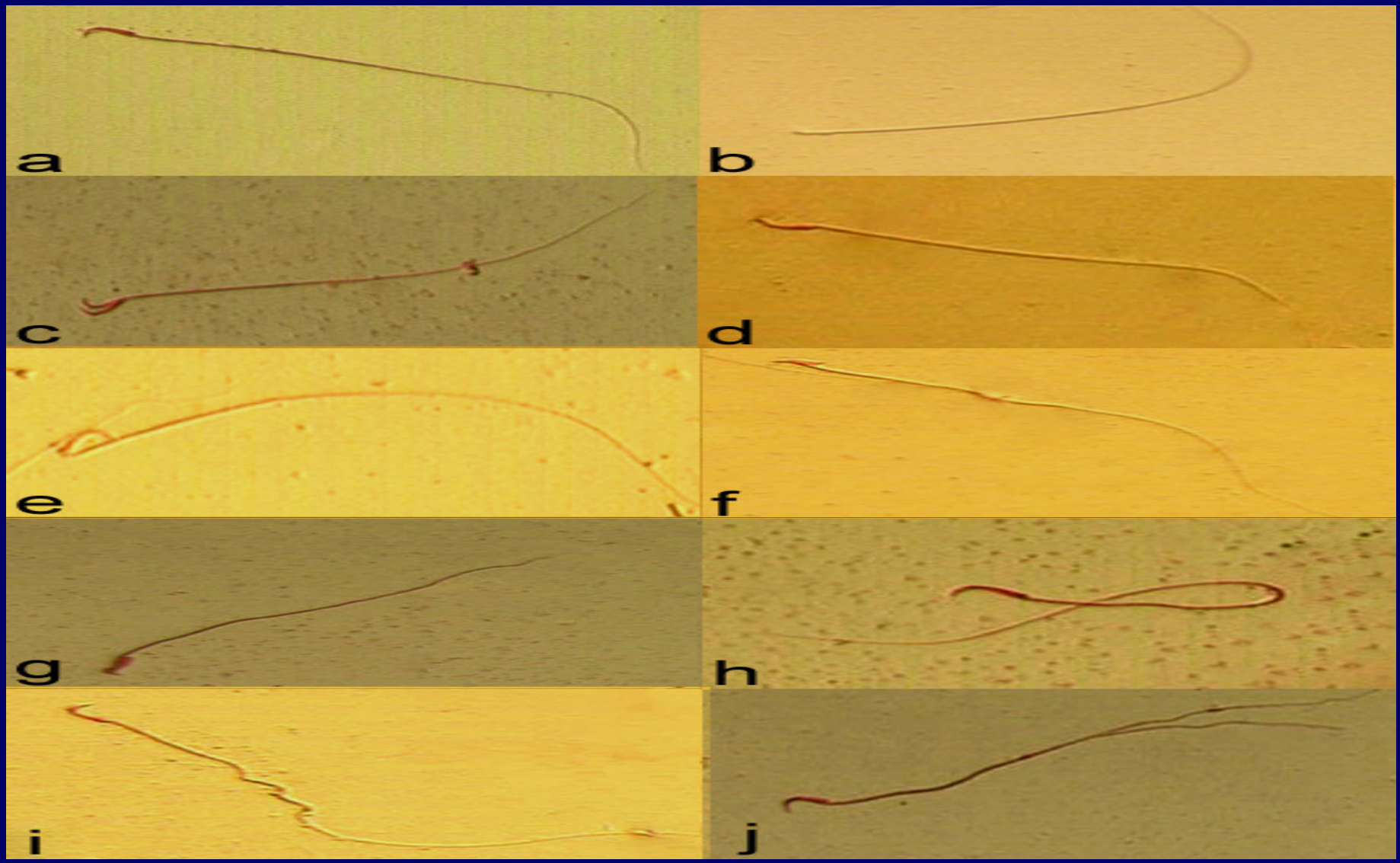
Degeneration of germ cells (arrows), epithelial sloughing (S) - PAS-H&E, 400X. B) Degrees of multinucleated cell formation and degeneration. (F) nuclear fusion (B). nuclear pyknosis (P), fragmentation and total degeneration of nuclei (D). PAS-H&E, 1300X.



Different steps involved in the formation and degeneration of multinucleated cells in DZN treated rat testis. a) A single cell, b) nuclear fusion (binucleate spermatid), c) trinucleate spermatid, d) a multinucleated giant cell with around 7 nuclei. e) 12 nuclei are seen and some of the nuclei present 'halo' appearance, f) 9 nuclei decrease in number and 'halo' appearance is clear, g) the chromatin has stained darkly but the 'halo' appearance is still pronounced, h) such nuclei show disintegration by breaking into pieces which are highly condensed, i) the chromatin fragments are extruded from the cell, and in j, k and l, they gradually disappear from the cell leaving an eosinophillic body as in l. PAS-H&E, (1300X).

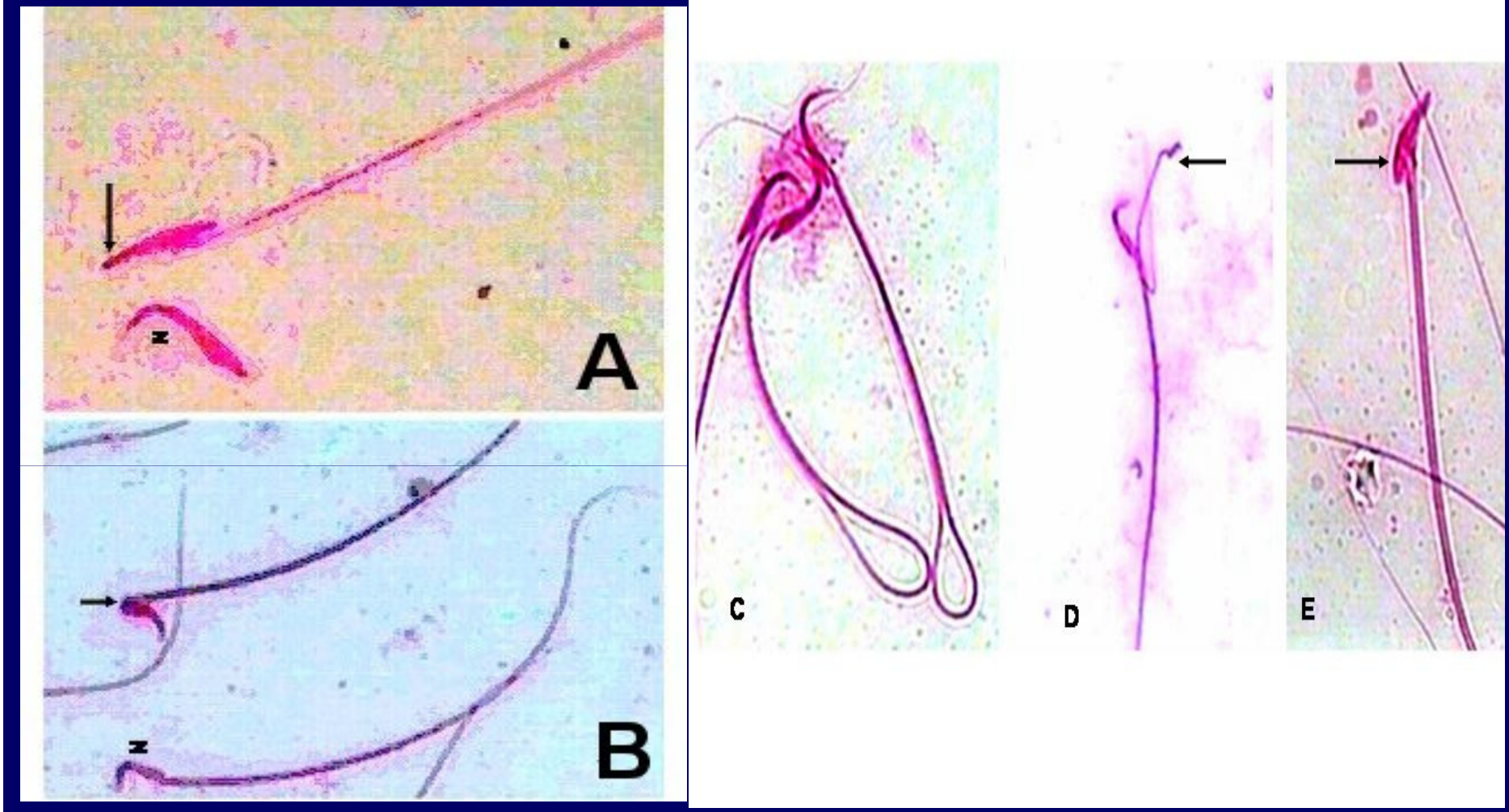
Sperm abnormality





(a) Normal sperm, (b) headless sperm, (c) double-headed sperm, (d) hookless sperm, (e) cephalo cauda junction, (f) microcephaly sperm, (g) banana shaped, (h) coiled tail, (i) broken tail, (j) double tail.

Abnormal sperm morphology



Sperm shape abnormalities in Pqt treated rats. A) A sperm showing cephalo-caudal junction bending (arrow). Head presents a straightened hook, 400X.
B)Sperm showing a straight head without a hook (arrow)
Normal sperms (N). 1300X. C) Folded-tailed sperm, D) Sperm showing microcephaly E) Sperm showing amorphous head (arrow). 400X, eosin Y.

Sperm abnormality - head

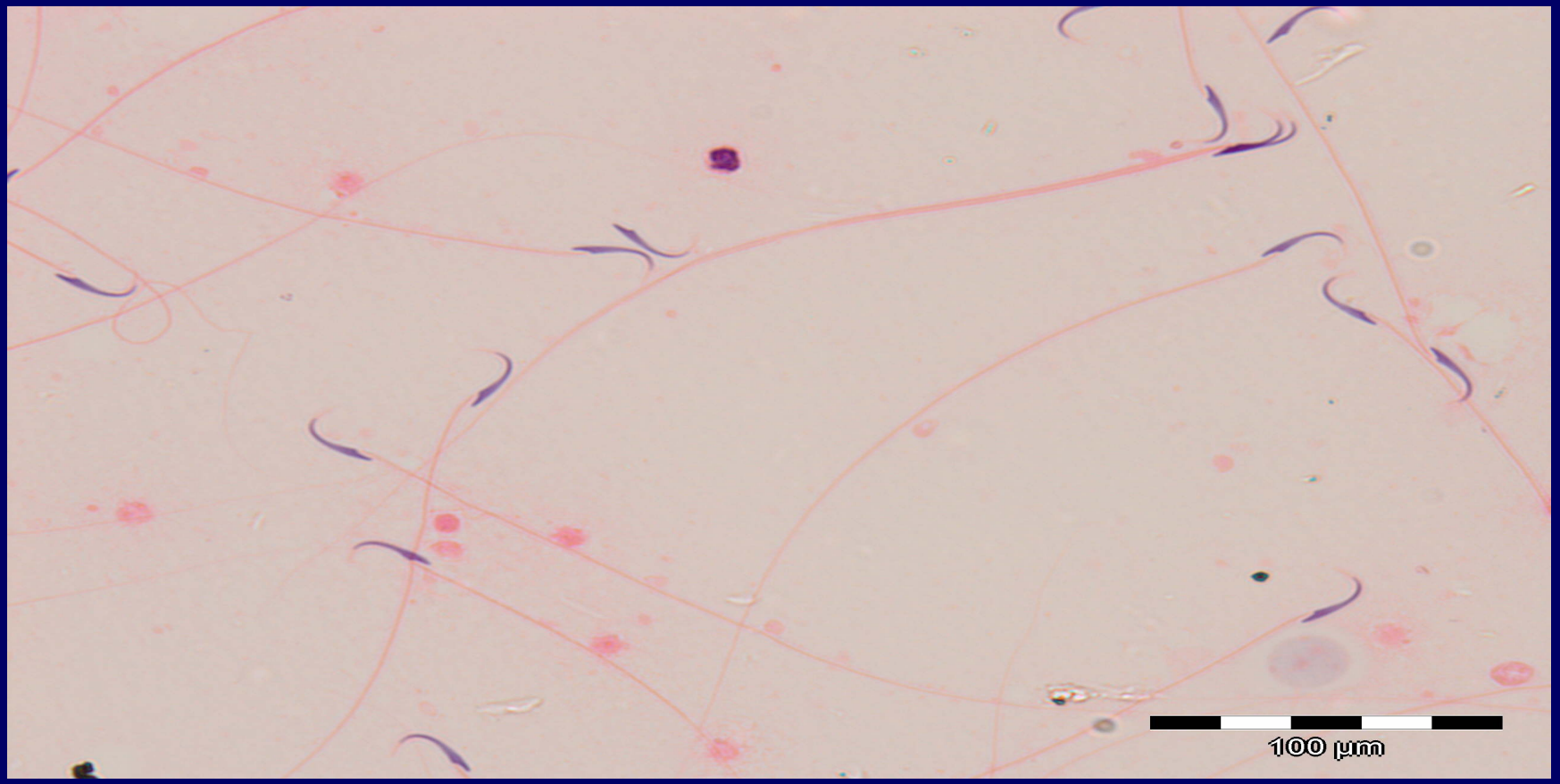
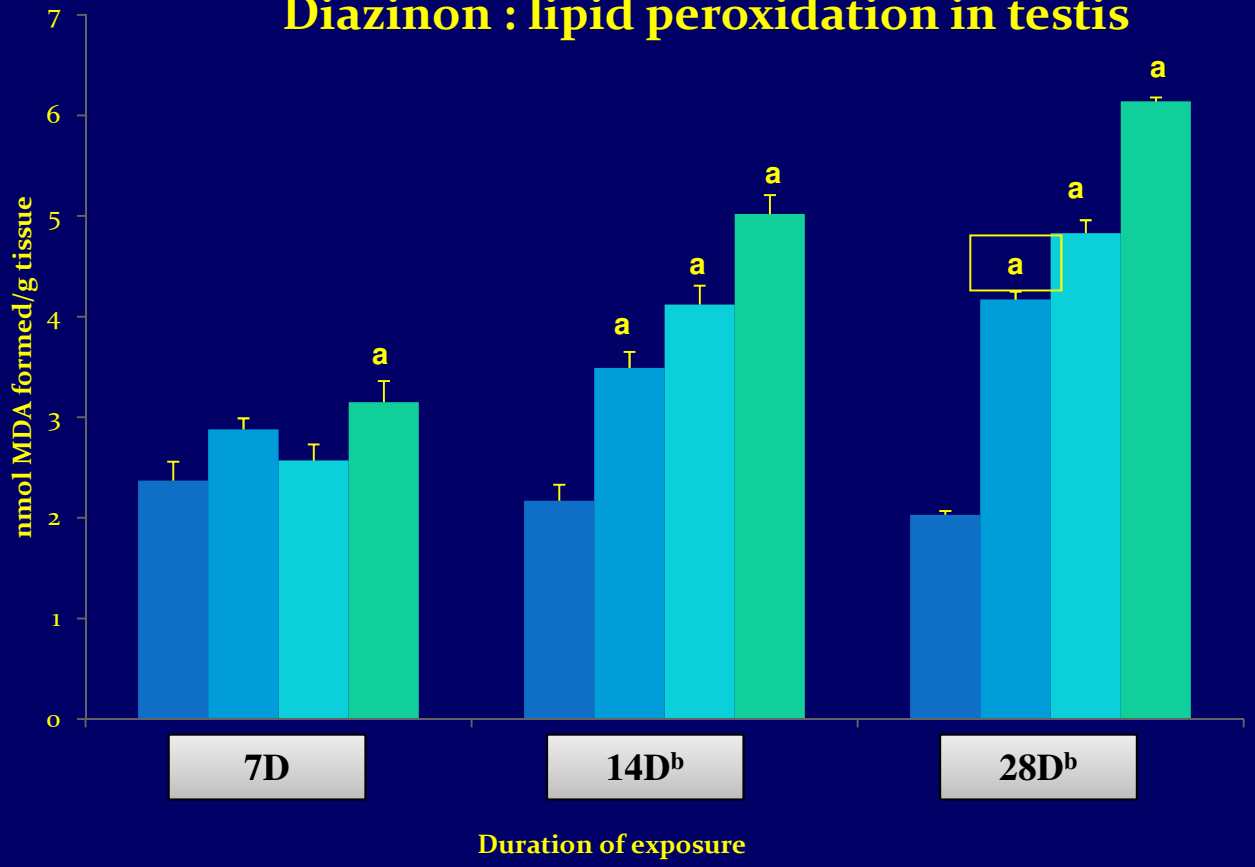


Figure 5

Diazinon : lipid peroxidation in testis

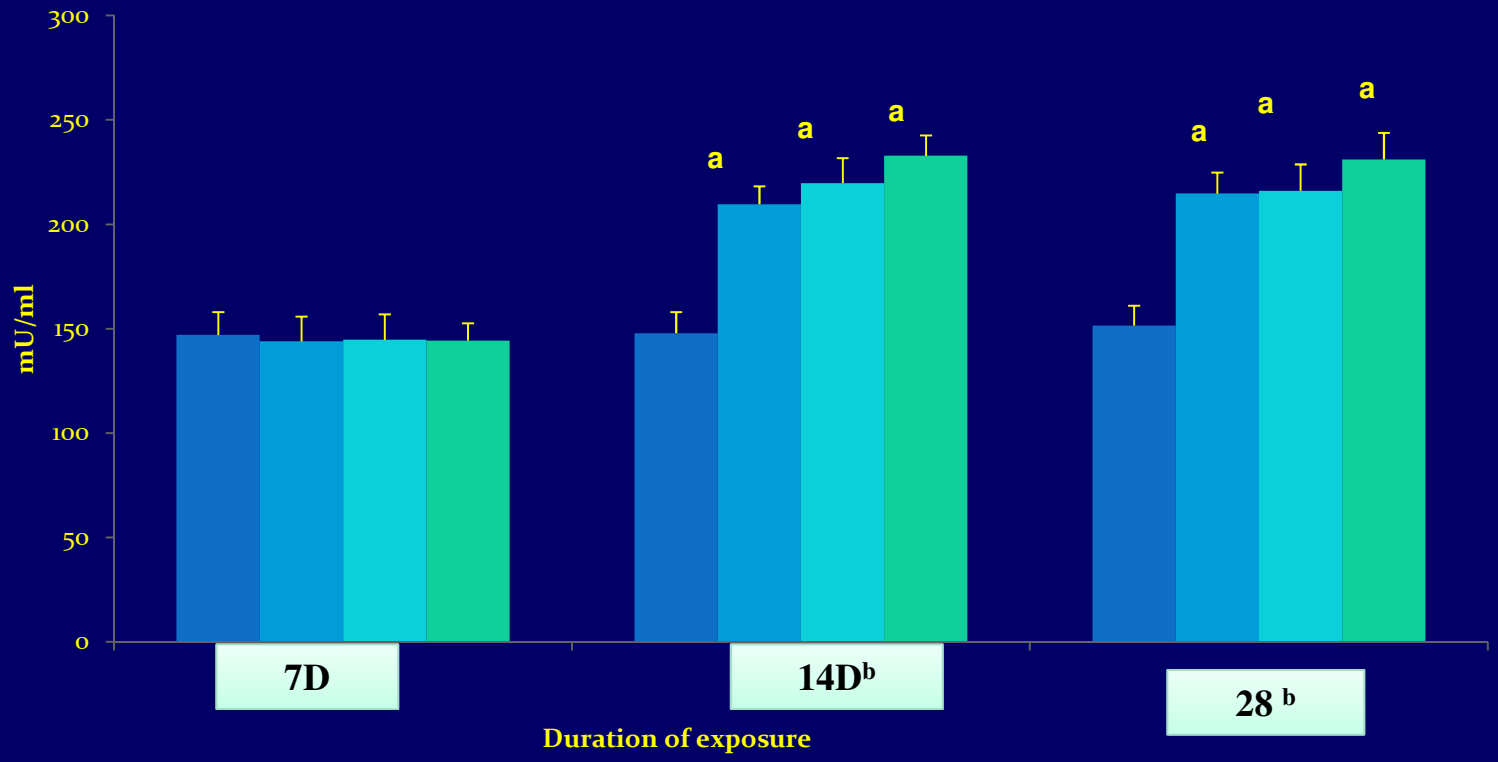


Results were shown as mean ± standard error mean.

a – for dose response relationship, indicates significant ($p<0.05$) when compared to control group

b – for time response relationship, indicates significant ($p<0.05$) when compared to 1 week exposure

Diazinon: Level of serum lactate dehydrogenase

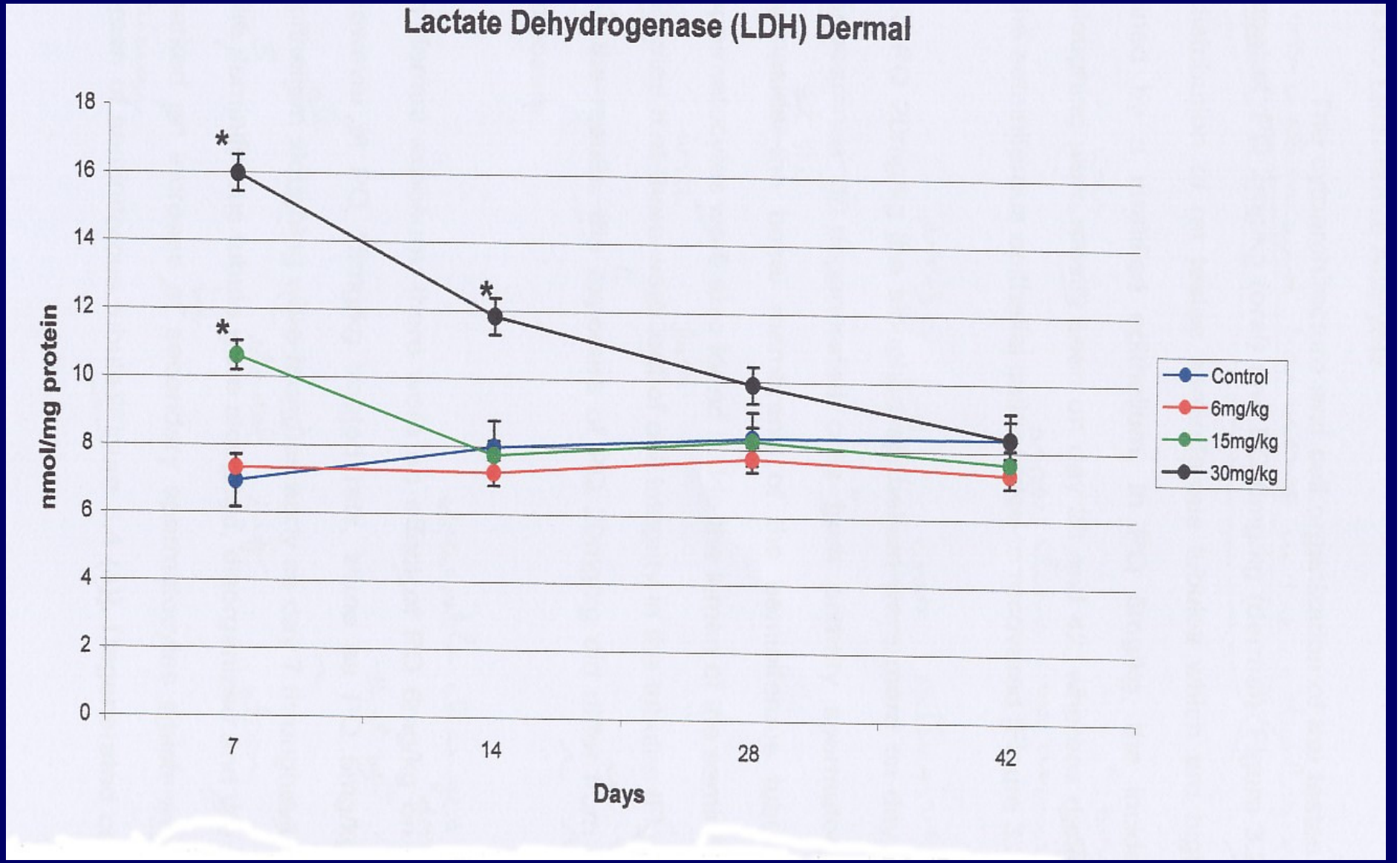


Results were shown as mean ± standard error mean.

a – for dose response relationship, indicates significant ($p<0.05$) when compared to control group

b – for time response relationship, indicates significant ($p<0.05$) when compared to 1 week exposure

LDH - Paraquat



Discussion & Conclusion

- Pesticides pose a threat to human/animal health.
- Low doses of both organochlorine (Paraquat)& organophosphate (Diazinon) compounds induce acute and chronic toxicities in male reproductive system.
- They interfere with spermatogenesis leading into oligo/azoospermia/teratospermia
- Induce testicular marker enzyme alteration as well as histopathological anomalies.

Discussion & Conclusion

- Pituitary-gonadal hormone levels are affected.
- Hormonal mechanisms are involved in the toxicity, evidenced by a significant decrease on exposure.
- FSH, LH, Prolactin and Testosterone levels.
- Reactive oxygen species - LPO increased on exposure.

Conclusion

- Present study concludes that, pituitary – gonadal hormonal alteration and lipid peroxidation were the contributors for pesticide – induced fertility problems.
- Organochlorines/phosphates toxic to human health, induce inheritable changes compromising to fertility index.
- Inhalation/dermal/oral exposures of pesticide residues alter the hypothalamo-pituitary-gonadal axis

Limitations

- Atmospheric dose level study, Measurement of atmospheric levels - air, water, food grains and initiating studies with equivalent atmospheric doses.
- Metabolic product levels & their effects.
- Long-term studies
- Toxic effects reversible? Irreversible?
- Generation study: F1, F2, F3
- Behavioral study: Mating behavior, Sexual performance, libido etc.
- Clinical study.

Recommendations

- Measurement of atmospheric levels – air, water, food grains and initiating studies with equivalent atmospheric doses.
- Generation studies.
- Cock-tail study.
- Clinical studies.
- Generation study: F1, F2, F3
- Behavioral study: Mating behavior, Sexual performance, libido
- Estrogenic effects: Sex-orientation: Female behavior of male- female type of brain in a male?

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- MSc students: Mr Chong Leong, Feroz Hossain , Anuar, Nizam
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