THE EVALUATION OF THE TOXIC EFFECT OF PARAQUAT AND ITS MECHANISM OF ACTION ON REPRODUCTIVE SYSTEM OF MALE RATS

By

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DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been acknowledged.

27th May 2007

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THE EVALUATION OF THE TOXIC EFFECT OF PARAQUAT AND ITS MECHANISM OF ACTION ON REPRODUCTIVE SYSTEM OF MALE RATS

ABSTRACT

Paraquat® - PQ (1,1' – dimethyl 4,4' – bipyridillium dichloride) is a non-selective contact herbicide. Its herbicidal and toxicological properties are dependent on the ability of the parent cation to undergo a single electron addition to form a free radical. The present study was conducted to evaluate the toxic effect of low dose of PQ and its mechanism of action on the reproductive system of adult male rats. Two routes of administration were selected; oral and dermal which are the most common exposure routes in human being. Groups of six male Sprague-Dawley rats (n=6) were orally gavaged in selected dose of 2mg/kg, 5mg/kg and 20mg/kg (1/80, 1/32 and 1/8 of oral LD_{50}) and dermally applied in selected dose of 6mg/kg, 15mg/kg and 30mg/kg (1/15, 1/6, and 1/3 of dermal LD_{50}) for five consecutive days separately and scheduled for 7, 28, 42, 84 and 105 days for oral, and 7, 14, 28 and 42 days for dermal applications in separate groups after the last dose. Body weight did not show any change in the treated groups compared to the control. Reproductive organ weight (testis, epididymis, seminal vesicles and prostate), seminiferous tubular diameter (STD), seminiferous epithelial height (SEH) and epididymal epithelial height (EEH) decreased significantly ($p<0.05$) in all treated groups except in 2mg/kg dose. STD was significantly decreased only in 20mg/kg dose following 7, 28 and 42 days oral treatment and in 15 and 30mg/kg dermal dose following 7 and 14 days. A significant decline ($p<0.05$) in SEH and EEH were observed in most of the groups. Epithelial

sloughing, number of degenerated cells as well as loss of cell integrity in the seminiferous tubules of the testis was observed, except in 2mg/kg treatment group. Number of spermatogonia, spermatocytes, spermatids, 'dividing cells' and Leydig cells were decreased on exposure to PQ ($p<0.05$). Increase in sperm mortality and abnormal sperm morphology was significant ($p<0.05$). The hormones testosterone, Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH) and prolactin were decreased significantly in most of the doses as well as testicular marker enzyme lactate dehydrogenase (LDH) were increased significantly ($p<0.05$). But exposure to paraquat did not change the level of Acid Phosphatase (ACP) in oral treated rats. In conclusions, PQ is a toxic substance both by dermal and oral route affecting the male reproductive function on exposure to a short duration of five consecutive treatment. Most of the parameters exhibited a transient change with an initial response such as decline in hormone level, increase in LDH level and decreased STD, SEH and member of different cell population of spermatogenic cycle. Based on our findings we propose that PQ is toxic to male reproductive functions both by oral and dermal route of exposures. Our hypothesis, for the possible mechanism of PQ toxicity in male reproductive system is by inducing a derangement in pituitary-gonadal-hormone synthesis/secretion, and elevation of testicular marker enzyme-LDH indicative of cell damage/destruction/death and also due to the direct toxicity of free radicals generated as reported earlier by other researchers.

PENILAIAN KESAN TOKSIK PARAQUAT DAN MEKANISMA TINDAKANNYA KE ATAS SISTEM PEMBIAKAN TIKUS JANTAN

ABSTRAK

Paraquat® (1,1' – dimetil 4,4' – bipiridilium diklorida) merupakan sejenis racun herbisid bukan selektif. Kesan herbisid dan toksikologinya bergantung kepada keupayaan kompoun utamanya untuk mengubah kepada elekron tunggal dan membentuk radikal bebas. Kajian ini dijalankan untuk menilai kesan toksik paraquat dan mekanisma tindakannya ke atas sistem reproduktif tikus jantan dewasa. Sebagai sebahagian daripada penilaian kesan herbisid terhadap sistem reproduktif dan juga melalui beberapa kes pendedahannya terhadap manusia, dua pendedahan dipilih, iaitu pendedahan secara oral dan kulit. Kumpulan enam ekor tikus Sprague-Dawley bagi satu kumpulan (n=6) telah diberi oral dalam dos dedahan sebanyak 2mg/kg, 5mg/kg dan 20mg/kg (1/80, 1/32 dan 1/8 dari LD_{50}) dan secara dedahan kulit dalam dos dedahan sebanyak 6mg/kg, 15mg/kg dan 30mg/kg (1/15, 1/6 dan 1/3 dari LD_{50}) selama 5 hari berturutan bagi jangkamasa 7, 28, 42, 84 dan 105 hari bagi dedahan oral dan 7, 14, 28 dan 42 dari bagi dedahan kulit. Berat badan tikus tidak menunjukkan sebarang perubahan berbanding kumpulan tikus kawalan. Berat organ reproduktif (testis, epididimis, vesikel semen dan prostat), STD, SEH dan EEH menunjukkan penurunan yang signifikan ($p<0.05$) didalam semua kumpulan rawatan kecuali kumpulan 2mg/kg. Didapati STD menurun secara signifikan didalam kumpulan 20mg/kg pada 7, 28 dan 42 hari dedahan oral dan juga didalam kumpulan 15mg/kg dan 30mg/kg pada hari 7

dan 14 hari dedahan dermal. Penurunan EEH & SEH yang signifikan ($p<0.05$) didapati dalam kesemua kumpulan rawatan. Kerosakan epitelium, peningkatan bilangan sel degenerasi selain daripada kehilangan integriti tubul seminiferous didapati pada semua kumpulan rawatan kecuali pada kumpulan dedahan 2mg/kg. Bilangan spermatogonia, spermatosit, spermatid, sel membahagi, dan sel Leydig didapati menurun didalam semua kumpulan rawatan PQ ($p<0.05$). Hormon testosteron, FSH, LH dan prolaktin didapati menurun secara signifikan pada kesemua kumpulan rawatan selain daripada peningkatan aras enzim Laktat dehidrogenase ($p<0.05$). Kesimpulannya, PQ merupakan bahan toksik bagi kedua-dua dedahan dermal dan oral dan juga kesannya terhadap fungsi sistem reproduktif pada jangkamasa pendek selama 5 hari dedahan. Kebanyakkan parameter menunjukkan perubahan yang mendadak seperti penurunan aras hormon, peningkatan aras LDH, penurunan STD, SEH dan juga bilangan populasi sel didalam kitaran spermatogenesis. Berdasarkan penemuan ini, mencadangkan bahawa PQ merupakan bahan toksik bagi dedahan oral dan juga dermal. Hipotesis kesan toksik ialah mekanisma toksik PQ terhadap fungsi sistem reproduktif ialah melalui gangguan pada sistem pituitari-gonadal-hormon, peningkatan aras penanda LDH terhadap kerosakan sel dan juga kesan langsung radikal bebas sepertimana dilaporkan pada kajian terawal.

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ABBREVIATIONS

ABP	-	Androgen binding protein
ACP	-	Acid Phosphatase
ALP	-	Alkaline Phosphatase
ALT	-	Alanine Aminotransferase
AST	-	Aspartate Aminotranferase
EEH	-	Epididymes Epithelial Height
EPA	-	Environmental Protection Agency
FP	-	Fluorescent pigmentation
FSH	-	Follicle Stimulating Hormone
G6PDH	-	Glucose-6-phosphate dehydrogenase
GSH	-	Glutathione
i.p.	-	intraperitoneal
IPCS	-	International Programme on Chemical Safety
IRMA	-	Immunoradiometricassay
LDH	-	Lactate dehydrogenase
LH	-	Luteinizing Hormone
MDA	-	malonialdehyde
Mg/kg	-	milligram per kilogram
NIOSH	-	National Institute of Occupational Safety and Health
OSHA	-	Occupational Safety and Health Administration
PQ	-	Paraquat

RIA	-	Radioimmunoassay
ROS	-	Reactive Oxygen Species
SDH	-	Sorbitol dehydrogenase
SEH	-	Seminiferous Epithelial Height
STD	-	Seminiferous Tubule Diameter
TDS	-	Testicular Dysgenesis Syndrome

CHAPTER 1

INTRODUCTION

General

Modern technology, including the use of pesticides to control insects, weeds, and disease-inducing agents, enables food production to support the world population of nearly 6.5 billion. Without the use of pesticides, food production would be further reduced and the number of individuals suffering from malnutrition would increase.

Since the early development of agricultural practices, people have always sought different ways to increase their crop yield. The early use of pesticides included a variety of substances, such as urine, lime, soap, vinegar, tobacco, and similar simple compounds. Agrochemical production began as a relatively simple process, based primarily on combinations of a few chemical substances such as copper, mercury salts, elemental sulphur, arsenic, and cyanide (BMA 1992).

The development of highly complex, chemical methods of pest control started around World War II, with the introduction of the first synthetic organochlorine (OC) insecticides, which included DDT, lindane (HCH), aldrin and dieldrin. Thousands of different pesticides manufactured today fall roughly into the following chemical categories; organochlorines, halogenated hydrocarbons, carbamates, heterocyclic compounds, organophosphates, chlorinated phenoxy substances, amines and ureas, benzonitrics, phenolic compounds and pyrethroids. They consist of a mixture of active ingredients; designed to destroy the pests, together with many other chemical additives, such as solvents, combined into usable products.

The growth of the agrochemical industry since World War II has been enormous, and now covers the globe. It is estimated that the industry worldwide produces about 45-50,000 different pesticides based on about 600 active ingredients. In one year alone 23,504 tonnes of active ingredients were sold by UK pesticide manufacturers, which amounts to nearly 420g for a person in the UK population (Robbins 1991). The actual sales of pesticides by UK manufacturers increased from £30 million a year from the late 1940s to £150 million in the mid-1970s. In 1985, total sales of pesticides amounted to almost £900 million, of which approximately 60% was accounted for export purposes (BAA 1989; Robbins 1991; BMA 1992). Pesticide manufacture is dominated by a few large chemical companies worldwide, including Ciba Geigy, Bayer and ICI. Many of these companies also have interests in other chemical productions, including the manufacture of pharmaceuticals (Brian 1988; BMA 1992).

The number of people who produce food has changed dramatically during the past 200 years. In 1787, 90 percent of the U.S. population lived on the farm and produced enough food for themselves and one other individual. By 1950, the percentage of individuals who lived on the farm had decreased to 16 percent, but those individuals produced enough food for themselves and 27 others. In 1990, only 2 percent of the U.S. population remained on the farm, but produced food for 120 people, in addition to themselves. Ninety-five of these 120 people live in the United States and the other 25 live overseas (Anonymous 1991).

Mechanization and technological advances of the early 1900's created a need for employees in factories and at manufacturing sites. High wages, combined with less physical labor, attracted laborers from the farm to the factory. Fewer and fewer laborers remained on the farm to plant, cultivate, and harvest crops. Fortunately, along with this decrease in labor came farm mechanization, which replaced horses with tractors and cultivators, and herbicides replaced hoes for weed control. These advancements enabled farmers to grow and manage more acres of crops with the reduced labor force.

Since pesticides play an important role in every day of life, their unwanted toxicities are of much concern as there are a number of diseases affecting humankind. Since fertility is one of the important aspects to carry forward the progeny, it is important to study any toxic effects affecting the fertility adversely.

In spite of gaps in our knowledge of reproductive vulnerability to xenobiotics across species, data and theoretical approaches are not available that would allow more rational prediction of human reproductive and developmental risk. Conducting physiologically based toxicological studies are essential to understand the exact mechanism of action of chemical compounds especially pesticides. A more rational approach for risk assessment would be to determine the physiologic, pharmacologic, toxicologic, cellular, and molecular characteristics that control reproductive and developmental toxicity in experimental models and translate that information into predicted human risk based on human characteristics that are similar.

Besides that, most of the farming population is directly exposed to pesticides, and as the fertility index by means of sperm count declining throughout the world, a probable cause may be the usage of these chemicals. This study helps to explore the possibilities of any toxic effects of PQ exposed either by oral or dermal route in very low concentration on male reproductive system and further, extrapolation of the same on human model may yield a supportive evidence. Usage of this chemical eventhough known to be fatal, low doses of PQ in environment may have adverse effect on reproductive system and may become a major threat to humankind affecting their reproductive ability. Eventhough there are few reports of PQ on male reproductive system, a thorough systematic investigation with low doses by oral and dermal route exposure were found to be scanty. Systematic exploration with different dose and time levels by dermal and oral route

exposure is importance since the use of PQ in agriculture is still in practice and exposure by means of oral and dermal routes are common. Hence the objectives of the present study were:

To elucidate the possible adverse toxic effects of low doses of PQ on different aspects of male reproductive system. More specifically we have aimed to investigate the toxic effect of PQ by means of oral and dermal routes on:

1. Body weight, reproductive organ weights – testes, epididymis, seminal vesicles and prostate.
2. Histopathological evaluation of the testes and epididymis – Qualitative changes like integrity of seminiferous epithelium, sloughing of epithelial cells, atrophy of germ cells if any. Quantitative estimation of seminiferous tubular diameter (STD), seminiferous epithelial height (SEH), epididymal epithelial height (EEH), count of Leydig cell number, count of spermatogonium, spermatocytes, spermatids and dividing cells.
3. Sperm analysis – Epididymal sperm count, sperm morphology, sperm motility and sperm mortality.
4. Estimation of testicular marker enzymes – serum levels of Acid Phosphatase (ACP) and Lactate dehydrogenase (LDH).
5. Estimation of hormones – serum levels of Follicle-Stimulating Hormone (FSH), Letuinizing Hormone (LH), Prolactin and Testosterone.

CHAPTER 2

REVIEW OF LITERATURE

2.1 General

Pests cause major problems and discomfort. Unfortunately, pests also invade fields and attack the crops cultivated for food and fiber. Large acreages of the same crops or concentrations of food-producing animals in a limited area favor buildup of insects, diseases, and certain weeds that must be controlled if usable food and fiber products are to be produced. For example, there are 80,000 to 100,000 diseases, 3,000 species of nematodes, 10,000 species of insects, and 1,800 species of weeds that damage the crop production process (Chambers 1992). Current estimates are that insects, diseases, and weeds destroy approximately one-third of the world food supply, even with the use of the most current pest management technology. Losses without this technology could soar to 60 to 80 percent.

For many years, pesticides have been used in conjunction with host plant resistance, cultural, mechanical, and biological tactics in an integrated pest management system to combat the battle against destructive pests. *The American Heritage Dictionary* (Davies 1979) defines pesticide as "a chemical that is used to control pests, especially insects, weeds and rodents". Pesticides may be naturally occurring substances or synthetic (man-made).

Pesticides can be categorized into various classes (Table 1) such as algicide, fungicides, herbicides, nematocides, insecticides, acarides and rodenticides.

2.2 The Role of Pesticides in Agriculture

The agricultural industry's use of pesticides amounts to approximately 83% of the whole pesticide manufacture (Robbins 1991). It utilizes pesticides in many ways. They are used during the crop growth as insecticides, herbicides and fungicides. It has been estimated that cereal crops receive approximately five to eight pesticide applications per growing season, while for high value crops, such as some vegetables and fruit, 10-15 applications are often the norm (Watterson 1989). After harvesting, during storage, most cereal, fruit and vegetable crops are dosed again with several pesticides to protect them from any storage diseases.

Consequently, even though the actual harvest may have been relatively uncontaminated with pesticides, this casual form of post-harvest storage treatment can add a considerable amount of pesticide residues to the finished product. Thus, pesticide residue levels on stored products can accumulate, as well as vary considerably from patch to patch (MAFF 1989). Pesticides are also used during livestock production, when they are either applied as 'animal medicines' such as sheep dips, lice/mange treatments, or as other 'veterinary pesticides' for controlling flies and other insects in livestock houses (Robbins 1991; BMA 1992).

2.3 General Properties of Paraquat (PQ)

Paraquat (1,1'-dimethyl, 4,4'-bipyridyllium dichloride) is a non selective contact herbicide. It is produced in several countries including China, Province of Taiwan, Italy, Japan, the United Kingdom and USA, and is used worldwide in approximately 130 countries. If not manufactured under strictly controlled conditions, it can contain impurities that are more toxic than the parent compound. It is almost exclusively used as a dichloride salt and is usually formulated to contain surfactant wetters.

Both its herbicidal and toxicological properties are dependent on the ability of the parent cation to undergo a single electron addition to form a free radical which reacts with molecular oxygen to reform the cation and concomitantly produce a superoxide anion. This oxygen radical may directly or indirectly cause cell death. Paraquat can be detected because of its ability to form a radical. Numerous analytical procedures are available.

2.4 Identity and Properties

2.4.1 Identity

Paraquat is a non-selective contact bipyridylium herbicide. The term has been applied to 2 technical products: 1,1'-dimethyl-4,4'-bipyridylium dichloride ($C_{12}H_{14}N_2Cl_2$) and 1,1'-dimethyl-4,4'-bipyridylium dimethylsulphate ($C_{12}H_{14}[CH_3SO_4]_2$).

2.4.2 Physical and Chemical Properties

Pure paraquat salts are white and the technical products yellow. They are crystalline, odourless, hygroscopic powders with a relative molecular mass of 257.2 for paraquat dichloride and 408.5 for paraquat dimethylsulphate. The relative molecular mass of the paraquat ion is 186.2 (Summers 1980). Some of the other physical properties of paraquat dichloride, the salt most used for herbicide formulations, are listed in Table 1.

Paraquat is slightly soluble in alcohol and practically insoluble in organic solvents (Haley 1979). The chemical structure of paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride) is:

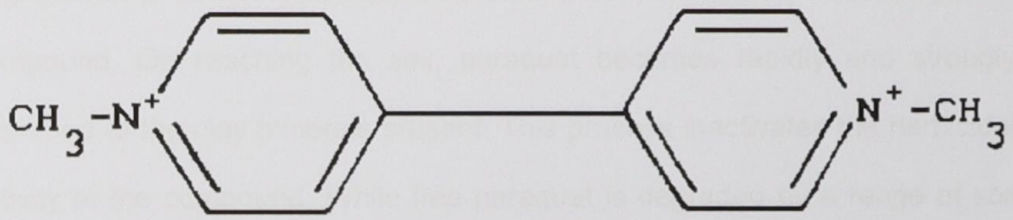


Table 2.1 Physical properties of Paraquat (Worthing 1979)

Specific gravity at 20°C	1.24 – 1.26
Melting point	175 - 180°C
Boiling point	Approximately 300°C with Decomposition
Solubility in water at 20°C	700g/litre
pH of liquid formulation	6.5 – 7.5
Vapour pressure	Not measurable

Paraquat is non-explosive and non-flammable in aqueous formulations. It is corrosive to metals and imcompatible with alkylarylsulfonate wetting agents. It is stable in acid or neutral solutions but is readily hydrolysed by alkali. Paraquat readily undergoes a single-electron reduction to the cation radical. The redox potential for this reaction is 446mv. This chemical property led to its use as a redox indicator dye (methyl viologen) as early as 1933 (Summers 1980).

2.5 Environmental Distribution and Transformation-Environmental Effects

Paraquat deposits on plant surfaces undergo photochemical degradation to compounds that have a lower order of toxicity than the parent compound. On reaching the soil, paraquat becomes rapidly and strongly adsorbed to the clay minerals present. This process inactivates the herbicidal activity of the compound. While free paraquat is degraded by a range of soil microorganisms, degradation of strongly-adsorbed paraquat is relatively slow. In long-term studies, degradation rates were 5-10% per year. Strongly –bound paraquat has no adverse effects on soil microfauna or soil microbial process.

Paraquat residues disappear rapidly from water by adsorption on aquatic weeds and by strong adsorption to the bottom mud. The toxicity of paraquat for fish is low, and the compound is not cumulative. Normal applications of paraquat for aquatic weed control are not harmful to aquatic organisms. However, care should be taken when applying paraquat to water

containing heavy weed growth to treat only a part of the growth, since oxygen consumed by subsequent weed decay may decrease dissolved oxygen levels to an extent that may be dangerous for fish. Treated water should not be used for overhead irrigation for 10 days following treatment.

Paraquat is not volatile and following spraying the concentrations of airborne paraquat have been shown to be very low. Under normal working conditions, the exposure of workers in spraying and harvesting operations remains far below present TLVs and the exposure of passers-by or of persons living downwind of such operations is lower still. Normal paraquat usage has been shown not to have any harmful effects on birds. Finite paraquat residues are to be expected only when a crop is sprayed directly. Cattle allowed to graze on pasture for 4 hours after spraying at normal application rates did not suffer any toxic effects. Consequent residues in products of animal origin are very low.

2.6 Kinetics and Metabolism

Although toxic amounts of paraquat may be absorbed after oral ingestion, the greater part of the ingested paraquat is eliminated unchanged in the faeces. Paraquat can also be absorbed through the skin, particularly if it is damaged. The mechanism of the toxic effects of paraquat are largely the result of a metabolically catalyzed single-electron reduction-oxidation reaction, resulting in depletion of cellular NADPH and the generation of potentially toxic forms of oxygen such as the superoxide radical. Absorbed paraquat is

distributed via the bloodstream to practically all organs and tissues of the body, but no prolonged storage takes place in any tissues. The lung selectively accumulates paraquat from the plasma by an energy-dependent process. Consequently, this organ contains higher concentrations than other tissues. Since the removal of absorbed paraquat occurs mainly via the kidneys, an early onset of renal failure following uptake of toxic doses will have a marked effect on paraquat elimination and distribution and on its accumulation in the lung.

2.7 Effects on Experimental Animals

A characteristics dose-related lung injury reported to be induced in the rat, mouse, dog and monkey, but not in the rabbit, guinea-pig and hamster (Butler & Kleinerman 1971). The pulmonary toxicity is characterized by initial development of pulmonary edema and damage to the alveolar epithelium, which may progress to fibrosis. Exposure to high doses of paraquat may also cause less severe toxicity to other organs, primarily the liver and kidney. Minor toxic effects have been noted only at high doses in the nervous, cardiovascular, blood, adrenal and male reproductive systems.

Paraquat has not been found to be teratogenic or carcinogenic in long-term studies on rats and mice (Bus et al. 1974). *In vitro* mutagenicity studies have been inconclusive although generally suggestive of weak potential activity, while *in vivo* studies were negative (Bateman 1966).

2.7.1 Gastrointestinal Tract and Liver

Daniel and Gage (1966) studied the absorption of ^{14}C-paraquat following oral and subcutaneous single-dose administration to rats. About 76 – 90% of the oral doses were found in the faeces, and 11 – 20% in the urine; most of the subcutaneous dose (73 – 88%) was found in the urine and only 2 – 14.2% in the faeces.

The clinical signs of acute and chronic oral poisoning (Kimbrough & Gaines 1970; Murray & Gibson 1972) or of intraperitoneal injection (Butler & Kleinerman 1971) include transient diarrhoea and body weight loss, decreased food intake and dehydration. Some of the animals emited soon after paraquat administration. Residual skin contamination after dermal toxicity studies in rabbits (McElligott 1972) caused severe tongue ulceration and an unwillingness to eat. ALT and AST- levels, determined in monkeys (Macaca fascicularis) given a single toxic oral dose of paraquat (20 – 25 mg/kg cation), did not indicate liver dysfunction (Murray & Gibson 1972). In another study (Baynova & Anadoliiska 1969) albino rats given a single peroral dose of 50 mg/kg paraquat, developed metabolic acidosis, a disturbance that was attributed to both renal and liver toxicity. According to (Butler & Kleinermann 1971), the macroscopical and histological aspect of the liver of rabbits that were given single or repeated toxic paraquat doses, was normal. Based on (Clark et al. 1966), the histopathological appearance of the liver in rats, mice, rabbits given single or repeated intraperitoneal or oral doses of the herbicide. Small areas of centrolobular cell necrosis were also found in

another study (Murray & Gibson 1972) in which rats were given a single oral dose of 143 mg/kg of paraquat cation and were sacrified from 1 to 14 days after the administration.

After intravenous administration of paraquat (27 mg/kg) to Sprague-Dawley rats, the histopathological appearance of the liver was normal 1 to 2 days after the administration. However, in animals surviving 3 days, a prominent loss of glycogen was noted near the central hepatic vein and interpreted as a possible effect of food intake reduction (Fisher et al. 1973a). There have been several reports of liver damage following exposure to high doses of paraquat (Clark et al. 1966; Bainova 1969a; Murray & Gibson 1972). Centrilobular necrosis of hepatocytes with proliferation of the Kupffer cells and bile canals have been described. In general, liver damage in experimental animals has not been severe compared with lung and kidney damage. Serum enzyme activities (SGOT & SGPT) only increased when large amounts of paraquat were given (Giri et al. 1979).

This, together with the absence of marked biliary excretion, evidence of poor absorption along the gut. This low rate of absorption was confirmed by Litchfield et al. (1973) and Conning et al. (1969). Rats, guinea-pigs, and monkeys orally administered LD50 doses of ^{14}C-paraquat had low peak serum concentrations (2.1 – 4.8 mg/litre) (Murray & Gibson 1974).

In fasting dogs, low oral doses of paraquat were rapidly but incompletely absorbed, the peak plasma concentration being attained 75

minutes after dosing (Bennett et al. 1976). After an oral dose of 0.12 mg/kg body weight, 46 – 66% was absorbed in 6 hours. For doses of 2 – 5 mg/kg, only 22 – 38% and 25 – 28% of the dose was absorbed, respectively. Dose-dependent data from dogs and whole body autoradiography suggest that absorption is facilitated in the small intestine. Some non-ionic surfactants (0.001%) increased ^{14}C-paraquat transport through isolated gastric mucosa models, but histological evaluation suggested that this was due to damage of the epithelial cell membranes (Walter et al. 1981).

2.7.2 Pulmonary Absorption

Absorption of paraquat following instillation and inhalation in the lung has been described in several studies (Gage 1968a, Kimbrough & Gaines 1970, Seidenfeld et al. 1978, Popenoe 1979). The uptake of ^{14}C-paraquat after an intratracheal injection of 1.86 nmol/lung was investigated in the isolated perfused rat lung by Charles et al. (1978). The efflux of ^{14}C-paraquat was biphasic with a rapid phase half-life of 2.65 minutes and a slow phase half-life of 356 minutes. Various doses of ^{3}H-paraquat (10^{-5} – 10^{-12} g) in 0.1 ml saline were introduced directly into the left bronchus of rats (Wyatt et al. 1981). Fifteen minutes after instilling 10^{-8} of ^{3}H-paraquat, 90% of the ion could accounted in the tissues and urine, 50% being present in the lung.

Zavala and Rhodes (1978) reported that the lung of the rabbit was highly sensitive to paraquat intrabronchial instillation in doses ranging from 0.1 g – 1 pg; moderately sensitive to intravenously administered paraquat (25

mg/kg body weight); resistant to the herbicide when given intraperitoneally or subcutaneously (25 mg/kg body weight). Clark et al. (1966) reported that, in rats in the earlier stages after a single toxic oral dose of paraquat, breathing was gasping or deep and fast, but some days after a single or repeated toxic doses, the respiration became increasingly laboured, and the hairs around the mouth and nares were soiled with a brownish liquid.

The extensive alveolar edema observed in severe intoxication was responsible for the development of hypoxia, cyanosis, and dyspnea. The progressive development of pulmonary fibrosis was accompanied by difficulty in breathing, gasping and hyperpnea (Smith et al. 1973). Exposure of rats to high concentration of respirable paraquat aerosols was accompanied by shallow respiration. Within 2 – 3 hours, the test animals became dyspnoeic, cyanotic, and inactive and there were signs of local eye and nose irritation (Gage 1968a).

2.7.3 Dermal and eye administration

Paraquat absorption through animal and human skin has been studied using an *in vitro* technique (Walker et al. 1983). Human skin was shown to be impermeable to paraquat, having a very low permeability constant of 0.73. Furthermore, human skin was found to be at least 40 times less permeable than animal skins tested (including rat, rabbit, and guinea-pig). In mice and rats, the application of 5 – 20 gm paraquat/ liter solutions in single and 21 days repeated dermal toxicity tests provoked dose-related toxic dermatitis

with erythema, edema, desquamation and necrosis (Bainova 1969b). No skin sensitization was observed in studies on guinea-pigs when paraquat was applied (Bainova 1969b; Fodri et al. 1977).

The instillation of dilutions of paraquat (up to 500 gm/liter) in rabbit eye induced inflammation within 24 hours and continued for 96 hours (Clark et al. 1966). Sinoi & Wei (1973) introduced 62.5, 125, 250, 500, and 1000 gm paraquat/liter into the rabbit eye. Concentration of 62.5 and 125 gm/liter caused severe conjunctival reactions; higher levels (250 – 500 gm/liter) provoked iritis and pannus, while at the 500 gm/liter concentration there was corneal opacification, iritis and conjunctivitis. All rabbits receiving 0.2 ml of paraquat at 1000 gm/liter in one eye or 0.2 ml of a concentration of 500 gm/liter in both eyes died within 6 days of application (Sinow & Wei 1973).

There are no *in vivo* studies on the rate of absorption of paraquat through the skin. However, observations of dose-related dermal toxicity in experimental animals and human percutaneous poisoning have provided some qualitative information concerning the dermal absorption of paraquat.

2.7.4 Renal System

In paraquat toxicity, kidney damage often precedes signs of respiratory distress (Clark et al. 1966; Butler & Kleinerman 1971; Murray & Gibson 1972). Paraquat is excreted mainly via the urine and the concentration of the herbicide in the kidneys are relatively high. BUN-measurements in

experimental animals poisoned by paraquat were performed only in one study (Murray & Gibson 1972): in monkeys (*Macaca fascicularis*) given single toxic doses of paraquat cation ranging from 20 to 25 mg/kg, BUN-levels were normal. Gross pathological and histological examinations of paraquat-poisoned rats, guinea-pigs, rabbits, and dogs revealed vacuolation of the convoluted renal tubules and proximal tubular necrosis (Bainova 1969a; Murray & Gibson 1972; Tsutsui et al 1976). The generation of the proximal tubular cells has also been confirmed by electron-optical studies (Fowler & Brooks 1971; Marek et al. 1981). Paraquat is actively secreted by the kidney base transport system. The nephrotoxicity caused by paraquat is pronounced and appears to be restricted to the proximal nephron (Ecker et al. 1975; Gibson & Cagen 1977; Lock & Ishmael 1979; Purser & Rose 1979).

2.7.5 Effects on Reproductive System

2.7.5.1 Testes

Some histological changes in the testes have been reported in a few paraquat toxicity studies. Butler & Kleinerman (1971) found multinucleated giant cells in rabbit testicular tubules. When paraquat was orally administrated at 4 mg/kg body weight to male rats for 60 days and the testes were examined, there were no significant deviations in the spermatozoa count or motility, nor were there any biochemical changes in the several enzymes of testes homogenates. The histoenzyme activity of lactate dehydrogenase, succinate dehydrogenase, DPN- diaphorase, alkaline phosphatase and acid phosphatase in the treated animals did not differ from that of the controls, nor

did quantitative and qualitative histological examination of the testicular tubule cells reveal any abnormality (Butler & Kleinerman 1971).

Degeneration of the seminiferous tubules with the formation of small multinucleated giant cells lying free in their lumen was observed in the testes of two of five rats that had been given a single large 200mg/kg or few repeated small peroral or peritoneal doses of paraquat (Clark et al. 1966). Other studies (Butler & Kleinermann 1971) also reported on the histological appearance of the testes of immature New Zealand rabbits that had been given doses of paraquat ranging from 2 to 100 mg/kg, administered in 1 – 5 intraperitoneal injections. Seven of the 20 animals receiving 50 mg/kg of paraquat or more, had multinucleated giant cells in the lumen of their testicular tubules. A decrease in route of spermatogenesis was also observed in rabbits that were given a single toxic dermal dose of 70 to 500 mg paraquat cation/kg during 24 hours (McElligott 1972). Impairment of spermatogenesis has also been detected with the dominant lethal test in male mice that were given a single intraperitoneal dose of 25mg/kg paraquat or diquat (Pasi et al. 1974; Pasi & Embree 1975).

On the other hand, the testes of rats, mice, guinea-pig, rabbits and dogs that had been exposed to paraquat aerosols concentrations of up to 0.4 μgm/liter for periods of up to three weeks were histologically normal (Gage 1968a). Also morphologically normal were the testes of a 15 year old boy (Matthew et al. 1968) and two adults (Nienhaus & Ehrenfeld 1971; Bronkhorst et al. 1968) fatally poisoned by paraquat as were epididymes, seminal

vesicles and prostate. A three generation reproductive study has been carried out in rats treated with paraquat ion at 100 mg/kg diet (FAO/WHO 1973). There were no significant abnormalities in fertility, fecundity and neonatal morbidity or mortality, nor were there any signs of gonadotoxicity or structural or functional lesions.

2.7.5.2 Ovaries

In 97 cases of paraquat poisoning reported in the literature there were no observations on the function and morphology of the ovaries. Experimental observations on the action of paraquat on this organ are also scarce. According to Molnar (1971), after the single peroral administration of 160 mg/kg paraquat on the 9th day of pregnancy, the ovaries and Fallopian tubes of rats, did not show any significant macroscopical or microscopical lesions.

2.7.6 Embryotoxicity and Teratogenicity

Oral and intraperitoneal administration of high doses of paraquat to mice and rats on various days of gestation produced significant maternal toxicity, evidenced by increased mortality rates (Bainova & Vulcheva 1974; Bus et al. 1975). Examination of the fetuses from the higher-dose groups revealed a reduction in fetal body weights, and increased resorption rate in mice, as a result of the maternal intoxication. The absence of a specific embryotoxic action of paraquat has also been observed and reported in other

studies in rats (Khera et al. 1968; Luty et al. 1978), mice (Selypes et al. 1980), and rabbits (FAO/WHO 1973).

In a perinatal toxicity study, Bus & Gibson (1975) administered paraquat at 50 or 100 mg/liter in the drinking water to pregnant mice beginning on day 8 of gestation, with continued treatment of the litters up to 42 days after birth. Paraquat treatment did not alter postnatal growth rate, although the mortality rate in the 100 mg/liter-treated mice increased to 33% during the first 7 days after birth. It was also noted that paraquat at 100 mg/liter significantly increased the sensitivity of the pups to oxygen toxicity on days 1, 28, and 42 after birth.

2.7.7 Mutagenicity and Genotoxicity

PQ has been found to have minimal to no genotoxic activity when evaluated in a variety of *in vitro* and *in vivo* test systems. In studies producing weakly positive results (Moody & Hassan 1982; Parry 1973; Bignami & Grebelli 1979), which were limited to *in vitro* studies, paraquat genotoxicity was accompanied by high cytotoxicity. These results were best explained by Moody & Hassan (1982), showed that the mutagenicity of paraquat in bacterial test systems (*Salmonella typhimurium* TA98 and TA100) was mediated by the formation of superoxide.

The genotoxic potential of the herbicide PQ was comparatively tested in various genotoxicity tests with V79 Chinese hamster cells. PQ clearly

induced cytotoxicity and chromosome aberrations but did not induce gene mutations at the HPRT locus or increased DNA migration in the comet assay under the same treatment conditions. Speit and co-workers (1998) suggest that PQ does not significantly induce DNA lesions relevant for HPRT gene mutations in cultivated V79 cells. Furthermore, paraquat was not mutagenic when evaluated in human leucocytes and in *in vivo* cytogenetic tests on mouse bone marrow (Selypes & Paldy 1978) and dominant lethal tests on mice (Pasi et al. 1974; Anderson et al. 1976).

2.7.8 Carcinogenicity

A carcinogenicity study was performed on mice at dietary levels of 25, 50 and 75 mg/kg per day for 80 weeks (FAO/WHO 1973). There were rediced weight gains among the animals receiving paraquat, but deaths during the study were associated with respiratory disease. Clinical and histopathological examination determined that paraquat was not tumorigenic in mice.

A two year study of exposure of rats to 1.3 and 2.6 mg/liter daily, in the drinking-water provoked histopathological changes in the lung, liver, kidney and myocardium. The lung lesion were dose-related; inflammation, atelectasis, reactive proliferation of the epithelium, pulmonary fibrosis, and pulmonary adenomatosis were noted, but no sign of tumour growth or atypism (Bainova & Vulcheva 1977). Nor was any increased tumour incidence reported in rats in a 2-year study with a maximum dietary level of 250 mg/kg diet (12.5 mg/kg body weight per day) (FAO/WHO 1971)

Bainova & Vulcheva (1977) did not discover any indication of tumourigenicity in a 2-year study in rats receiving paraquat at 1.3 or 2.6 mg/liter in their drinking-water. While testing the carcinogenicity of urethane in mice, Bojan et al. (1968), also attempted to evaluate the influence of paraquat on urethane-induced lung tumourigenesis. It is felt that the results of this study are not of relevance for the assessment of the carcinogenic potential of paraquat.

2.8 Clinical Signs of Paraquat Poisoning in Experimental Animals

2.8.1 1st Phase of Paraquat Intoxication: Latency & First Clinical Signs

According to (Molnar-Sebestyen 1971), the first clinical signs appeared 12 hours after the peroral administration of a saline solution of paraquat dichloride to adult albino rats in doses ranging from 80 – 320 mg cation/kg body weight. The treated animals looked sick, were hypoactive and their food intake was decreased.

After intraperitoneal administration of paraquat, the latency time of appearance of first clinical signs was surprisingly and unexplainably as long as 48 hours (Molnar-Sebestyen 1971), although administered doses were comparable or larger than the peroral doses mentioned in the previous study. In another study (Roujeau et al. 1974), however, performed on Wistar rats, latency time was considerably shorter.

Intravenous administration of a single toxic dose (27 mg/kg) of paraquat to rats, first clinical signs appeared as expected after a latency time of few hours (Fisher et al. 1970). In subhuman primates (Macaca fascicularis) given the chemical by gavage, early signs of paraquat poisoning included tachycardia and hyperpnea (Murray & Gibson 1972). Lethargy (possibly a central nervous system effect) as one of the first signs was observed in rats that had received a single oral dose of 143 mg/kg of paraquat (Murray & Gibson 1972). In mice that had received 200 ppm of paraquat with the drinking water for a period of 4 weeks, first signs included immobility, fur irregularities and avoidance of food and water intake. Respiratory difficulties appeared only at a level of 300 ppm (Brooks 1971).

2.8.2 2nd and 3rd Phase of Paraquat Intoxication

In both intrpertitoneally and orally treated rats, the first intoxication phase (Phase I) lasted for about 48 hours. Then (Phase II), a period of apparent remission occurred in both groups and continued for 1 – 10 days in the intraperitoneally treated animals and for only 16 hours in the orally treated rats. Subsequently, (Phase III) an abrupt onset of respiratory difficulty occurred in both orally and intraperitoneally treated rats. The animals became again hypoactive, their eyes remained closed and the respiratory rate was increased. White foam appeared around their mouths and this became bloody; blood was also noticed around the eyes and on the paws. Terminally animals exhibited an increased tendency to turn away from light. They all died apparently from respiratory distress (Molnar-Sebestyen 1971).

2.8.3 Dose-Effect of Paraquat

The acute LD_{50} values for paraquat in various species are given in Table 2. The acute toxicity studies of paraquat salts (dichloride, dimethylsulphate, dimethylphosphate) have shown significant differences in the acute oral and intraperitoneal LD_{50} in rats (Clark et al. 1966; Makovskii 1972).

There were no significant differences in the oral LD_{50} values obtained for the same species from different laboratories, but the acute oral LD_{50} values among the species examined varied. Paraquat, was administered, orally and in the diet, to rats, mice, guinea-pigs and dogs. The guinea-pigs appeared to be very sensitive (Makovskii 1972). According to Kimbrough & Gaines (1970), Makovskii (1972), and Bainova (1975), the herbicide has a moderate cumulative toxicity. The joint FAO/WHO meeting (1976) decided on a no-observed-adverse-effect level of 1.5 mg/kg body weight per day in the rat and a 25 mg/kg body weight per day in the dog. Guinea-pigs, monkeys, cattle, and human subjects were more sensitive, while rats and birds were less sensitive to paraquat through the gastrointestinal route.

2.9 Sources in the Environment

2.9.1 Industrial Technology

Paraquat does not occur naturally. It was originally synthesized by Weidel & Russo as reported in 1882 (Summers 1980). Its herbicidal properties were discovered only in 1955. The compound is produced by coupling pyridine in the presence of sodium in anhydrous ammonia and quartenizing the 4, 4'-bipyridyl with methyl chloride.

When bipyridyl is refluxed with methyl iodide, the iodide salt is obtained. Haley (1979) and Summers (1980) thoroughly reviewed the published methods for paraquat synthesis, and for the separation and purification of bipyridylium salts. The yields obtainable vary from 20% to 96% of pure product. The first commercial paraquat formulation approved for agricultural use was Gramoxone®.

2.9.2 Impurities

Aqueous solutions of paraquat used as herbicides must correspond to the FAO Specification Code 56/13/S/6 (FAO 1973). This requires a description of the active ingredient in the formulation, of the impurities, of the physical and chemical properties, and of the methods for determining the components. The only impurity permitted in paraquat is free 4,4'-bipyridyl at a maximum level of 0.25% of the paraquat content.

2.10 Production and Use

PQ is produced in several countries, including China, Province of Taiwan, Italy, the United Kingdom, and the U.S.A. Formulations of the active ingredients (mainly paraquat dichloride) are used in more than 130 countries worldwide. Paraquat dimethylphosphate is used in Russia. Since its introduction in agricultural use in 1962, PQ has been widely used for weed control and as a dessicant. In many countries, PQ is formulated locally, only the technical active ingredient being imported. Records of world production of PQ are not available.

Technical paraquat dichloride has been formulated in liquid concentrates or granules. Water-soluble granules containing PQ (25g/kg) and diquat (25g/kg) are used for weed control in private gardens. PQ is sold under a variety of trade names, for example Paracol (Malaysia, Indonesia, Philipines), Gramuron (Africa, Italy) and Tryquat (Australia).

PQ is a total contact herbicide used to control broad-leaved and grassy weeds. It should be sprayed when the weeds are young and less than 30cm high. It kills all green tissues, but does not harm the mature bark. PQ is used for plantation crops (banana, cocoa-palm, coffee, oil-palm, rubber, etc.) and for citrus fruits, apples, plums, vines, and tea. On certain crops (potato, pineapple, sugarcane, sunflower), it is used as a dessicant; it is also used as a cotton defoliant. It is applied around the trees in orchards and between the

rows of crops. Uncropped land on industrial sites, railways, roadsides etc. can be cleared of weeds by applying PQ at higher concentrations.

2.11 Mechanism of the Herbicidal Effect

The herbicidal activity of paraquat is dependent on the parent molecule undergoing a single-electron redox cycling in the presence of molecular oxygen, is immediately reoxidized forming the parent molecule and superoxide radicals (O_2^-) (Conning et al. 1969). As early as 1960, Mees had shown the oxygen was necessary for the herbicidal activity of paraquat, suggesting the importance of the redox cycling and O_2^- formation in mediating toxicity. Paraquat was not toxic to plant leaves incubated under anaerobic conditions, despite the continuation of photosynthetic reaction capable of forming paraquat radicals.

Exposure of the anaerobic incubates to air, however, resulted in immediate on set of toxicity. The generation of O_2^- may lead to many potentially cytotoxic reactions, including the membrane-damaging process of lipid peroxidation (Bus & Gibson 1979). When plant leaves were incubated with paraquat, there was rapid stimulation of the formation of malonialdehyde, which is an indicator of lipid peroxidation (Dodge 1971).

2.12 Occupational Exposure

Exposure to paraquat occurs by dermal and ocular contact, by inhalation or by oral intake. Early occupational exposure studies in Sri Lanka, Malaysia and the United States assessed exposure levels of knapsack and field tractor applicators (Swan 1969; Staiff et al. 1975; Chester & Woolen 1981; Wojeck et al. 1983) and occupational exposures for aerial applications (Chester & Ward 1981). More recently some exposure assessments studies on knapsack applicators have been performed in Sri Lanka (Chester et al. 1993) and Costa Rica (Van Wendel et al. 1996; Spruit & Van Puijvelde 1998). In general, dermal exposure levels seemed most important, whereas measured inhalation levels were relatively low. Four out of six studies assessing uptake of PQ found in urine at the end of one or more working days. However, the relations between exposure determinants, levels of external exposure, and levels in urine were far from clear and not investigated with much detail.

Studies carried out with collaboration with Imperial Chemical Industries (currently SYNGENTA) concluded that PQ was most unlikely to cause serious health problems under correct conditions of use (Howard 1980; Swan 1969; Chester & Woolen 1981; Chester et al. 1993) despite the fact that in several of these studies between 40 and 50% workers experienced topical injuries (Swan 1969; Howard et al. 1981). Spray operators were continuously at high risk of exposure that may lead to severe intoxication and injuries (Van Wendel et al 1996).

2.12.1 Dermal Exposure

PQ is poorly absorbed through intact skin, but penetration is considerably increased by damaged skin, which is of particular concern because PQ itself is a skin irritant (Tabak et al. 1990). On banana plantations, dermal exposures varied rather due to differences between plantations, than differences between applicators or between days (Van Wendel et al. 1996; Spruit and Van Puijvelde 1998). The body parts identified with highest exposure were hands, wrists, back and scrotum. They included splashing during preparation of the spray solution and open transportation, deposition of spraying mist, contact with spray solution when filling knapsack, leaking of knapsack on back and groin, adjustment of spray equipment, and walking through the sprayed vegetation.

Use of protective clothing is supposed to considerably reduce dermal exposure. However, few studies have evaluated the effectiveness of personal protection or other safety measures (Spruit & Van Puijvelde 1998; Machado-Neto et al. 1998). Swan (1969) compared exposure of applicators using normal clothing with workers using gloves, boots and respiratory protectors. Despite the use of gloves, hands remained exposed due to cross contamination by taking off and on. Occlusion of pesticides by protective devices may result in increased absorption (Brouwer et al. 2000; Meuling et al. 1997).

United States (EPA) concluded that PQ causes moderate to severe eye irritation (Toxicity category II) and minimal dermal irritation (Category IV), based on toxicity experiments in rabbits (US EPA 1997). In fact, dermal lesions observed in workers range from mild irritation to blistering and ulceration (2nd and 3rd degree chemical burn), often in genital area (IPCS-WHO 1984; Stevens & Sumner 1991). Eye injuries may range from blepharitis and conjunctivitis to ulcerations of keratosis of the cornea; and a nail damage due to prolonged hand contact with PQ ranges from localized discoloration to temporary nail loss. Skin, nail and eye lesions have been reported (Howard 1979; Hoffer & Taitel,am 1989; George 1989) including in children.

Workers in formulating factories were at high risk. A survey among 18 PQ formulation workers in the UK found that 14 (78%) had experienced nail damage, nose bleeding, blepharitis, or skin lesions with delayed healing. In Malaysia, 15 out of 18 formulators presented topical lesions, such as dermatitis or chemical burns (50%), and eye injury or blepharitis (Howard 1979). Another studies had also shown that in Malaysia, approximately 50% of PQ sprayers experienced skin or eye injuries during fourteen and twelve week spraying programs, respectively (Swan 1969; Howard et al. 1981).

In Costa Rica, a number of surveys on occupational injuries among wage-earning workers have been carried out by the Central American Institute for Studies on Toxic Substances between 1982 and 1996 (Wesseling et al. 1993; Wesseling & Castillo 1992; Wesseling et al. 2001a). In absolute numbers, hundreds of PQ injuries occur each year in Costa Rica, most of

them in the banana plantation. The majority (60%) of victims presented skin burns or dermatitis and 26% chemical eye injuries. The remaining 14% represented systemic poisonings; nose bleeding and nail damage (Wesseling et al. 2001b).

2.12.2 Inhalation

In general, inhalaton exposure is not considered a relevant exposure route, due to the low volatility of PQ and droplets being too large to enter the small airways during application (US EPA 1997). Ambient air concentrations are generally well below NIOSH and OSHA limits (0.1 mg/m^3 and 0.5 mg/m^3 TWA, respectively).

The low inhalation levels measured in the different studies seem inconsistent with the frequent episodes of epistaxis or nose bleeds reported among exposed workers (IPCS-WHO 1984; Howard 1980; Swan 1969), which were due to local irritation of the upper respiratory tract by PQ particles (US EPA 1997). However, it remains unclear at which inhalation exposure levels epistaxis occurs and whether these levels may be relevant for systemic uptake.

2.12.3 Oral Exposure

Oral exposure may occur during work when ingesting PQ solution by mistake, through splashes in the mouth during mixing and transporting, by

eating with contaminated hands, by blowing or sucking spray nozzles, or when eating contaminated food (Wesseling et al. 1997; Howard 1980; Chester & Woolen 1981; Fitzgerald et al. 1978).

In addition, oral ingestion may also occur as a result of swallowing the 'run off' on the face caused by droplets when the operator is working in the spray mist (Howard 1980). Retention of PQ particles in the nose and mouth, as evidenced by soar throat and nosebleed, may contribute to the internal dose by swallowing (Wesseling et al. 1997).

2.12.4 Non-Occupational Exposure: Risk for Children

Accidental oral exposure may occur under a variety of circumstances. Confusion of PQ concentrate or solution due to inappropriate storage in refreshment or liquor bottles has apparently diminished but still occurs. Accidental intake at home in Costa Rica is common, often in association with alcohol intake (Leon et al. 2001). Children's exposure are of special concern. In Costa Rica, between 1991 and 1995, the exposure circumstances of severe and fatal poisoning in children age 1 – 6 included the cases of two toddlers placing respectively a rinsed spray jet and a bottle top into their mouths, two cases of confusion of bottles stored in the kitchen, two cases of children playing with empty bottles, and a 7-year old sister giving 'cough medicine' to a younger brother (Leon et al. 2001).

2.13 Epidemiology of Severe and Fatal Paraquat Poisoning

Thousand of PQ poisonings and fatalities have been reported in case reports, case series, and surveys and through surveillance systems, in particular in developing countries. Incidence and mortality rates vary enormously according to patterns of PQ use, prevention and control programs, type of register and reporting practices.

Asia and Latin America including Malaysia, Fiji, Japan, Sri Lanka, Mexico, Costa Rica, Trinidad and Tobago and Samoa (Wong & Ng 1984; Taylor et al. 1985; Zinn 1995), For example, the incidence of fatal PQ poisonings, particularly suicides, has increased in Costa Rica during 1992 – 1998 as compared to the period time of 1980 – 1986 (Leon et al. 2001),

Seventy five percent were accidental and occupational poisonings. Forty-eight percent of the PQ fatalities with an identified cause were unintentional, mostly after accidental ingestion but also after occupational exposure (Wesseling et al. 1988; Wesseling et al. 1993; Wesseling et al. 1997). Severe PQ poisoinings and fatalities in children have been reported (Onyon & Volans 1987), ranging from accidents with extremely low doses (Wesseling et al. 1988; Fernando et al. 1990) up to homicides (Daisley & Simmons 1999; Kalabalikis et al. 2001).

Although by far, the majority of PQ poisonings occur by oral intake, a number of severe and fatal occupational and accidental poisoning reports

after skin absorption are available (Newhouse et al. 1978; Wohlfhart 1982; Athanaselis et al. 1983). One report concerned an unintended death following vaginal absorption (Ong & Glew 1989). It has been alleged that, systemic effects do not occur at recommended dilution rates (Howard 1980; Hart 1987). However, in 1983 a fatal case was reported of a farmer having applied a PQ solution diluted according label instructions (0.5% solution of PQ), during 3.5 hours that resulted in skin exposure due to a leaking knapsack (Athanaselis et al. 1983). The farmer died within a week after application due to PQ induced systemic intoxication. Wesseling et al. (1997) described fifteen unintentional fatal PQ poisonings, of which five were due to contact with diluted spray solution.

Xenoestrogen, pesticides and many industrial chemicals, freely avaiable in the atmosphere are the major threats to animal and human health. Over the years, eventhough the world is leaping forward in scientific and technological development, alarmning health adverse effects inducing mutagenic and carcinogenic sequalae in normal cell population is common. Scientific studies blame the environmental pollutants as the major ingredients for the observed health problems.

Pesticides as insecticides, fungicides, and herbicides are the common freely avaiable adverse chemicals in the atmosphere including bed rooms, lawns, fields, as well as all surrounding of human habitat. In most of the situations, they are recklessly handled and applied without the exercise of common precautious. Literature reveals volumes of reports on these

substances, use of these chemicals are still a major problem, affecting the health. Reproductive system is one of the most vulnerable system which is a major site for the toxic effects of these chemicals as the germ cells are available at different stages of cell division.

Male reproductive system, involving different stages of spermatogenesis throughout the life following puberty is one of the most vulnerable system affected adversely by these chemicals. Most of the chemicals were known to disrupt these function directly by affecting the different cell cycles at different stagesof their development and maturation. Apart from the direct adverse effect, indirect toxicities affecting the hypothalamo-hypophyseal-gonadal axis, hormonal system and target enzymes regulating the spermatogenesis may be disrupted. PQ is a commonly used herbicides in agriculture. Toxic effects of PQ on human health were well studied. There are only few reports available with reference to the toxic effects on male reproductive system.

CHAPTER 3

METHODOLOGY

3.1 Animals

In the present study, male albino rats of Sprague-Dawley strain aged between 11 to 13 weeks old and weighing 200 – 220 gm were purchased from Animal House Unit, School of Medical Sciences, Universiti Sains Malaysia (USM) Kelantan. Animals were housed in polypropylene cages (29 x 22 x 14 cm) with wood husk bedding at 26 ± 2°C room temperature and 50 ± 5% humidity in animal room, Physiology Laboratory, USM Kelantan. In each cage, maximum of 3 animals were housed to avoid any stress due to overcrowding.

Animals were fed on laboratory rat feed (Gold Coin Sdn. Bhd., Malaysia) and water *ad libitum*. The number of animals per group, age, sex, dose, duration and routes of administration of the chemical were given in Table 3.1. In all cases, the animals were maintained according to the specified duration of post exposure and sacrificed by cervical dislocation at the time of experiment. Instituitional ethical guidelines were strictly followed during the handling and experimentation.

No.	Routes of Administration	Dose level (mg/kg)	Sample Time (days)	Number of animals per group (n)
1.	ORAL & DERMAL	Control	7d	n=6
		Control	14d	n=6
		Control	28d	n=6
		Control	42d	n=6
		Control	84d	n=6
		Control	105d	n=6
2.	ORAL	1A – 2mg/kg	7d	n=6
		1B – 5mg/kg	7d	n=6
		1C – 20mg/kg	7d	n=6
		2A – 2mg/kg	28d	n=6
		2B – 5mg/kg	28d	n=6
		2C – 20mg/kg	28d	n=6
		3A – 2mg/kg	42d	n=6
		3B – 5mg/kg	42d	n=6
		3C – 20mg/kg	42d	n=6
		4A – 2mg/kg	84d	n=6
		4B – 5mg/kg	84d	n=6
		4C – 20mg/kg	84d	n=6
		5A – 2mg/kg	105d	n=6
		5B – 5mg/kg	105d	n=6
		5C – 20mg/kg	105d	n=6
3.	DERMAL	6D – 6mg/kg	7d	n=6
		6E – 15mg/kg	7d	n=6
		6F – 30mg/kg	7d	n=6
		7D – 6mg/kg	14d	n=6
		7E – 15mg/kg	14d	n=6
		7F – 30mg/kg	14d	n=6
		8D – 6mg/kg	28d	n=6
		8E – 15mg/kg	28d	n=6
		8F – 30mg/kg	28d	n=6
		9D – 6mg/kg	42d	n=6
		9E – 15mg/kg	42d	n=6
		9F – 30mg/kg	42d	n=6

Table 3.1 The routes of administration, dose level, duration and total number of animals per group were shown.

3.2 Routes of Exposure

Two routes of PQ exposure were adopted; oral and dermal application. These were the major exposure routes of PQ in human and were selected based on many case reports among human as they were exposed to PQ either by occupational exposure (sprayers), suicidal failures or environmental exposure.

3.3 Preparations

For both oral and dermal treatment groups, animals were kept in their environment cages under the experimental housing and feeding conditions for at least five days prior to the experiment. Twenty four hours before the test, rats (as mentioned in Table 3.1 Dose selection) were labeled and weighed. The doses selected for oral route were 2, 5, and 20 mg/kg of body weight and for dermal route were 6, 15 and 30 mg/kg of body weight.

For dermal application, approximately 24 hours before the test, fur was removed by clipping and shaving from the dorsal area of the trunk of the animals. Caution was taken during clipping and shaving the fur, to avoid abrading the skin which could alter the permeability of PQ. Less than 10% of the body surface area was cleared for the dermal application. For oral application, a 20G-oral gavage was used. The needle was cleaned before every test from any debris.

Any irritant or corrosive effects of the main compound (PQ) were taken into account while dose level selection. The post – exposure animal groups were observed for their healthy clinical signs - toxic reactions, rate of onset and the length of the recovery period (for dermal application). The time at which signs of toxicity appear and disappear, their duration and the time of death if any were important and recorded.

3.4 Dermal and Oral Application procedure

For dermal study, animals were caged individually. The test substance (PQ) was applied uniformly over the area which is approximately 10% of the total body surface area. The test compound was applied as a thin and uniform layer, area of application was covered in order to get an optimum exposure.

PQ solution was applied to the skin with a porous gauze dressing and non – irritating tape throughout four hour exposure period. The area of application was further covered with a tape to retain the gauze and test substance and also to ensure the animal from reaching the test substance. Restrainers were used to prevent the ingestion of the test substance with a complete immobilization of rat. At the end of the exposure period, residual test substance was removed and the skin was cleaned with distilled water. Dermal application was continued for 5 consecutive days with a gap of 24 hours between each application.



Fig 3.1 Oral administration – gavage



Fig 3.2 Dermal application for PQ dermal exposure

For oral exposure, the test substance was administrated through oral route by using a non - irritating 20G – oral gavage. Rats were treated at the same time, in gaps of twenty four hours for five consecutive days.

Careful observations of animals were made for changes in fur, skin, mouth, eyes and mucous membrane and also the respiratory system. Particular attention was taken directly to signs of tremors, convulsions, salivation, diarrhea, lethargy and coma. The time and date of death of animals if any were recorded as precisely as possible. Assessment of body weight on weekly basis was done (Digital Weight Balancer, Ohaus Navigator Co., USA) with pre and post weight after the administration of PQ. The final body weight of animal represents a good marker from any systemic toxic effect of pesticide.

3.5 Chemicals

3.5.1 Paraquat dichloride (1,1' – dimethyl – 4,4' – bipyridylium dichloride)

Paraquat ($C_{12}H_{14}N_2$) is a bipyridyl contact herbicides most commonly marketed as paraquat dichloride (PQ). According to Shirasu & Takahashi (1977), the oral LD_{50} of PQ in male rat was 161 mg/kg while in female rat was 187 mg/kg. Dermal routes LD_{50} of PQ was 90 mg/kg for male rat and 80 mg/kg for female rat (Kimbrough & Gaines 1970). In this study, the doses used were 1/8, 1/32, and 1/80 of LD_{50} oral treatment and 1/3, 1/6, and 1/15 of LD_{50} for dermal treatment.

Species/Sex	Oral LD50	Dermal LD50	Inhalation LC50 Respirable paraquat aerosol
Rat	200[a]		1[c]
Rat (F)	100[e]	90[e]	10[f]
Rat (M)	***161***[e]	***90***[k]	10[f]
Mouse		62[d]	
Rabbit		500[a]	
Rabbit		236[b]	
Rabbit		240[h]	
Guinea-pig	40 – 80[a]		
Guinea-pig (M)	30[b]		
Guinea-pig	22[i]		
Guinea-pig	42[g]	319[g]	4[g]
Monkey	50[i]		
Cat	40 – 50[a]		
Cat (F)	35[b]		
Hen	300 – 380[a]		
Hen	262[b]		
Turkey	250 – 280[j]	375[j]	
Cow	50 – 75[a]		
Sheep	50 – 75[a]		

.a Howe & Wright (1965)
.b Clark et al. (1966)
.c Gage (1968)
.d Bainova (1971)
.e Shirasu & Takahashi (1977)
.f Bainova & Vulcheva (1972)
.g Makovskii (1972)
.h McElliot (1972)
.i Murray & Gibson (1972)
.j Smalley(1973)
.k Kimbrough & Gaines (1970)

Table 2. Paraquat LD_{50} (mg/kg body weight) and LC_{50} (mg/m^3) in various species.

3.5.2 Chemical Solutions

Following were the different chemical solutions employed in various assay procedures:

3.5.2.1 Phosphate Buffered Saline (PBS)

- *Solution A* – Sodium chloride (NaCl) – 9 gm/L
    1. Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) – 2.34 gm/100ml distilled water.
    2. Disodium hydrogen monophosphate (Na_2HPO_4) – 2.13 gm/100ml distilled water.
- *Solution B* – 18 ml of solution (1) was mixed with 82 ml of solution (2).

Note: Equal parts of Solution A and B were mixed and pH was adjusted to approximately 7.2

3.5.2.2 Haematoxylin & Eosin (H&E)

- **Haematoxylin**

  Erlich's Haematoxylin was used to stain the testis. It was prepared as follows:

  - Haematoxylin powder 2 gm
  - Absolute Ethanol (100%) 100ml
  - Glycerin 100 ml
  - Distilled water 100 ml
  - Glacial Acetic Acid 10 ml
  - Potash alum [$K_2SO_4.Al_2(SO_4)_3.24H_2O$] 14 gm

The stain was prepared according to the standard procedure (Drury et al. 1976) and naturally ripened before use.

- **Eosin Y**

- Eosin powder	1 gm
- Tap water	100 ml
- Thymol	required amount

The staining solutions were filtered before use.

3.5.2.3 Bouin's Solution - Fixative

This fixative was prepared according to the standard procedure (Hess & Moore 1993).

- Saturated Picric Acid	750 ml
- Formaldehyde (Methanol free)	250 ml
- Glacial Acetic Acid	50 ml

The solution was filtered and stored at room temperature.

3.6 Laparatomy: Dissection of Animals

Rats were sacrificed by cervical dislocation as mentioned earlier and blood was collected by cardiac puncture. Blood was centrifuged at 4500rpm for 15 minutes (Avanti JHC Centrifuge, Beckmann Coulter, USA) and serum was collected and stored in deep freezer (-80°C) (Heto-Ultra Freezer, USA) for hormone estimation (Testosterone, FSH, LH and Prolactin). The animals were dissected from the lower abdomen to the thoracic cavity and caution

was taken to avoid from cutting through the testis cavity, blood vessels and other organs. During dissection, the vas deferens was removed carefully from the cauda epididymis and precaution was taken to avoid any damage to the testis. Vas deferens was immersed in 2ml of PBS, pH 7.2 and used for the sperm motility and sperm mortality assays. The left testis, epidydimis, prostate and seminal vesicles were fixed in Bouin's and were preserved for 24 hours in room temperature.

After two hours of fixing in Bouin's, the poles of the testis were pricked using 18-gauge needle so as to allow the fixative to enter the depth of testis and the testis was placed back into the fixative for complete 24 hour immersion. After 24 hours, all tissues were weighed using a digitalized top balancer (AA-160 Top Balancer, Denver Instrument Co., USA) and all data were presented for 100 gm of body weight. The right testis was dissected and homogenized (T25 Tissue Homogenizer, Ika LaborTechnik, Germany) at 9500rpm for two to three minutes in 2ml of normal saline (0.9% NaCl with additional Triton X-100 as a preservative). The tissue sample were centrifuged at 6500rpm for 30 minutes and the supernatant was collected in eppendorf tubes and kept in deep freezer (-20°C) for testicular enzyme estimation.

Right epididymes was collected and the cauda epididymes was placed in 1 ml of PBS. Minced thoroughly and one drop of Eosin Y was added and left for 30 minutes. Then it in filtered through nylon mesh (80μm) and filtrate was used for sperm count and sperm morphology assay.

3.7 Histopathology & Tissue Preparation for Evaluation of the Testis

(Culling et al. 1983; D'Souza & Narayana 1993)

The histopathological examination of reproductive organs especially testis is important in evaluating the adverse effect on male reproductive system. This is a mandatory step in regulatory testing protocols and an important step in research protocols. The histopathological evaluation of testis and other reproductive organs needs immersion fixation of tissue in Bouin's fixative, paraffin embedding and staining with hematoxylin and eosin (H&E) (Preece 1972).

After 24 hours of Bouin's fixation, tissues were sliced and cut into small blocks and were put together in histocassette (Alto Co., Italy). The cassette were labelled with pencil and washed with 70% ethanol (I to VI solutions), 12 hours for each solutions to clean from fixative before the tissues were ready to process. Tissue processing was done immediately to reduce the shrinkage of seminiferous tubules and cell lining within the tubules. All protocols were done manually in Physiology Laboratory, USM Kelantan (Table 3.2).



Fig. 3.3 Laparatomy surgical technique



Fig 3.4 Exposure of right and left testes intact with epididymis

No.	Solutions	Duration
1.	70% Ethanol (I – VI) – for Washing	12 hour each
2.	70% Ethanol I	1 hour
3.	70% Ethanol II	1 hour
4.	70% Ethanol III	1 hour
5.	95% Ethanol I	1 hour
6.	95% Ethanol II	1 hour
7.	100% Ethanol I	1 hour
8.	100% Ethanol II	1 hour
9.	100% Ethanol III	1 hour
10.	Xylene I	1 hour
11.	Xylene II	1 hour
12.	Xylene III	1 hour
13.	Warmed Paraffin I	1 hour
14.	Warmed Paraffin II	1 hour
15.	Warmed Paraffin III	1 hour

Table 3.2 Standard protocols for tissue processing (Preece 1972)

3.7.1 Paraffin Embedding

Immersion fixed testes and epididymis tissue were then processed - embedding in paraffin, sectioning and H&E staining. The tissues were kept warm at 56 ± 3°C and were embedded in paraffin using a mould and embedding ring. Each tissue was labelled separately and was kept in - 4°C to cool the paraffin. The paraffin block was kept in cold condition to make it easier for tissue slicing. Paraffin tissue block was placed carefully into the microtome (Leica Co., Germany) block holder and 5 μm sections were selected. All sections were placed gently into the hot water bath (50 ± 5°C) and placed onto a clean slide. The slides were placed onto the hot plate (60 ± 5°C) to remove all the water and also to make sure that the section was fully covered on the glass slides during tissue staining. Details of procedure for H&E staining were shown in Table 3.3.

Before tissue staining, the slides were placed into a slide rack. For staining, the wax was removed and the sections were rehydrated. The wax was removed by using xylene. Slides were left in xylene I and II for 4 minutes each and into 100% of ethanol, 90% ethanol, 80% ethanol and 70% ethanol for rehydrating the sections. Paraffin wax was removed completely to avoid alterations during the staining procedure. Sections were stained with haematoxylin and eosin. After the staining, the slides were mounted with DPX to avoid the slides from scratches and also to give a good contrast under microscopic observation.

3.7.2 Slide Analysis

3.7.2.1 Qualitative Analysis

Slides were screened for both qualitative and quantitative observation by light microscope and only the best sections were selected for histopathology analysis. Slides were then analysed microscopically qualitative and quantitatively changes using an Image Analyzer (Leica Co., Germany 1998). All procedures were done in Pathology Laboratory, School of Medical Sciences, USM, Kelantan. Testis sections from control and treated animals were analysed for the presence/absence of vacuoles, gaps, and abnormal cells in the seminiferous epithelium as well as the intertubular spaces. Qualitative measures such as sloughing of epithelium and morphological changes in both germ and somatic cells were conducted.

3.7.2.2 Quantitative Analysis

(a) Seminiferous Tubular Diameter (STD)

Diameter of 20 round transversely cut stage VII tubules were measured using an Image Analyzer. In each tubule, two measurements were made one perpendicular to the other and their average was taken.

No.	Solutions	Durations
1.	Xylene 1	**2 minutes**
2.	Xylene 2	**2 minutes**
3.	100% Ethanol 1	**2 minutes**
4.	100% Ethanol 2	**2 minutes**
5.	95% Ethanol	**2 minutes**
6.	80% Ethanol	**2 minutes**
7.	70% Ethanol	**2 minutes**
8.	Bring section to water	**5 minutes**
9.	Hematoxylin	**20 minutes**
10.	Running water	**3 minutes**
11.	1% Acid alcohol	**10 seconds**
12.	Running water	**2 minutes**
13.	Eosin	**3 minutes**
14.	95% Ethanol 1	**2 minutes**
15.	95% Ethanol 2	**2 minutes**
16.	100% Ethanol 1	**2 minutes**
17.	100% Ethanol 2	**2 minutes**
18.	Xylene 1	**2 minutes**
19.	Xylene 2	**2 minutes**
20.	Mounting with DPX	

Table 3.3 Standard protocol for H&E staining.

(b) Seminiferous Epithelial Height (SEH)

The epithelial height was measured in 10 stage VII tubules. In each tubule, the SEH was measured from the basement membrane to the surface of the epithelium at two different regions and the mean was taken.

(c) Count of Leydig cells/intertubular space

The number of Leydig cells in 10 intertubular spaces/section and 10 sections/animal were recorded and expressed as Leydig cells/intertubular space (Goldberg et al. 1997)

(d) Count of Spermatogonium, Spermatocytes, Spermatids & 'Dividing cells' in seminiferous tubule

By using Image Analyzer, a total of 100 tubules were screened continuously to record the count of spermatogonium, spermatocytes, spermatids as well as 'dividing cells'.

3.8 Sperm Analysis

3.8.1 Epididymal Sperm Count

The epididymal sperm count was carried out in control and treatment groups. Cauda epididymal suspension was made. The sperm count was carried out according to the standard procedure (Vega et al. 1988). The epidydimal sperm suspension was prepared in 1 ml PBS at pH 7.2. An aliquot from the suspension was diluted in 1:100. A sample of the diluted suspension was charged into both side of the Neubauer's chamber. Total sperm count in

8 squares (except the central erythrocyte area) of 1 mm^2 area was determined and multiplied by 10^4 (standard multiplication factor, Vega et al. 1988).

$$\text{Number of sperm/ml} = N \times 10^4$$

3.8.2 Sperm Morphology Assay

This assay was conducted in control and treatment groups. The method followed was similar to the procedure previously explained by Wyrobek & co-workers (Wyrobek & Bruce 1975; Wyrobek et al. 1983; Narayana & D'Souza 2002). The epidydimis was minced by using a standard laboratory scissor in 1 ml of PBS and then filtered into a clean petry dish using 80 micron nylon mesh. One drop of 1% of Eosin Y was added to the filtrate and kept for 15 minutes under room temperature.

One drop of stained suspension (remaining suspension was used for sperm count) was placed on a clean glass tube for dilution of 1:20 (one drop of filtrate into 19 drops of 0.9% normal saline). One drop of the filtrate was put onto a clean glass slide and the smear was prepared by using a clean cover slip (22 x 22mm). Slides were dried at room temperature, coded and analyzed. The slides were screened under x100 and x400 magnification of light microscope.

Analysis

- The smears were analyzed as follows: -
  a) One thousand sperm per animal were screened and counted manually by using Telly Counter (Line Co., Japan) under 400x magnification of light microscope.
  b) Classified into normal and abnormal types.
  c) The abnormal sperm were headless, double-heads, coiled, double-tails, microcephaly, cephalo-caudal junction abnormalities, broken-tail, hookless, banana-shaped as well as amorphous head (Figure 4.5 – Chapter 4).

3.8.3 Sperm Motility and Mortality (Qualitative assessment)

These assays were also conducted in control and treatment groups. The Makler's chamber (Sefi Medical Instrument, USA) was used for sperm motility as well as sperm mortality. The grading of sperm motility and also the percentages of sperm mortality were studied. The sperm motility and mortality assay were carried out according to the standard procedure (Makler 1980a & 1980b). The vas deferens was removed from each animal and placed in 2 ml of PBS solution for 5 minute at room temperature. Since the viability of sperm is of short duration, this assay was done immediately to avoid death of sperm.

With the aid of a rod or small stick, a small drop of semen sample from vas deferens was directly placed onto the center of the lower disc of the Makler's chamber. The Makler's cover slip was placed onto the lower disc and

the sample was left for at least 5 minutes for uniform spreading of the sperm to the entire disc. The chamber was lifted by its handles and placed on the stage of the microscope. It was important not to use the x40 objective with this chamber to avoid breaking the chamber's lens. For safety recommendation, a combination of x20 objective and x10 eyepiece were used and the sperm motility and mortality were observed clearly according to the motility grading and percentage of mortality (Patrick et al. 2000).

Definition	Description
0 – None	An absence of forward progression
+1 – Poor	Weak or sluggish forward progression
+2 – Moderate	Definite forward progression
+3 – Good	Good forward movement with progression
+4 – Excellent	Vigorous rapid forward progression

Table 3.4 Methods describing the quality of the forward movement (progressive activity) of sperm for a semen analysis.

Calculation for Sperm Mortality:

$$\text{Sperm Mortality} = \frac{\text{Total number of 'dead' sperm}}{\text{Total number of sperm in 10 chambers}}$$

3.9 Hormone Analysis

3.9.1. Testosterone Radioimmunoassay (RIA)

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 antibody binding 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. The separation of the free and bound antigen was achieved by decanting or aspirating the antibody-coated tubes.

Summary & Explanation of the Test

Testosterone, (17β-hydroxyl-4-androsten-3-one), a C_{19} steroid, is a potent circulating naturally-secreted androgen (Dorfman & Shipley 1956). In normal postpubertal males, testosterone is secreted primarily by the testes with only a small amount derived from peripheral conversion of Δ^4androsten-3,17-dione (ASD) (Horton & Tait 1966). In adult women, it has been estimated that over 50% of serum testosterone is derived from peripheral conversion of ASD secreted by the adrenal and ovary, with the remainder from direct secretion of testosterone by these glands (Horton & Tait 1966; Pang & Riddick 1990).

Test Procedure

A. Materials Supplied:

Materials supplied in the DSL ACTIVE® Testosterone Coated-Tube Radioimmunoassay Kits, Catalog No.DSL-4000 (100 tubes) was:

Material	Quantity
Testosterone Standard A	One Vial
Testosterone Standard B	One Vial
Testosterone Standard C	One Vial
Testosterone Standard D	One Vial
Testosterone Standard E	One Vial
Testosterone Standard F	One Vial
Testosterone [I-125] Reagent	One Bottle
Anti-Testosterone-Coated Tubes	100 Tubes
Testosterone Control I	One Vial
Testosterone Control II	One Vial

Table 3.5 Materials supplied in the Testosterone RIA.

B. All reagents were allowed to reach room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. The Standards and Controls were reconstituted and mixed thoroughly to avoid foam. Standards, Controls and unknowns were assayed in duplicate.

1. Two plain uncoated tubes were labeled for Total Count (TC). All Anti-Testosterone-Coated tubes were labeled and arranged in duplicate for Standards, Controls, and unknowns.
2. 50 μl of Testosterone Standards, Controls and unknowns were pipetted to the appropiate and directly through the bottom of the tubes.

3. 500 µl of Testosterone [I-125] Reagent were added immediately to all tubes.
4. The tubes were mixed gently and incubated in a water bath at 37 ± 2°C for 60 minutes.
5. Except for TC, all tubes were decanted or aspirated by simultaneous inversion with a sponge rack into a radioactive waste receptacle.
6. The tubes were stroke sharply on a absorbent material for a minimum of 2 minutes. The tubes were blotted to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
7. All tubes were counted in a gamma counter for one minute.

C. Results

All performance characteristics from the gamma counter were stated in ng/ml. All values must be converted to nmol/L based on this calculations:

$$ng/ml \times 3.47 = nmol/L$$

3.9.2. ImmunoradiometricAssay (IRMA)

The procedure employs a two-site immunoradiometric assay (IRMA) principle described by Miles et.al. (1974). The IRMA is a non-competitive assay in which the analyte to be measured is 'sandwiched' between two antibodies. The first antibody is immobilized to the inside walls of the tubes. The other antibody is radiolabeled for detection. The analyte present in the unknown, Standard and Control is bound by both of the antibodies to form a 'sandwich' complex. Unbound materials were removed by decanting and washing the tubes.

3.9.2.1. Follicle-Stimulating Hormone (FSH-IRMA)

Summary & Explanation of the Test

Follicle-stimulating hormone (FSH, Follitropin) is a 33kDa glycoprotein hormone secreted by the anterior pituitary gland. FSH production and secretion are stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH release is normally pulsatile, resulting in episodic release of FSH (Levine et al. 1985). In men, FSH stimulates testicular synthesis of inhibin and androgen binding protein in Sertoli cells, and acts with Luteinizing Hormone (LH) and Testosterone to stimulate spermatogenesis.

Test Procedure

A. Materials supplied in the DSL ACTIVE® FSH Coated-Tube IRMA Kits, Catalog No.DSL-4700 (100 tubes) were:

Material	Quantity
FSH Standard A	One Vial
FSH Standard B	One Vial
FSH Standard C	One Vial
FSH Standard D	One Vial
FSH Standard E	One Vial
FSH Standard F	One Vial
FSH Standard G	One Vial
FSH Control Level I	One Vial
FSH Control Level II	One Vial
Anti-FSH-Coated Tubes	100 Tubes
Anti-FSH [I-125] Reagent	One Vial
Wash Concentrate G	One Bottle

Table 3.6 Materials supplied for FSH IRMA.

B. **Preparation of Reagents:**

Wash Solution

The Wash-Concentrate were diluted with 50-fold of deionized water in a clean container. The Wash Solution is stable for one month at room temperature (~ 25°C) in a tightly sealed container.

C. **Assay Procedure**

All reagents were allowed to reach room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. All Standards, Controls and unknowns were labeled in duplicate.

1. Two plain uncoated tubes were labeled for Total Counts (TC). All Anti-FSH-Coated tubes were arranged and

labeled in duplicate for Standards, Controls and unknowns.

2. 100 µl of the Standards, Controls and unknowns were pipetted to the bottom of the appropiate tubes.
3. 100 µl of the Anti-FSH [I-125] reagent were added immediately to each tube. All tubes were inversed gently for 1-2 seconds.
4. All tubes were incubates for 2 hours at room temperature (~ 25°C) on a shaker set at 180 rpm.
5. Except for TC, all tubes were decanted or aspirated by simultaneous inversion with a sponge rack into a radioactive waste receptacle.
6. The tubes were stroke sharply on absorbent material for a minimum of 2 minutes. The tubes were blotted to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
7. By using a repeating pipette, 2 ml of Wash Solution were added to all tubes except TC.
8. Step 5,6, and 7 were repeated for a total of three washing.
9. All tubes were counted in a Gamma Counter for one minute.

D. Results

All values were given as ng/ml. To convert to mIU/ml, the values had to multiplied by 9:

$$ng/ml \times 9 = mIU/ml$$

3.9.2.2. Luteinizing Hormone (LH-IRMA)

Summary & Explanation of the Test

Luteinizing hormone (LH, Lutropin) is a 29.4 kDa glycoprotein secreted by the anterior pituitary gland. Both LH and FSH production and secretion are stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH release is normally pulsatile, resulting in episodic release of both LH and FSH (Levine et al. 1985).

LH production and secretion are regulated by a complex balance of endocrine feedback systems involving a number of hormones, including estrogen and progesterone (Sherman & Korenman 1975; Liu & Yen 1983). LH binds to specific cell membrane receptors on the ovarian theca cells and testicular Leydig cells. In men, LH stimulates production of testosterone by the Leydig cells and acts with FSH and testosterone to stimulate spermatogenesis.

Test Procedure

A. Materials Supplied

Materials supplied in the DSL ACTIVE® LH Coated-Tube IRMA Kits, Catalog No.DSL-4600 (100 tubes) contained:

Materials	Quantity
LH Standard A	One Vial
LH Standard B	One Vial
LH Standard C	One Vial
LH Standard D	One Vial
LH Standard E	One Vial
LH Standard F	One Vial
LH Standard G	One Vial
LH Control Level I	One Vial
LH Control Level II	One Vial
Anti-LH-Coated Tubes	100 tubes
Anti-LH-[I-125] Reagent	One Vial
Wash Concentrate G	One Bottle

Table 3.7 Materials supplied for LH IRMA.

B. Preparation of Reagent

Wash Solution

The Wash-Concentrate were diluted with 50-fold of deionized water in a clean container. The Wash Solution is stable for one month at room temperature (~ 25°C) in a tightly sealed container.

C. Assay Procedure

All reagents were allowed to reach room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. All Standards, Controls and unknowns were labeled in duplicate.

1. Two plain uncoated tubes were labeled for Total Counts (TC). All Anti-LH-Coated tubes were arranged and labeled in duplicate for Standards, Controls and unknowns.
2. 100 µl of the Standards, Controls and unknowns were pipetted to the bottom of the appropiate tubes.
3. 100 µl of the Anti-FSH [I-125] reagent were added immediately to each tube. All tubes were inversed gently for 1-2 seconds.
4. All tubes were incubates for 2 hours at room temperature (~ 25°C) on a shaker set at 180 rpm.
5. Except for TC, all tubes were decanted or aspirated by simultaneous inversion with a sponge rack into a radioactive waste receptacle.
6. The tubes were stroke sharply on a absorbent material for a minimum of 2 minutes. The tubes were blotted to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
7. By using a repeating pipette, 2.5 ml of Wash Solution were added to all tubes except TC.
8. Step 5,6, and 7 were repeated for a total of three washing.

9. All tubes were counted in a Gamma Counter for one minute.

D. **Results**

All values were given as ng/ml. To convert to mIU/ml, the values had to multiplied by 4.5 :

$$ng/ml \times 4.5 = mIU/ml$$

3.9.2.3. Prolactin IRMA

Summary & Explanation of the Test

Prolactin is a 198-amino acid, 23kDa polypeptide hormone secreted in significant amounts by the anterior pituitary gland and the placenta. Prolactin, in synergy with estrogen, plays an important physiologic role in the initiation and maintenance of mammary gland growth and lactation in humans (Nicoll et al. 1986; Lambert & MacLeod 1990). Relative elevations in plasma prolactin concentrations occur during ovulation, pregnancy, nursing and during stress (Liu & Zhou 1990; Sinha 1995). Abnormal elevations in plasma prolactin levels, or hyperprolactinemia, can occur as a result of pituitary adenomas, and may also be seen in other anatomic and traumatic abnormalities involving the pituitary gland, as a consequences of certain pharmacologic agents and in hypothyroidism. Low prolactin levels, or hypoprolactinemia, are observed in cases of hypopituitarism.

Test Procedure

A. Materials Supplied

Materials supplied in the DSL ACTIVE Prolactin Coated-Tube IRMA Kits, Catalog No.DSL-4500-5 (100 tubes) contained:

Materials	Quantity
Prolactin Standard A	One Vial
Prolactin Standard B	One Vial
Prolactin Standard C	One Vial
Prolactin Standard D	One Vial
Prolactin Standard E	One Vial
Prolactin Standard F	One Vial
Anti-Prolactin-Coated Tubes	100 Tubes
Anti-Prolactin [I-125] Reagent	One Bottle
Prolactin Control Level I	One Vial
Prolactin Control Level II	One Vial
Wash Concentrate F	One Bottle

Table 3.8 Materials supplied for Prolactin IRMA.

B. Preparation of Reagent

Wash Solution

The Wash-Concentrate were diluted with 50-fold of deionized water in a clean container. The Wash Solution is stable for one month at room temperature (~ 25°C) in a tightly sealed container.

C. Assay procedure

All reagents were allowed to reach room temperature (~25°C) and were mixed thoroughly by gentle inversion before use. All Standards, Controls and unknowns were labeled in duplicate.

10.1. Two plain uncoated tubes were labeled for Total Counts (TC). All Anti-LH-Coated tubes were arranged

and labeled in duplicate for Standards, Controls and unknowns.

11. 25 µl of the Standards, Controls and unknowns were pipetted to the bottom of the appropiate tubes.
12. 200 µl of the Anti-FSH [I-125] reagent were added immediately to each tube. All tubes were inversed gently for 1-2 seconds.
13. All tubes were incubates for 90 minutes at room temperature (~ 25°C) on a shaker set at 180 rpm.
14. Except for TC, all tubes were decanted or aspirated by simultaneous inversion with a sponge rack into a radioactive waste receptacle.
15. The tubes were stroke sharply on a absorbent material for a minimum of 2 minutes. The tubes were blotted to remove any droplets adhering to the rim before returning them to the upright position. Failure to blot tubes adequately may result in poor replication and spurious values.
16. By using a repeating pipette, 2.5 ml of Wash Solution were added to all tubes except TC.
17. Step 5,6, and 7 were repeated for a total of three washing.
18. All tubes were counted in a Gamma Counter for one minute.

D. **Result**

All values were given as ng/ml. To convert to μIU/ml, the values had to multiplyied by 27 :

$$ng/ml \times 27 = \mu IU/ml$$

3.10 Testicular Marker Enzyme Assays

3.10.1. Acid Phosphatase (Pesee 1987)

Serum acid phosphatase hydrolyzes thymolphtalein monophosphate at pH 6.0 to yield thymolphtalein and inorganic phosphate ions. The addition of alkali stops enzymatic activity and simultaneously converts thymolphtalein to a blue chromogen that is measured photometrically at 596 nm.

Acid Phos Thymolphtalein $\longrightarrow$ Thymolphthalein Alkali + $(PO_4)3-$

Monophosphate (blue chromogen)

Reagents Required:

- Acid Phosphate Substrate

  0.14% Thymolphtalein monophosphate with buffer pH 6.0 ± 0.05 at 25 °C

- Acid Phosphate Color developer

  2% w/v Sodium carbonate

- Standard

A solution of Thymolphtalein in 70% v/v –(Propanol/water); equivalent to 5 IU/L of acid phosphatase activity.

Materials Required:

- Test tubes/rack
- Accurate pipetting devices
- Distilled/deionized water
- Timer
- Spectrophotometer (Beckmann Coulter Co. USA) capable of reading at 595 nm

Procedure

1. 5 ml of acid phosphatase Substrate was pipetted into 5 ml test tube.
2. The tube was incubated in water bath at 37± 2°C for 5 minutes.
3. 0.2 ml of sample (unknown) was added in the tube and mixed.
4. The tube was incubated for exactly 30 minutes.
5. 2 ml of Acid Phosphatase color developer was added to the tube and mixed.
6. All tubes were recorded at 595 nm versus distilled water (as blank).

Note: The final color produced in the reaction was stable for at least 60 minutes at room temperature.

Calculation:

Acid Phosphatase (IU/L) = (A – B) x (C / D)

Note: A : Test or unknown (nm)
B : Blank (nm)
C : Standard (IU/L)
D: Standard (nm)

3.10.2 Lactate dehydrogenase (LDH) (Appleby & Morton 1959)

The rate of reduction of potassium ferricyanide was determined by spectrophotometrically at 420 nm. The molar absorbancy of potassium ferricyanide is 1.04×10^3 at this wavelength whereas the reduced ferricyanide has negligible absorption. One unit reduces one μmol of ferricyanide per minute at 25°C and pH 8.4 under specified conditions.

Reagents

- 0.067 M Sodium phosphate, pH 7.4 with 0.001 M EDTA
- 0.01 EDTA
- 0.0083 M Potassium ferricyanide (Cautious)
- 0.1 M Sodium pyrophosphate, pH 8.4
- 0.5 M Sodium lactate, pH 7.0

Procedure

1. The spectrophotometer (Beckmann Coulter Co. USA) was adjusted at 420 nm and 25°C.

2. 2 ml of 0.1 M Sodium pyrophosphate, 0.5 ml of 0.5 M Sodium lactate, 0.3 ml of 0.01 M EDTA and 0.1 ml of potassium ferricyanide were pipetted into a cuvette.
3. The cuvette was incubated in the spectrophotometer at 25°C for 2 minutes before the absorbance was recorded.
4. 0.1 ml of test or unknown sample was pipetted into the cuvette and all of the data collection were recorded.
5. A_{420} per minute were counted from the initial linear curve of standard.

Calculation

$$\text{LDH (IU/ml)} = \frac{A_{420}/\text{min} \times 1000 \times \text{dilution} \times 2.9}{1040 \times 0.1 \text{ ml (sample vol.)}}$$

3.11 Statistical Analysis

All data including the body and organ weight, sperm analysis, hormone and enzymes estimation were analysed by using the One – Way ANOVA followed by Bonferroni's post – hoc test. The statistical tests were obtained from Statistical Package of Social Sciences (SPSS) version 11.5. P values that are less than 0.05 ($p<0.05$) are significant.

CHAPTER 4

RESULTS

4.1 Body weight

The body weight was recorded once a week to detect for any changes during the pre and post exposure of PQ administration. There was no change in body weight in both oral and dermal exposure groups (Table 4.1 and 4.2). Body weight of control and PQ treated groups did not show any change except for the normal increase due to the growth overtime in dermal and oral treatment groups.

4.2 Organ weight

4.2.1 Testes weight

Testis weight in the oral exposure group did not show any significant differences with the control animals (Table 4.3) but dermal route exposure group exhibited a significant decrease in the weight on day 14 post exposure ($p<0.05$, Table 4.4, Fig. 4.1).

4.2.2 Epididymes weight

Weight of epidydimes was significantly increased on day 42 in oral groups with 5 and 20 mg/kg doses of PQ ($p<0.05$) and also on day 84 post exposure with 2 and 20 mg/kg doses (Table 4.5, Fig. 4.2). Epidydimal weight showed a significant decrease ($p<0.05$) on day 7 in dermal route exposure groups of PQ at all the dose levels studied (Table 4.6, Fig. 4.3).

4.2.3 Seminal Vesicle weight

A significant increase ($p<0.05$) in the weight of seminal vesicle was observed following day 42, oral route exposure of PQ (Table 4.7, Fig. 4.4). But there were no significant changes of seminal vesicle weight in dermal exposure rats (Table 4.8, Fig 4.5).

4.2.4 Prostate weight

PQ 20mg/kg body weight dose showed a significant increase ($p<0.05$) in the weight of prostate gland following day 42, 84 and 105, oral route exposure (Table 4.9, Fig 4.6). For dermal groups, the prostate weight were increased significantly ($p<0.05$) on day 28 in PQ 15mg/kg and 30mg/kg (Table 4.10, Fig 4.7).

Table 4.1 Effect of PQ (Oral) on body weight of the rats. n=6 for each group, Mean ± SD (gm). *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	Weight after 7 days (gm)	Weight after 28 days (gm)	Weight after 42 days (gm)	Weight after 84 days (gm)	Weight after 105 days (gm)
Control	205.06 ± 10.12	280.10 ± 17.42	318.23 ± 10.87	388.68 ± 7.14	404.46 ± 14.30
PQ – 2 mg/kg	211.83 ± 8.36	303.33 ± 14.58	348.12 ± 10.34	380.83 ± 5.84	401.68 ± 21.14
PQ – 5 mg/kg	208.96 ± 16.39	325.84 ± 17.44	338.82 ± 10.68	382.50 ± 17.84	398.33 ± 12.25
PQ – 20 mg/kg	224.54 ± 6.46	300.44 ± 12.14	328.50 ± 17.72	370.88 ± 15.66	380.12 ± 18.12

Table 4.2 Effect of PQ (dermal) on body weight of the rats, n=6 for each group, Mean ± SD. *One-way ANOVA followed by Bonferroni's Post-hoc test ($p<0.05$).

Dose/Duration	Weight after 7 days (gm)	Weight after 14 days (gm)	Weight after 28 days (gm)	Weight after 42 days (gm)
Control	205.06 ± 10.12	240.18 ± 14.78	280.10 ± 17.42	318.23 ± 10.87
PQ – 6 mg/kg	212.30 ± 6.58	243.42 ± 17.56	294.72 ± 13.56	341.70 ± 13.96
PQ – 15 mg/kg	219.95 ± 5.30	263.63 ± 19.59	288.52 ± 8.78	363.85 ± 13.38
PQ – 30 mg/kg	217.55 ± 9.36	251.50 ± 16.72	270.25 ± 10.30	331.82 ± 18.40

Table 4.4 Effect of PQ on testis weight in PQ (dermal) treated rats. Each time point at particular dose level represents Mean ± SD (gm/100gm of body weight), n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc procedure ($p<0.05$).

Dose/Duration	7 days (gm)	14 days (gm)	28 days (gm)	42 days (gm)
Control	0.5626 ± 0.04	0.6603 ± 0.06	0.5070 ± 0.06	0.4692 ± 0.05
PQ – 6 mg/kg	0.6126 ± 0.06	0.5471* ± 0.06	0.5111 ± 0.03	0.4580 ± 0.03
PQ – 15 mg/kg	0.5707 ± 0.04	0.5214* ± 0.03	0.4877 ± 0.02	0.4735 ± 0.02
PQ – 30 mg/kg	0.6494 ± 0.12	0.4797* ± 0.07	0.5995 ± 0.18	0.4122 ± 0.12

Table 4.3 Effect of PQ on testis weight in PQ (oral) treated rats. Each time point at particular dose level represents Mean ± SD (gm/100gm of body weight) n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days (gm)	28 days (gm)	42 days (gm)	84 days (gm)	105 days (gm)
Control	0.6087 ± 0.10	0.5070 ± 0.06	0.4855 ± 0.06	0.4563 ± 0.05	0.4447 ± 0.08
PQ – 2 mg/kg	0.5104 ± 0.04	0.4992 ± 0.02	0.4482 ± 0.03	0.4713 ± 0.03	0.4062 ± 0.07
PQ – 5 mg/kg	0.6513 ± 0.08	0.4499 ± 0.03	0.4330 ± 0.02	0.3958 ± 0.14	0.3720 ± 0.09
PQ – 20 mg/kg	0.5577 ± 0.03	0.4662 ± 0.03	0.5107 ± 0.05	0.5045 ± 0.03	0.4040 ± 0.07

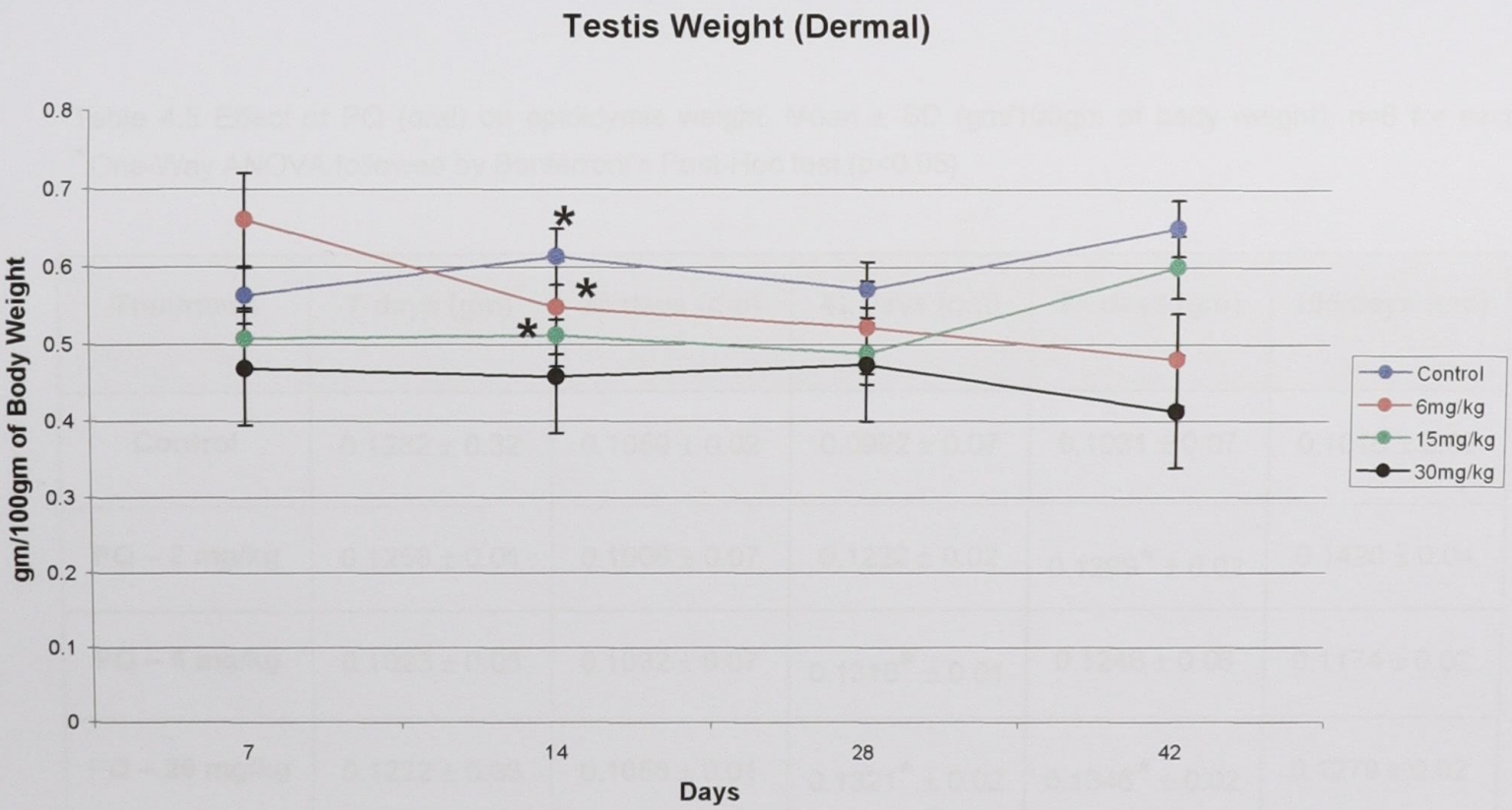


Figure 4.1 Time – response relationship of PQ (dermal) on testis weight. The mean difference is significant at the level of $p<0.05^{*}$. All data were shown as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.5 Effect of PQ (oral) on epididymis weight. Mean ± SD (gm/100gm of body weight), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days (gm)	28 days (gm)	42 days (gm)	84 days (gm)	105 days (gm)
Control	0.1282 ± 0.32	0.1059 ± 0.02	0.0992 ± 0.07	0.1031 ± 0.07	0.1016 ± 0.02
PQ – 2 mg/kg	0.1258 ± 0.01	0.1008 ± 0.07	0.1222 ± 0.02	0.1299* ± 0.02	0.1420 ± 0.04
PQ – 5 mg/kg	0.1023 ± 0.03	0.1032 ± 0.07	0.1316* ± 0.01	0.1246 ± 0.06	0.1174 ± 0.02
PQ – 20 mg/kg	0.1222 ± 0.33	0.1058 ± 0.01	0.1321* ± 0.02	0.1346* ± 0.02	0.1279 ± 0.02

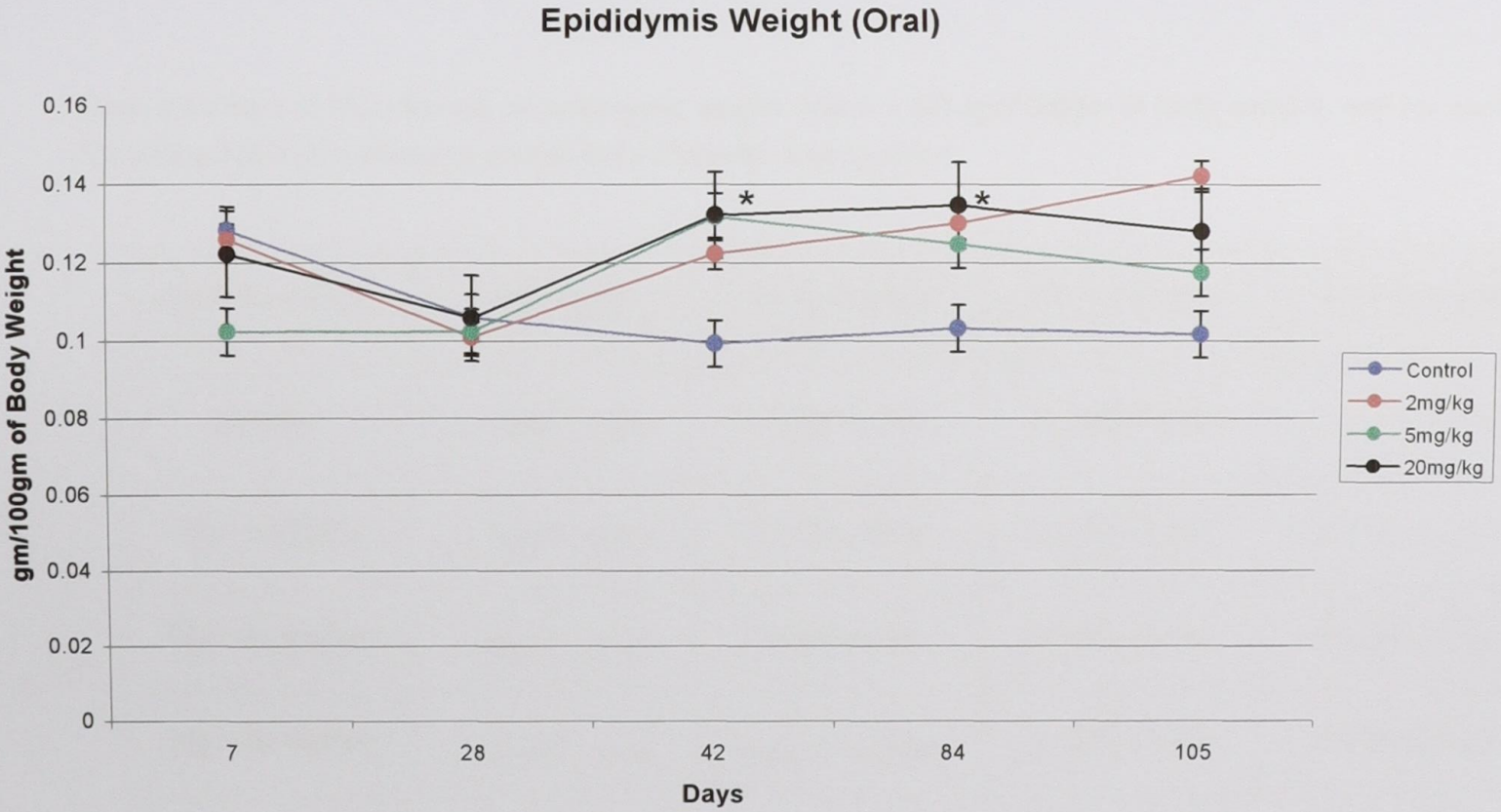


Figure 4.2 Time – response relationship of PQ (oral) on epididymis weight. The mean difference is significant at the level of $p<0.05^{*}$. All point presentes as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.6 Effect of PQ (dermal) on epididymis weight. Mean ± SD (gm/100gm of body weight), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose/Duration	7 days (gm)	14 days (gm)	28 days (gm)	42 days (gm)
Control	0.1282 ± 0.32	0.1020 ± 0.01	0.1059 ± 0.03	0.1017 ± 0.02
PQ – 6 mg/kg	0.1049* ± 0.07	0.1220 ± 0.02	0.1221 ± 0.07	0.1167 ± 0.07
PQ – 15 mg/kg	0.1099* ± 0.01	0.1065 ± 0.01	0.1084 ± 0.06	0.1327 ± 0.02
PQ – 30 mg/kg	0.1129* ± 0.02	0.0947* ± 0.04	0.1269 ± 0.02	0.1084 ± 0.02

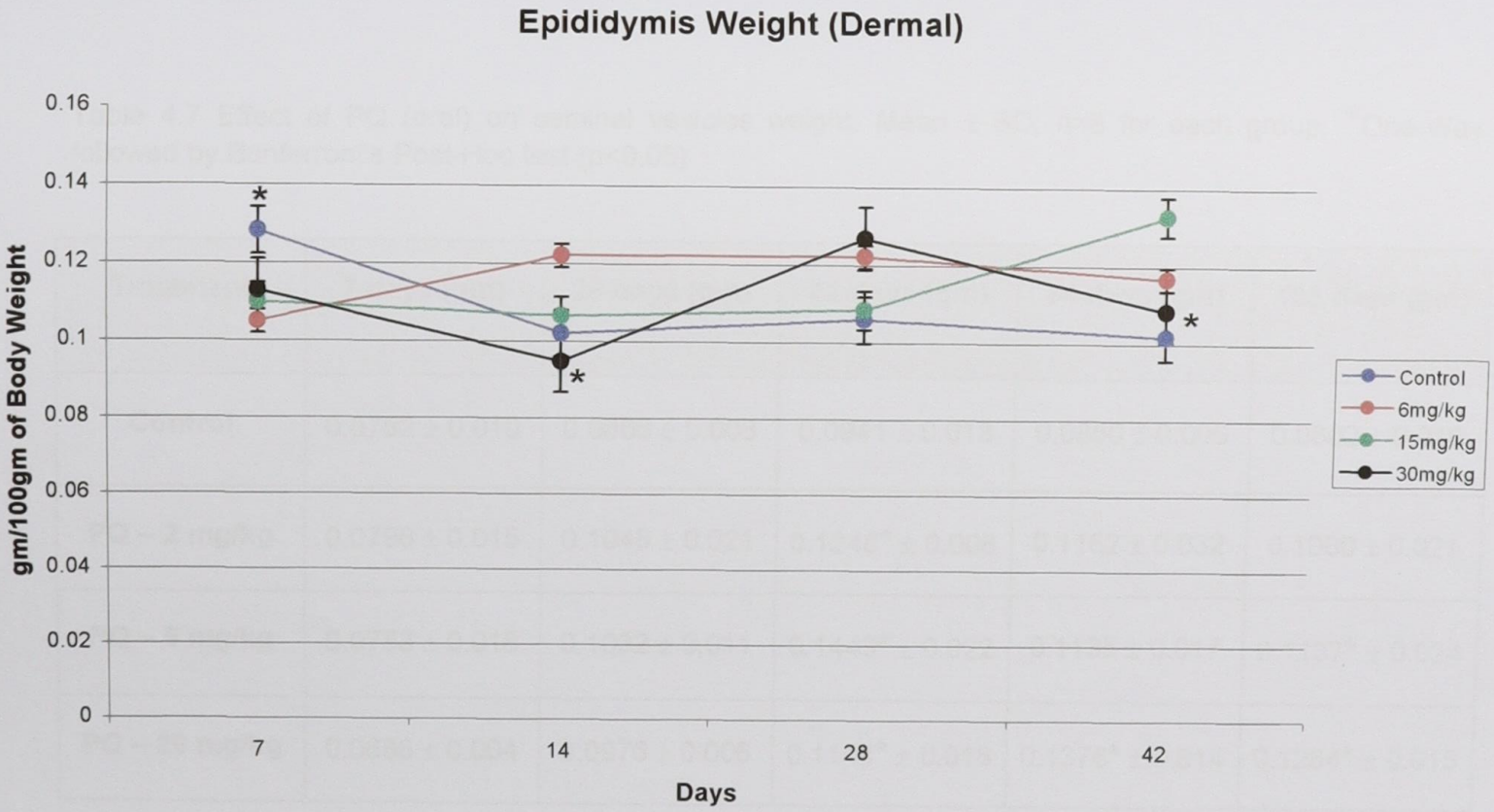


Figure 4.3 Time – response relationship of PQ (dermal) on epididymis weight. The mean difference is significant at the level of $p<0.05^{*}$. Data were shown as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.7 Effect of PQ (oral) on seminal vesicles weight. Mean ± SD, n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days (gm)	28 days (gm)	42 days (gm)	84 days (gm)	105 days (gm)
Control	0.0782 ± 0.010	0.0866 ± 0.008	0.0941 ± 0.013	0.0850 ± 0.006	0.0802 ± 0.016
PQ – 2 mg/kg	0.0798 ± 0.015	0.1048 ± 0.021	0.1248* ± 0.008	0.1162 ± 0.032	0.1060 ± 0.021
PQ – 5 mg/kg	0.0753 ± 0.016	0.1032 ± 0.011	0.1443* ± 0.022	0.1135 ± 0.017	0.1137* ± 0.024
PQ – 20 mg/kg	0.0686 ± 0.004	0.0976 ± 0.006	0.1144* ± 0.018	0.1376* ± 0.014	0.1264* ± 0.015

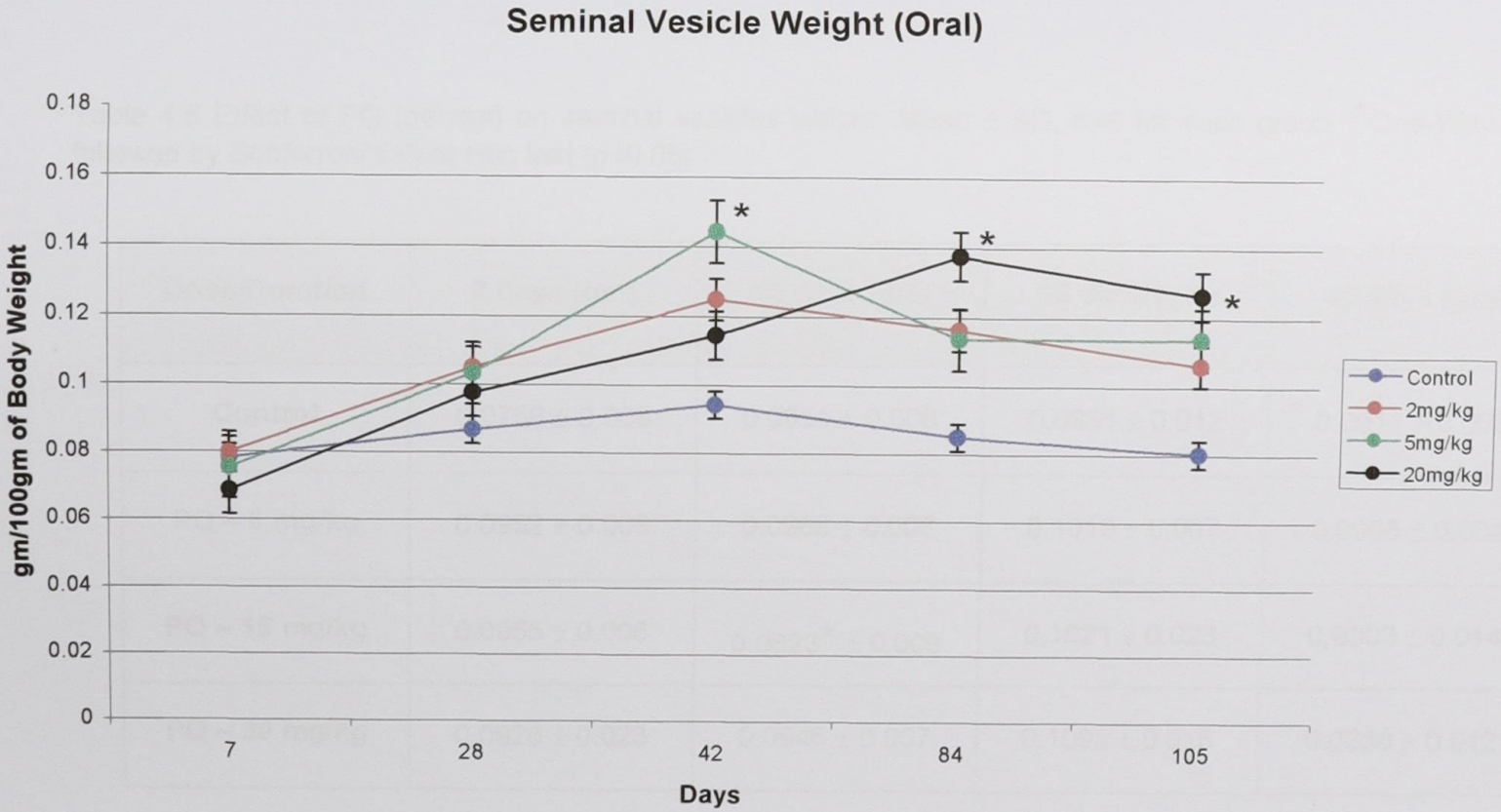


Figure 4.4 Time – response relationship of PQ (oral) on seminal vesicle weight. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.8 Effect of PQ (dermal) on seminal vesicles weight. Mean ± SD, n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose/Duration	7 days (gm)	14 days (gm)	28 days (gm)	42 days (gm)
Control	0.0758 ± 0.008	0.0951 ± 0.006	0.0931 ± 0.012	0.0911 ± 0.008
PQ – 6 mg/kg	0.0952 ± 0.008	0.0968 ± 0.006	0.1019 ± 0.007	0.0966 ± 0.004
PQ – 15 mg/kg	0.0955 ± 0.006	0.0823* ± 0.008	0.1021 ± 0.026	0.0903 ± 0.014
PQ – 30 mg/kg	0.0926 ± 0.023	0.0946 ± 0.007	0.1092 ± 0.016	0.0838 ± 0.012

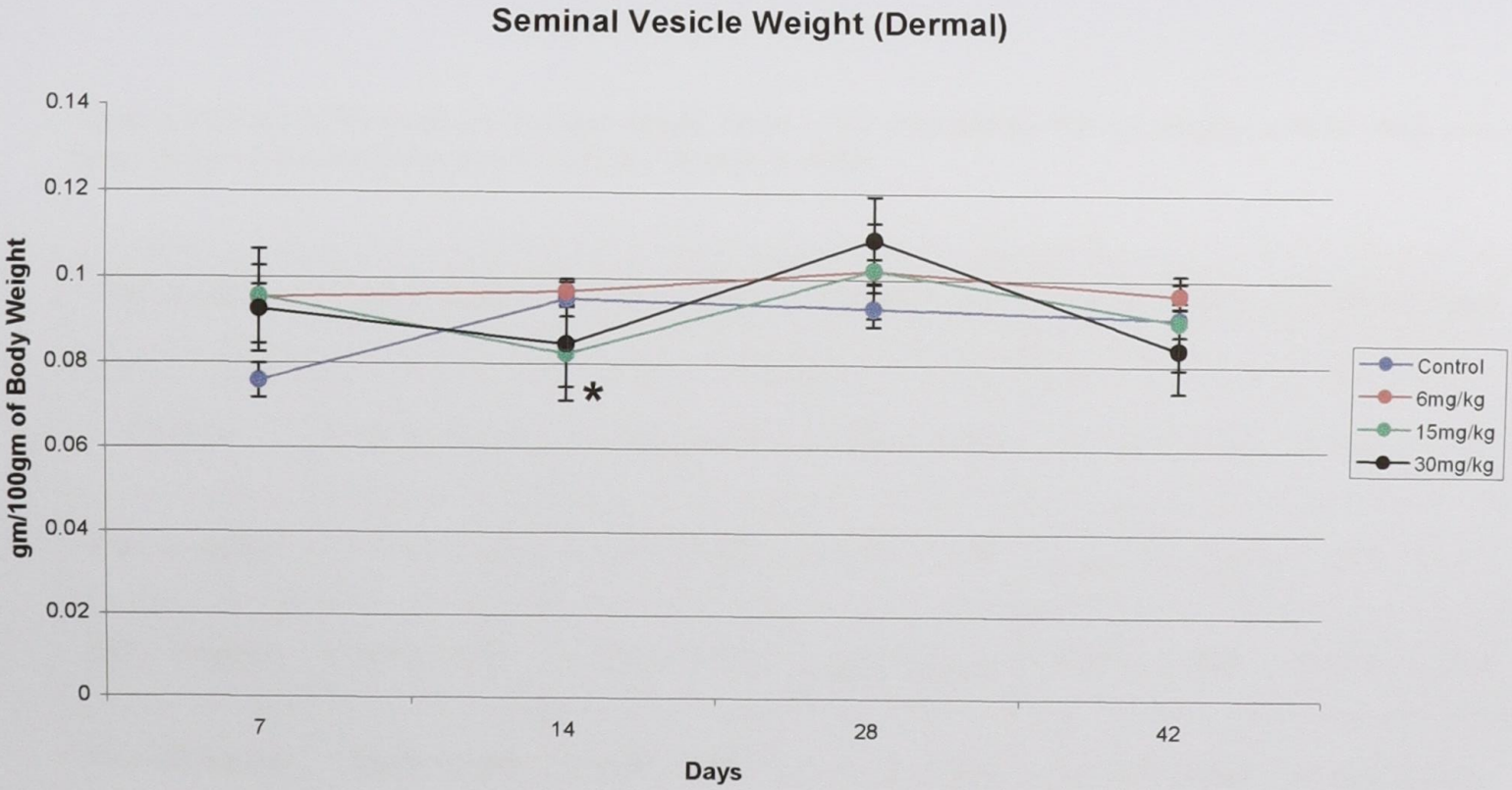


Figure 4.5 Time – response relationship of PQ (dermal) on seminal vesicle weight. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.9 Effect of PQ (oral) on prostate weight. Mean ± SD (mg/100mg of body weight), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days (gm)	28 days (gm)	42 days (gm)	84 days (gm)	105 days (gm)
Control	0.1504 ± 0.034	0.1762 ± 0.018	0.1763 ± 0.052	0.1749 ± 0.024	0.1636 ± 0.035
PQ – 2 mg/kg	0.1073 ± 0.026	0.1837 ± 0.032	0.2765 ± 0.046	0.3021* ± 0.055	0.2406 ± 0.051
PQ – 5 mg/kg	0.1243 ± 0.011	0.1653 ± 0.012	0.2872* ±0.045	0.2551 ± 0.066	0.2546 ± 0.068
PQ – 20 mg/kg	0.0975 ± 0.006	0.1493 ± 0.021	0.3132* ± 0.055	0.3058* ± 0.058	0.2846* ±0.061

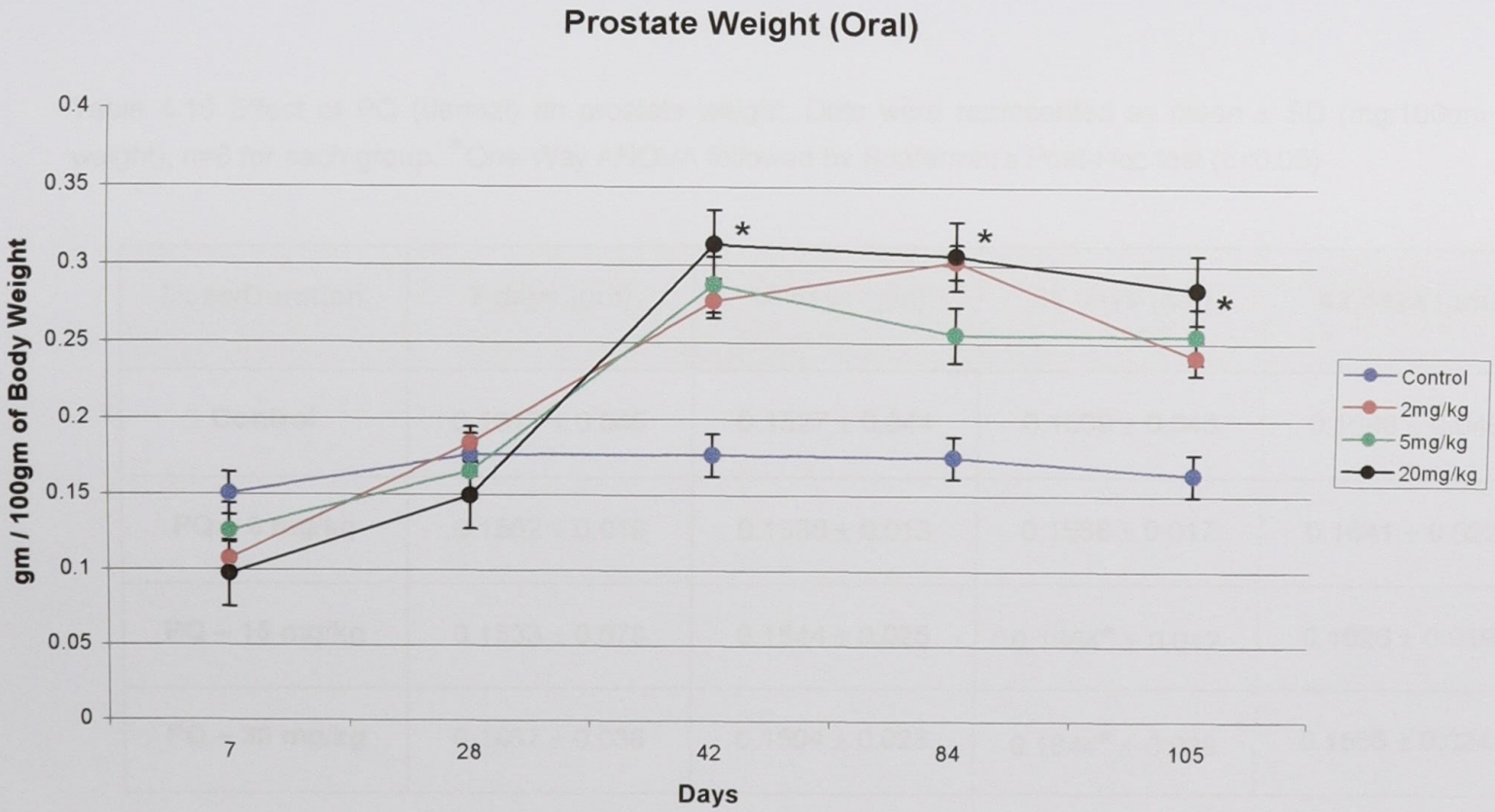


Figure 4.6 Time – response relationship of PQ (oral) on prostate weight. The mean is difference at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.10 Effect of PQ (dermal) on prostate weight. Data were represented as mean ± SD (mg/100gm of body weight), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose/Duration	7 days (gm)	14 days (gm)	28 days (gm)	42 days (gm)
Control	0.1617 ± 0.045	0.1527 ± 0.044	0.1609 ± 0.048	0.1608 ± 0.048
PQ – 6 mg/kg	0.1562 ± 0.019	0.1338 ± 0.013	0.1588 ± 0.017	0.1641 ± 0.022
PQ – 15 mg/kg	0.1533 ± 0.078	0.1544 ± 0.025	0.1954* ± 0.012	0.1626 ± 0.019
PQ – 30 mg/kg	0.1487 ± 0.038	0.1504 ± 0.023	0.1844* ± 0.028	0.1666 ± 0.024

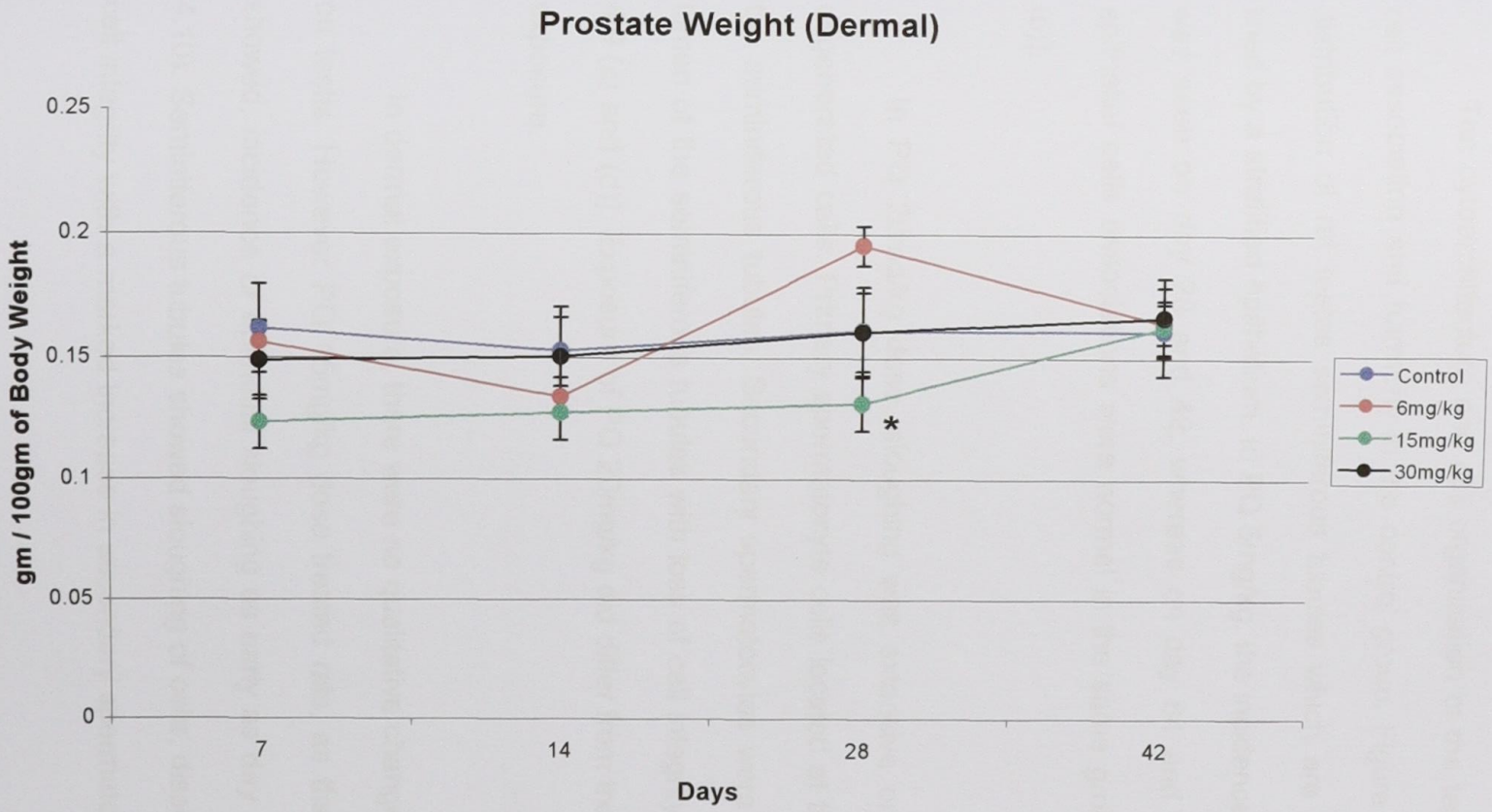


Figure 4.7 Time – response relationship of PQ (dermal) on prostate weight. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

4.3 Histopathological Evaluation

4.3.1 Qualitative Analysis

The cytoarchitecture and cell organization of the testis exhibited a normal cell association and harmony in the control group. Figure 4.8 shows the normal distribution of rat testes seminiferous tubules which are highly convoluted and lined by a stratified epithelium. In PQ 5mg/kg, the incidence of epithelial sloughing was seen on day 28 and 42, whereas on day 84 and 105, the seminiferous epithelial cells associations were normal in the same group [Figure 4.9 (a) and (b)].

In PQ 20mg/kg dose, sloughing was extensive on day 28 and 42 with degenerated cells. Primary spermatocyte cells located at the basal membrane of the seminiferous tubules. Secondary spermatocytes were also found inside the lumen of the seminiferous tubules with loss of cell integrity in the tubules [Figure 4.9 (c) and (d)]. Exposure of PQ 20mg/kg did differ from the PQ 5mg/kg oral dose exposure.

In dermal exposure, there were no qualitative changes by PQ 6mg/kg dose on testis. However PQ 15mg/kg dose treated rats, as that of PQ 5mg/kg have showed, incidence of epithelial sloughing as early as day 7 through day 14 (Fig 4.10). Seminiferous tubules showed sloughing of cells, disorganization and loss of cell integrity with a marked increase in secondary spermatocytes found inside the

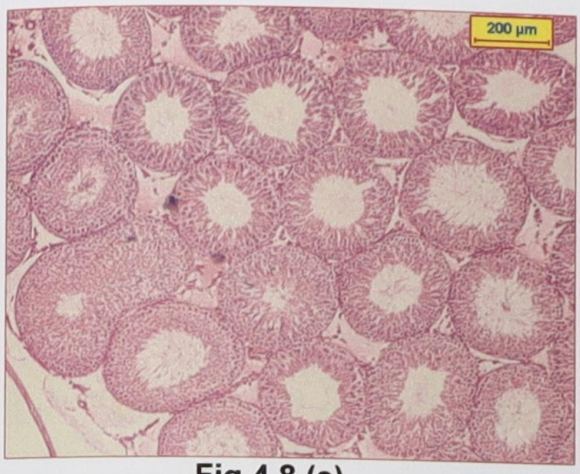


Fig 4.8 (a)

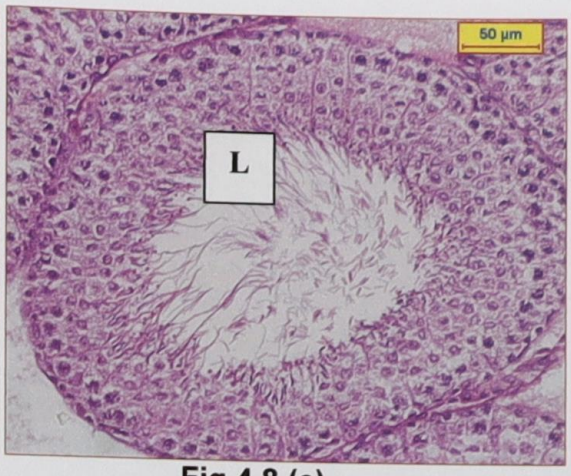


Fig 4.8 (c)

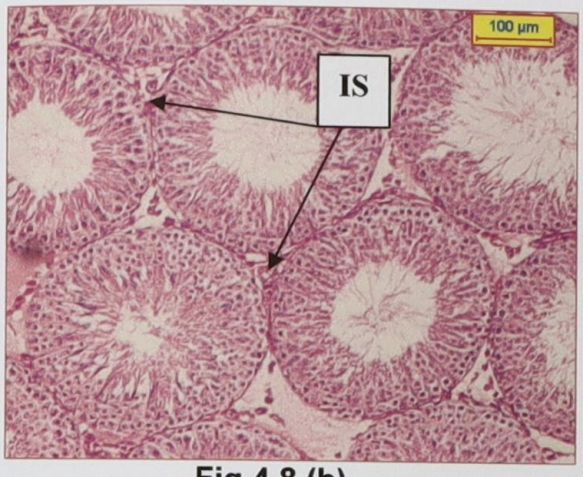


Fig 4.8 (b)

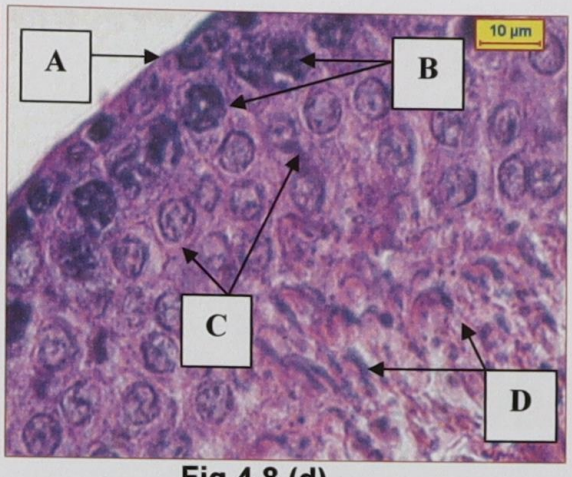


Fig 4.8 (d)

Fig 4.8 Photomicrographs of a control rat testis in various magnifications [H&E (a) x 25, (b) x 50, (c) x 100 and (d) x 400.

Fig 4.8 (b) illustrates seminiferous tubules cut in various planes. Seminiferous tubules are highly convoluted and lined by a stratified epithelium. In the interstitial spaces **(IS)** between the tubules, Leydig cells are found.

Fig 4.8 (c) shows a single tubule with a lumen **(L)** contains spermatozoa or sperms, ready to be released into the epididymis for maturation.

Fig 4.8 (d) shows normal cell population in seminiferous tubule. **(A)** Spermatogonia, **(B)** Primary spermatocyte, **(C)** Secondary spermatocyte, and **(D)** Elongate spermatid.

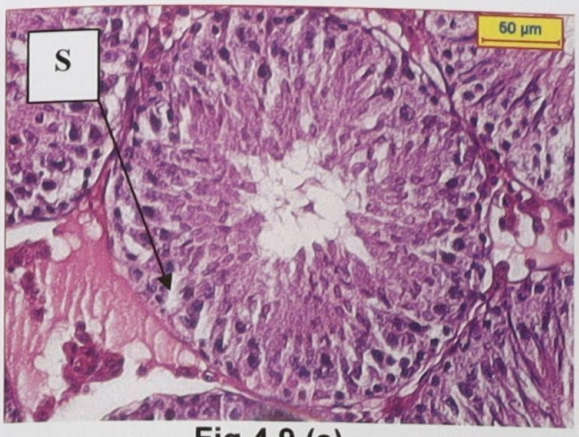


Fig 4.9 (a)

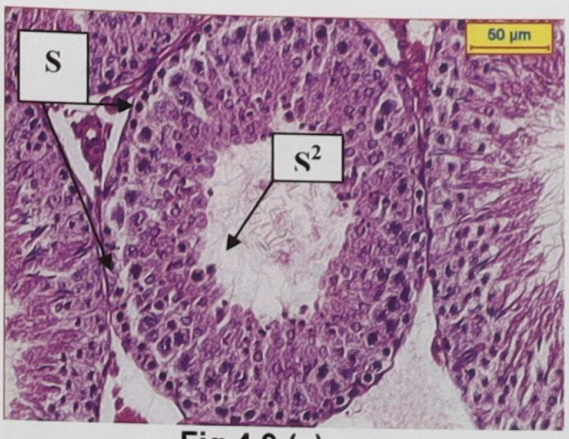


Fig 4.9 (c)

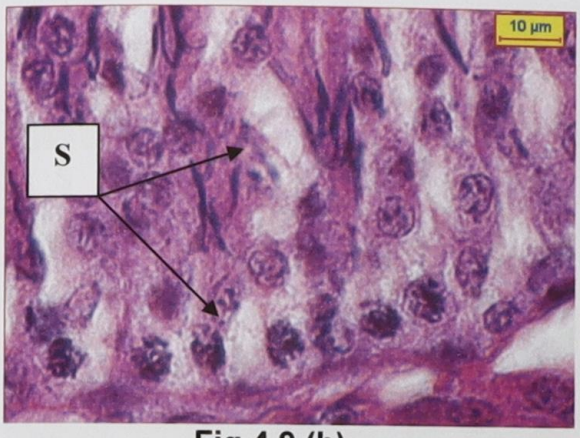


Fig 4.9 (b)

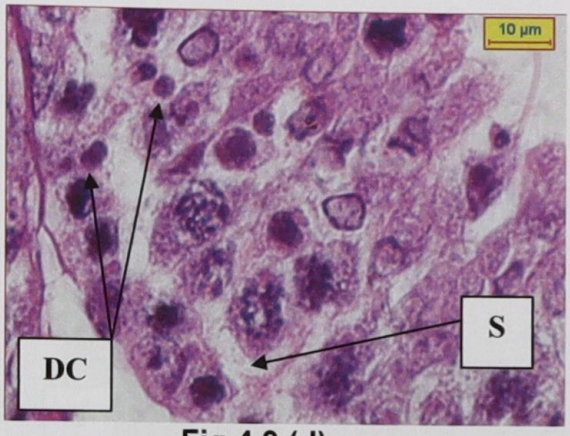


Fig 4.9 (d)

Fig 4.9 Photomicrographs of testis sections from PQ (oral) treated rats [H&E **(a)** x 100, **(b)** x 400, **(c)** x 100, and **(d)** x 400].

Fig 4.9 (a) and **(b)** shows adverse effect of PQ 5mg/kg (oral) on cell integrity caused sloughing **(S)** and disorganization of cell population in the seminiferous tubule.

Fig 4.9 (c) and **(d)** also shows sloughing **(S)** of tubule with gaps of appearance of degenerated cells **(DC)** after exposure to PQ 20mg/kg (oral). Secondary spematocytes **(S^2)** were also found inside the lumen of seminiferous tubule.

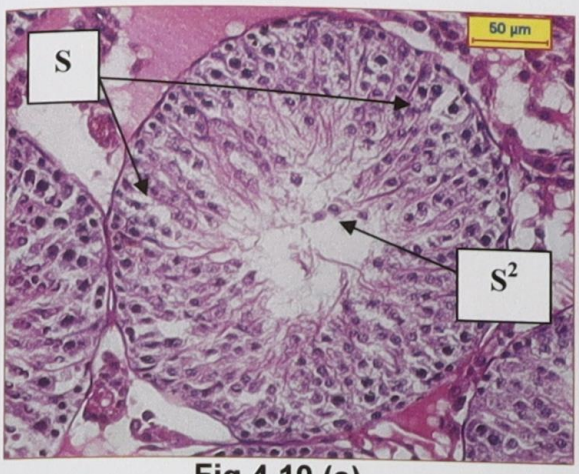


Fig 4.10 (a)

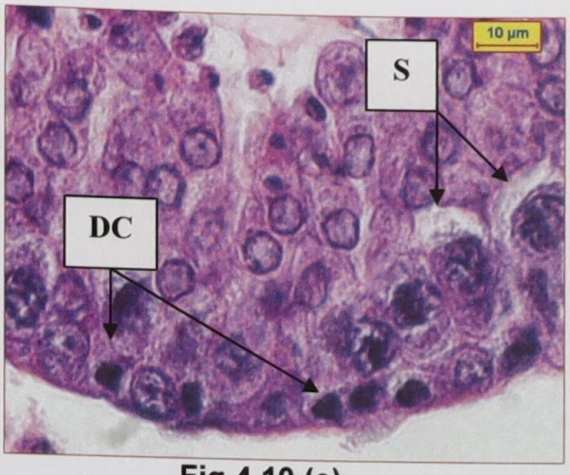


Fig 4.10 (c)

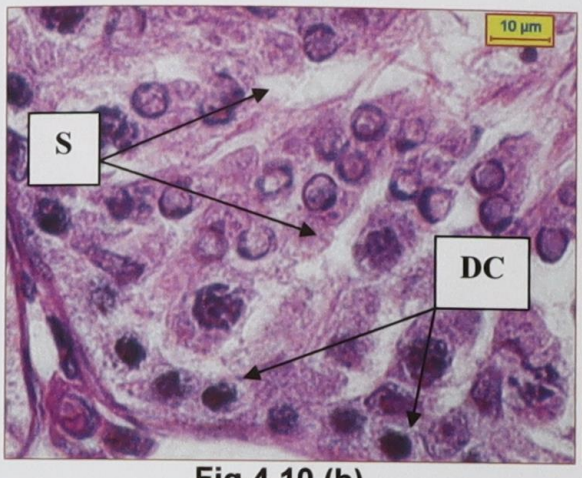


Fig 4.10 (b)

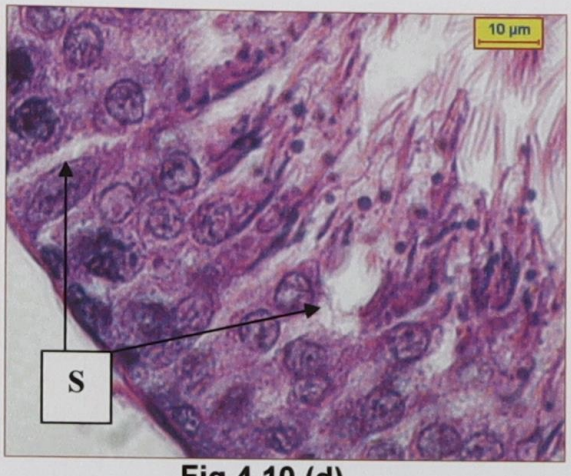


Fig 4.10 (d)

Fig 4.10 Photomicrographs of testis sections from PQ (dermal) treated rats [H&E **(a)** x 100, **(b), (c)** and **(d)** x 400.

Fig 4.10 (a) and **(b)** shows effect of PQ 15mg/kg (dermal) on cell integrity and organization. Tubules were sloughed **(S)**, disorganized and the secondary spermatocytes **(S^2)** moved into the lumen because of loss of cell integrity. As a result of PQ treatment, degenerated cells **(DC)** were also found in the seminiferous tubules of treated rats (PQ 15mg/kg).

Fig 4.10 (c) and **(d)** shows the effect of PQ 30mg/kg (dermal) with an increased bunmber of degenerated cells **(DC)** found mostly inside the seminiferous tubules of treated rats.

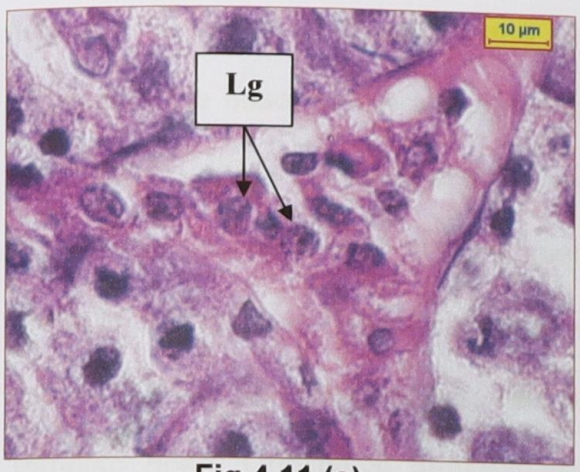


Fig 4.11 (a)

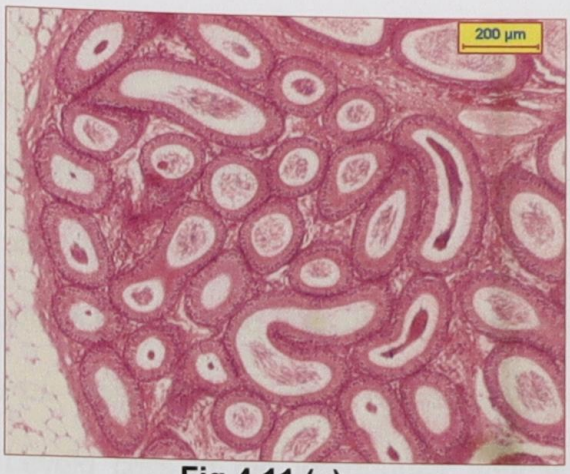


Fig 4.11 (c)

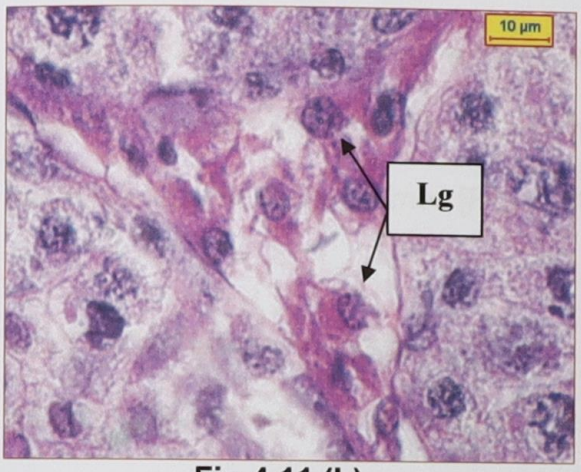


Fig 4.11 (b)



Fig 4.11 (d)

Fig 4.11 Photomicrographs of testis and epididymis sections.

Fig 4.11 (a) shows an normal intertubular space with Leydig cells **(Lg)** (H&E, x 400).

Fig 4.11 (b) shows decreased number of Leydig cells **(Lg)** in the intertubular space of testis (H&E, x 400).

Fig 4.11 (c) and **(d)** shows the integrity and tubular of epididymis with the lumen **(L)** full of spermatozoa. Pseudostratified epithelium cells is intact. [H&E, (c) x 100 and (d) x 400].

lumen of seminiferous tubules [Fig 4.10 (a)]. Degenerated cells were also found along side the basal membrane of seminiferous tubules [Fig. 4.10 (b) and (c)]. In PQ 30mg/kg dose, the testis sections exhibited a marked increase in the number of degenerating cells from primary spermatocyte cell lines and the incidence of epithelial sloughing in the seminiferous tubules [Figure 4.10 (c) and (d)]. In dermal exposure , PQ 30mg/kg dose seems to be more adverse when compared with 15mg/kg dose. Qualitative analysis of Leydig cells in the intertubular spaces, showed a normal morphology and there were no infiltration of intertubular cells into the seminiferous tubules [Fig. 4.11 (a) and (b)]. In the epididymal sections, there were no changes produced by PQ on the pseudostratified epithelial cells and the basal membrane of epididymes [Fig. 4.11 (c) and (d)].

4.3.2 Quantitative Analysis

4.3.2 (a) Seminiferous Tubule Diameter (STD) Analysis

In oral treatment of PQ 20mg/kg dose, a significant decrease in STD ($p<0.05$) throughout day 7, 28 and 42 were observed (Table 4.11). There were no significant change in STD of PQ 2mg/kg and 5mg/kg dose groups. All data were illustrated in Chart 4.12. In dermal exposure group of PQ 15mg/kg and 30mg/kg, STD was decreased significantly ($p<0.05$) on day 7 and 14 (Table 4.12). No change in STD in PQ 6mg/kg dose. All data were illustrated in Chart 4.13.

Table 4.11 Effect of PQ (oral) on Seminiferous Tubule Diameter (STD). Data were presented as Mean ± SD (μm); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	268.44 ± 5.44	276.03 ± 18.40	286.84 ± 16.26	288.48 ± 12.38	285.56 ± 16.96
PQ – 2 mg/kg	282.24 ± 9.10	280.96 ± 15.76	282.34 ± 15.28	285.49 ± 12.70	285.67 ± 13.16
PQ – 5 mg/kg	282.02 ± 22.16	276.14 ± 11.24	288.45 ± 12.88	292.96 ± 10.58	317.76 ± 19.18
PQ – 20 mg/kg	260.80* ± 11.54	261.44* ± 12.79	278.24* ± 15.12	284.22 ± 10.53	291.44 ± 16.20

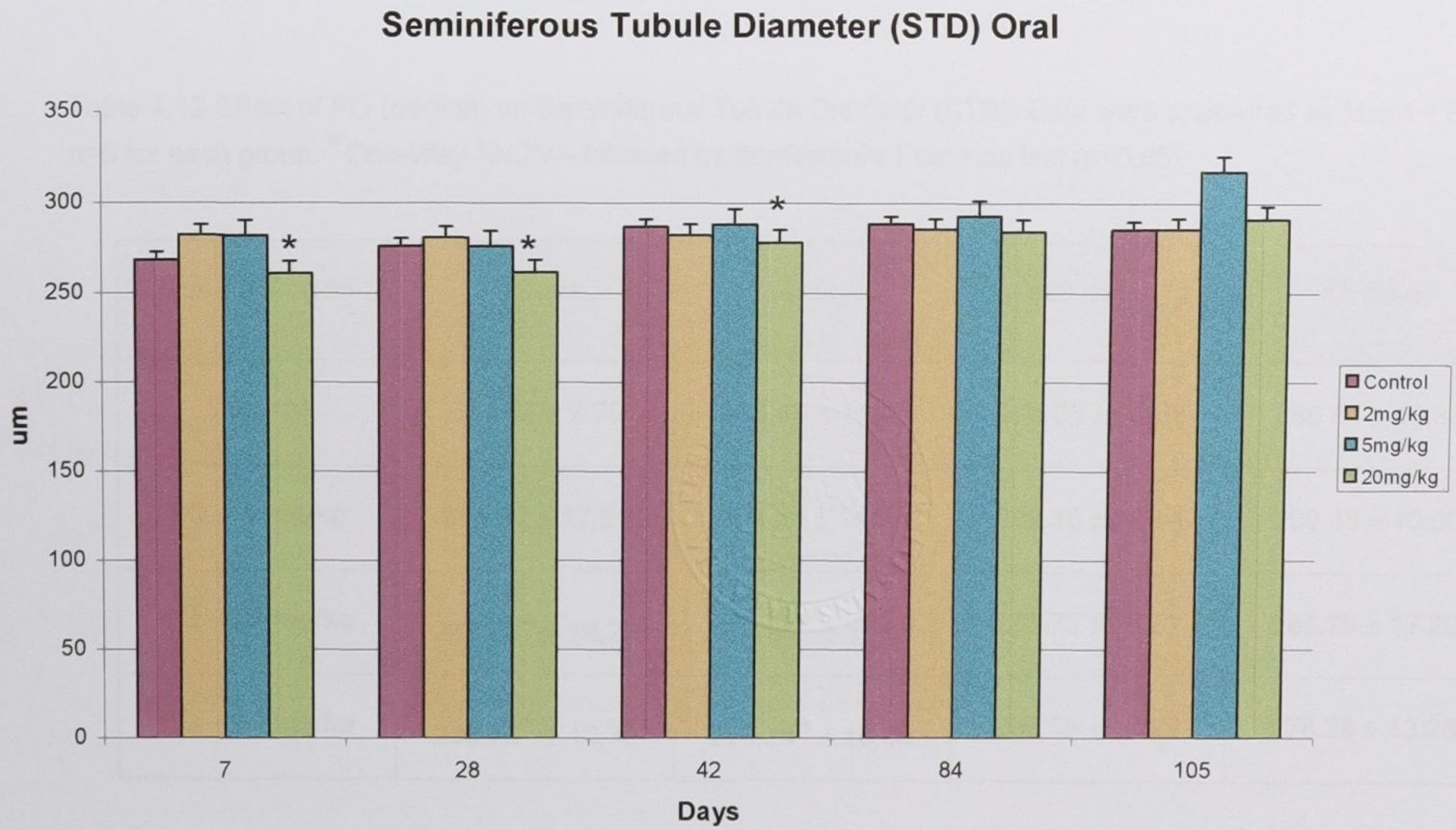


Figure 4.12 Time – response relationship of STD in control and PQ (Oral) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.12 Effect of PQ (dermal) on Seminiferous Tubule Diameter (STD). Data were presented as Mean ± SD (μm); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	314.02 ± 7.70	306.48 ± 14.68	276.03 ± 14.68	286.84 ± 13.33
PQ – 6 mg/kg	321.52 ± 17.61	298.84 ± 11.98	289.16 ± 18.64	299.48 ± 10.06
PQ – 15 mg/kg	287.23* ± 18.27	272.94* ± 15.81	273.72 ± 14.87	285.78 ± 17.20
PQ – 30 mg/kg	269.75* ± 10.70	270.24* ± 16.06	267.56 ± 17.29	278.28 ± 13.98

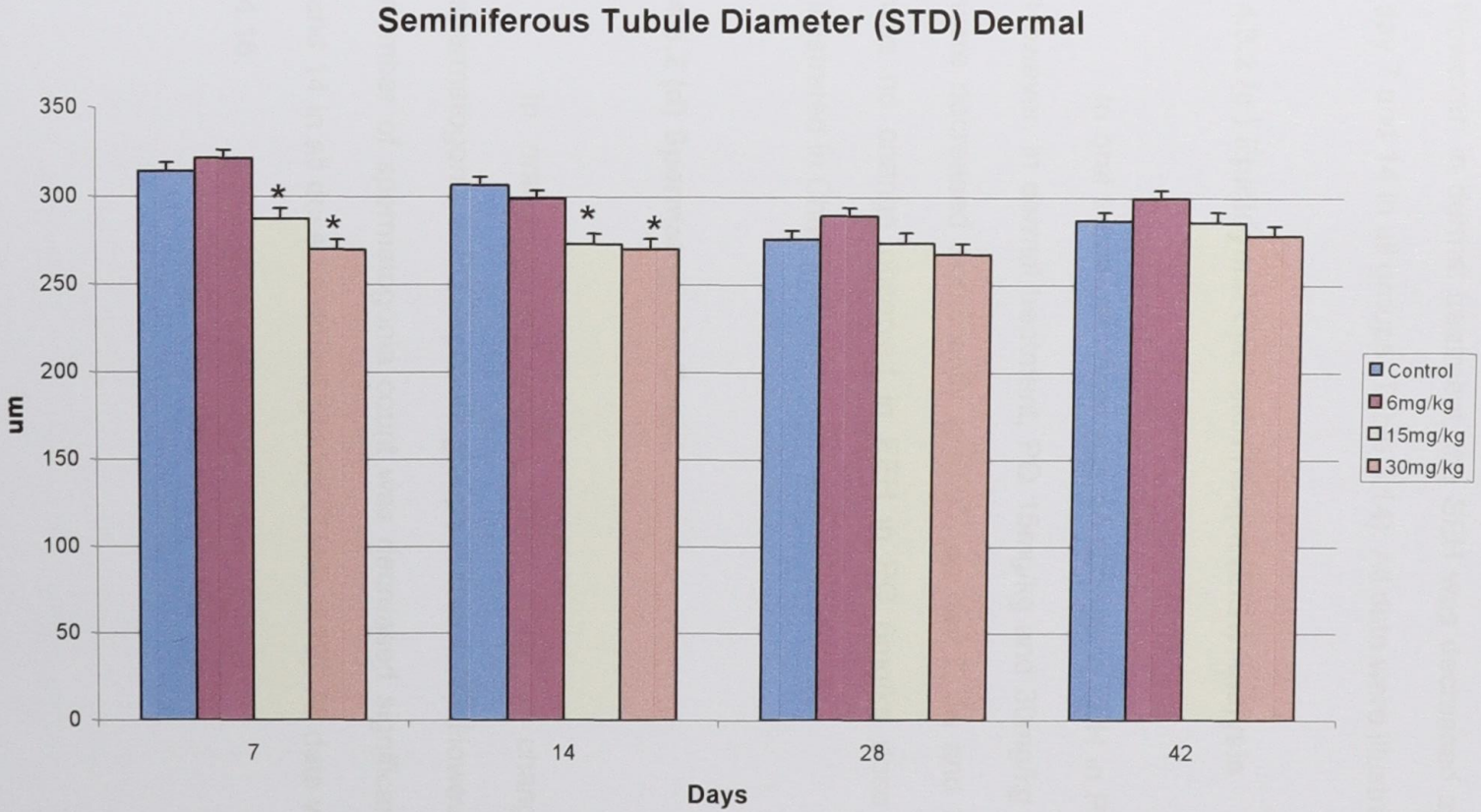


Figure 4.13 Time – response relationship of STD in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

4.3.2 (b) Seminiferous Epithelial Height (SEH) Analysis

There was no change in SEH in PQ oral exposure groups (Table 4.13). However, in dermal treatment, the SEH was decreased significantly ($p<0.05$) on day 7 and 14 in all groups (Table 4.14). All data were illustrated in Chart 4.14.

4.3.2 (c) Epididymal Epithelial Height (EEH) Analysis

In oral treatment, there was no change in EEH in PQ groups (Table 4.15). However, in dermal treatment, PQ 15mg/kg and 30mg/kg dose groups, the EEH were decreased significantly ($p<0.05$) on day 7, 14 and 28 (Table 4.16). There was no change observed in EEH in PQ 6mg/kg dose group. All data were illustrated in Chart 4.15.

4.3.2 (d) Spermatogonia Count

In oral treatment groups, there were no change in the number of spermatogonia in PQ exposure groups (Table 4.17). However in dermal treatment, number of spermatogonia count was decreased significantly ($p<0.05$) on day 7 and 14 in all dose treatment groups (Table 4.18). All data were illustrated in Chart 4.16.

Table 4.13 Effect of PQ (oral) on Seminiferous Epithelial Height (SEH). Data were presented as Mean ± SD (μm); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days	28 days	42 days	84 days	105 days
Control	67.07 ± 4.58	62.92 ± 9.54	66.08 ± 4.26	69.57 ± 4.76	71.08 ± 6.18
PQ – 2 mg/kg	64.52 ± 6.08	63.44 ± 8.58	65.74 ± 6.72	68.16 ± 9.86	70.32 ± 3.88
PQ – 5 mg/kg	65.61 ± 6.59	64.84 ± 7.01	66.92 ± 8.42	67.28 ± 9.06	69.08 ± 7.14
PQ – 20 mg/kg	65.11 ± 3.45	69.77 ± 3.10	68.78 ± 8.53	66.07 ± 10.13	68.22 ± 3.56

Table 4.14 Effect of PQ (dermal) on Seminiferous Epithelial Height (SEH). Data were presented as Mean ± SD (μm); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	67.07 ± 4.58	71.58 ± 5.32	62.92 ± 9.54	66.08 ± 4.26
PQ – 6 mg/kg	56.99* ± 9.51	53.36* ± 6.51	58.78 ± 7.56	60.38 ± 7.48
PQ – 15 mg/kg	57.62* ± 8.80	54.79* ± 5.52	61.43 ± 7.36	68.36 ± 8.18
PQ – 30 mg/kg	57.34* ± 3.42	61.81* ± 7.20	62.09 ± 5.62	64.66 ± 4.02

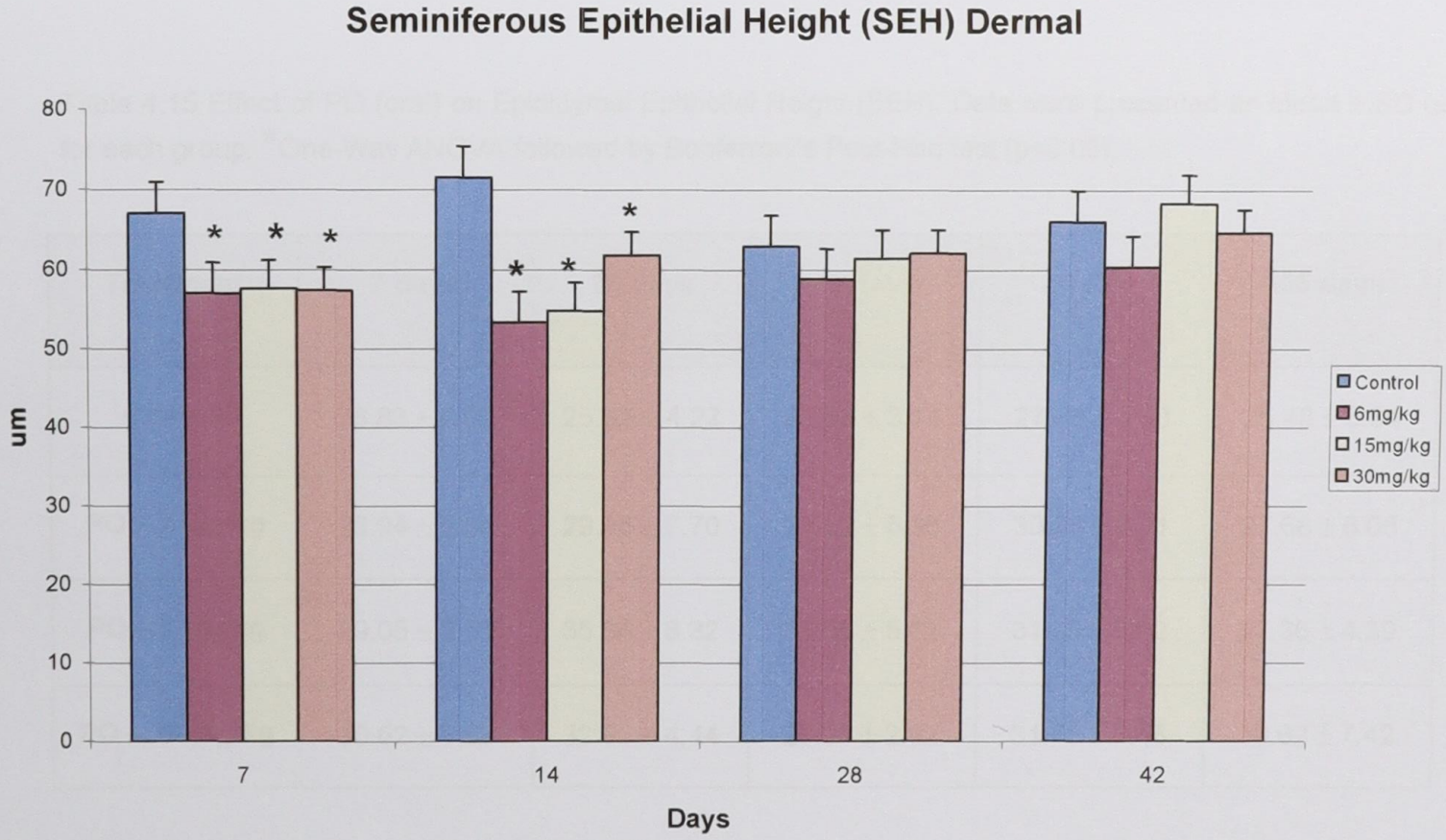


Figure 4.14 Time – response relationship of SEH in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.15 Effect of PQ (oral) on Epididymal Epithelial Height (EEH). Data were presented as Mean ± SD (μm); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	28.83 ± 4.67	25.62 ± 4.22	31.58 ± 3.14	27.88 ± 4.08	25.48 ± 5.34
PQ – 2 mg/kg	26.94 ± 5.55	29.08 ± 7.70	28.82 ± 5.36	30.25 ± 5.21	27.68 ± 6.08
PQ – 5 mg/kg	29.05 ± 5.51	35.34 ± 8.32	30.58 ± 5.11	31.25 ± 9.02	27.36 ± 4.39
PQ – 20 mg/kg	30.62 ± 1.62	32.92 ± 4.44	28.91 ± 2.82	31.78 ± 8.46	30.62 ± 7.42

Table 4.16 Effect of PQ (dermal) on Epididymal Epithelial Height (EEH). Data were presented as Mean ± SD (μm); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	28.83 ± 4.67	28.81 ± 6.45	30.20 ± 4.22	31.58 ± 3.14
PQ – 6 mg/kg	25.20 ± 4.86	26.48 ± 4.22	29.82 ± 1.76	32.56 ± 8.49
PQ – 15 mg/kg	23.24* ± 2.93	25.46* ± 2.19	22.85* ± 1.91	30.65 ± 5.06
PQ – 30 mg/kg	23.56* ± 5.18	23.72* ± 4.11	22.68* ± 3.50	26.59 ± 2.08

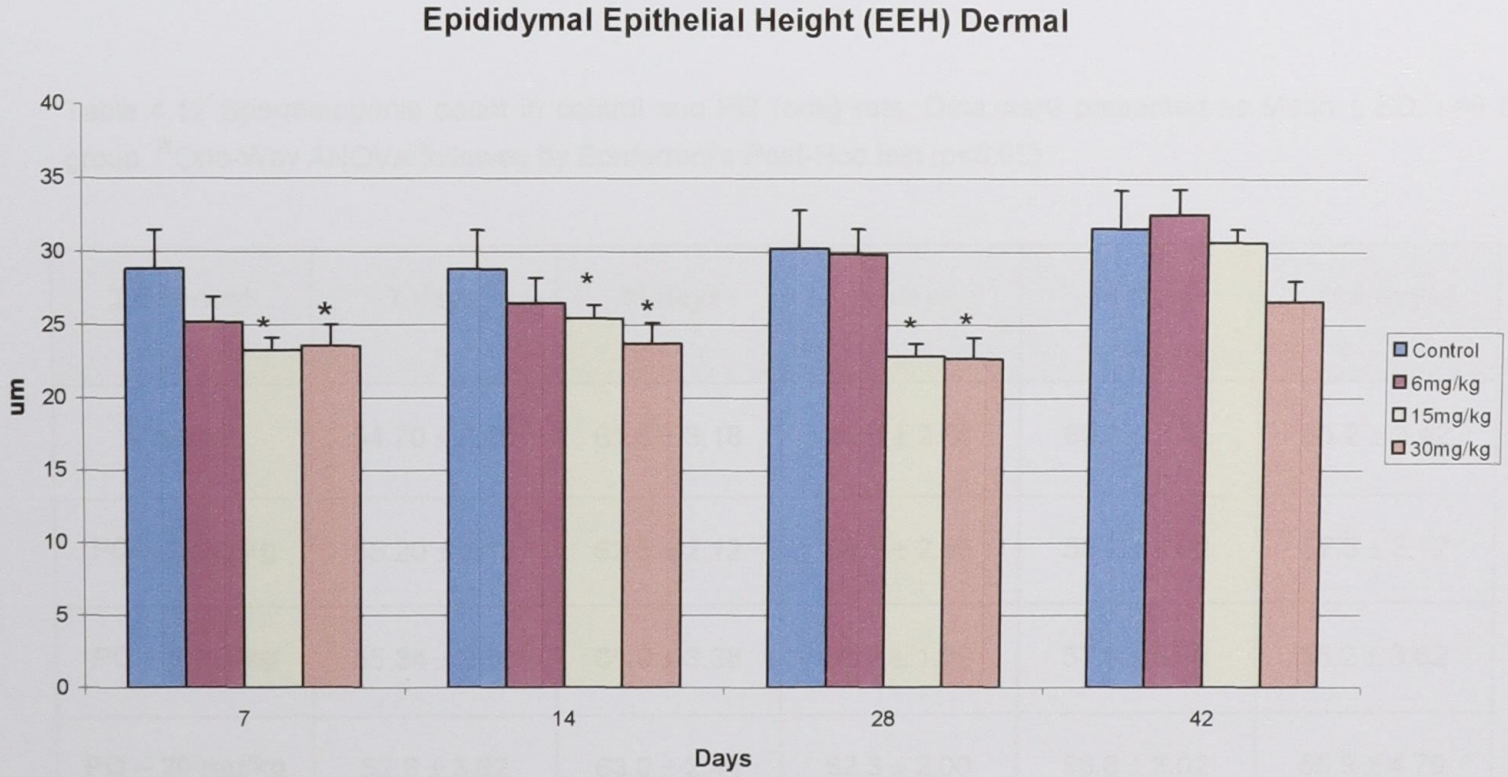


Figure 4.15 Time – response relationship of EEH in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05$*. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.17 Spermatogonia count in control and PQ (oral) rats. Data were presented as Mean ± SD; n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	54.70 ± 2.62	61.5 ± 3.18	65.0 ± 2.66	60.9 ± 3.41	55.2 ± 3.42
PQ – 2 mg/kg	55.20 ± 3.12	62.5 ± 2.12	64.1 ± 2.56	58.8 ± 4.62	57.3 ± 3.12
PQ – 5 mg/kg	55.34 ± 3.50	61.9 ± 3.38	63.4 ± 1.89	57.9 ± 5.18	55.2 ± 3.62
PQ – 20 mg/kg	52.8 ± 3.62	63.0 ± 2.44	62.3 ± 2.00	56.8 ± 3.02	55.9 ± 4.79

Table 4.18 Spermatogonia count in control and PQ (dermal) rats. Data were presented as Mean ± SD; n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	54.70 ± 2.62	56.4 ± 2.88	61.5 ± 3.18	65.0 ± 2.66
PQ – 6 mg/kg	45.9* ± 3.96	48.4* ± 3.30	60.4 ± 2.16	62.9 ± 2.56
PQ – 15 mg/kg	40.5* ± 3.56	43.4* ± 2.02	67.3 ± 4.48	65.3 ± 3.40
PQ – 30 mg/kg	39.4* ± 6.02	40.9* ± 2.28	64.7 ± 3.12	65.5 ± 4.52

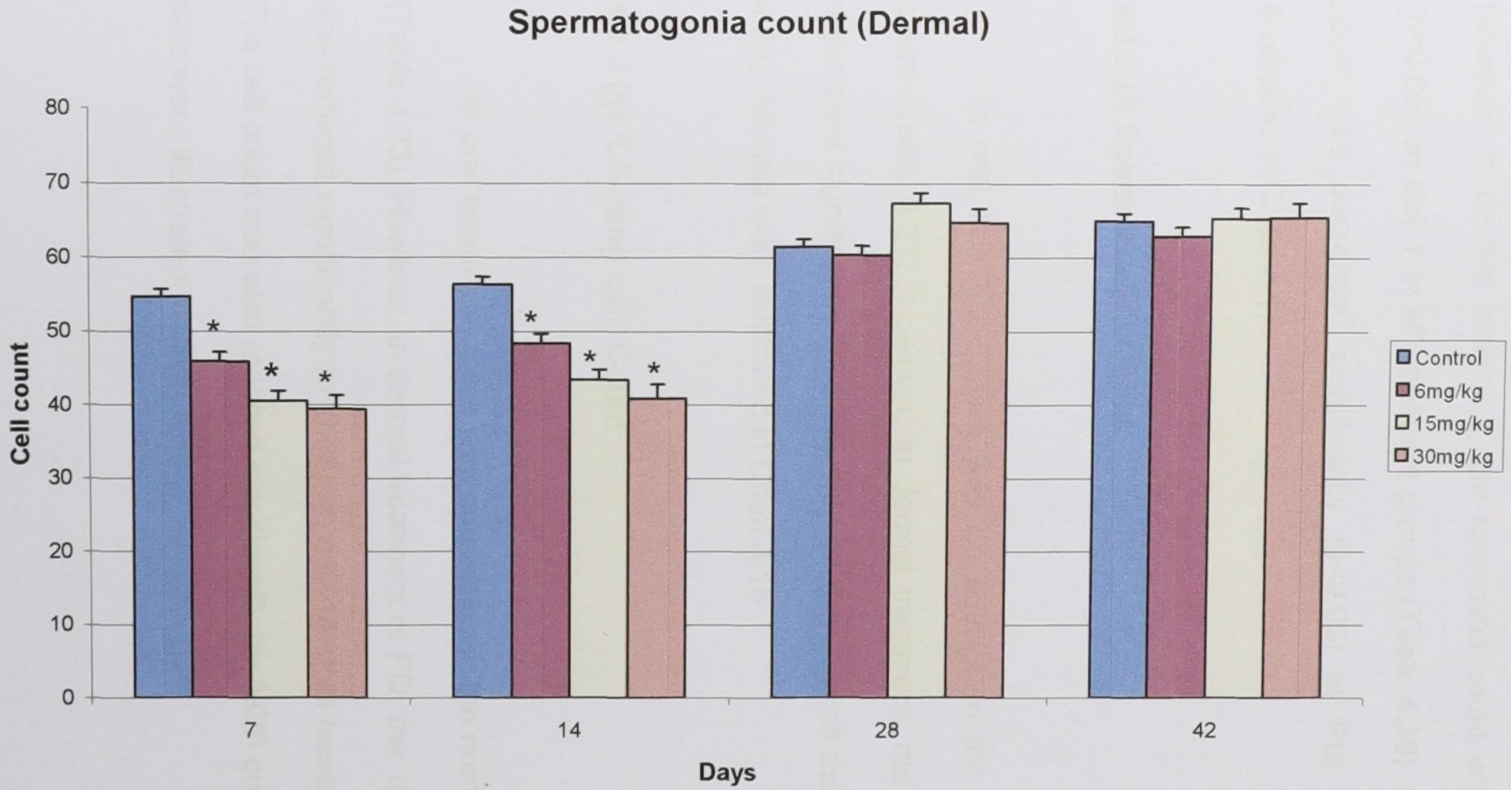


Figure 4.16 Time – response relationship of spermatogonia count in control and PQ (dermal) treated rats. The mean difference is significant at the level of $p<0.05$*. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

4.3.2 (e) Spermatid Count

In oral treatment, there was no change in the spermatid count (Table 4.19). However, in dermal treatment, the spermatid count was decreased significantly ($p<0.05$) on day 7 in all treatment groups (Table 4.20). On day 14, the spermatid count was decreased significantly ($p<0.05$) in PQ 30mg/kg. All data were illustrated in Chart 4.17.

4.3.2 (f) Spermatocyte Count

In oral treatment, there was no change in the number of spermatocyte count (Table 4.21). However, in dermal treatment, the spermatocyte count was decreased significantly ($p<0.05$) only on day 7 in all the treatment groups (Table 4.22). All data were illustrated in Chart 4.18.

4.3.2 (g) 'Dividing cell' Count

In oral treatment, there was no change in the number of 'dividing cell' count (Table 4.23). However, in dermal treatment of PQ, the 'dividing cell' count number was reduced significantly ($p<0.05$) on day 14 in all treatment groups (Table 4.24). The cell count was also reduced significantly ($p<0.05$) on day 7 in PQ 30mg/kg. All data were illustrated in Chart 4.19.

Table 4.19 Spermatid count in control and PQ (oral) rats. Data were presented as Mean ± SD ; n=6 for each group.
*One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	255.7 ± 4.42	240.6 ± 3.09	233.6 ± 5.39	219.3 ± 5.16	209.5 ± 5.08
PQ – 2 mg/kg	251.6 ± 3.92	240.4 ± 4.40	236.3 ± 5.42	224.2 ± 11.32	220.1 ± 10.32
PQ – 5 mg/kg	258.9 ± 3.10	242.4 ± 2.59	243.8 ± 6.34	219.8 ± 7.26	216.1 ± 8.02
PQ – 20 mg/kg	261.2 ± 4.26	247.1 ± 7.82	236.8 ± 7.19	231.8 ± 15.29	210.9 ± 6.56

Table 4.20 Spermatid count in control and PQ (dermal) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	155.7 ± 4.42	196.5 ± 8.93	240.6 ± 3.09	233.6 ± 5.39
PQ – 6 mg/kg	146.8* ± 5.98	199.8 ± 4.52	239.9 ± 5.17	241.1 ± 6.70
PQ – 15 mg/kg	149.6* ± 4.67	195.5 ± 2.46	237.0 ± 5.97	240.9 ± 12.22
PQ – 30 mg/kg	143.4* ± 4.12	188.1* ± 2.08	249.7 ± 4.27	247.3 ± 8.02

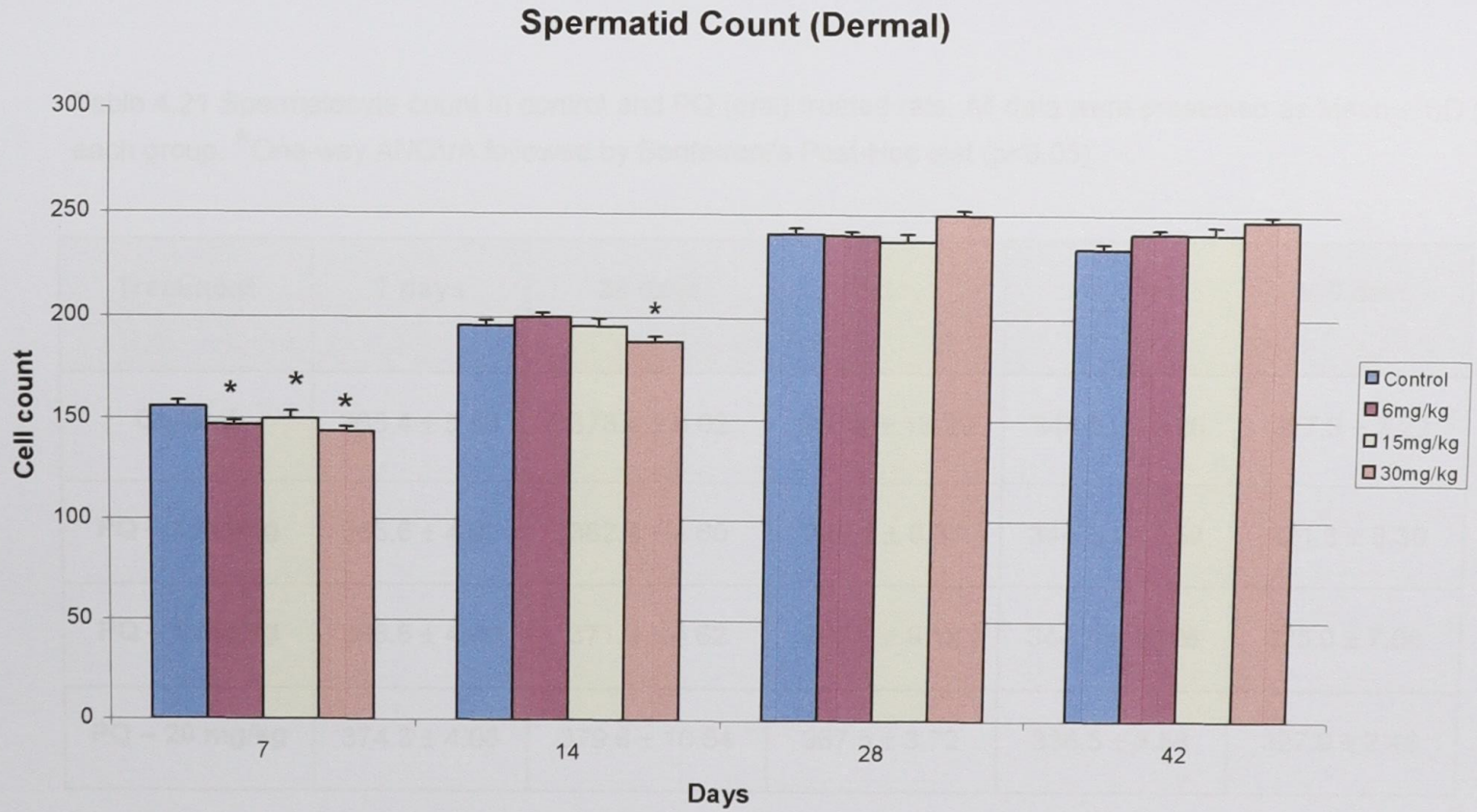


Figure 4.17 Time – response relationship of spermatid count in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.21 Spermatocyte count in control and PQ (oral) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	365.4 ± 5.58	378.4 ± 6.02	351.2 ± 18.20	344.6 ± 10.26	327.3 ± 7.22
PQ – 2 mg/kg	365.6 ± 4.92	382.4 ± 4.60	366.1 ± 9.34	344.8 ± 13.59	321.3 ± 8.30
PQ – 5 mg/kg	368.6 ± 4.60	371.6 ± 4.62	363.9 ± 9.12	344.7 ± 10.26	325.0 ± 7.08
PQ – 20 mg/kg	374.3 ± 4.06	379.8 ± 10.54	367.5 ± 3.72	336.5 ± 8.58	327.9 ± 2.46

Table 4.22 Spermatocyte count in control and PQ (dermal) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	365.4 ± 5.58	375.5 ± 4.90	378.4 ± 6.02	351.2 ± 8.20
PQ – 6 mg/kg	346.1* ± 6.26	382.4 ± 5.03	378.3 ± 6.09	356.3 ± 8.89
PQ – 15 mg/kg	349.0* ± 4.16	386.71 ± 2.75	373.4 ± 6.26	351.4 ± 11.85
PQ – 30 mg/kg	345.7* ± 4.34	385.5 ± 4.50	372.8 ± 7.14	355.7 ± 12.98

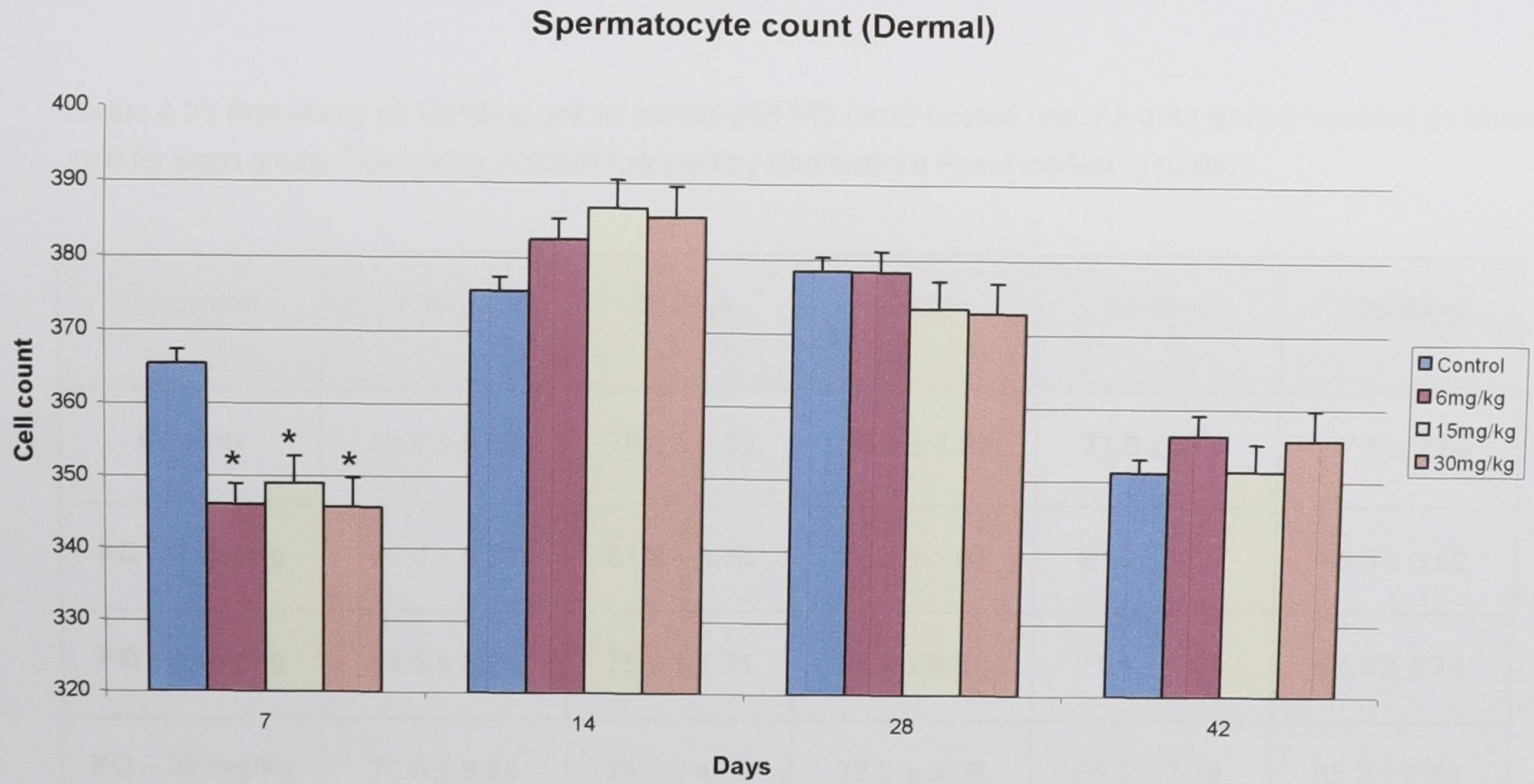


Figure 4.18 Time – response relationship of spermatocyte count in control and PQ (dermal) treated rats. The mean difference is significant at the level of $p<0.05$*. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.23 Frequency of 'Dividing cell' in control and PQ (oral) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	68.2 ± 5.69	78.6 ± 2.02	76.1 ± 4.88	71.0 ± 2.44	67.5 ± 2.06
PQ – 2 mg/kg	70.0 ± 5.33	81.2 ± 3.55	73.8 ± 1.94	61.9 ± 4.22	66.3 ± 3.02
PQ – 5 mg/kg	64.5 ± 5.28	75.0 ± 4.71	74.9 ± 3.51	71.1 ± 7.03	65.3 ± 4.74
PQ – 20 mg/kg	70.5 ± 3.89	76.2 ± 4.02	72.0 ± 3.09	64.3 ± 7.18	65.2 ± 2.89

Table 4.24 Frequency of 'Dividing cell' in control and PQ (dermal) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	68.2 ± 5.62	67.0 ± 7.45	78.6 ± 2.01	76.1 ± 4.88
PQ – 6 mg/kg	60.6 ± 7.24	51.4* ± 2.68	78.2 ± 2.52	72.7 ± 4.27
PQ – 15 mg/kg	62.8 ± 5.88	44.5* ± 3.77	71.3* ± 5.86	76.0 ± 7.16
PQ – 30 mg/kg	48.9* ± 2.33	43.5* ± 5.04	73.9 ± 3.44	74.2 ± 5.02

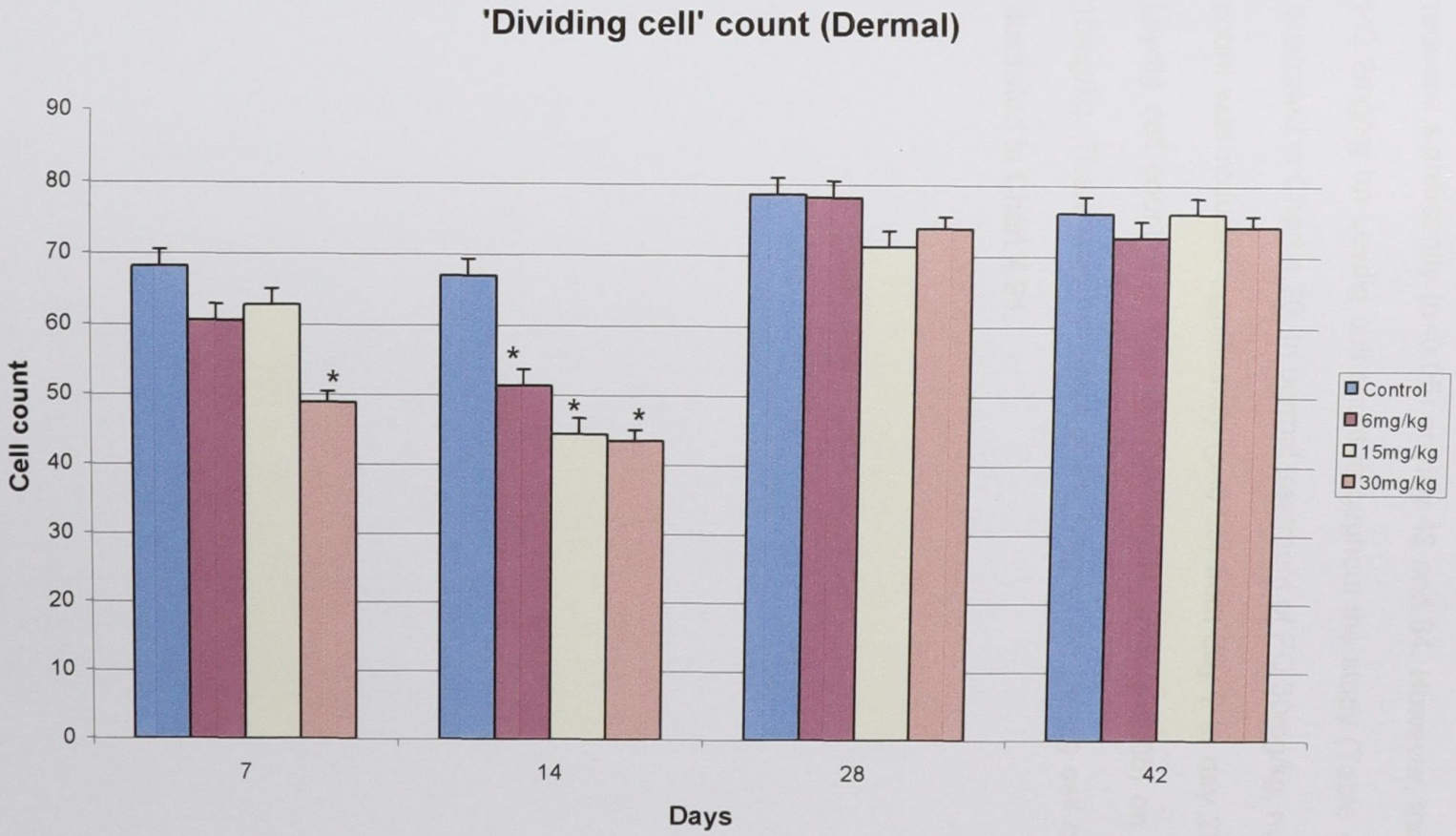


Figure 4.19 Time – response relationship of 'Dividing cell' count in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-Hoc test).

4.3.2 (h) Leydig Cell Count

In oral treatment of PQ 5mg/kg and 20mg/kg, number of Leydig cell were reduced significantly ($p<0.05$) on day 42 and 84. However, there was no effect of PQ 2mg/kg on Leydig cell count throughout the study (Table 4.25). All data were illustrated in Chart 4.20. In dermal treatment of PQ 30mg/kg, number of Leydig cell count was reduced significantly ($p<0.05$) from day 7 to day 28 (Table 4.26). The Leydig cell count was also reduced significantly ($p<0.05$) on day 14 only in PQ 15mg/kg. There was no effect of PQ 6mg/kg on Leydig cell count. All data were illustrated in Chart 4.21.

Table 4.25 Leydig cell count in control and PQ (oral) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	5.5 ± 1.96	5.3 ± 1.49	6.0 ± 2.72	6.1 ± 2.84	5.8 ± 2.39
PQ – 2 mg/kg	5.3 ± 1.18	5.7 ± 1.44	5.5 ± 1.94	6.0 ± 1.94	5.9 ± 1.60
PQ – 5 mg/kg	5.4 ± 1.78	6.1 ± 1.37	4.4* ± 1.08	4.3* ± 2.02	5.1 ± 1.19
PQ – 20 mg/kg	5.6 ± 1.34	5.8 ± 1.82	3.5* ± 2.42	4.9* ± 1.79	5.4 ± 1.18

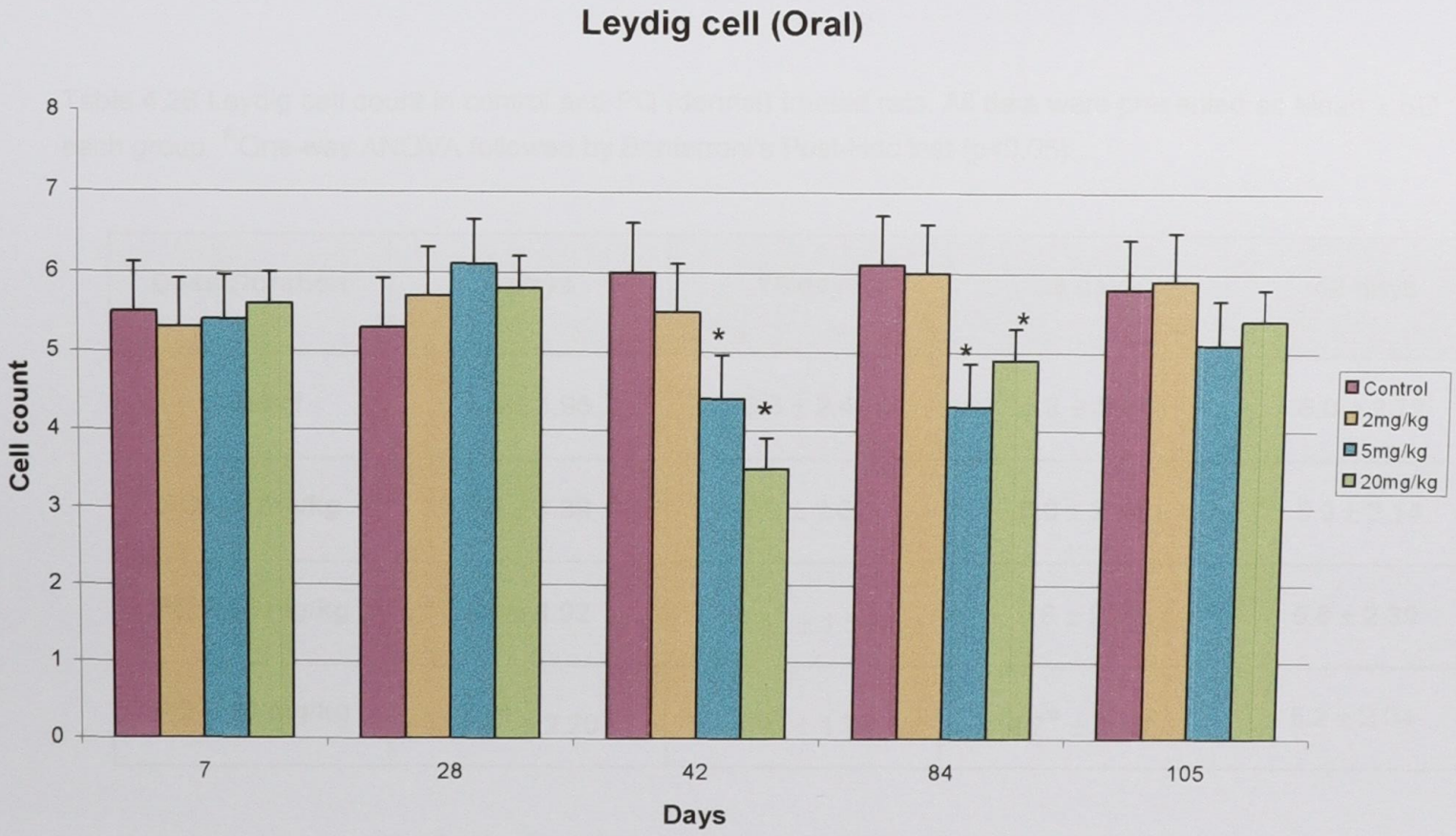


Figure 4.20 Time – response relationship of Leydig cell count in control and PQ (Oral) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-hoc test).

Table 4.26 Leydig cell count in control and PQ (dermal) treated rats. All data were presented as Mean ± SD ; n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	5.5 ± 1.96	6.3 ± 2.49	5.3 ± 1.49	6.0 ± 2.72
PQ – 6 mg/kg	5.8 ± 1.39	7.0 ± 2.62	6.0 ± 2.44	5.9 ± 2.14
PQ – 15 mg/kg	5.1 ± 1.92	5.5* ± 1.54	5.6 ± 2.27	5.8 ± 2.39
PQ – 30 mg/kg	4.2* ± 2.20	4.0* ± 1.75	4.7* ± 2.83	5.2 ± 2.04

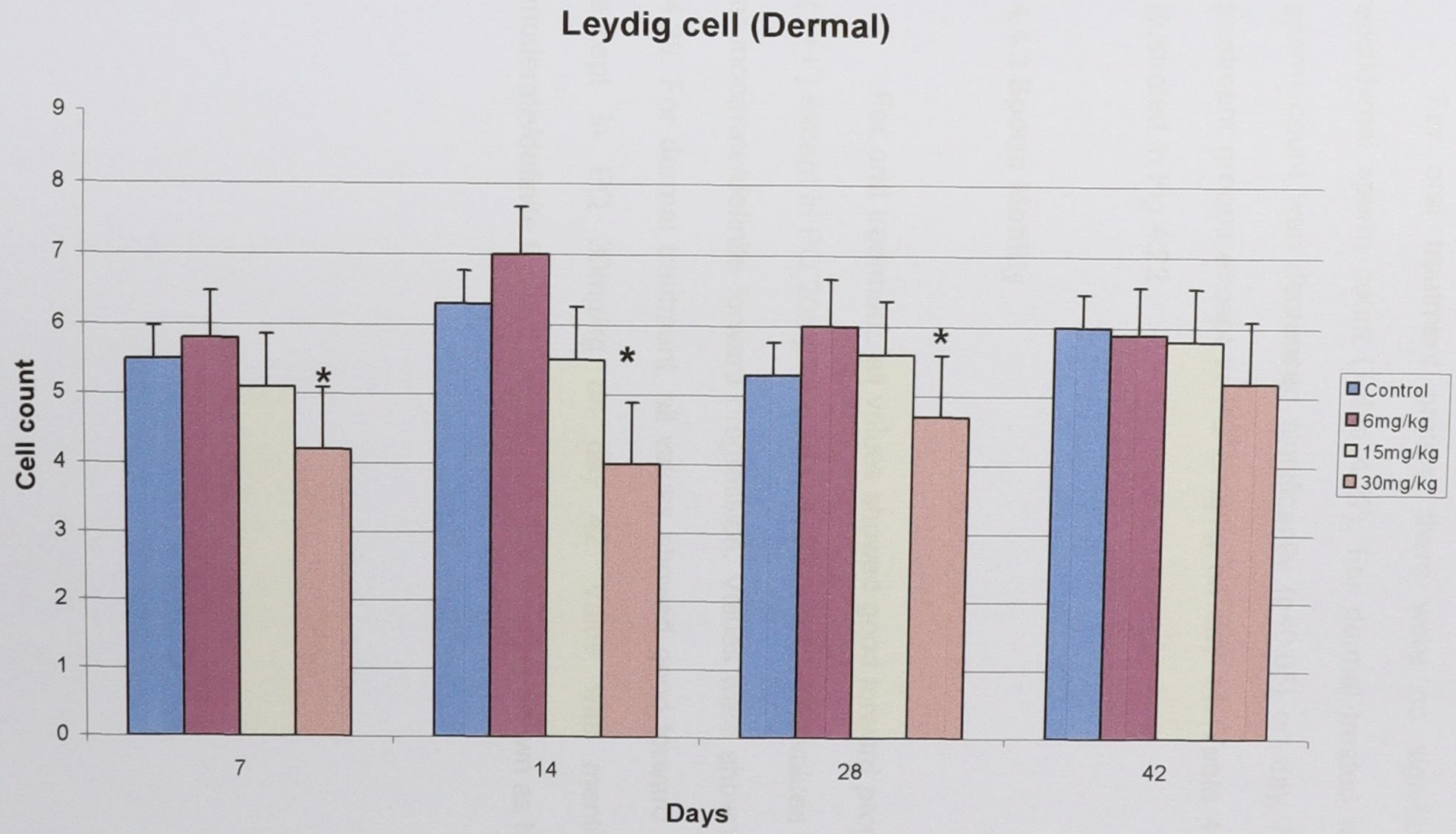


Figure 4.21 Time – response relationship of Leydig cell count in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-hoc test).

4.4 Sperm Assays

4.4.1 Sperm Count

For oral treatment groups, there were no significant differences of epididymal sperm count (Table 4.27). For dermal treated rats, the epididymal sperm count was decreased significantly ($p<0.05$) on day 7 and 14 in all the treatment groups except in PQ 6mg/kg on day 14 (Table 4.28). All data were illustrated in Fig 4.22.

4.4.2 Sperm Motility

For oral treatment, all values showed good forward progression movement ('+++') except in PQ 20mg/kg on day 7, 28 and 42. The values were shown as '++' or moderate/definite forward progression. Values were shown as Mode in Table 4.29. For dermal treatment, all values showed good forward progression ('+++') except in PQ 30mg/kg on day 42. Value was mentioned as '++' or moderate/definite forward progression. Values were shown as Mode in Table 4.30.

Table 4.27 Epididymal sperm count in control and PQ (oral) rats. Data were represented as mean ± SEM (x 10^6), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days	28 days	42 days	84 days	105 days
Control	23.02 ± 1.19	23.95 ± 1.69	23.65 ± 0.64	23.95 ± 3.06	25.38 ± 3.03
PQ – 2 mg/kg	22.68 ± 1.86	22.86 ± 2.82	23.80 ± 2.74	27.42 ± 1.11	25.89 ± 2.96
PQ – 5 mg/kg	23.36 ± 2.02	22.40 ± 3.61	25.46 ± 1.42	24.66 ± 2.64	22.66 ± 1.96
PQ – 20 mg/kg	22.35 ± 1.84	22.96 ± 2.31	28.82 ± 1.06	28.02 ± 1.09	25.32 ± 2.42

Table 4.28 Epididymal sperm count in control and PQ (dermal) rats. Data were pointed as mean ± SEM (x 10^6) , n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose/Duration	7 days	14 days	28 days	42 days
Control	23.02 ± 1.19	22.56 ± 1.29	23.95 ± 1.69	23.62 ± 0.64
PQ – 6 mg/kg	13.74* ± 2.69	22.92 ± 1.63	22.96 ± 0.81	24.38 ± 1.49
PQ – 15 mg/kg	13.26* ± 2.66	18.32* ± 0.57	22.62 ± 0.55	23.00 ± 1.44
PQ – 30 mg/kg	9.93* ± 1.06	13.26* ± 0.37	23.68 ± 1.92	22.98 ± 0.97

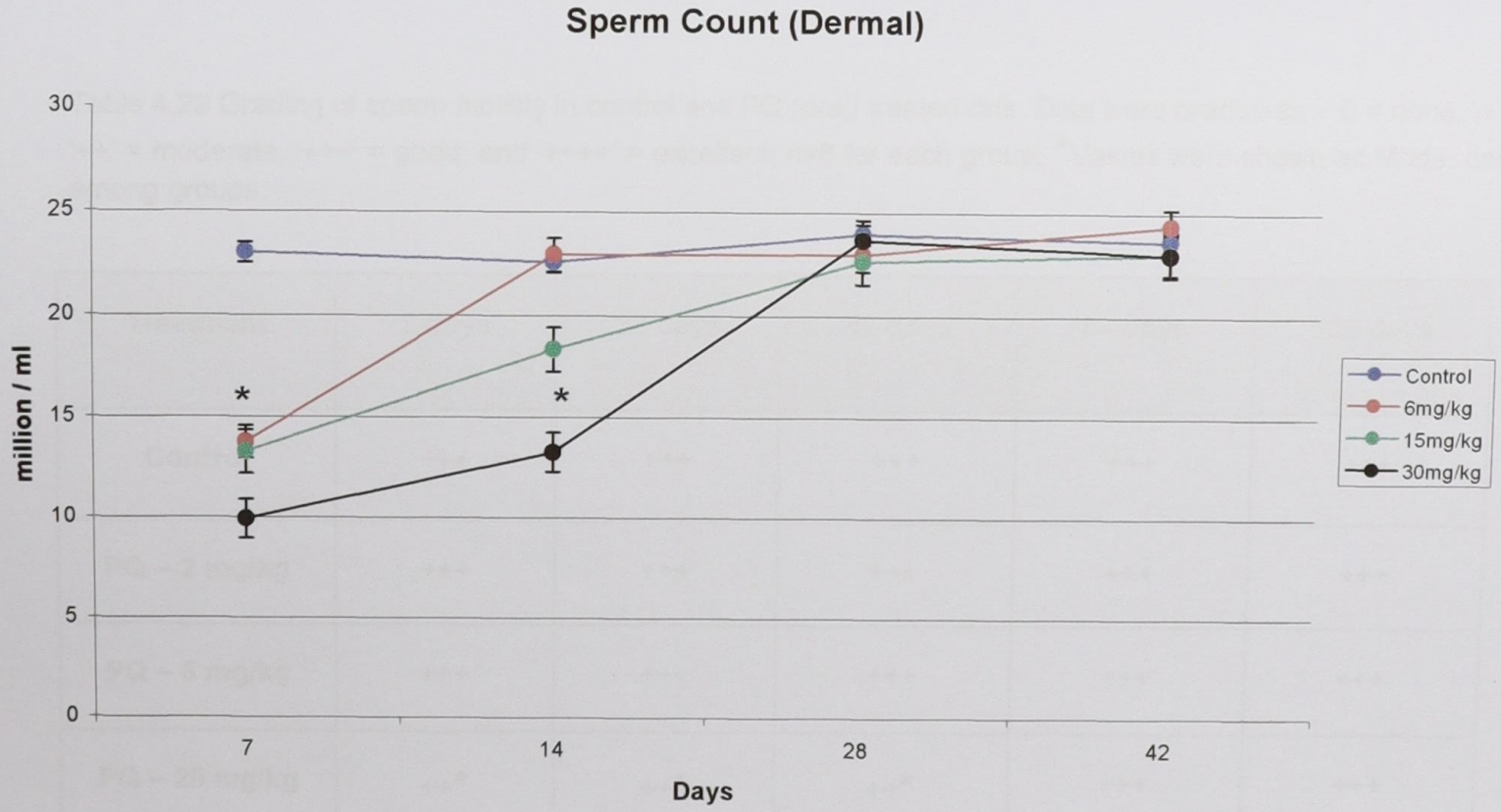


Figure 4.24 Time – response relationship of epididymal sperm count in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were pointed as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.29 Grading of sperm motility in control and PQ (oral) treated rats. Data were graded as - 0 = none, '+' = poor, '++' = moderate, '+++' = good, and '++++' = excellent; n=6 for each group. *Values were shown as Mode, comparing among groups.

Treatment	7 days	28 days	42 days	84 days	105 days
Control	+++	+++	+++	+++	+++
PQ – 2 mg/kg	+++	+++	+++	+++	+++
PQ – 5 mg/kg	+++	+++	+++	+++	+++
PQ – 20 mg/kg	++*	++*	++*	+++	+++

Table 4.30 Grading of sperm motility in control and PQ (dermal) treated rats. Data were graded as - 0 = none, '+' = poor, '++' = moderate, '+++' = good, and '++++' = excellent; n=6 for each group. *Values were shown as Mode, comparing among groups.

Dose/Duration	7 days	14 days	28 days	42 days
Control	+++	+++	+++	+++
PQ – 6 mg/kg	+++	+++	+++	+++
PQ – 15 mg/kg	+++	+++	+++	+++
PQ – 30 mg/kg	+++	+++	+++	++*

4.4.3 Sperm Mortality

For oral treated rats, the percentage of sperm mortality were increased significantly ($p<0.05$) in PQ 20mg/kg on day 28 and 42 (Table 4.31). There were no effect in the percentage of sperm mortality in PQ 2mg/kg and 5mg/kg. All data were illustrated in Chart 4.23. For dermal treatment, the percentage of sperm mortality were increased significantly ($p<0.05$) on day 7, 14, and 28 in PQ 15mg/kg and 30mg/kg (Table 4.32). All data were illustrated in Chart 4.24.

4.4.4 Sperm Morphology

4.4.4 (a) Qualitative analysis

The abnormal rat spermatozoa exhibited either head or tail deformities. The different types of head abnormalities were hookless, microcephaly, cephalo-caudal junction deformities, double headed, banana-shaped, amorphous head and headless. The different types of tail deformities are double tailed, coiled and broken tail. The sperm head and tail abnormalities were shown in Figure 4.25.

4.4.4 (b) Quantitative analysis

Treatment of PQ 2mg/kg (oral) induced an increase in the incidence of abnormal sperm on day 42 and 84 ($p<0.05$). Numerous hookless sperm were seen mostly on day 42 (Table 4.33), whereas sperm with broken tail were more on

day 84. In PQ 5mg/kg (oral) treated rats, the percentage of abnormal sperm were elevated at all sample period ($p<0.05$) with a highest abnormality percent recorded on day 42 (Table 4.34). Sperm with microcephaly, cephalo-caudal junction defect, hookless and banana-shaped were the predominant types seen on day 42. In contrast to the head defects, the tail deformities were found strikingly on day 105.

Treatment of PQ 20mg/kg induced the formation of abnormal sperm at all sample period with the highest percent recorded on day 42 ($p<0.05$) (Table 4.35). Sperm with hookless and cephalo-caudal junction defects were numerous on day 42, whereas the tail deformities were found mostly on day 105. All data were illustrated in Chart 4.26.

In dermal treatment of PQ 6mg/kg, percentage of abnormal sperm were elevated significantly ($p<0.05$) at all sample period, with the highest percent abnormality recorded on day 28 (Table 4.36). Hookless sperm were found mostly on day 14, whereas cephalo-caudal junction defect were seen mostly on day 7. Broken tail abnormalities were found predominantly among other tail deformities on day 42.

In dermal treament of PQ 15mg/kg, the incidence of abnormal sperm were elevated significantly ($p<0.05$) at all sample period (Table 4.37), with the highest percent abnormality found on day 14. Sperm with hookless, cephalo-caudal junction defect and banana shape were found predominantly, whereas sperm with broken tail was ranked the highest among tail deformities on day 14.

The percent of abnormal sperm in dermal treatment of PQ 30mg/kg were increased significantly ($p<0.05$) at all sample period, with the highest percentage of abnormal sperm found on day 14 (Table 4.38). Hookless forms appeared on day 7 with a hike through day 14. The sperm with broken tail were more on day 28 and 42, whereas the coiled were more on day 14. All data were illustrated in Chart 4.27.

Table 4.31 Percentage of sperm mortality in control and PQ (oral) treated rats. Each treatment at particular time point represents as mean (%) ± SD, n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days	28 days	42 days	84 days	105 days
Control	22.28 % ± 2.54	24.36 % ± 3.24	22.98 % ± 1.56	23.64 % ± 3.38	23.44 % ± 2.21
PQ – 2 mg/kg	23.84 % ± 2.79	24.15 % ± 1.98	23.94 % ± 2.40	24.66 % ± 2.06	25.80 % ± 2.52
PQ – 5 mg/kg	23.73 % ± 2.02	27.85 % ± 2.18	24.76 % ± 2.16	23.52 % ± 1.90	24.63 % ± 2.40
PQ – 20 mg/kg	24.75 % ± 2.65	29.46 %* ± 2.36	28.47 %* ± 2.08	24.41 % ± 1.50	23.46 % ± 2.22

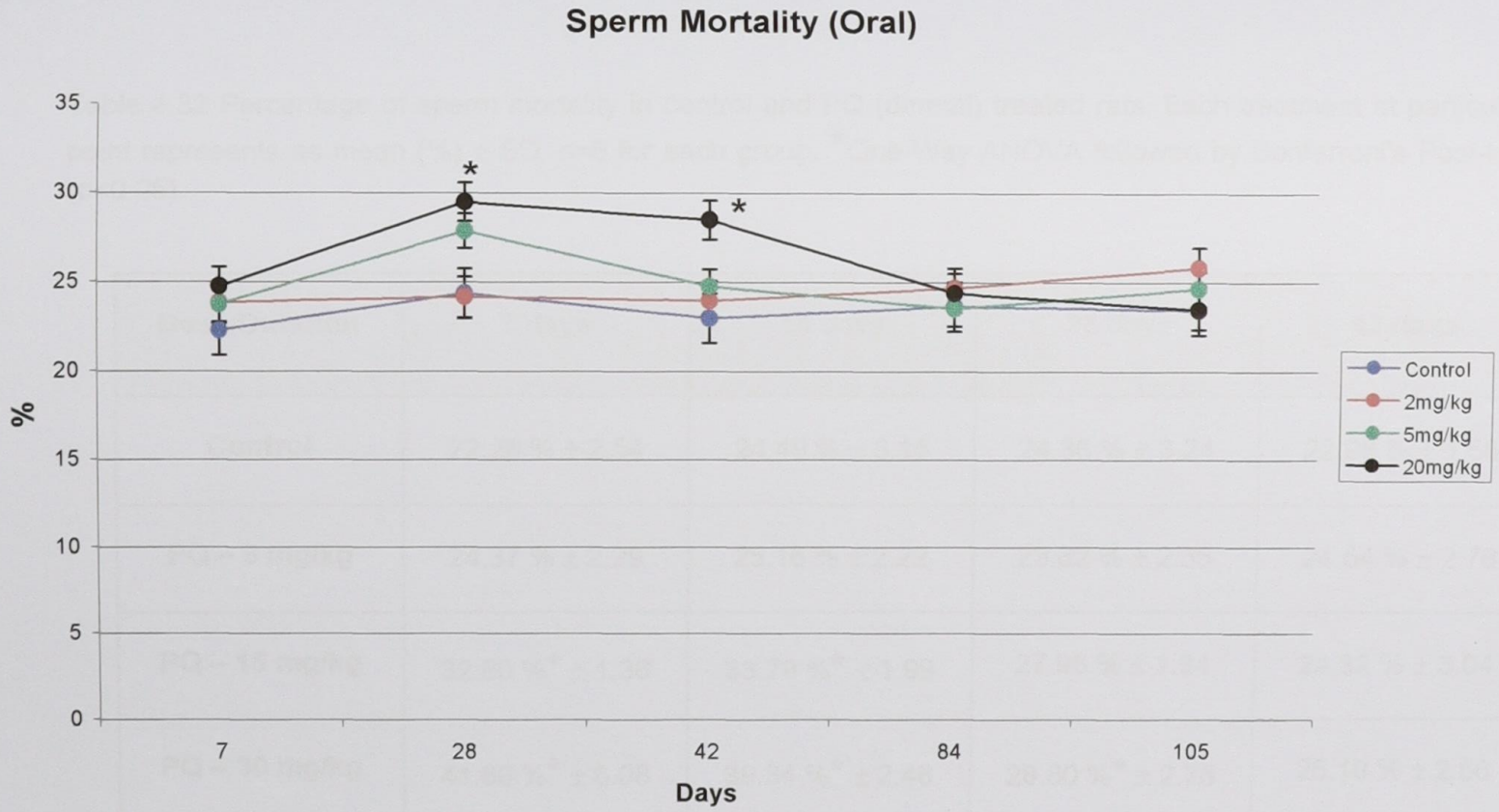


Figure 4.25 Time – response relationship - Percentage (%) sperm mortality in control and PQ (Oral) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.32 Percentage of sperm mortality in control and PQ (dermal) treated rats. Each treatment at particular time point represents as mean (%) ± SD, n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose/Duration	7 days	14 days	28 days	42 days
Control	22.28 % ± 2.54	24.49 % ± 3.15	24.36 % ± 3.24	22.98 % ± 1.56
PQ – 6 mg/kg	24.37 % ± 2.29	25.16 % ± 2.22	23.82 % ± 2.36	24.54 % ± 2.76
PQ – 15 mg/kg	32.80 %* ± 1.36	33.79 %* ± 1.99	27.98 % ± 1.84	24.32 % ± 3.04
PQ – 30 mg/kg	41.60 %* ± 6.08	39.34 %* ± 2.48	28.80 %* ± 2.78	25.19 % ± 2.56

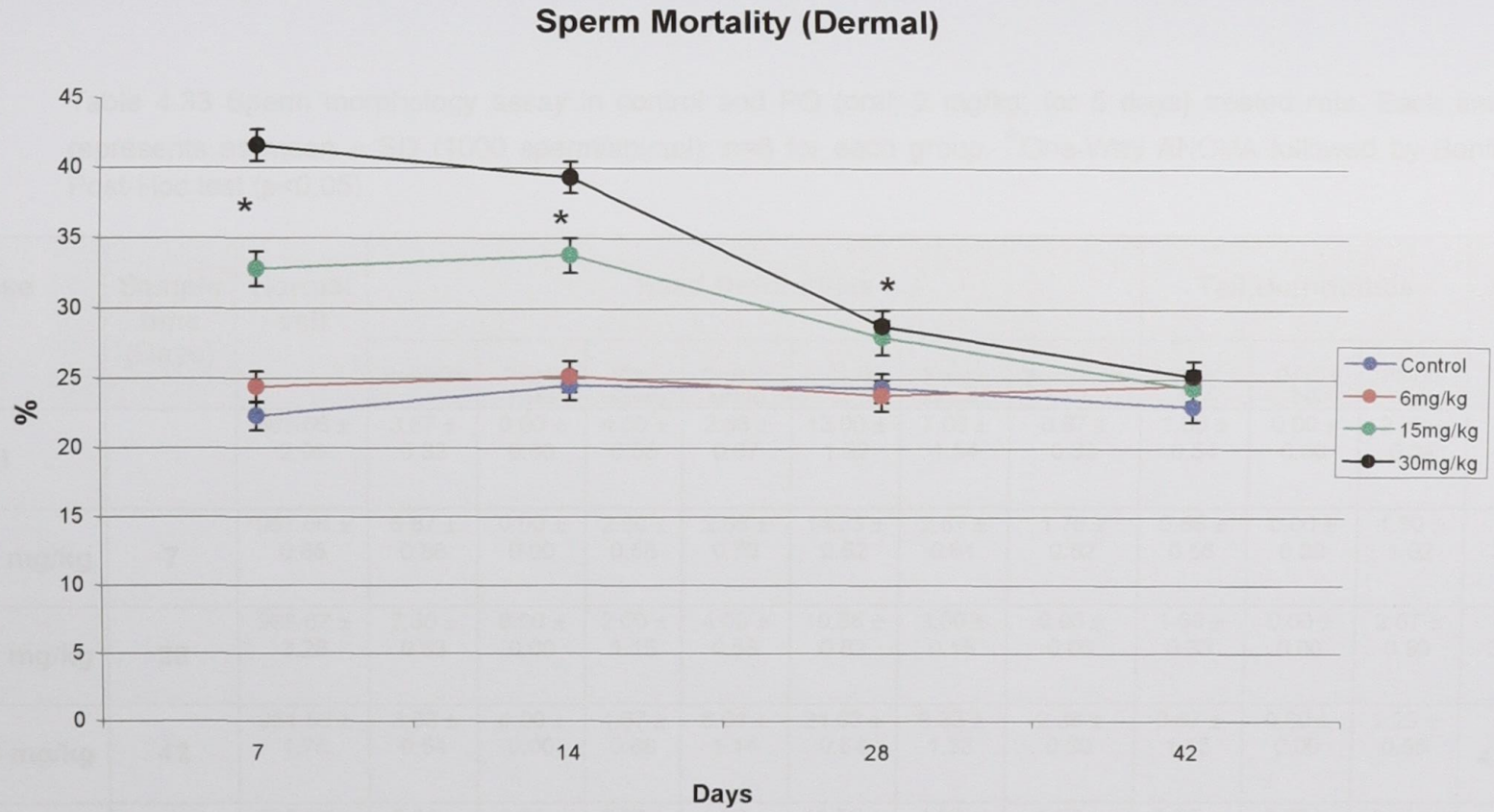


Figure 4.26 Time – response relationship - Percentage (%) sperm mortality in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.33 Sperm morphology assay in control and PQ (oral; 2 mg/kg; for 5 days) treated rats. Each time point represents as mean ± SD (1000 sperm/animal), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose	Sample time (Days)	Normal cell	Head Deformities							Tail Deformities			Abnormal Cells (%)
			Headless	Double Head	Micro cephaly	Cephalo caudal	Hookless	Banana shaped	Amorphous	Coiled Tail	Double Tail	Broken Tail	
Control		965.66 ± 2.96	3.67 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
PQ – 2 mg/kg	7	967.66 ± 0.88	6.67 ± 0.88	0.00 ± 0.00	2.00 ± 0.58	2.66 ± 0.73	14.33 ± 0.62	2.67 ± 0.61	1.76 ± 0.82	0.66 ± 0.56	0.00 ± 0.00	1.30 ± 1.02	3.32 ± 1.78
PQ – 2 mg/kg	28	968.67 ± 3.28	7.30 ± 0.33	0.00 ± 0.00	2.00 ± 1.15	4.00 ± 0.58	10.68 ± 0.82	3.00 ± 0.15	0.00 ± 0.00	1.68 ± 0.33	0.00 ± 0.00	2.67 ± 0.89	3.42 ± 1.84
PQ – 2 mg/kg	42	951.86 ± 1.76	3.03 ± 0.54	0.00 ± 0.00	4.67 ± 0.68	6.01 ± 1.14	21.03 ± 0.54	8.33 ± 1.33	2.36 ± 0.30	0.67 ± 1.15	0.00 ± 0.00	2.23 ± 0.58	4.72* ± 1.92
PQ – 2 mg/kg	84	965.33 ± 1.66	5.01 ± 0.58	0.00 ± 0.00	3.03 ± 0.58	4.32 ± 0.82	13.33 ± 0.80	4.04 ± 1.53	1.52 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	3.68 ± 0.63	3.92* ± 2.78
PQ – 2 mg/kg	105	964.37 ± 3.33	6.68 ± 0.88	0.00 ± 0.00	3.00 ± 1.52	2.34 ± 0.89	13.32 ± 1.22	3.05 ± 0.88	1.56 ± 0.53	1.76 ± 0.89	0.00 ± 0.00	2.63 ± 0.58	3.52 ± 1.74

Table 4.34 Sperm Morphology assay in control and PQ (oral; 5 mg/kg; for 5 days) treated rats. Data values were presented as mean ± SD (1000 sperm/animal); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose	Sample time (Days)	Normal cell	Head Deformities							Tail Deformities			Abnormal Cells (%)
			Headless	Double Head	Micro cephaly	Cephalo caudal	Hookless	Banana shaped	Amorphous	Coiled Tail	Double Tail	Broken Tail	
Control		965.66 ± 2.96	3.67 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
PQ–5 mg/kg	**7**	956.34 ± 4.06	7.00 ± 0.58	0.33 ± 0.34	2.00 ± 1.16	4.68 ± 1.29	14.02 ± 1.73	7.68 ± 0.68	2.00 ± 0.33	2.33 ± 1.21	0.00 ± 0.00	3.68 ± 0.58	4.28* ±1.72
PQ–5 mg/kg	**28**	951.63 ± 1.76	9.33 ± 0.88	0.00 ± 0.00	2.02 ± 0.58	5.52 ± 0.54	19.76 ± 0.35	7.01 ± 2.64	2.34 ± 0.80	2.02 ± 1.17	0.00 ± 0.00	2.28 ± 1.73	4.70* ±1.74
PQ–5 mg/kg	**42**	938.02 ± 0.58	3.34 ± 0.54	1.33 ± 0.66	4.67 ± 0.82	13.30 ± 0.58	22.01 ± 0.54	9.66 ± 1.57	3.00 ± 0.51	2.21 ± 1.16	0.00 ± 0.00	3.80 ± 1.73	6.26* ±1.06
PQ–5 mg/kg	**84**	957.33 ± 3.38	7.01 ± 0.54	0.00 ± 0.00	3.67 ± 0.88	5.11 ± 1.16	15.01 ± 0.58	3.33 ± 0.58	2.67 ± 0.60	2.67 ± 0.33	0.00 ± 0.00	3.33 ± 0.35	4.98* ±4.44
PQ–5 mg/kg	**105**	952.67 ± 2.61	8.00 ± 0.54	0.00 ± 0.00	3.34 ± 0.81	5.30 ± 0.67	15.33 ± 1.21	4.67 ± 0.34	2.88 ± 0.62	3.67 ± 0.55	0.00 ± 0.00	4.35 ± 0.56	4.32* ±3.62

Table 4.35 Sperm Morphology assay in control and PQ (oral; 20 mg/kg; for 5 days) treated rats. Data values were presented as mean ± SD (1000 sperm/animal), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose	Sample time (Days)	Normal cell	Head Deformities							Tail Deformities			Abnormal Cells (%)
			Headless	Double Head	Micro cephaly	Cephalo caudal	Hookless	Banana shaped	Amorphous	Coiled Tail	Double Tail	Broken Tail	
Control		965.66 ± 2.96	3.67 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
PQ-20 mg/kg	7	960.23 ± 2.33	8.03 ± 0.54	0.00 ± 0.00	3.32 ± 0.85	4.68 ± 1.52	14.04 ± 0.54	4.33 ± 0.23	2.36 ± 0.81	1.68 ± 0.22	0.00 ± 0.00	1.68 ± 0.44	4.88* ±1.28
PQ-20 mg/kg	28	945.78 ± 5.17	7.07 ± 0.58	2.01 ± 0.11	3.34 ± 0.65	7.69 ± 1.47	21.03 ± 0.55	5.33 ± 0.39	2.20 ± 1.40	2.12 ± 1.15	0.66 ± 0.33	3.03 ± 0.45	5.44* ±2.78
PQ-20 mg/kg	42	931.66 ± 3.71	2.66 ± 1.30	1.68 ± 0.35	6.33 ± 1.54	14.03 ± 1.47	23.32 ± 1.03	11.68 ± 1.35	1.78 ± 0.88	1.56 ± 0.52	0.66 ± 0.26	5.52 ± 1.73	6.48* ±2.64
PQ-20 mg/kg	84	963.31 ± 1.45	5.34 ± 0.35	0.00 ± 0.00	2.33 ± 0.37	4.04 ± 0.54	14.83 ± 0.66	3.35 ± 0.35	0.68 ± 1.15	2.23 ± 0.56	0.00 ± 0.00	4.43 ± 0.98	5.72* ±2.78
PQ-20 mg/kg	105	944.11 ± 4.97	6.68 ± 1.20	0.68 ± 1.45	4.33 ± 0.89	6.33 ± 0.88	19.68 ± 1.45	5.11 ± 0.51	3.33 ± 1.54	4.02 ± 1.58	0.68 ± 0.26	5.00 ± 0.45	5.22* ±3.82

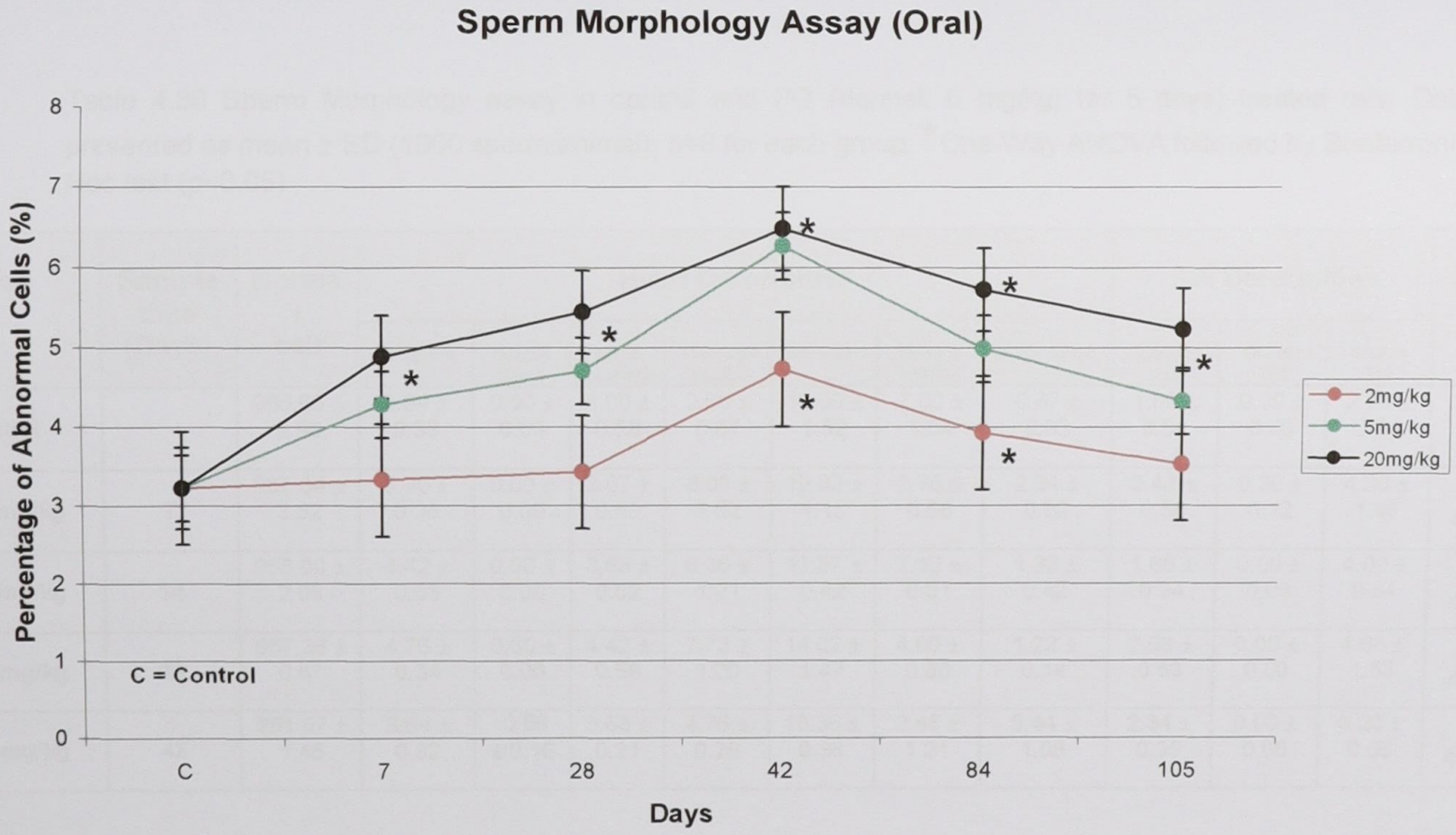


Figure 4.27 Time – response relationship in sperm morphology assay (Percentage of Abnormal cells) in control and PQ (Oral) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were shown as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.36 Sperm Morphology assay in control and PQ (dermal; 6 mg/kg; for 5 days) treated rats. Data were presented as mean ± SD (1000 sperm/animal); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose	Sample time (Days)	Normal cell	Head Deformities							Tail Deformities			Abnormal Cells (%)
			Headless	Double Head	Micro cephaly	Cephalo caudal	Hookless	Banana shaped	Amorphous	Coiled Tail	Double Tail	Broken Tail	
Control		965.66 ± 2.96	3.64 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
PQ-6 mg/kg	7	957.34 ± 3.52	5.35 ± 0.38	0.00 ± 0.00	3.67 ± 0.89	8.03 ± 1.52	12.02 ± 1.15	3.76 ± 0.66	2.34 ± 0.82	3.43 ± 0.58	0.30 ± 0.12	4.36 ± 1.46	4.34* ± 2.66
PQ-6 mg/kg	14	955.00 ± 2.08	4.42 ± 0.58	0.00 ± 0.00	3.68 ± 0.62	6.36 ± 1.21	17.27 ± 0.42	7.12 ± 0.51	1.32 ± 0.42	1.66 ± 0.34	0.00 ± 0.00	4.00 ± 0.54	4.66* ± 2.50
PQ-6 mg/kg	28	957.36 ± 0.67	4.76 ± 0.34	0.00 ± 0.00	4.42 ± 0.58	7.73 ± 1.20	14.02 ± 1.47	4.00 ± 0.50	1.22 ± 0.14	2.68 ± 0.53	0.00 ± 0.00	4.68 ± 1.53	4.84* ± 2.38
PQ-6 mg/kg	42	951.67 ± 1.45	3.64 ± 0.32	0.68 ± 0.16	3.68 ± 0.21	4.76 ± 0.26	18.36 ± 0.36	3.48 ± 1.21	5.44 ± 1.08	2.34 ± 0.35	0.00 ± 0.00	6.23 ± 0.56	4.08* ± 3.68

Table 4.37 Sperm Morphology assay in control and PQ (dermal; 15 mg/kg; for 5 days) treated rats. Data were presented as Mean ± SD (1000 sperm/animal), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Dose	Sample time (Days)	Normal cell	Head Deformities							Tail Deformities			Abnormal Cells (%)
			Headless	Double Head	Micro cephaly	Cephalo caudal	Hookless	Banana shaped	Amorphous	Coiled Tail	Double Tail	Broken Tail	
Control		965.66 ± 2.96	3.67 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
PQ-15 mg/kg	**7**	940.32 ± 5.46	7.64 ± 1.21	2.24 ± 0.36	0.68 ± 1.20	10.88 ± 0.38	22.46 ± 1.14	6.68 ± 0.62	3.68 ± 1.28	2.06 ± 0.18	2.48 ± 0.58	4.78 ± 1.28	5.48* ± 1.66
PQ-15 mg/kg	**14**	935.77 ± 2.84	4.36 ± 0.35	2.08 ± 0.83	5.68 ± 0.84	6.48 ± 0.32	21.82 ± 0.66	9.38 ± 2.80	4.44 ± 1.14	2.66 ± 1.48	2.88 ± 0.28	6.38 ± 0.94	6.38* ± 1.92
PQ-15 mg/kg	**28**	949.02 ± 1.15	7.66 ± 0.35	1.48 ± 0.58	4.06 ± 0.60	5.38 ± 0.58	16.88 ± 1.52	2.36 ± 0.54	3.66 ± 0.34	2.63 ± 0.38	1.68 ± 0.22	6.06 ± 1.16	5.54* ± 2.48
PQ-15 mg/kg	**42**	942.03 ± 0.58	6.02 ± 0.51	1.34 ± 0.58	5.12 ± 0.23	5.02 ± 0.54	19.66 ± 1.45	5.00 ± 1.75	3.00 ± 0.34	2.54 ± 0.32	3.34 ± 0.62	7.02 ± 0.89	5.02* ± 2.32

Table 4.38 Sperm Morphology assay in control and PQ (dermal; 30 mg/kg; for 5 days) treated rats. Data were presented as Mean ± SD (1000 sperm/animal), n=6 for each group. *One-Way ANOVA followed by Bonferroni's Poat-Hoc test ($p<0.05$).

Dose	Sample time	Normal cell	Head Deformities							Tail Deformities			Abnormal Cells
			Headless	Double Head	Micro cephaly	Cephalo caudal	Hookless	Banana shaped	Amorphous	Coiled Tail	Double Tail	Broken Tail	
Control		965.66 ± 2.96	3.67 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
PQ-30 mg/kg	7	943.67 ± 2.02	5.88 ± 0.68	1.66 ± 0.12	3.33 ± 1.22	9.08 ± 2.08	17.66 ± 0.89	6.28 ± 0.34	2.44 ± 0.26	1.36 ± 0.34	1.33 ± 1.20	4.36 ± 0.88	5.64* ± 2.98
PQ-30 mg/kg	14	933.07 ± 2.66	4.62 ± 0.36	3.34 ± 1.16	4.88 ± 0.82	9.68 ± 1.34	24.22 ± 0.52	6.22 ± 0.98	3.34 ± 0.88	3.68 ± 0.68	3.08 ± 0.54	4.28 ± 1.86	6.68* ± 1.68
PQ-30 mg/kg	28	953.35 ± 2.41	4.66 ± 0.33	0.00 ± 0.00	4.48 ± 0.82	4.24 ± 0.58	17.04 ± 1.58	3.66 ± 0.82	4.33 ± 0.58	2.68 ± 0.66	0.68 ± 1.02	4.66 ± 1.15	5.68* ± 2.34
PQ-30 mg/kg	42	937.06 ± 3.78	5.58 ± 0.36	2.02 ± 0.26	5.52 ± 1.74	6.78 ± 0.58	21.22 ± 0.54	2.68 ± 0.36	4.48 ± 0.78	3.08 ± 0.34	3.36 ± 0.14	6.38 ± 0.88	4.52* ± 1.59

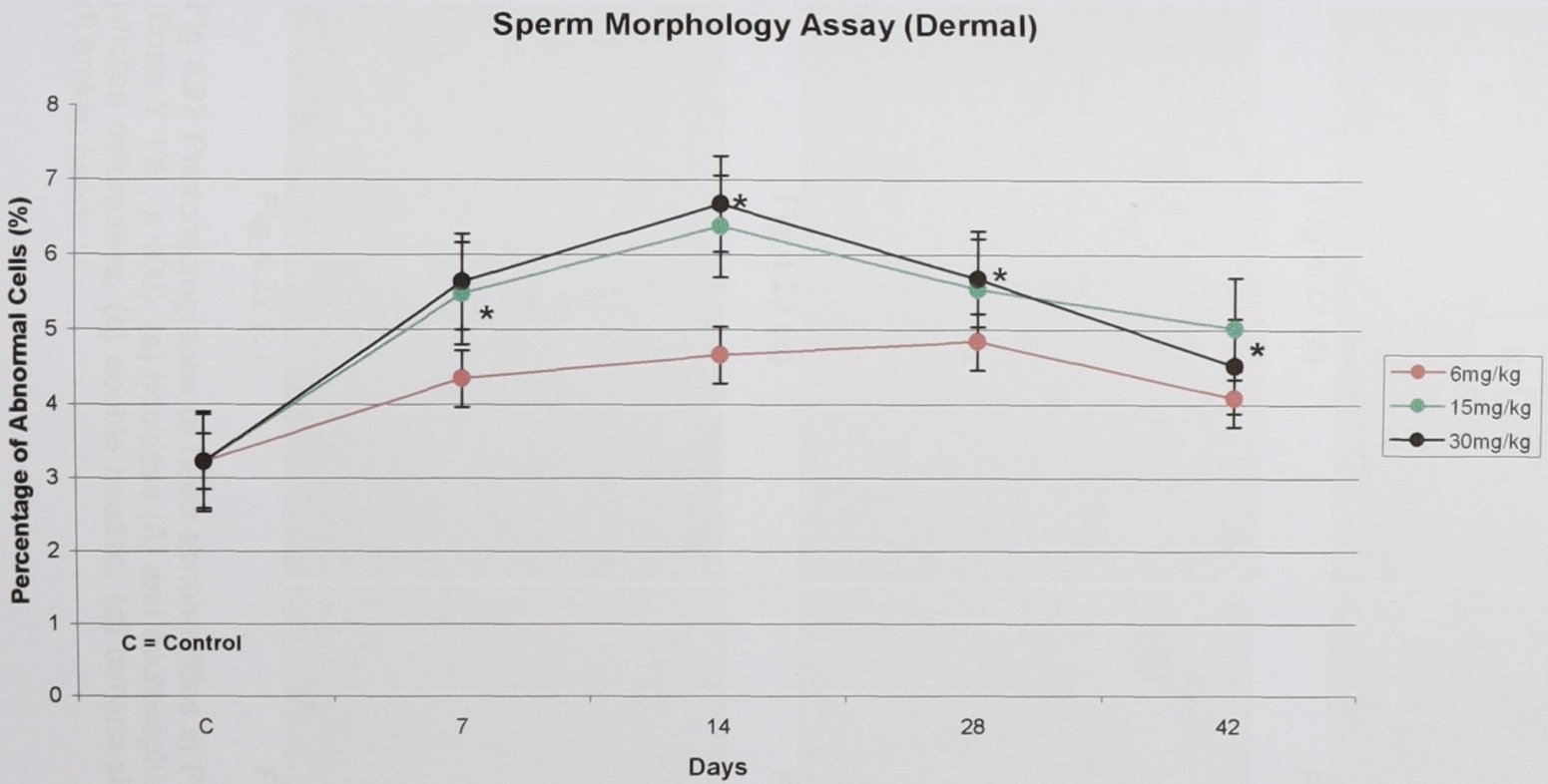


Figure 4.28 Time – response relationship in sperm morphology assay (Percentage of Abnormal cells) in control and PQ (Dermal) treated rats. The mean difference is significant at the level of $p<0.05^{*}$. Data were shown as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

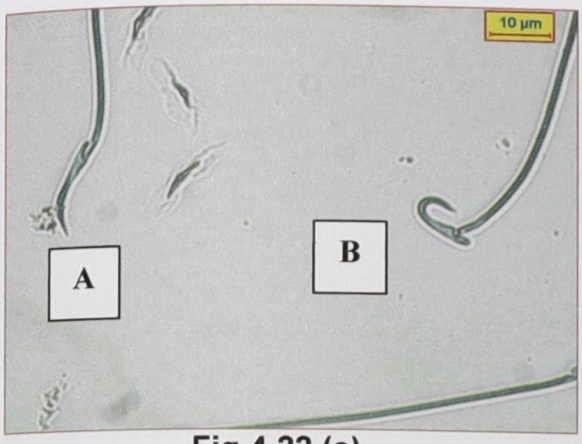


Fig 4.22 (a)

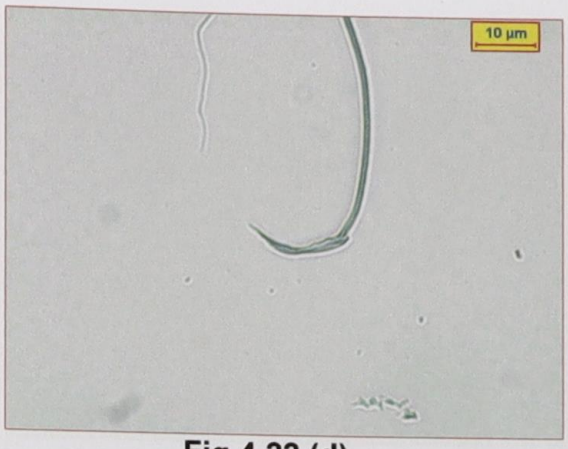


Fig 4.22 (d)

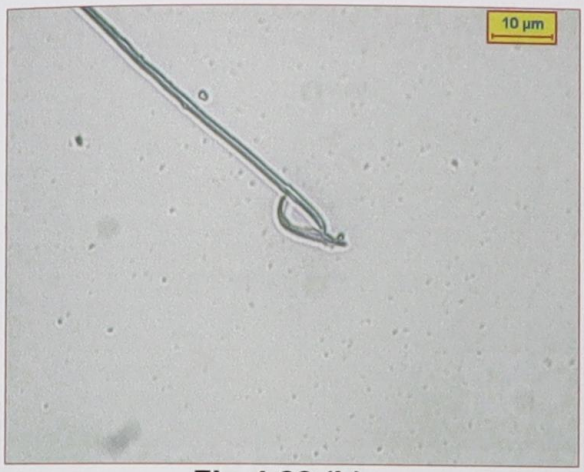


Fig 4.22 (b)

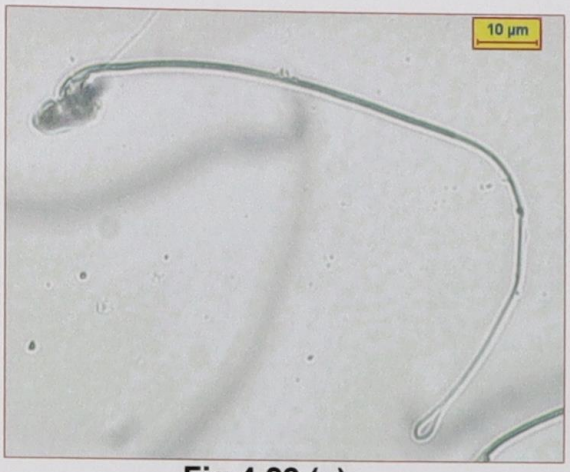


Fig 4.22 (e)



Fig 4.22 (c)

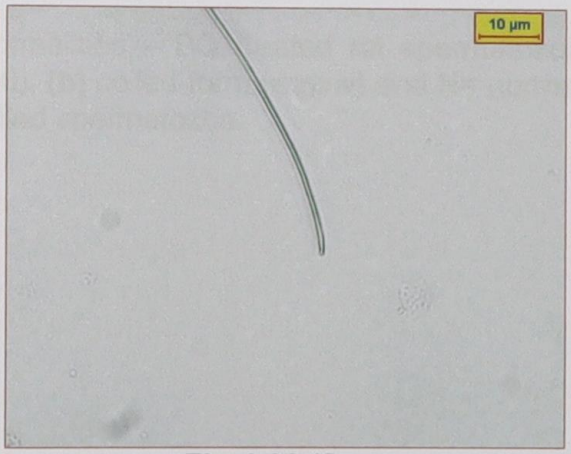


Fig 4.22 (f)

Fig 4.22 Photomicrographs of head abnormalities in PQ treated rat spermatozoa (Eosin Y 1% ; x 400), **(a)** hookless **(A)** and microcephaly **(B)**, **(b)** cephalo caudal-junction deformities, **(c)** double headed, **(d)** banana-shaped, **(e)** amorphous and **(f)** broken head.

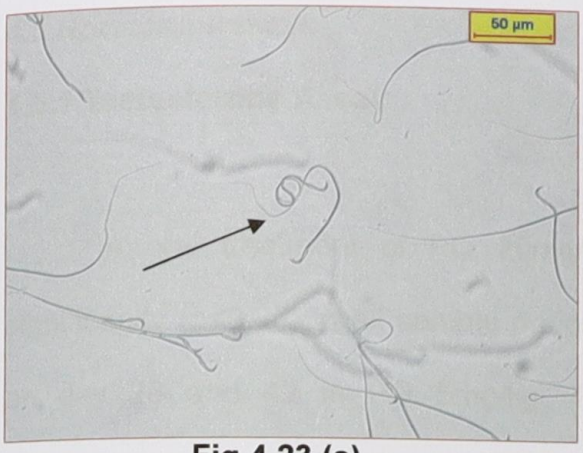


Fig 4.23 (a)



Fig 4.23 (c)

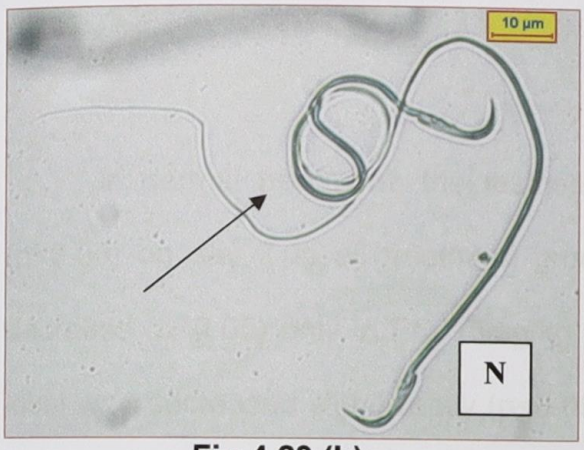


Fig 4.23 (b)



Fig 4.23 (d)

Fig 4.23 Photomicrographs of tail abnormalities – PQ treated rat spermatozoa (Eosin Y 1%; x 400), **(a)** coiled-form (x100), **(b)** coiled-form **(arrow)** and **N**= normal spermatozoa, **(c)** bent, and **(d)** double-tailed spermatozoa.

4.5 Hormone Assays

4.5.1 Testosterone Assay

In oral treatment of PQ 20mg/kg, testosterone level were decreased significantly ($p<0.05$) in all sample period and also significantly different ($p<0.05$) on day 28 and 42 in PQ 5mg/kg. There were no significant difference of testosterone level in PQ 2mg/kg dose (Table 4.39). All data were illustrated in Chart 4.28.

In dermal treatment, the testosterone level were decreased significantly ($p<0.05$) on day 7 in all treatment groups. On day 14, it showed a significant decrease ($p<0.05$) only in PQ 15mg/kg and 30mg/kg. However, the testosterone level was decreased significantly ($p<0.05$) on day 28 only for PQ 30mg/kg (Table 4.40). All data were illustrated in Chart 4.29.

4.5.2 Follicle Stimulating Hormone (FSH) Assay

In oral treatment, the FSH level was decreased significantly ($p<0.05$) at all sample period in PQ 20mg/kg. A significant difference was also observed ($p<0.05$) on day 28, 84, and 105 in PQ 5mg/kg. There was no significant difference in FSH level in PQ 2mg/kg (Table 4.41). All data were illustrated in Chart 4.30. In dermal treatment, the FSH level decreased significantly ($p<0.05$) on all sample period in

PQ 30mg/kg, whereas a significant decrease was observed ($p<0.05$) on day 7 in PQ 15mg/kg (Table 4.42). All data were illustrated in Chart 4.31.

4.5.3 Luteinizing Hormone (LH) Assay

The LH level was decreased significantly ($p<0.05$) at all sample period in PQ 20mg/kg and also on day 105 in PQ 5mg/kg (Table 4.43). There was no significant difference in LH level in PQ 2mg/kg dose. All data were shown in Chart 4.32. In dermal treatment, the LH level decreased significantly ($p<0.05$) only on day 7 and 14 in PQ 30mg/kg dose (Table 4.44). All data were shown in Chart 4.33.

4.5.4 Prolactin Assay

In oral treatment of PQ 20mg/kg, prolactin level decreased significantly ($p<0.05$) in all sample period except on day 7. In PQ 5mg/kg, it was decreased significantly ($p<0.05$) on day 42, 84 and 105. There were no significant difference in prolactin level in PQ 2mg/kg (Table 4.45). All data were illustrated in Chart 4.34.

In dermal treatment of PQ 30mg/kg, prolactin level decreased significantly ($p<0.05$) at all sample period except on day 7. In PQ 15mg/kg, it was decreased significantly ($p<0.05$) on day 14. There was no significant difference in prolactin level in PQ 6mg/kg (Table 4.46). All data were illustrated in Chart 4.35.

Table 4.39 Effect of PQ (oral) on testosterone level. Each data at particular time point represents as Mean ± SD (nmol/L); n=6 for each group. *One-way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	29.70 ± 1.87	30.21 ± 1.86	29.85 ± 3.04	28.92 ± 1.48	31.56 ± 1.22
PQ – 2 mg/kg	29.92 ± 0.92	29.82 ± 2.28	29.86 ± 1.08	30.82 ± 3.04	32.60 ± 1.86
PQ – 5 mg/kg	28.52 ± 0.68	22.88* ± 1.96	24.34* ± 0.82	27.88 ± 2.34	31.66 ± 2.26
PQ – 20 mg/kg	21.56* ± 2.36	22.78* ± 5.16	23.66* ± 2.02	24.98* ± 2.78	28.78* ± 0.82

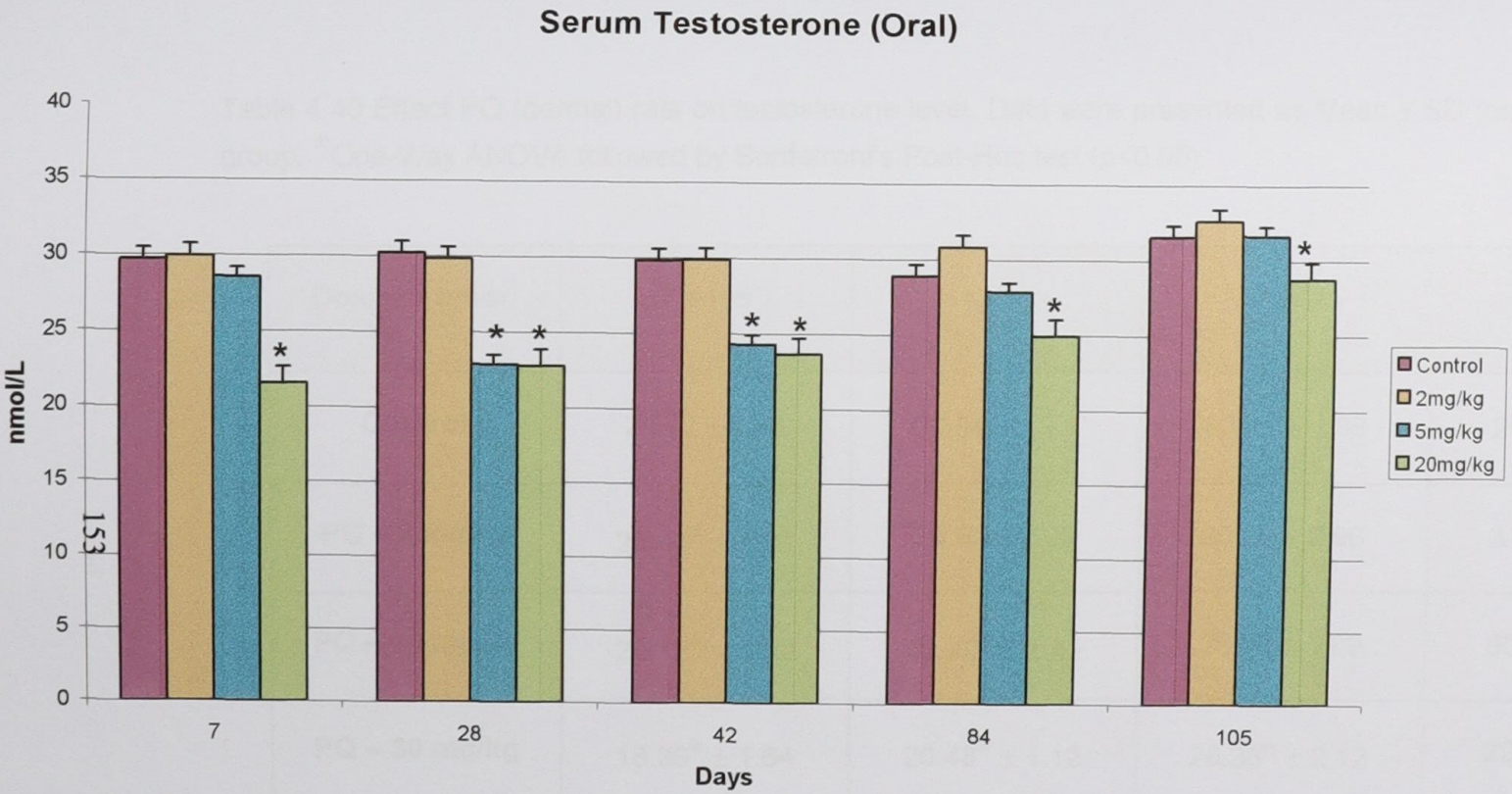


Figure 4.29 Time – response relationship of PQ (Oral) rats on Testosterone level. The mean difference is significant at the level of $p<0.05$*. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.40 Effect PQ (dermal) rats on testosterone level. Data were presented as Mean ± SD (nmol/L); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	29.72 ± 1.88	30.54 ± 1.74	30.21 ± 1.86	29.85 ± 1.08
PQ – 6 mg/kg	25.44* ± 1.22	29.82 ± 2.02	30.52 ± 0.96	31.38 ± 1.44
PQ – 15 mg/kg	20.12* ± 1.02	23.22* ± 1.42	28.98 ± 1.09	30.14 ± 1.43
PQ – 30 mg/kg	18.36* ± 1.64	20.48* ± 1.12	26.38* ± 2.12	29.06 ± 2.64

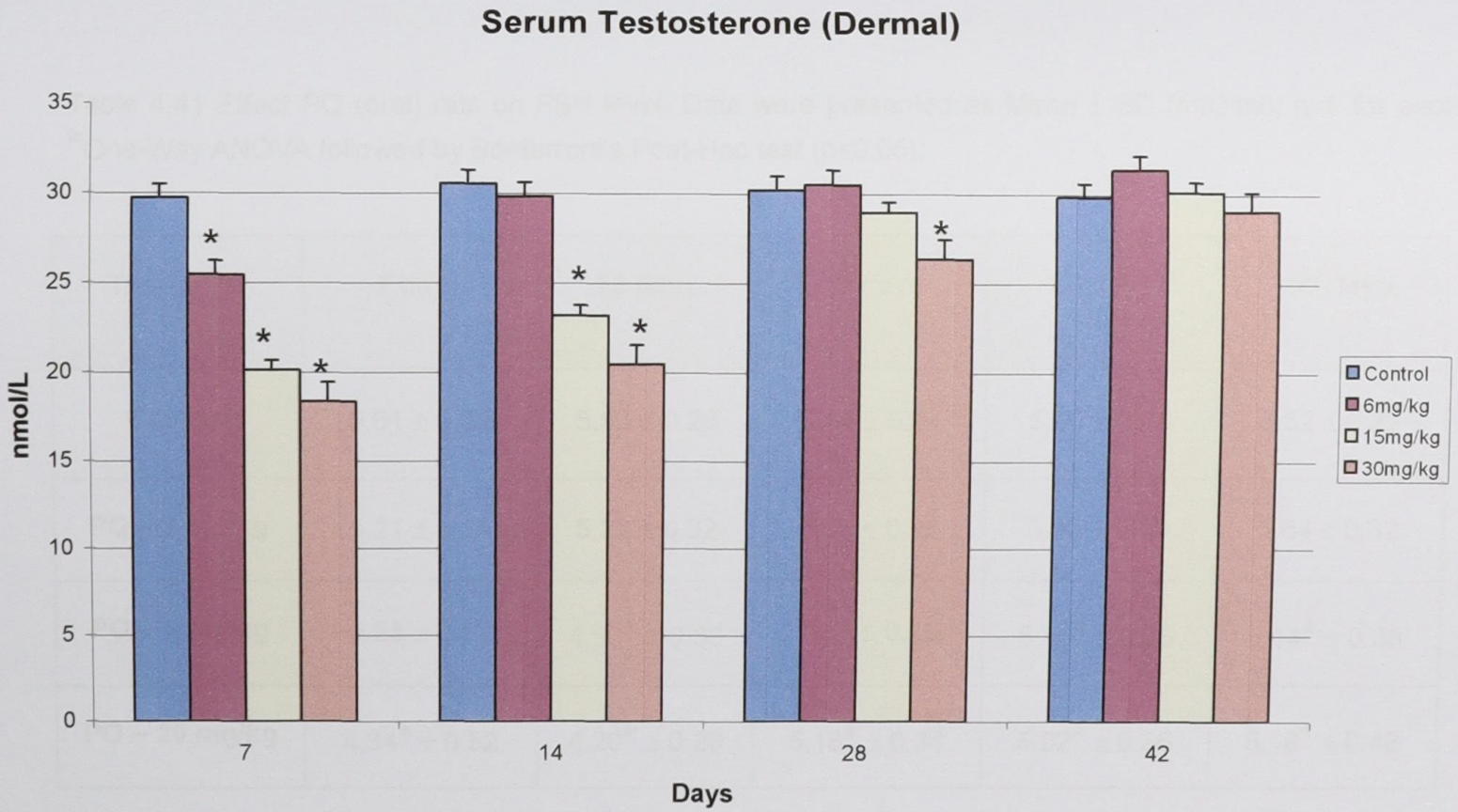


Figure 4.30 Time – response relationship of PQ (Dermal) rats on Testosterone level. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.41 Effect PQ (oral) rats on FSH level. Data were presented as Mean ± SD (mIU/ml); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	5.61 ± 0.32	5.63 ± 0.28	5.44 ± 0.24	5.60 ± 0.28	5.52 ± 0.22
PQ – 2 mg/kg	5.21 ± 0.14	5.22 ± 0.32	5.56 ± 0.22	5.32 ± 0.53	5.64 ± 0.32
PQ – 5 mg/kg	5.28 ± 0.12	4.96* ± 0.38	5.36 ± 0.16	5.14* ± 0.26	5.14* ± 0.38
PQ – 20 mg/kg	4.34* ± 0.52	4.28* ± 0.28	5.18* ± 0.34	5.02* ± 0.36	5.18* ± 0.48

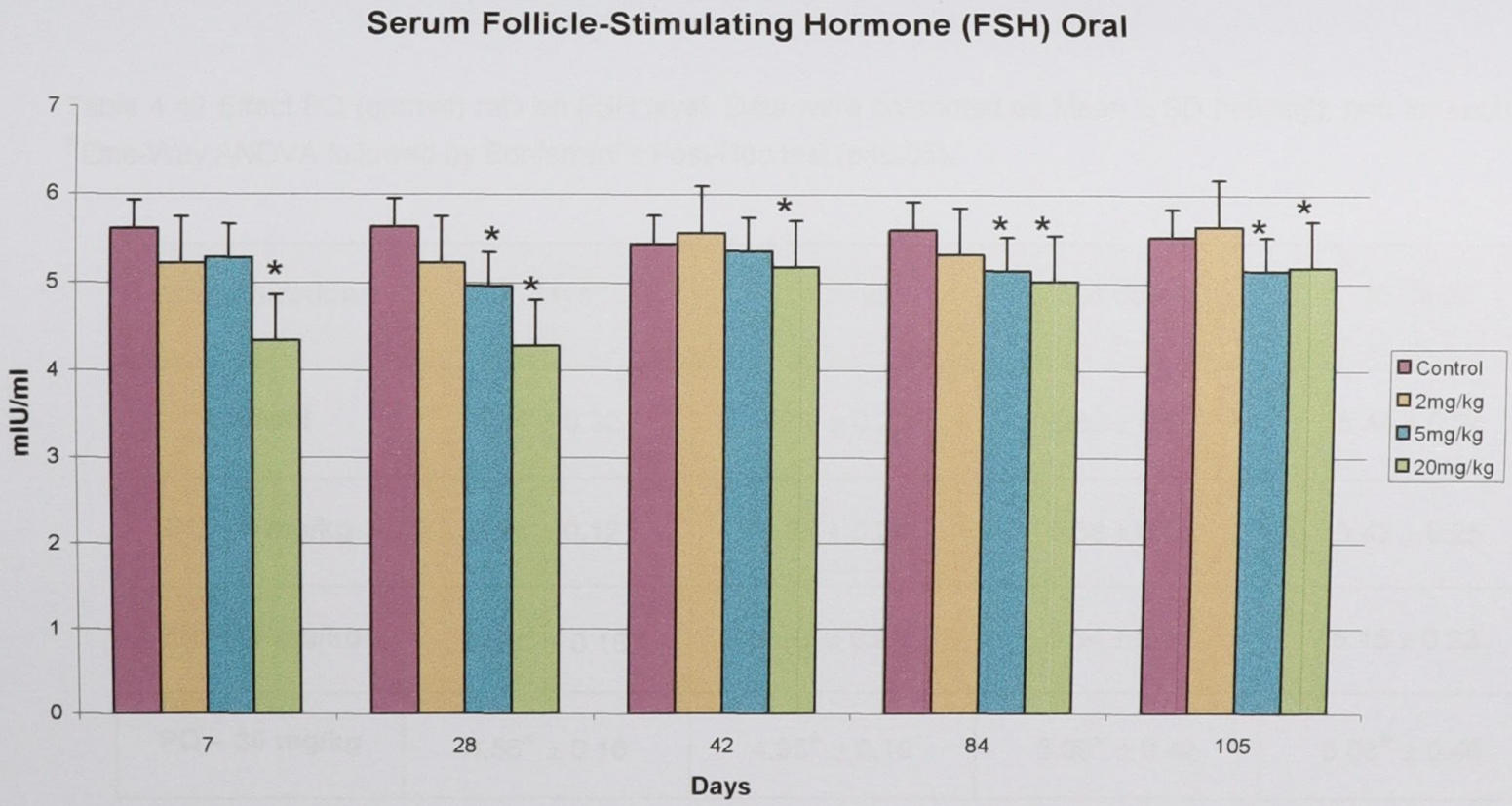


Figure 4.31 Time – response relationship of PQ (oral) rats on FSH level. The mean difference is significant at the level of $p<0.05$*. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.42 Effect PQ (dermal) rats on FSH level. Data were presented as Mean ± SD (mIU/ml); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	5.61 ± 0.32	5.66 ± 0.22	5.63 ± 0.28	5.44 ± 0.24
PQ – 6 mg/kg	5.34 ± 0.12	5.29 ± 0.24	5.38 ± 0.16	5.42 ± 0.25
PQ – 15 mg/kg	5.20* ± 0.18	5.38 ± 0.22	5.34 ± 0.18	5.15 ± 0.23
PQ – 30 mg/kg	4.56* ± 0.16	4.95* ± 0.19	5.09* ± 0.42	5.03* ± 0.46

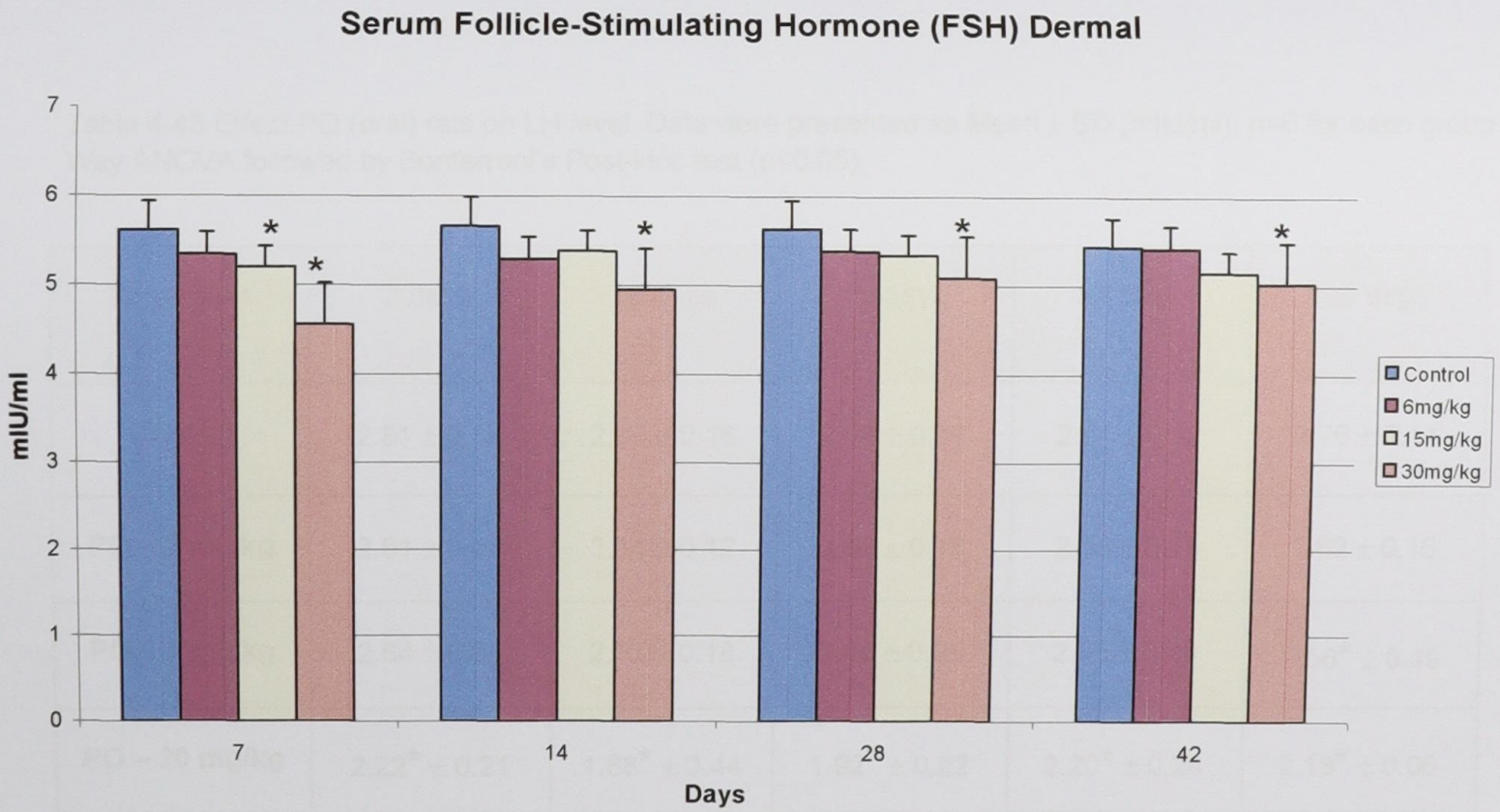


Figure 4.32 Time – response relationship of PQ (Dermal) rats on FSH level. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.43 Effect PQ (oral) rats on LH level. Data were presented as Mean ± SD (mIU/ml); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	2.81 ± 0.16	2.60 ± 0.16	2.58 ± 0.09	2.51 ± 0.12	2.76 ± 0.11
PQ – 2 mg/kg	2.61 ± 0.08	3.14 ± 0.12	2.86 ± 0.18	2.66 ± 0.26	2.82 ± 0.16
PQ – 5 mg/kg	2.64 ± 0.06	2.57 ± 0.18	2.48 ± 0.08	2.65 ± 0.20	2.56* ± 0.19
PQ – 20 mg/kg	2.22* ± 0.21	1.88* ± 0.44	1.92* ± 0.22	2.20* ± 0.24	2.18* ± 0.06

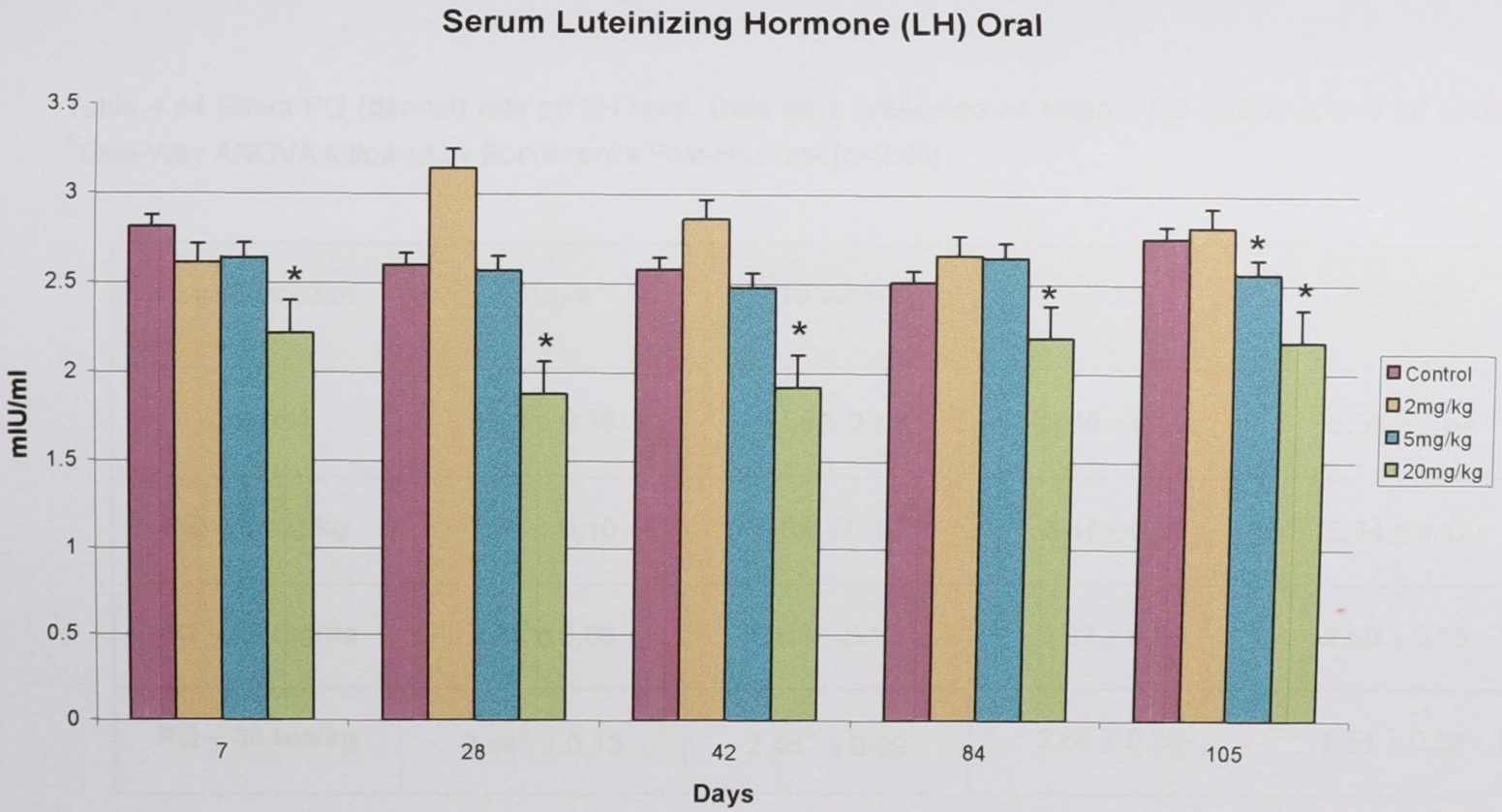


Figure 4.33 Time – response relationship of PQ (Oral) rats on LH level. The mean difference is significant at the level of $p<0.05$*. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.44 Effect PQ (dermal) rats on LH level. Data were presented as Mean ± SD (mIU/ml); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	2.64 ± 0.16	2.58 ± 0.15	2.60 ± 0.18	2.58 ± 0.94
PQ – 6 mg/kg	2.56 ± 0.10	2.64 ± 0.12	2.64 ± 0.08	2.54 ± 0.12
PQ – 15 mg/kg	2.62 ± 0.08	2.62 ± 0.17	2.67 ± 0.09	2.60 ± 0.16
PQ – 30 mg/kg	2.48* ± 0.13	2.48* ± 0.09	2.66 ± 0.20	2.51 ± 0.22

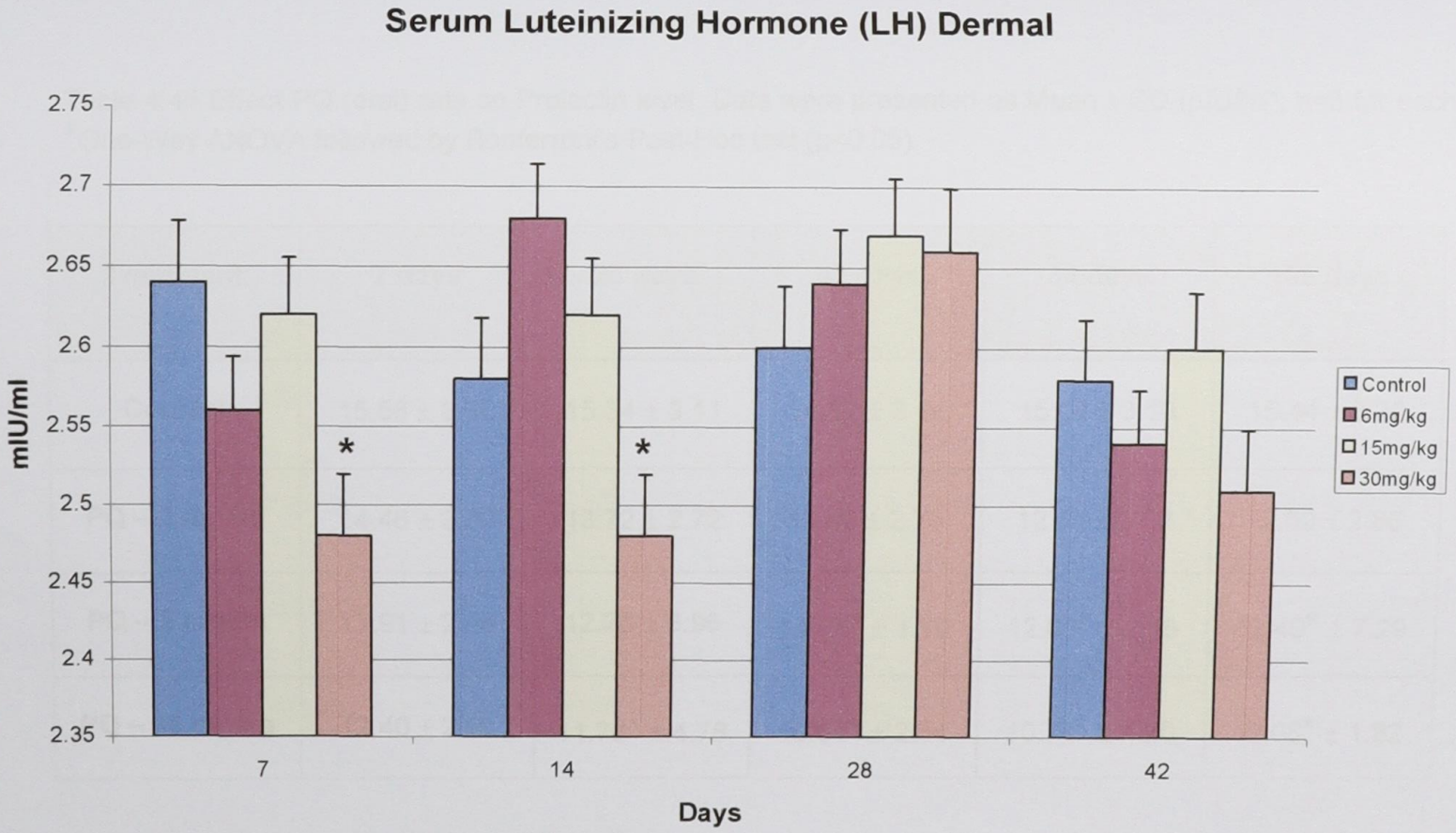


Figure 4.34 Time – response relationship of PQ (Dermal) rats on LH level. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.45 Effect PQ (oral) rats on Prolactin level. Data were presented as Mean ± SD (μIU/ml); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	15.58 ± 3.90	15.34 ± 3.11	14.52 ± 3.44	15.02 ± 3.10	15.44 ± 3.20
PQ – 2 mg/kg	14.46 ± 3.32	13.72 ± 2.72	12.46 ± 2.76	12.91 ± 5.32	12.52 ± 2.86
PQ – 5 mg/kg	13.91 ± 2.88	12.26 ± 3.96	11.77* ± 1.70	12.68* ± 2.18	12.40* ± 7.29
PQ – 20 mg/kg	12.40 ± 2.56	11.78* ± 4.78	10.64* ± 2.54	10.71* ± 4.20	12.08* ± 1.82

Table 4.46 Effect PQ (dermal) rats on Prolactin level. Data were presented as Mean ± SD (μIU/ml); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	15.58 ± 3.90	15.88 ± 1.76	15.34 ± 3.11	14.52 ± 3.44
PQ – 6 mg/kg	16.08 ± 2.72	12.38 ± 1.48	14.92 ± 3.78	14.68 ± 4.08
PQ – 15 mg/kg	15.77 ± 1.08	10.88* ± 3.44	14.57 ± 2.79	13.72 ± 1.02
PQ – 30 mg/kg	14.88 ± 2.82	10.02* ± 5.62	10.56* ± 2.38	10.07* ± 0.74

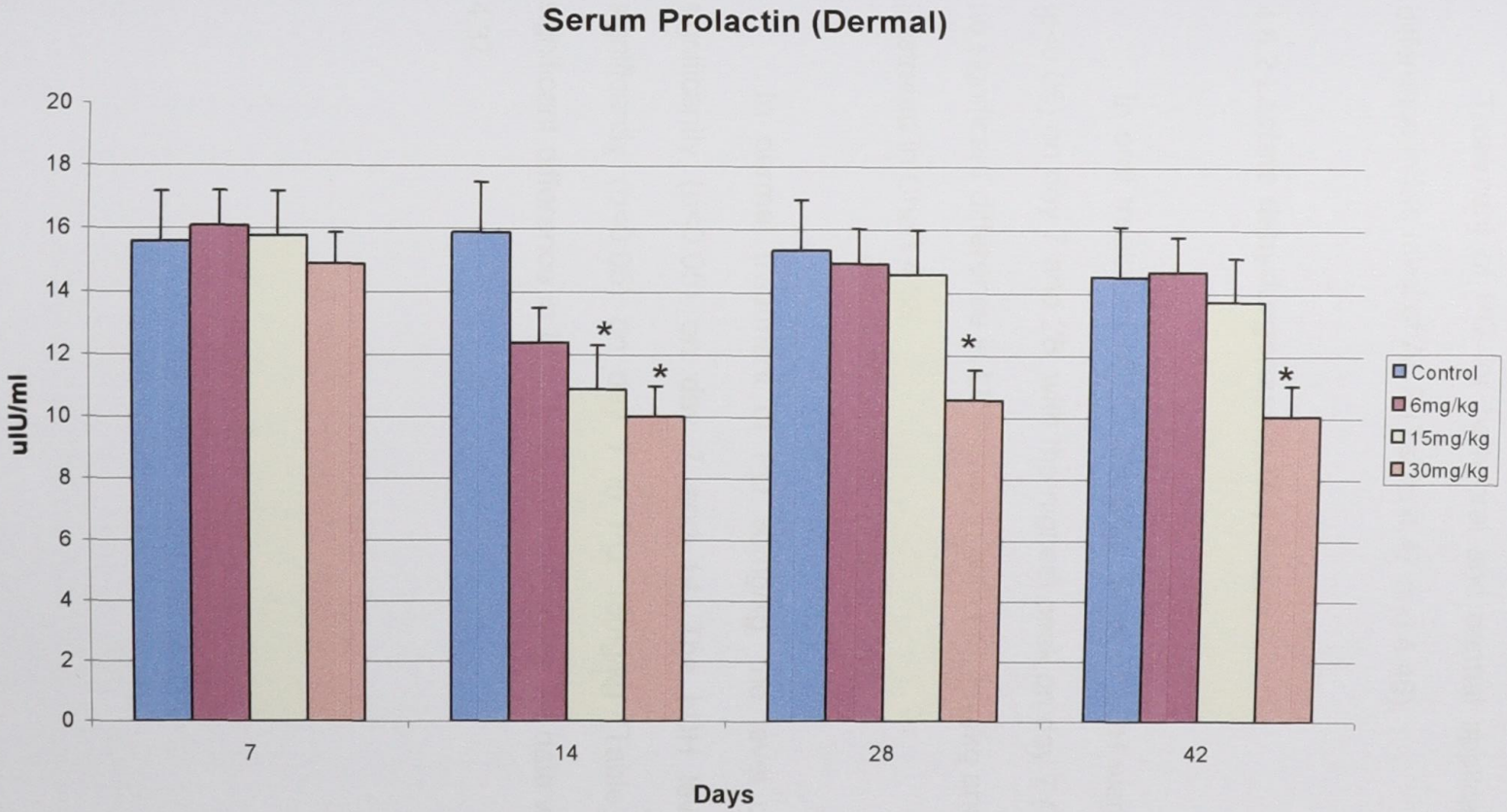


Figure 4.36 Time – response relationship of PQ (Dermal) rats on Prolactin level. The mean difference is significant at the level of $p<0.05^{*}$. Data were presented as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

4.6 Testicular Marker Enzyme Assays

4.6.1 Acid Phosphatase (ACP) Assay

Treatment of PQ for both oral and dermal application did not show any difference in the level of ACP (Table 4.47 and 4.48).

4.6.2 Lactate dehydrogenase (LDH) Assay

In oral treatment of PQ 20mg/kg, level of LDH were elevated significantly ($p<0.05$) on day 7 and 28, with the highest peak on day 7 (Table 4.49). There was no significant difference in LDH level in both PQ 2mg/kg and 5mg/kg. All data were illustrated in Chart 4.36.

In dermal treatment of PQ 30mg/kg, the level of LDH were elevated significantly ($p<0.05$) on day 7 and 14. The LDH level was also elevated significantly ($p<0.05$) on day 7 in PQ 15mg/kg (Table 4.50). There was no significant difference in LDH level in PQ 6mg/kg. All data were illustrated in Chart 4.37.

Table 4.47 Effect of PQ (oral) rats on Acid Phosphatse level. Data were presented as Mean ± SD (mg of p-nitrophenol/mg protein); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Treatment	7 days	28 days	42 days	84 days	105 days
Control	11.73 ± 0.68	12.55 ± 0.60	12.66 ± 0.42	12.56 ± 0.42	12.47 ± 0.63
PQ – 2 mg/kg	11.69 ± 0.78	12.44 ± 0.54	12.48 ± 0.44	12.49 ± 0.44	12.56 ± 0.34
PQ – 5 mg/kg	11.61 ± 0.97	12.47 ± 0.63	12.44 ± 0.26	12.36 ± 0.26	12.40 ± 0.14
PQ – 20 mg/kg	11.72 ± 0.82	12.42 ± 0.44	12.76 ± 0.53	12.60 ± 0.53	12.92 ± 0.75

Table 4.48 Effect of PQ (dermal) rats on Acid Phosphatase level. Data were presented as Mean ± SD (mg of p-nitrophenol/mg protein); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	11.73 ± 0.68	12.48 ± 0.69	12.55 ± 0.60	12.66 ± 0.42
PQ – 6 mg/kg	11.86 ± 1.15	12.94 ± 0.87	13.72 ± 1.18	12.83 ± 0.58
PQ – 15 mg/kg	13.16 ± 1.30	13.01 ± 1.34	13.18 ± 1.02	12.82 ± 0.82
PQ – 30 mg/kg	13.09 ± 1.04	13.05 ± 0.78	13.19 ± 1.58	13.64 ± 1.14

Table 4.49 Effect of PQ (oral) rats on LDH level. Data were presented as Mean ± SD (nmol/mg protein); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$)

Treatment	7 days	28 days	42 days	84 days	105 days
Control	6.94 ± 1.92	8.32 ± 1.14	8.38 ± 1.32	8.50 ± 1.34	8.56 ± 2.69
PQ – 2 mg/kg	8.02 ± 1.08	7.50 ± 0.60	8.08 ± 0.72	7.84 ± 0.75	7.72 ± 0.51
PQ – 5 mg/kg	9.12 ± 0.96	8.44 ± 0.64	7.77 ± 0.64	8.16 ± 0.44	8.31 ± 0.48
PQ – 20 mg/kg	13.86* ± 2.88	11.24* ± 1.12	8.76 ± 0.89	9.02 ± 0.74	9.23 ± 0.56

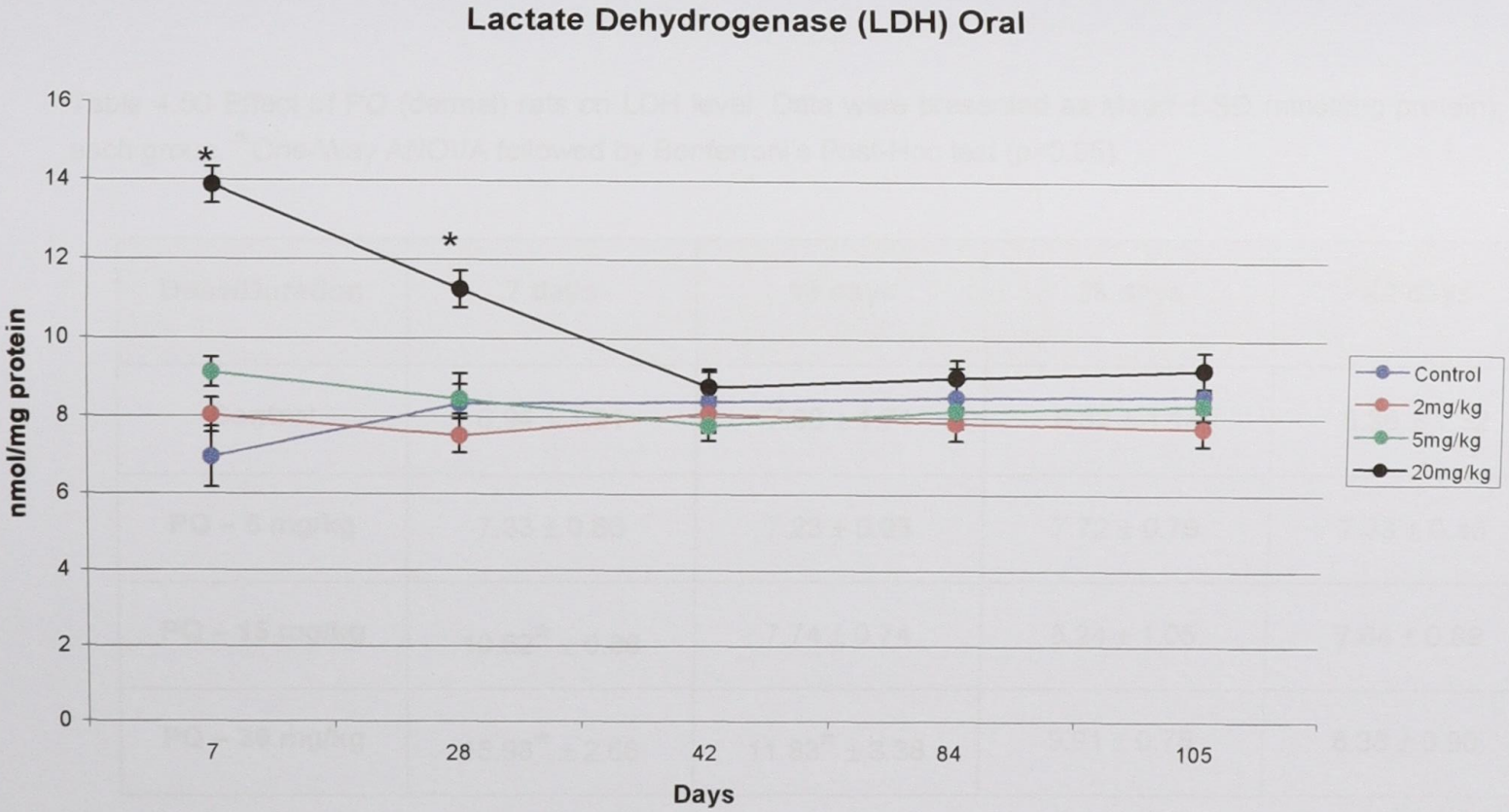


Figure 4.37 Time – response relationship of PQ (Oral) rats on LDH level. The mean difference is significant at the level of $p<0.05^{*}$. Data were pointed as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

Table 4.50 Effect of PQ (dermal) rats on LDH level. Data were presented as Mean ± SD (nmol/mg protein); n=6 for each group. *One-Way ANOVA followed by Bonferroni's Post-Hoc test ($p<0.05$).

Dose/Duration	7 days	14 days	28 days	42 days
Control	6.94 ± 1.91	7.98 ± 1.71	8.32 ± 1.14	8.38 ± 1.32
PQ – 6 mg/kg	7.33 ± 0.66	7.23 ± 0.93	7.72 ± 0.79	7.33 ± 0.43
PQ – 15 mg/kg	10.62* ± 0.86	7.74 ± 0.74	8.24 ± 1.05	7.64 ± 0.89
PQ – 30 mg/kg	15.98* ± 2.66	11.83* ± 3.38	9.91 ± 0.78	8.38 ± 0.90

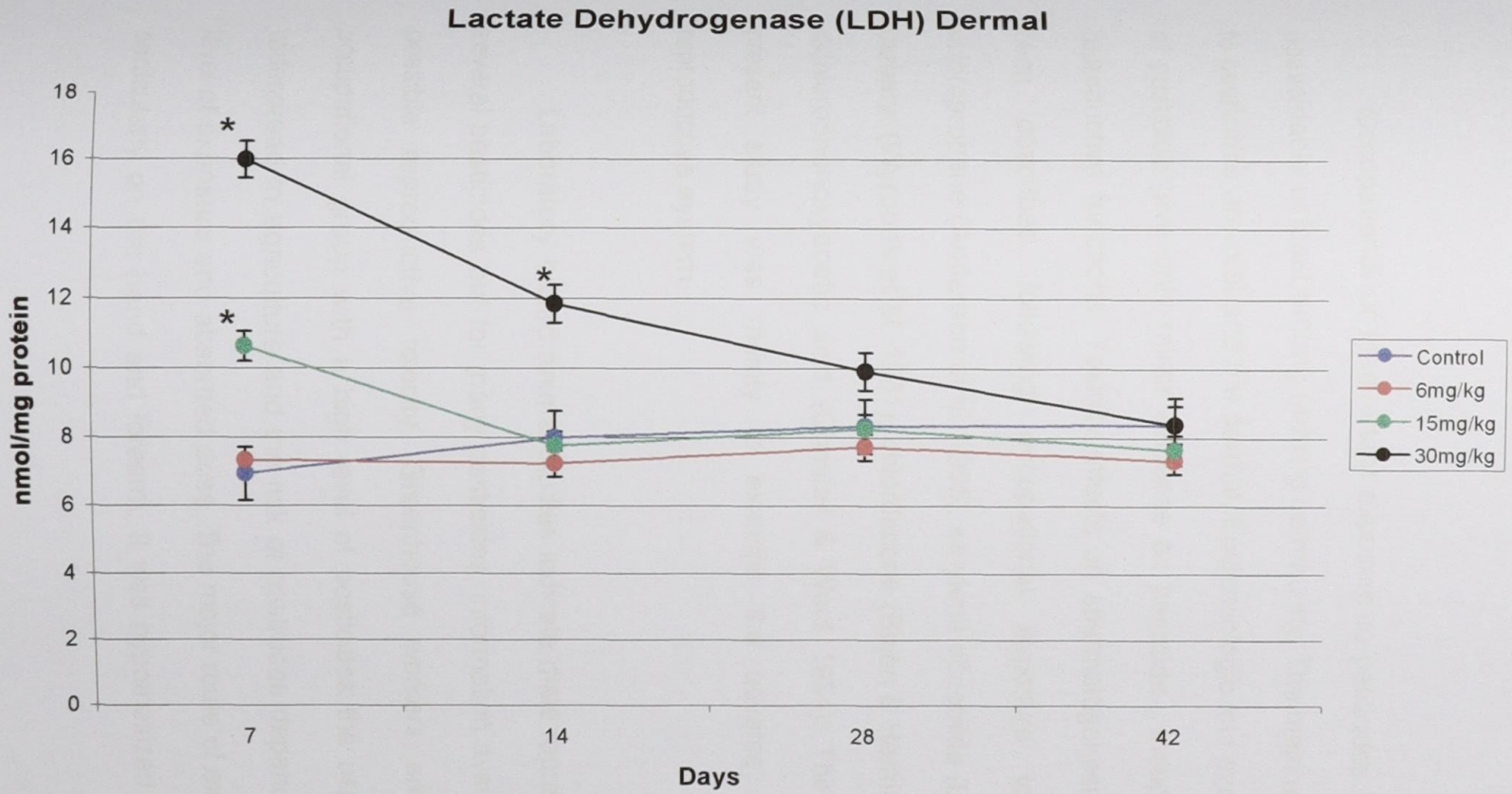


Figure 4.38 Time – response relationship of PQ (Dermal) rats on LDH level. The mean difference is significant at the level of $p<0.05$*. Data were pointed as Mean ± SD (*One-Way ANOVA followed by Bonferroni's Post-Hoc test).

CHAPTER 5

DISCUSSION

Occupational or inadvertent exposure to pesticides has been seldom unavoidable at least among farming community. The main routes of exposure to pesticides are oral and the dermal. Epidemiologic and experimental studies of pesticide give only minor evidence on pesticide - induced alterations on reproductive functions. Toxicity effects on spermatogenesis in human have been described following occupational exposure to 1,2-dibromo-3-chloropropane (Anderson et al. 1995), ethylene dibromide (Baker et al. 1986), carbaryl (Wyrobek et al. 1981), chlordecone (Bojan & Herman 1978) and 2,4-dichlorophenoxyacetic acid (Chester & Ward 1984). The objective of the present study was mainly to examine the adverse effects on male reproductive system.

Laboratory based animal studies indicate male reproductive toxicity of several pesticides but for many pesticides, information available is scanty on possible reproductive toxicity. Greenhouse workers are considered an occupational group with a high level of pesticides the use of pesticides is widespread in agriculture and the risk of pesticides depend on their toxicity, level of exposure and absorbed dose. The major route of exposure is dermal, particularly on the hand and forearm. It was hypothesized that occupational

exposure to pesticides during a spraying season may cause short-term changes in semen quality (Daniel & Gage 1966).

5.1 Body & Organ Weight Evaluation

Our present study revealed that there were no significant changes in body weight for both treatment routes in all the doses (oral and dermal). These finding may be considered due to the lower dose level of PQ exposure and physiological normal growth of rats over a prolonged period (Kar et al. 1989). The exposure of PQ following 7, 14, 28, 42, 84 and 105 days revealed that the food and water consumption in both control and treated rats were unaffected by low dose of PQ administration for 5 days regime of exposure. Similarly, influence of 30mg/kg of methyl parathion on healthy male Wistar rats did not affect their body weight (Huckin & Oakberg 1978). A study done by Kar and co-workers (1989) showed no significant alterations in body weight on low dose of methyl isocyanate exposure (insecticide) in mice.

Besides these findings, statistically significant changes were also observed in testis, epidydimis, seminal vesicle and prostate weight in PQ-treated rats. The weight of testis shows a significant decrease ($p<0.05$) on day 14 post-exposure by dermal route compared to oral group. Significant decrease in testicular weight in PQ-dermal treated rats may be due to a decrease in the number of spermatozoa and the increase of testicular atrophy (Lee et al. 1999; Leon et al. 2001). Xenobiotics can disturb testicular function and structure by interfering with several pathways operating within the testis.

Testicular lesions may be characterized by early detachment of spermatocytes and spermatids from the germinal epithelium leading to a disruption in the normal interaction between Sertoli cells and germ cells that are responsible for maintaining the integrity of the epithelium (Litchfield et al. 1973). Since this study also indicated disorganization of the normal structure of seminiferous tubules the resultant decrease in testicular weight may be attributed to the damage of spermatogenic cells as observed in our histology study.

In most of the previously reported studies, testis weight as well as epididymis, seminal vesicle and prostate weight have been used as a quantitative indicator of testis normality (Mathew et al. 1992; Takahashi et al. 2004). Unless and until other circumstances for an increase in testis weight such as tumor or edema, there is usually a strong correlation between testis weight and the number of germ cells present in the testis (Sinha et al. 1989; Zenick & Clegg 1989). In our present study, there were no changes in the number of spermatogonia in PQ oral-exposure groups (Table 4.17). However in dermal treatment group, number of spermatogonia count was decreased significantly ($p<0.05$) on day 7 and 14 in all treatment groups (Table 4.18) (All data were illustrated in Figure 4.16).The testis weight could decrease when there is an extensive sloughing and atrophy of the tubules (Allard et al. 1993; D'Souza & Narayana 2001). However, extensive sloughing can also increase the testis weight due to the blockage of efferent ductules and resultant back flow of exfoliated cells (Nakai et al. 1992). The latter phenomenon was also

observed following the ligation of efferent ductules, due to the accumulation of seminiferous tubular fluid (Anton 1979).

In our study PQ-oral treated rats has shown a significant decrease results in epidydimis, seminal vesicles and prostate weight, and a decrease in the reproductive organ weight in PQ-dermal treated rats. In 42 days post-exposure sample of PQ, data shows a significant increase ($p<0.05$) of epidydimis weight in 5mg/kg and 20mg/kg oral group but significant decrease ($p<0.05$) in dermal groups as early as day 7 post-exposure. However, the organ weight changes in PQ-oral treated rats are considered not to be treatment-related because these were not accompanied by histopathological alterations. A similar study done by Takahashi and co-workers (2004) support these findings of reproductive organ weight. Even though PQ is well known to create reactive oxygen species (ROS) in many tissues, oral treatment of low dose of PQ did not induce major changes in reproductive organ weight and histopathology (Sorensen & Loft, 2000).

Increase in epididymis weight may be due to an alteration in secretion of seminiferous fluid that is transfered to epididymis. PQ generally known to affect the contractility of peritubular cells (pseudostratified epitheliums) and alter the transportation of sperm & seminiferous fluid to the epididymis. Weight of epididymis was significantly increased in day 42 sample in oral groups with 5 and 20 mg/kg doses of PQ ($p<0.05$) and also on day 84 post exposure with 2 and 20 mg/kg doses (Table 4.5, Fig. 4.2). Nevertheless, these effects were also seen in the seminal vesicles and prostate weight in

both treatment groups. Instead, A significant increase ($p<0.05$) in the weight of seminal vesicle was observed following day 42, oral route of PQ (Table 4.7, Fig. 4.4). However, PQ also showed a significant increase ($p<0.05$) in prostate weight in both oral & dermal exposure groups (Table 4.9, Fig 4.6), (Table 4.10, Fig 4.7). According to Tripiciano et al. (1998), the potential regulators of cell contractions are oxytocin, oxytocin-like substances, prostaglandins and androgenic hormones such as testosterone. The male sex accessory glands compound with tubuloalveolar glands whose ducts converge to form a number of terminal ducts which empty into the urethra. The epithelium ranges from simple cuboidal/columnar to pseudostratified columnar depending on the hormonal status and such chemicals could alter the normal function of these cells (Tripiciano et al. 1988). Nevertheless, any chemical substances including PQ could increase the secretions and alter the cell lining of each sex accessory glands of the subjects.

Conversely, decrease in epidydimes weight in 6, 15 and 30mg/kg PQ-dermal treated rats proved to be a dose-dependent and time-response effect. In our study, the epididymal weight showed a significant decrease ($p<0.05$) on day 7 in dermal route exposure groups of PQ at the entire dose levels studied (Table 4.6, Fig. 4.3). And to support this hypothesis, our study also showed a significant decrease ($p<0.05$) in testosterone levels in both group treatments (Table 4.39 & 4.40). Reduction in the weight of reproductive organs directly support the reduced availability of androgens especially testosterone secretions (Molnar 1971; Pant et al. 1995). Besides the alterations of testosterone level, the consequence of increase in rapid fluid

movement by seminiferous tubule is known to be another reason for a decrease in reproductive organ weight. In normal physiological condition, the seminiferous (seminal) fluid act as a media to spermatozoa and their movement towards the epididymes for maturation (Tsang et al. 1981). Under pathological changes, the myoid cells which are lining between seminiferous tubules and other reproductive organs will start to increase the peristaltic movement of seminal fluid alongside with immature spermatozoa. As a result, the reproductive organs contain less or none seminal fluid and this conditions will affect the reproductive organ weight (Huang et al. 1983). A similar pattern of reproductive organ weight reduction has been seen after estrogen administration (Weber et al. 1988) and after treatment of phenolic compounds like gossypol (Tso & Lee 1982).

5.2 Histopathological Evaluation

Histopathology evaluation is the most reliable and sensitive method for detecting effects on sperm production, whereas fertility index is the least sensitive (Takayama et al.1995). Damage to the seminiferous tubule of the testes can be caused by direct chemical damage to the cells of the tubules or indirectly by chemical interference with the Leydig cells of the testes resulting in decreased testosterone production and secondary testicular degeneration (Steinberger & Klinefelter 1993).

In our study, the administration of 2mg/kg of PQ orally did not induce any alteration nor histopathological changes which may be because of the

lower dose which does not affect the cellular integrity and seminiferous tubular structure. Conversely, the epithelial sloughing and formation of degenerative cell revealed in higher dose (both oral and dermal exposure) groups. In 5 and 20 mg/kg PQ, showed a remarkble alteration of seminiferous tubule as well as increased numbers of degenerative cells. This dose-dependant effect revealed a significant change in histopathology of testis. In 6mg/kg dermal dose, there were no changes in histopathological parameters on rat testis due to low dose of PQ. However, in 15 and 30mg/kg PQ dermal groups exposure showed a significant effect with altered structure of seminiferous tubules (Fig 4.10, page 122). Furthermore there were no significant effects of PQ detected qualitatively on epidydimes in both oral & dermal groups (Fig 4.11, page 123).

Our histopathological evaluation in PQ groups defined that qualitative alterations occur when germ cells are altered structurally. However, Sertoli and Leydig cells seem to sense certain kinds of qualitative abnormalities occurring during spermatogenesis. Nevertheless, in our study there was a significant decline in the number of Leydig cells during the early & period of time frame ($p<0.05$) (Table 4.25 & 4.26). During the process of degeneration, germ cells change qualitatively. Phagocytosis of germ cells by Sertoli cells may eliminate all or only a few defective cells of a cell population (Weber et al. 1988). Our findings shows that a remarkable increase in degenerative cells occured as early 14 days for dermal exposure groups (Fig 4.10, page 122). However, the number of degenerative cells in oral exposure groups found only on 84 days post exposure and were based on dose-dependent effect (Fig

4.9, page 121). Our study suggests that PQ exposure altered the function of Sertoli cells including the integrity of seminiferous tubules. The end result is a quantitative change reflected in the number of cells. Most alterations in spermatogenesis are due to degeneration of germ cells (Parvinen 1982). Since it takes four or more cycles (one cycle consists of 65 days) of the seminiferous epithelium for germ cells to complete spermatogenesis, germ cell degeneration can occur at any time during one or more of the many developmental phases throughout the life span.

The predominant mode of failure of immature germ cells to produce spermatozoa is through degeneration of germ cells. In the present study, PQ has resulted in degeneration of cells as well as in the number of spermatocytes and spermatids in the seminiferous tubules. To a certain extent, some loss of germ cells also occurs in normal animal (Barr et al. 1971; Russell & Clermont 1977). Degenerating cells are usually phagocytized by Sertoli cells. Retardation of spermatogenesis, although theoretically possible (Russell et al. 1983), is an abnormality that eventually leads to cell degeneration.

In the present study, degenerating cells were found near the basal membrane of the seminiferous tubules. In our study, PQ oral-treated groups showed no changes in the number of spermatogonia (Table 4.17). However in dermal treatment, number of spermatogonia count was decreased significantly by 10 – 12% ($p<0.05$) on day 7 and 14 in all dose treatment

groups (Table 4.18). Since spermatogonial cells normally reside near the basement membrane of the tubule, degenerations of these cells are commonly seen in that position. Very early degenerating cells are in contact with the basal lamina and demonstrate one flattened surface leaving a vacuole. More advanced degenerating cells were round up and became only slightly displaced from the basal membrane of the seminiferous tubule before being engulfed and phagocytized by the Sertoli cell. The nucleus of most degenerating differentiated spermatogonia is highly characteristic [Fig 4.10 (b), page 122]. The chromatin marginates along the nuclear envelope, appearing as large planoconvex area (Sprando & Russell 1987) (Fig 4.10, page 122). Newly degenerating cells are very similar to their viable cell counterparts. At first, they reveal characteristic features of the cell type. However, with time, they may no longer be identifiable because their characteristic features are progressively distorted (Russell 1983). As degeneration progresses, these cells tend to move toward the base of the tubule where they are phagocytized by Sertoli cells normally. Some may move closer to the lumen, although this is a far less common occurrence.

The stem cell spermatogonia periodically divide to repopulate themselves and also to give rise to cells that are committed to undergo spermatogenesis (Huckins & Oakberg 1978). Stem cell spermatogonia are usually less vulnerable to a variety of agents that affect the testis. However, a study by Dym & Clermont (1970) shows that the proliferative spermatogonia are slightly vulnerable to certain chemical agents than are differentiating spermatogonia. PQ is a potent free radical and superoxide generating agents

available and the exposure of PQ directly could have altered the number of cells inside the testis.

Spermatogenesis can return to its former level if stem cell spermatogonia remain (De Rooij 1983), that is, after the influence of the causative agent has been removed, assuming there is no other damage except to germinal cells. In many instances the testis remains at a steady state with the only germ cells present being the stem cell spermatogonia. The reason for the inability of spermatogonia to repopulate themselves in some instances is not known. Blockage of the division pattern of spermatogonia can have a major impact on spermatogenesis, since one stem cell has the potential to divide and differentiate to produce thousands of sperm in each cycle (Ettlin et al. 1984).

If stem cell spermatogonia are partially depleted by treatment of the testis with an agent especially PQ, it has been hypothesized that there may be a lag period in which spermatogonia repopulate tubules, although this has not been proven (De Rooij 1983). This implies that inhibitory factors prevent the stem cells from becoming differentiating cells. Spermatogonia will begin to initiate spermatogenesis when the effect of an agent has been removed, but full spermatogenesis will not occur until the population of spermatogonia has completely renewed itself (Pant et al. 1995).

Spermatocytes frequently degenerate in metaphase of the second meiotic division in normal animals (Russell & Clermont 1977; Russell et al.

1981). Qualitatively, our study revealed that the secondary spematocytes have entered the lumen of seminiferous tubules as a result of alteration in the structure and the integrity of seminiferous tubules [Fig 4.10 (a), page 122]. In our study, there were no significant differences in the number of spermatocyte count (Table 4.21). However, in dermal treatment, the spermatocyte count was decreased significantly by 18.5% ($p<0.05$) only on day 7 in all the treatment groups (Table 4.22). Degenerating preleptotene and pachytene cells have been identified and these type of cells are initially appeared more densely stained than normal spermatocytes (Sharpe et al. 1988). Upon degeneration, the cells frequently shrink slightly and the features of the nucleus remain. In the advanced phases of degeneration, the cellular components are markedly distorted (Russell & Clermont 1977).

In our study, there was no significant change in the spermatid count (Table 4.19). However, in dermal treatment, the spermatid count was decreased significantly by 9.1% ($p<0.05$) on day 7 in all treatment groups (Table 4.20). On day 14, the spermatid count were decreased significantly by 8.4% ($p<0.05$) in PQ 30mg/kg. Degenerating elongate spermatids can be identified by their characteristic shape and also by their position near the tubular lumen (Russell et al. 1981). Numerous agents have been shown to cause phagocytosis of late spermatids. On the other hand, the Sertoli cells may sense the presence of abnormal spermatids. In the normal rat, occasional morphologically abnormal sperm are not released with the cohort of normal sperm. They are then engulfed by the Sertoli cell and phagocytized (Russell 1984).

Degenerating spermatids usually developed a pyknotic acrosome and irregularly infolded nuclei. As degeneration progresses, the cytoplasmic constituents become heavily stained, whereas the acrosomal contents become less stained. However, in the present study, there were no such effects were observed with elongate spermatids in the seminiferous tubules (Fig 4.9, page 121). Failure of sperm or spermatid release is a frequently seen abnormality in the testis (Russell & Clermont 1977; Russell et al. 1981). The cause of failure of release is not known. According to Huang & Marshall (1983), wherever delayed spermiation take place, the late spermatids could be seen at various levels within the germinal epithelium, having been taken up by Sertoli cells and in the process of being phagocytized. The spermatids that are phagocytized by the Sertoli cell are clearly defective (Russell 1984). However, there were no reports indicate that PQ or any agents eventually have caused the Sertoli cells to phagocytize the cells. The frequent presence of morphologically abnormal sperm in the epididymis indicates that not all morphologically abnormal sperm are phagocytized by the Sertoli cells.

Degenerating elongate spermatids can be identified by their characteristic shape and also by their position near the tubular lumen (Russell et al. 1981). Degenerating spermatids usually developed a pyknotic acrosome and irregularly infolded nuclei. As degeneration progresses, the cytoplasmic constituents become heavily stained, whereas the acrosomal contents become less stained. However, in the present study, there were no such effects in elongate spermatids in the seminiferous tubules.

On the other hand, our study also shows that the number of 'dividing cells' were decreased significantly ($p<0.05$) on day 14 in all dermal groups (Fig 4.19 page 149). Our findings revealed that PQ has interfered the entire cellular compartment and reduced the number of dividing cells which might be due to the free radical generating effect of PQ. Occasionally, germ cells will be released from contacts with other germ cells of the same type by breakage of intercellular bridges. There will also be freed from attachments to Sertoli cell by disengagement of Sertoli-germ cell junctions (Russell & Peterson 1985) and will be sloughed individually or as a group into the lumen of the tubule. This condition frequently occurs when degeneration of germ cells is massive, but it has also been seen when only minor changes in the testis were noted. Sometimes germ cells are sloughed with fragments of attached Sertoli cells (Russell et al. 1981; Russell et al. 1987).

In the present study, 5mg/kg and 20mg/kg of PQ oral-treated groups showed a significant reduction of Leydig cell count ($p<0.05$) on day 42 and 84. However, there was no such effect of PQ 2mg/kg on Leydig cell count throughout the study (Table 4.25). In dermal treatment of PQ 30mg/kg, number of Leydig cell count was reduced significantly ($p<0.05$) from day 7 to day 28 (Table 4.26). The Leydig cell count was also reduced significantly ($p<0.05$) on day 14 only in PQ 15mg/kg. There was no such effect of PQ 6mg/kg on Leydig cell count could have an impact on Leydig cells to secrete the major testosterone hormone to support normal spermatogenesis. Although Leydig cell damage causes a non-specific damage to the germ cells,

it preferentially affects more advanced germinal elements. Spermatocytes and spermatids are thought to be more dependent on the Leydig cell for physical and nutritional support (Russell 1980).

Sertoli cells are thought to be notoriously resistant to many treatments that affect germ cells. Nevertheless, some reports also indicating Sertoli cell injury due to environmental substances (Foster et al. 1987; Creasy et al. 1983; Boekelheide 1988). In the present study PQ might have affected the structure and function of Sertoli cells and support our results on histopathological evaluation. Sertoli cells injury appears to be more prominent in prepurbertal animals than in adult animals (Gray & Beamand 1984; Dostal et al. 1988; Anderson et al. 1989).

The normal structure and function of Sertoli cells and germinal cells are mutually dependent. In any condition that targets germ cells can cause the cells to be moderately or severely depleted, the form and probably the size of the Sertoli cell will change as a consequence. Some agents are targeted primarily to the Sertoli cell. However there is no intense study of PQ on Sertoli cells so far in the literature. Sertoli cells can survive without germ cells even after suffering initial damage (Dym 1977; Russell 1980). Besides, germ cells have never been shown to survive for any length of time without Sertoli cells and the complete loss of Sertoli cells from the tubule is rare. Therefore, it is thought that after Sertoli cell damage, germ cell damage is probably inevitable. Insult to the Sertoli cell often produces a histological derangement with a great reduction germ cell population (Russell 1980).

Although Sertoli cell damage causes a non-specific damage to the germ cells, it preferentially affects more advanced germinal elements. Spermatocytes and spermatids are thought to be more dependent on the Sertoli cell for physical and nutritional support since both cells population reside on the adluminal side of the Sertoli cell barrier (Russell 1980). In our study there is a significant dose – dependant change in Sertoli cells and the growth of spermatocytes and spermatids. Qualitative alterations occur when germ cells are altered structurally. However, Sertoli cells seem to sense certain kinds of qualitative abnormalities occurring during spermatogenesis. During the process of degeneration, germ cells change qualitatively. Phagocytosis of germ cells by Sertoli cells may eliminate all or only a few defective cells of a cell population (Weber et al. 1988). The end result is a quantitative change reflected in the number of cells. Most alterations in spermatogenesis are due to degeneration of germ cells (Parvinen 1982). Since it takes four or more cycles of the seminiferous epithelium for germ cells to complete spermatogenesis, germ cell degenerations can occur at any time during one or more of the many developmental phases throughout the life span.

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after suffering initial damage (Dym 1977; Russell 1980). Besides, germ cells have never been shown to survive for any length of time without Sertoli cells and the complete loss of Sertoli cells from the tubule is rare. Therefore, it is thought that after Sertoli cell damage, germ cell damage is probably inevitable. Insult to the Sertoli cell often produces a derangement in cell associations with a greatly reduce germ cell population (Russell 1980).

Newly degenerating cells are very similar to their viable cell counterparts. At first, they reveal characteristic features of the cell type. However, with time, they may no longer be identifiable because their characteristic features are progressively distorted (Russell 1983). As degeneration progresses, these cells tend to move toward the base of the tubule where they are phagocytized by Sertoli cells. Some may move closer to the lumen, although this is a far less common occurrence. In the present study, degenerating cells were found near the basal membrane of the seminiferous tubules. Since spermatogonial cells normally reside near the basement membrane of the tubule, degenerations of these cells are commonly seen in that position. Very early degenerating cells are in contact with the basal lamina and demonstrate one flattened surface. More advanced degenerating cells were round up and became only slightly displaced from the basal membrane of the seminiferous tubule before being engulfed and phagocytized by the Sertoli cell. The nucleus of most degenerating

differentiated spermatogonia is highly characteristics. The chromatin marginates along the nuclear envelope, appearing as a large planoconvex areas (Sprando & Russell 1987).

In our study, histopathological evaluation showed a major impact of epithelial sloughing in seminiferous tubules. Our results shows a dose dependant effect whereby there were a significant effect of epithelial cell sloughing in 5 and 20 mg/kg in oral, and also 15 and 30 mg/kg in dermal exposure (Fig 4.9 page 121; Fig 4.10 page 122). It seem that PQ has direct damaging effects to the cell integrity and lining and many of the spermatocytes were detached from their connection to Sertoli cells. Our study concluded that the seminiferous tubules were sloughed and a dose-dependant effect were observed in 5, 15, 20 and 30mg/kg. Eventually the epithelial sloughing recovered at 84 and 105 days post exposure. Nakai et al. (1992) reported that around 80% tubules showed sloughing in carbendazim treated rats, and the authors proved that the inhibition and destruction of mirotubules as the reason for it. There is no doubt about this factor underlying this mechanism. However, 5-fluorouracil also caused high level of epithelial sloughing (Narayana et al. 2000) probably due to the disruption of ectoplasmic specialization of Sertoli cells (Russell & Russell 1991). This indicates that there are several mechanisms involved with the induction of epithelial sloughing, but all are mediated through the Sertoli cells (Nakai & Hess 1994; D'Souza & Narayana 2001).

Knowledge of the size and content of the tubular lumen provides valuable information. In that case, the seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) are used for measurements throughout the study. In our study STD was decreased significantly ($p<0.05$) with time-response with 7 to 28 days post exposure of PQ oral-treated groups (Fig 4.12 page 129; Fig 4.13 page 131). No change in STD in PQ 6mg/kg dose. In dermal exposure groups of PQ 15mg/kg and 30mg/kg, STD was decreased significantly by 3 – 4% ($p<0.05$) on day 7 and 14 (Table 4.12). A smaller than normal tubular lumen usually indicates a decrease in fluid secretion by the Sertoli cells and if the tubular lumen appears larger than usual, this feature may signify a blockage in the duct system (Russell et al. 1988). Decrease in STD was also observed following the treatment of a variety of chemicals, for example, 1,3-dinitrobenzene (Hess et al. 1988) and 5-fluorouracil (D'Souza & Narayana 2001) due to extensive epithelial sloughing induced by these chemicals. However the results show a recovery to normal level on 28, 42, 84 and 105 days post exposure.

In the present study SEH was decreased significantly ($p<0.05$) in 7 and 14 days treatment groups of dermal study (Table 4.14 page 133). However there were no significant difference found in PQ oral groups. Immature germ cells or Sertoli cells or cell debris within the tubular lumen indicate that sloughing phenomena have taken place. This parameter is therefore very sensitive (Sun et al. 1990) and functions as an early indicator of gonadotoxicity. A nonspecific injury to Sertoli cells could result in experimental damage to the seminiferous epithelial cells and therefore results in epithelial

sloughing of seminiferous tubules (Bergh 1981; Hoffer 1983; Chapin et al. 1984). Epithelial sloughing appear in testis exposed to a variety of chemicals due to loss of cell integrity and cell-junction within Sertoli cells (Allard et al. 1993; Gol'dberg et al. 1997).

Our study also shows a significant decrease in EEH ($p<0.05$) on 7, 14 and 28 days post exposure in 15 and 30 mg/kg dermal groups (Fig 4.15 page 137). This suggest that PQ generated free radicals might have resulted in a remarkble reduction in EEH. However, there were no significant changes in EEH in PQ oral groups. The toxic effect of PQ might have similar response as administration of methoxychlor which has shown to decrease the antioxidant enzyme level and reduction in the height of epidydimal epithelial lining in rats (Latchoumycandane et al. 2001).

5.3 Sperm Analysis Evaluation

On the other hand, pesticides also influence other fertility parameters such as sperm count, motility, and seminal volume. This has a direct positive correlation with the advancement of pesticides and other chemical industries (Stillman 1982). In continuation with this, recent years have seen plenty of discussions regarding the precipitious drop in semen quality (Gopalakrishnan 1997). This issue has been raised by Carlsen et al. (1992) in their controversial report on drastic drop in sperm concentration to 50% in men for the last 50 years. For methodological reasons, the report was criticized extensively (Farrow 1994; Auger et al. 1995; Bromwich et al. 1994), otherwise

predicted the increased male reproductive dysfunction due to chemical exposure (Sharpe & Skakkebaek 1993; Keiding et al. 1994).

Our study supported the previous reports that claimed pesticides have such effect on sperm quality. In the present study, sperm count, sperm morphology, sperm mortality and sperm motility were decreased significantly ($p<0.05$) at a dose dependant manner in all groups. Several agents such as cadmium chloride, cyclophosphamide and estradiol known to affect the sperm count in rat and decreased the count due to cytotoxicity (Wyrobek et al. 1983). Chemicals that affect Sertoli cells are also known to reduce the sperm count, since Sertoli cells regulate and orchestrate the spermatogenesis (Sharpe & Skakkebaek 1993).

Disturbances of Sertoli cells therefore may reduce the sperm count. Such destructive effects of PQ on intracellular harmony of these cells are probably due to the ability of generation of ROS and act indirectly on the germ cells as well as the Sertoli cell population. Nevertheless, PQ exposure has a major influence on rat spermatogenesis and toxic to the entire male reproductive system. However, during 42, 84 and 105 days post exposure, the sperm count as well as other sperm assays were back to normal as the cells recovered from toxic insults.

In rats, metamorphosis of spermatids occurs in Golgi, cap, acrosome and maturation phases called spermiogenesis (Clermont & Leblond 1955). Several structural components like ectoplasmic specialization of Sertoli cell,

chromatin condensation (Fawcett et al. 1971) and microtubular manchette are known to affect the spermatid head shaping. Of these, the manchette monitors the head shaping caudal to the acrosome and the latter imparts head shape changes in the region covered by it, though the entire mechanism is not clear at present (Russell et al. 1994).

Similarly the factors responsible for dysmorphogenesis of sperm are also not clear (Mathew et al. 1992; Jayashree et al. 1994), though several mechanisms were attributed. Bruce and co-workers (1974) proposed that chromosomal aberrations were responsible for sperm abnormalities. Subsequently, the involvement of that phenomenon was denied by same authors and others as well (Wyrobek & Bruce 1975; Martin & Rademarker 1988). However, some authorities subsequently found that deletion of segment of Y-chromosome (Styrna et al. 1991) and disomy of Y chromosome (Rubens et al. 1998) have positive correlation with the sperm abnormality. Moreover, protein abnormalities (Hedge & Sujatha 1995) or destruction of somatic cells in the testis (Anderson et al. 1995) were also known to produce dysmorphologic sperm.

In the rat, about three weeks are required for cells to evolve from young spermatids to spermatozoa (Fawcett 1958). Any disruptions within this duration could cause an alteration of normal sperm morphology. The sperm might resulted either a reversible or permanent effects and shows as a head or tail defects or both. PQ has resulted a major effect on sperm morphology with an increasing percentage of abnormal sperm in all treatment groups (Fig

4.33, 4.34, 4.35, 4.36, 4.37, 4.38). Among these abnormalities, the hookless and the banana-shaped abnormalities were the most that can be found during this study. Though this study suggest that PQ might affect the normal spermiogenesis and cell formation, the exact mechanism of effect remain unclear.

However, several studies conducted on several pesticides suggest that these chemicals induced the formation of abnormal spermatozoa by alterations through their normal spermiogenesis (Mishra et al. 1993;Pant et al. 1995). The head defects could be resulted from an alterations of chemicals on the development of an acrosome. Eventough there are no extensive studies of PQ on sperm morphology, in the present study, an increase formation of head defects among treated rats suggest that PQ has a toxic effects on formation of rat spermatozoa. Nevertheless, PQ induces the formation of double-tail, amorphous and broken tail, but these abnormalities were less than the head defects.

The increased incidence of abnormal sperm morphology in the cauda epididymes might result due to the effect of PQ on peritubular cells of seminiferous tubules. The seminiferous tubules are covered by lamina propria which consists of a basal membrane, a layer of collagen and the peritubular cells (myofibroblasts). Myofibroblasts are poorly differentiated myocytes with the capacity of spontaneous contraction (Holstein et al. 1996). Peritubular cells produce several factors that are involved in cellular contractility such as panactin, desmin, gelsolin, smooth muscle myosin and actin (Holstein et al.

1996). Rat studies showed that periodical contractions occur in the peritubular cells leading to a peristaltic contraction of the seminiferous tubules (Harris & Nicholson 1998).

It is believed that mature sperm are transported towards the exit of the seminiferous tubules by contraction of these cells. This study suggests that PQ indirectly affect the myofibroblasts and increased the contraction pulses of the peritubular cells and consequently increased the transportation of the spermatozoa including the non – mature and abnormal sperm. The increase contraction of peritubular cells also affect the seminiferous fluid and this could affect the fluid in the epididymes.

Conversely, sperm transport through the epididymes may also be accelerated, resulting in ejaculates containing fewer or immature sperm (Cooper & Yeung 1999). In the present study, the epididymal epithelial height and the evaluation of epididymes section shows no such affect of PQ toxicity on epididymes. However, PQ exposure shows an adverse affect on sperm motility and mortality throughout the study. Some chemicals act on the epididymal epithelium and interfere with epididymal fluid secretion. Compounds such as α - chlorhydrin and the 6 – chloro – 6-deoxysugars (Tsang et al. 1981) or ornidazole have highly specific effects on rat epidydimal sperm, resulting in complete loss of motility and hence sterility (Oberlander et al. 1994). However the toxicological mechanism of actions of these chemicals remains unknown.

5.4 Biochemical (Hormones & Enzymes) Evaluation

Some environmental pollutants including pesticides are capable of mimicking the critical functions of natural hormones with dire and often unpredictable consequences to wildlife and human. According to Colborn et al. (1993), there were 48 chemicals with widespread distribution in the environment reported to have reproductive and endocrine-disrupting effect, including Atrazine, Amitrole, 2,4-D, Mancozeb, Maneb, Carbaryl, DDT, endosulfan.

In 97 cases of PQ poisoning there were only exceptionally few observations on the morphology or function of pituitary glands. In two cases, ended fatally early after the PQ ingestion (Hargreave et al. 1969; Nienhaus & Ehrenfeld 1971) the pituitary gland was morphologically normal. The pituitary gland is the central structure controlling gonadal function and is regulated by the hypothalamic -gonadotropin-releasing hormone (GnRH). The gonadotropins-luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are produced and secreted by the gonadotropic cell of the anterior pituitary. In males, these hormone control steroidogenesis and gametogenesis in the testis (Weinbauer & Nieschlag 1998).

The luteinizing hormone (LH) has an indirect effect on spermatogenesis via stimulation of Leydig cells. Our study shows lowered LH level ($p<0.05$) in 20mg/kg & 30mg/kg dose which was time dependant (Fig 4.33 page 187; Fig 4.34 page 189). The Leydig cell contains surface

receptors for LH and responds to stimulation by producing and releasing testosterone (Steinberger 1971). One of the probability may be a reduction in number LH receptors of Leydig cells which might have been affected by PQ. Testosterone either diffuses directly into the tubule or bound to a carrier, such as albumin, that delivers across the lymphatic space into the seminiferous tubule. Receptors for testosterone are present in the Sertoli cells (Sanborn et al. 1977; Tindall et al. 1977), but it is not clear if receptors are also present on some germ cells.

In the present study, PQ exposure has resulted a decrease of Leydig cell count in the intertubular space which may have a direct correlation for the level of testosterone in the serum which was decreased significantly ($p<0.05$) in both oral and dermal groups. The disruption of LH receptors and Leydig cell integrity by the ROS generated by PQ might be the reason of lower level of testosterone and Leydig cell count. PQ also might affect the LH level in the serum via indirectly inhibiting or biochemical alterations in the GnRH-hypothalamo-pituitary axis. A similar study by Wyrobek and Bruce (1975) shows that chemicals such as DDT, endosulfan, mancozeb and carbaryl induced an elevated levels of sperm abnormalities and a lowered level of testosterone in the serum. However, the exact mechanism of this alterations is still unknown. Lowered testosterone level in the serum could have decreased the spermatogenesis in rats and might have affected the entire process of spermatozoa formation. However the level of testosterone started to recover at 28, 42, 84 and 105 days post exposure to PQ.

The second major pituitary hormone FSH, which act directly in the process of spermatogenesis in the seminiferous tubules also affected by PQ exposure. In the present study, lowered level of FSH was observed in a dose and time dependant manner in 5, 20 and 30mg/kg treated rats. The importance of FSH in initiation and maintenance of spermatogenesis is well recognized (Russell et al. 1987b; Raj & Dym 1977). Spermatogonia probably have receptors for FSH (Means & Vaitukaitis 1972; Orth & Christensen 1978) but it is not clear regarding the effect of FSH on these cells. The Sertoli cells also has receptors for FSH on its surface and the appearance of hormone copycats are strongly associated with these types of receptors. In previous study, there were no such effect of PQ reported on hypothalamo-pituitary axis, either directly or indirectly. But its ablitity to produce strong free radicals might have an effect on the hypothalamus or pituitary or both through indirect mechanism of alterations of normal FSH receptor (Wyrobek & Bruce1975).

In normal condition, the Sertoli cells also suppress the production of FSH by secreting the inhibins which selectively inhibits the secretion of FSH. An increased production of inhibin might stop the entire hypothalamo-pituitary system to produce FSH as well as LH. Indirectly, this could stop the entire male reproductive system function and specifically the spermatogenesis. The presence of PQ in the body might have affected the normal level of inhibin by an increased secretion of inhibin via Sertoli cells into the hypothalamo-pituitary-gonadal axis which needs further study to confirm this hypothesis.

Eventhough there were no extensive studies on male hormones earlier, PQ exposure has resulted a non-specific and indirect mechanism of alteration in the hypothalamo-pituitary axis and this remains as an unanswered question till date on the mechanism of PQ in this pathway. In the present study, the level of testosterone, FSH, LH, and Prolactin were decreased significantly ($p<0.05$) and this suggests that PQ exposure affect the normal level of hormones in the male rats. However, this study suggest several reasons including the hormone copycats and increase level of inhibin in the serum which need to be addressed in future.

Prolactin affects a number of sexual functions in the male. Studies in rats demonstrated prolactin receptors in the Leydig cells of the testis, the prostate gland and the seminal vesicles (Aragona & Friesen 1975). Prolactin helps to maintain LH receptor number on the surface of Leydig cells & thereby maintains normal testosterone levels (Aragona et al. 1977). Prolactin also affects the growth of accessory sex glands in male rats both directly and indirectly (Nevalainen et al. 1991). Decreased level of prolactin and other male hormone in this study ($p<0.05$) revealed its function and contribution via stimulation of spermatogenesis. The lower number of Leydig cells which have been exposed to PQ signify the decreased number of receptors at the surface of Leydig cell and this is possibly might have effected the consistency of prolactin towards spermatogenesis. Contrary to this, elevated prolactin levels also inhibit sexual activity in male rats (Doherty et al. 1985) and in humans it may result in increased libido, impotence and secondary hypogonadism. These effects may not be reversible with testosterone therapy alone (Drago

1984). Prolactin is present in human semen at slightly higher levels than in serum and is probably synthesized by cells of the male reproductive tract.

A number of enzymes have been reported to be markers of germ cell maturation as well as spermatozoa. Among these enzymes, lactate dehydrogenase (LDH) and acid phosphatase (ACP) plays a major role. LDH has been studied in more detail and its increased level reveals alteration or disruption of cells. LDH is an unique enzyme, present in the testis of most species at times when spermatozoa are actively being formed (Hodgen 1977). This enzyme is not present in Leydig cells but it appears specifically in the midpiece of spermatozoa and the mitochondrial fraction of testicular homogenates. In the present study, LDH level was increased significantly ($p<0.05$) at a dose dependant manner in 20 and 30 mg/kg doses on 7, 14 and 28 days post exposure. This finding suggest that PQ had altered the cell population within the seminiferous tubules during 7, 14 and 28 day post exposure period. During this duration, the cell population were also decreased and this might be the response of cell fraction to released the LDH enzyme as a marker of biochemical alteration on PQ exposure. The cell started to recover on day 42 and the LDH level gradually reduced.

ACP is also one of the most important testicular marker in evaluating the function of spermatogenesis. ACP is present in both interstitial cells and germ cells as well in other organs such as prostate and epididymes associated with spermatogenesis (Prasad et al. 1974). In the present study, there were no significant changes in ACP level in both treatment groups. Our

study suggest that ACP level change was not significant and not reached a potent biochemical assessment level to detect abnormal cellular response in seminiferous tubules at the dose levels selected for PQ in this study.

Paraquat (PQ), an oxidative-stress inducing substance, is a herbicide that is highly toxic to all animals and to man as it is a well-characterized pneumotoxicant (Brooks 1971). It generates free radicals and leads to multiorgan toxicity with necrotic damage to the lung, myocardial muscles, liver and kidney. PQ also induces extensive haemorrhagic incidence throughout the body and consequently leads to death (Adachi 2000; Minakata et al. 2000; Sorensen & Loft 2000).

This study suggest an oxidative-induced mechanism on PQ exposure by both oral and dermal route administration. PQ accepts electron from reductases and forms PQ radicals. These radicals transfer the extra electron to molecular oxygen forming superoxide anion radicals and regenerating the parent compound that is ready to gain a new electron (Kappus 1986). Superoxide radical changes or detoxified by catalase or glutathione peroxidase in the presence of glutathione (GSH) to form water and oxidized glutathione (GSSG) (Gragus & Klaassen 1996).

These reactive oxygen species (ROS) especially hydroxyl radicals react with polyunsaturated fatty acids to yield lipid hydroperoxide. This initiates a lipid radical chain reaction, which may cause oxidative damage to cell leading to increased membrane fluidity, permeability, and loss of

membrane integrity. In addition, dysfunction of mitochondria and sarcoplasmic reticulum and altered calcium homeostasis may occur as a result of oxidative damage to the cells (Berisha et al. 1994).

Free radicals are known to play a crucial role in PQ-induced toxicity (Tampo et al. 1999; Minakata et al. 2000; Tomita et al. 2001). It has been reported that the toxicity is related to redox cycling of an iron-PQ complex, which in turn catalyzes the formation of ROS with the ultimate progression of lipid peroxidation (Karakashev et al. 2000; Tsukamoto et al. 2000). In general, the generation of free radicals provokes deleterious effects on the membrane lipids, leading to an extensive process of lipid peroxidation (Adachi 2000). In the present study, the appearance of epithelial sloughing, degenerating cells together with the increase in LDH levels suggest that the toxicokinetics effects of PQ might be attributed to the ability of PQ to generate oxygen free radicals. Nevertheless, a study conducted by Rios and co-workers (1995) claimed in mice germ cells, PQ induced an increase of sperm-shape abnormalities when the animals were treated at three stages of cell development, spermatozoa, spermatid and preleptotene spermatogonial cells.

As an organ the testis is probably unique in terms of the number and diversity of morphological and biochemical events taking place. Mitotic cell divisions, meiotic cell divisions, genetic recombinations, flagellar formation, acrosomal formation, nuclear condensation, head shape determination and cytoplasmic elimination are just a few of the special processes taking place during spermatogenesis. PQ exposure and production of free radicals have

contributed a major changes in the spermatogenesis and for the entire process of male reproductive system.

Testicular function including androgen secretion and the production of fertile spermatozoa can be adversely affected by pre-testicular, testicular and post-testicular mechanisms (Kerr et al. 1972). For pre-testicular toxicity, the mechanism takes part in the hypothalamo-pituitary-gonadal axis. The testicular function is regulated by the pituitary gonadal hormones such as FSH, LH, and toxicity that disturb this level of control is considered to be pre-testicular toxicity.

There are at least five mechanisms by which environmental contaminants are able to disrupt vital functions of the reproductive and endocrine system (Colborn et al. 1993). Some contaminants are similar enough in structure to hormones that they are able to bind to cellular receptors designed to be targets for natural hormones. This causes unpredictable and abnormal cell activity. Other contaminants appear to block these binding sites so that hormones are unable to bind to them, thus impairing normal cell activity. Some contaminants induce the creation of extra receptor sites in the cell. The consequences can be an amplification of the impact of hormones on cell activity. Contaminants can interact directly and indirectly with natural hormones, changing the hormones messages and thus altering cell activity. The natural pattern of hormone synthesis can be disrupted by contaminants, resulting in an improper balance or quantity of

circulating hormones. However, the exact mechanism by which environmental contaminants could be reducing sperm production is not fully understood.

Direct testicular toxicity may affect any of the various cell types within the testis including Leydig, Sertoli and germ cells. In the present study, the Leydig cell count were affected by PQ exposure. Selective Leydig cell damage is well known experimentally in animals given the drug ethane dimethane sulphonate (EDS). This is an alkylating agent that selectively destroys Leydig cells by an unexplained mechanism, leading subsequently to degeneration of the most androgen dependant germ cells, pachytene spermatocytes in rats (Bartlett et al. 1986).

Sertoli cells fulfill an enormous array of functions, playing a cardinal role in regulating and supporting spermatogenesis as well as forming the scaffolding of the seminiferous tubule and creating its unique internal milieu. Consequently, direct toxicity to Sertoli cells should have marked effects on sperm production and function (Boekelheide 1993). Dibromoacetic acid has recently been found to be a further example of a Sertoli cell toxin (Linder et al. 1997). The molecular mechanisms of Sertoli cell toxicity from these chemical, however remain unclear. The germ cell loss that is always consequent upon Sertoli cell damage may be mediated through phagocytosis in order to eliminate the germ cells that can no longer be supported (Lee et al. 1999).

Germ cells are mostly located within the diffusion tight blood-testis barrier, thereby achieving some protection from extrinsic chemicals. The best

established evidence for direct toxicity to human germ cells is the exquisite sensitivity of spermatogonia to ionizing irradiation and alkylating agents. Spermatocytes and spermatogonia are the germ cell types involved in meiosis, a unique genetic process involving one round of DNA replication (spermatogonia) and two of cellular division (spermatocytes), making these cells vulnerable to certain toxins as well as to genetic mutations.

Diploid germ cells may be more vulnerable than their haploid equivalents to spontaneous cell death (Kerr 1992) and possibly also to apoptosis induced by testicular toxicants. Round spermatid can also be lost by germinal exfoliation, a process whereby large numbers are released prematurely, although their subsequent fate is unclear. Sertoli cell toxicity can also sometimes have the same effect as the stricken Sertoli cell becomes unable to maintain its attachment to the spermatids.

In contrast to testicular toxicity, post-testicular toxic effects are on the sperm after leaving the rete testis. In experimental animals, mature spermatozoa are more resistant to DNA strand breaking by high-dose ionizing radiation than are testicular germ cells (Rousseaux et al. 1993). The neuromusculature of the epididymes can be affected by adrenolytic drugs such as guanethidine or methoxamine that lead to stasis of sperm in the spididymes, which may even swell and rupture.

Disorders of sperm production are seen commonly in clinical practice. Because normal spermatogenesis is dependent upon the complex

coordination of cellular divisions and differentiation, it is sensitive to disruption by external influences, such as environmental toxins, irradiation, cytotoxic agents and excessive heats (Matsumoto 1991). Infertility affects approximately 6% of men in the reproductive age group and the vast majority (~ 90%) of these men have impaired sperm production (Baker et al. 1986). Idiopathic oligozoospermia or azoospermia remains the most common diagnosis that results from as evaluation of male infertility.

This present study revealed an overall disturbance to male reproductive system inducing early effects on spermatogenesis which progressed up to 14 days of post-exposure with a cytotoxic effect on spermatozoa which may be due to the generation of free radicals. Sperm abnormality was significantly increased at all dose-levels and sampling time indicative of an inteference in metamorphosis of germ cells into mature spermatozoa. Sperm abnormality observed on exposure to PQ may be due to a direct effect of free radicals, imbalance of pituitary-gonadal hormonal secretions with a decline in most of the doses studied. A combined effect of free radical and decrease in testosterone, LH and FSH must have indirectly affected the spermatogenesis there by altering the metamorphosis and an increase incidence of abnormal spermatozoa.

Overall, the results of this study indicate a downward fall in male reproductive functions resulting in a fall in spermatozoa numbers. This hindered reproductive function is due to a derangement in pituitary-gonadal-

hormone deficiency, cytoarchitecture, death of different cells of spermatogenic cycle and abnormal production of spermatozoa.

In conclusion, findings of this study confirms that PQ at low doses both by oral and dermal routes results in adverse reproductive damage especially spermatogenesis. Since our study did not adopt the same dose and sample regimen for oral and dermal route studies, the result are not directly comparable. Our study in total confirms the low dose toxicity of PQ on male reproductive functions which is induced by hormonal and direct toxicities on spermatogenic cell population.

CHAPTER 5

CONCLUSIONS

On October, 3rd 2003, European United (EU) claimed that PQ will still be in use in agricultural sector for ten more years. This gives a great impact on this study to keep on searching the pro and con for the use of PQ in the future. Although, PQ is still the best, economically and effective herbicide ever produced, it safety and reliability specifically on male reproductive system remain equivocal. In this study, it is clear that PQ exposure has resulted a complete disruption to the male reproductive system of adult male rats, either by directly or indirectly mechanism is still remaining unclear. PQ caused impairment in testicular functions and altered it morphological appearances through germinal epithelium sloughing and increased of degenerating cells in the seminiferous tubules.

The production of reactive oxygen species (ROS) through metabolic reaction of PQ with oxygen in the body is known to be the main mechanism of action to male rat reproductive system. Although the effects of oral administration of PQ were less prominent than dermal application because of less absorption through gastrointestinal system, both routes of exposure resulted merely the same affect. The sperm count, which is the main parameter in this study, shows that PQ exposure decreased the number of

sperm as well as the mortality, motility and various abnormalities of sperm were also discovered.

The histopathological evaluation of testis also shows the gonadotoxic affect of PQ exposure for both oral and dermal administration with the evidence of germinal epithelium sloughing and degenerating cells. The evaluation on hormones and testicular marker enzymes revealed as well as increased of sperm abnormalities during the study proved that PQ is remain toxic to animals. However, all parameters were fully recovered after 2 weeks post exposure.

As a suggestions to this study, another study will be conducted on the evaluation of PQ of male pups of Sprague-Dawley rat. The pregnant female rat will be given an intraperitoneal (i.p.) injection to a various dose of PQ before mating and continuously until the rat give birth to pups. PQ will be given continuously through the lactation and male pups will be separated until their reach 70 and 170 days of life span. The evaluation of male reproductive system will be added for several parameters to extend the study.

Sertoli cell count will be added to the previous cell population study and a new technique of tissue fixation, embedding and staining will be introduced according to the standard procedure of Weinbauer & Nieschlag (1999). Testis will be fixed into the glutaraldehyde-osmium-tetraoxide fixation, embedded with epoxy-resin, stained with toluidine and will be cut through 1μm thickness. The special characterization and differentiation of cell population are clearly

visible and also it can reduced the error of tissue breaking. As the major mechanism of PQ in producing the ROS, malonialdehyde (MDA) and fluorescent pigmentation (FP) will be study for the evaluation of free radical parameters as well as in testicular marker enzymes, several enzymes such as sorbitol dehydrogenase (SDH), glucose-6-phosphate dehydrogenase (G6PDH), alkaline phosphatase (ALP) will be added.

As a conclusions, PQ is gonadatoxic to the male reproductive system and the production of free radical and ROS affects the normal spermatogenesis of male rats. However, the effects of PQ on male reproductive is reversible and it is safe for use under protection and regulation law. Uncontrolled and undermistaken of PQ solution can be fatal and until now, there is no antidote for PQ poisoning.

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APPENDIX 1

ORIGINAL ARTICLE

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Dermal exposure to the herbicide-paraquat results in genotoxic and cytotoxic damage to germ cells in the male rat

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The effects of exposure to low doses of paraquat, a herbicide, via the dermal route were studied on the spermatozoa of Sprague-Dawley rats. Paraquat (1, 1'-dimethyl-4, 4'-bipyridinium dichloride) was administered once a day for five days, at intervals of 24 h at 0, 6, 15 and 30 mg/kg, and the rats were sacrificed on days 7, 14, 28, and 42 after the last exposure. The sperm suspensions were obtained by mincing the caudae epididymes and ductus deferens for the purpose of performing a sperm morphology test, sperm count and analysis of sperm mortality and sperm motility, as per the standard procedures. The sperm count was decreased ($p < 0.05$) only on days 7 and 14 but sperm abnormalities increased on all days ($p < 0.05$). Sperm mortality increased at higher dose-levels ($p < 0.05$) except on day 42, and motility was affected by 30 mg/kg only on day 42. In conclusion, paraquat is a genotoxic and cytotoxic agent to germ cells in the male rat.

Key words: herbicides, pesticides, sperm abnormality, sperm count, sperm mortality, testicular function

INTRODUCTION

Paraquat (Paraquat dichloride; 1, 1'-dimethyl-4, 4'-bipyridinium dichloride) is a non-selective redox-cycling agroherbicide commonly used in weed control. Its herbicidal properties are enhanced by the addition of a single electron to the parent cation, thus generating the free radicals, owing to which it is highly toxic in almost all *in vitro* and *in vivo* systems [2, 8]. Consequently it has been observed as a mutagenic agent under different test conditions involving, for example, micro-organisms and cultured mammalian cells [9, 12]. Increased frequencies of sister-chromatid exchanges and chromosome aberrations have been observed in Chinese hamster pneumocytes exposed to paraquat [15, 16]. Intraperitoneal injection of paraquat to adult male ICR mice induced the formation of micronuclei in the peripheral blood and bone marrow cells [6]. Moreover, a recent study revealed that paraquat increased the incidence of micronuclei in polychromatic erythrocytes in the bone marrow of rats after a single dermal exposure [3]. Gene mutations and chromosomal aberrations have also been reported in several organisms living in a waste water reservoir contaminated by a paraquat manufacturing factory [4] and also in human lymphocytes *in vitro* [14].

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Its germ cell mutagenic potential in terms of increased sperm abnormalities was reported at dose-levels of 0.5, 1.5 and 3 mg/kg, after 1 to 3 weeks of intraperitoneal exposure in BALB/c mice [13]. Occupational or environmental exposure to paraquat or other pesticides is a daily affair for farmers mainly via oral or/and dermal routes. The latter route is a common means of exposure to paraquat and this pesticide is in agricultural use in more than 130 countries. However, there are few studies on its effects on male germ cells. The aim of the present study was to investigate the toxic effects of paraquat via the dermal route on germ cells in the male rat.

MATERIAL AND METHODS

Chemicals

Paraquat was purchased from Sigma Chemicals, USA (Lot No. 092K1359). All other chemicals and reagents used were of analytical grade.

Animals

Adult male Sprague Dawley rats (200–220 g body weight) were procured from the institutional animal house of University Sains Malaysia and housed in polypropylene cages with 3–4 animals per cage on paddy husk bedding under a controlled temperature of 21–25°C. All the animals received standard laboratory chow and water *ad libitum* during the experimental period.

Preparations of animals and paraquat treatment

The animals were allowed to acclimatise for a week and then were segregated into control and treatment groups. The control group consisted of 24 rats, which were further segregated into four groups of six animals each. The treatment group had 72 rats, which were segregated again into 12 groups of 6 rats each. The treatment groups received 6, 15 or 30 mg/kg of paraquat, equivalent to 1/15, 1/6 and 1/3 of LD_{50} for 5 consecutive days at intervals of 24 h. Approximately 24 h prior to the first application of paraquat the fur on the back of each rat was carefully shaved without causing any skin abrasions. This procedure was performed only once before the first treatment and was not carried out during the treatment. Paraquat was applied uniformly over the shaved area of skin with porous gauze for 4 hours. The area of paraquat application was further covered by an adhesive tape and restrainers were used to prevent the rat from reaching to the area of application. At the end of the fourth hour the skin was cleaned with distilled water. The control groups of rats also underwent similar preparation but were exposed to distilled water. Following the last treatment the rats were sacrificed by cervical dislocation on days 7, 14, 28 and 42.

Sperm count

A laparatomy was conducted and the reproductive system was exposed. The epididymis was separated from the testis. The cauda epididymis was further minced in 1 mL of phosphate buffered saline (pH 7.4) and the suspension thus obtained was filtered through a 80 μ nylon mesh. To the filtrate one drop of 1% eosin Y was added and left for 30 minutes. The stained sperm suspension was sucked slowly into a leukocyte haemocytometer exactly up to the 0.5 mark and then further diluted with phosphate buffered saline up to the 11 mark and mixed thoroughly. The diluted suspension was charged into a Neubauer counting chamber. The sperm count was performed according to the standard procedure [10, 17]. Briefly, the sperm in 8 squares, excluding the central erythrocyte area, were counted and the total count was then multiplied by 5×10^4 to obtain total sperm per epididymis.

Sperm morphology test

For the evaluation of abnormal sperm morphology smears were prepared from the filtrate on clean glass slides and dried. One thousand sperm per animal were screened and classified into normal and different types of abnormal spermatozoa as described earlier [11, 18]. The different types of abnormal spermatozoa were headless, double headed, microcephalous, defective at the cephalocaudal section, hookless, banana-shaped, amorphous, coiled, double tailed and broken tailed. Total sperm abnormality was expressed as percentage incidence per group.

Sperm motility and mortality

In order to estimate the effects on these parameters the ductus deferens was removed and placed in 1 mL of normal saline. A sample of this sperm suspension was charged into Makler's counting chamber, sperm motility and mortality were recorded as per standard procedure, and sperm motility was graded [5]. Dead and immotile sperm were considered equivalent in recording the sperm motility.

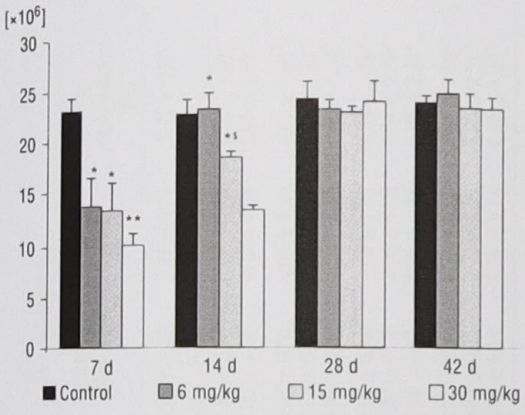


Figure 1. The effect of dermal exposure to paraquat on epididymal sperm count in the rat. Data are expressed as means ±SD. from 6 animals per group; $^{*}p < 0.05$: vs. control; $^{\#}p < 0.05$: 6 mg/kg vs. 15 mg/kg and 30 mg/kg on day 7; $^{\$}p < 0.05$: 15 mg/kg vs. 30 mg on day 14; one-way ANOVA followed by Bonferroni's post hoc test.

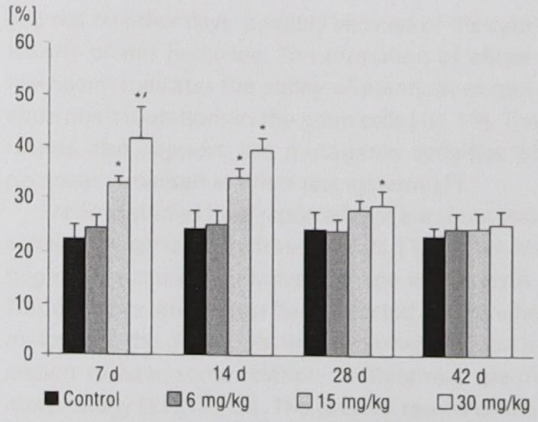


Figure 2. The effect of dermal exposure to paraquat on sperm mortality in the rat. Data are represented as means ±SD from 6 animals per group; $^{*}p < 0.05$: vs. control; $^{\#}p < 0.05$: 15 mg/kg vs. 30 mg/kg on day 7; one-way ANOVA followed by Bonferroni's post hoc test.

Statistical analysis

Data were expressed as means ±SD for each group (six rats per group). Differences were compared for statistical significance by one-way ANOVA followed by Bonferroni's post hoc test. The differences were considered as significant when $p < 0.05$.

RESULTS

The treatment with paraquat via the dermal route caused a decrease in sperm count on days 7 and 14 ($p < 0.05$; Fig. 1). The effect was in a dose-dependent pattern on day 7, whereas only two higher doses showed such an effect on day 14. Paraquat significantly induced the formation of abnormal sperm ($p < 0.05$; Table 1). On day 14 a dose-dependent increase was observed. On other sampling days however, only two lower doses showed a dose-dependent increase and the effect was less marked at a higher than at a lower dose-level, although not significantly so. At 6 mg/kg the abnormal sperm increased in a time-dependent pattern up to day 28 but decreased on day 42, whereas at 15 mg/kg the effect was greater on day 7 and at 30 mg/kg the effect was greater on day 14 and so on, with the effect subsiding in a time-dependent pattern (Table 1). Sperm mortality was increased in the paraquat-treated groups up to day 28. However, the induced mortality was significant only at higher dose-levels ($p < 0.05$). Sperm motility was just affected by 30 mg/kg only and unaffected by other doses (Fig. 2).

DISCUSSION

For many years occupational or inadvertent exposure to pesticides has been unavoidable, at least for farmers. The main routes of exposure to these treacherous chemicals are the oral and the dermal, although the latter may be the more common. The objective of the present study was to examine the adverse effects on spermatozoa of a commonly used herbicide, namely paraquat. The results showed that paraquat exerted cytotoxic effects on germ cells, especially on epididymal sperm (on day 7) and late spermatids (on day 14), thus decreasing the sperm count. The lack of effect on days 28 and 42 indicates that paraquat did not affect spermatocytes and spermatogonia. The cytotoxicity was further evident in terms of increased incidences of sperm mortality. The cytotoxicity must have been exerted via the free radical generation, as this herbicide is known to have such properties [7]. Paraquat, however, does not seem to affect sperm motility, although 30 mg/kg showed some positive effect, which we believe may not be biologically important.

On the other hand, paraquat affected the morphogenesis of spermatozoa at all dose-levels and on all sampling days, indicating that there was interference in the metamorphosis of germ cells into mature structurally perfect sperm. Sperm abnormality increased in a dose-dependent pattern only on day 14

Table 1. *The effect of dermal exposure to paraquat on sperm morphology in the rat. Data are represented as means ±SD*

Sample time (days)	Dose PQ [mg/kg]	Head deformities					Tail deformaties			Total abnormalities (%)
		Headless	Double headed	Micro-cephalous	Cephalo-caudally defective	Hookless	Banana shaped	Amorphous	Coiled tailed	Double tailed	Broken tailed	
	Control	3.64 ± 0.33	0.00 ± 0.00	4.00 ± 0.58	2.66 ± 0.67	13.00 ± 1.52	7.00 ± 1.54	0.67 ± 0.33	1.00 ± 0.54	0.00 ± 0.00	2.33 ± 0.34	3.22 ± 2.94
7	6	5.35 ± 0.38	0.00 ± 0.00	3.67 ± 0.89	8.03 ± 1.52	12.02 ± 1.15	3.76 ± 0.66	2.34 ± 0.82	3.43 ± 0.58	0.30 ± 0.12	4.36 ± 1.46	4.34 ± 2.66*
	15	7.64 ± 1.21	2.24 ± 0.36	0.68 ± 1.20	10.88 ± 0.38	22.46 ± 1.14	6.68 ± 0.62	3.68 ± 1.28	2.06 ± 0.18	2.48 ± 0.58	4.78 ± 1.28	6.35 ± 1.66*
	30	5.88 ± 0.68	1.66 ± 0.12	3.33 ± 1.22	9.08 ± 2.08	17.66 ± 0.89	6.28 ± 0.34	2.44 ± 0.26	1.36 ± 0.34	1.33 ± 1.20	4.36 ± 0.88	5.64 ± 2.98*
14	6	4.42 ± 0.58	0.00 ± 0.00	3.68 ± 0.62	6.36 ± 1.21	17.27 ± 0.42	7.12 ± 0.51	1.32 ± 0.42	1.66 ± 0.34	0.00 ± 0.00	4.00 ± 0.54	4.66 ± 2.50*
	15	4.36 ± 0.35	2.08 ± 0.83	5.68 ± 0.84	6.48 ± 0.32	21.82 ± 0.66	9.38 ± 2.80	4.44 ± 1.14	2.66 ± 1.48	2.88 ± 0.28	6.38 ± 0.94	6.38 ± 1.92*
	30	4.62 ± 0.36	3.34 ± 1.16	4.88 ± 0.82	9.68 ± 1.34	24.22 ± 0.52	6.22 ± 0.98	3.34 ± 0.88	3.68 ± 0.68	3.08 ± 0.54	4.28 ± 1.86	6.68 ± 1.68*
28	6	4.76 ± 0.34	0.00 ± 0.00	4.42 ± 0.58	7.73 ± 1.20	14.02 ± 1.47	4.00 ± 0.50	1.22 ± 0.14	2.68 ± 0.53	0.00 ± 0.00	4.68 ± 1.53	4.84 ± 2.38*
	15	7.66 ± 0.35	1.48 ± 0.58	4.06 ± 0.60	5.38 ± 0.58	16.88 ± 1.52	2.36 ± 0.54	3.66 ± 0.34	2.63 ± 0.38	1.68 ± 0.22	6.06 ± 1.16	5.54 ± 2.48*
	30	4.66 ± 0.33	0.00 ± 0.00	4.48 ± 0.82	4.24 ± 0.58	17.04 ± 1.58	3.66 ± 0.82	4.33 ± 0.58	2.68 ± 0.66	0.68 ± 1.02	4.66 ± 1.15	4.68 ± 2.34*
42	6	3.64 ± 0.32	0.68 ± 0.16	3.68 ± 0.21	4.76 ± 0.26	18.36 ± 0.36	3.48 ± 1.21	5.44 ± 1.08	2.34 ± 0.35	0.00 ± 0.00	6.23 ± 0.56	4.08 ± 3.68*
	15	6.02 ± 0.51	1.34 ± 0.58	5.12 ± 0.23	5.02 ± 0.54	19.66 ± 1.45	5.00 ± 1.75	3.00 ± 0.34	2.54 ± 0.32	3.34 ± 0.62	7.02 ± 0.89	5.02 ± 2.32*
	30	940.92 ± 3.78	5.58 ± 0.36	2.02 ± 0.26	5.52 ± 1.74	6.78 ± 0.58	21.22 ± 0.54	2.68 ± 0.36	4.48 ± 0.78	3.08 ± 0.34	3.36 ± 0.14	4.52 ± 1.59*

*$p < 0.05$: vs. control; one-way ANOVA followed by Bonferroni's post hoc test

and not on other days, possibly because of the cytotoxicity of this herbicide. The formation of abnormal sperm indicates the ability of paraquat to generate point mutations in the germ cells [10, 19]. The results also support the mutagenic activities of paraquat visualised in other test systems [1].

Previous studies have reported that paraquat was capable of generating free radicals [1, 7], which might have caused oxidative damage in the testis. This oxidative stress must have affected the genetic material of the germ cells, and this phenomenon is known to have some relation to abnormal sperm morphology [11, 18, 19]. The present results of the sperm morphology test are in consensus with that of an earlier report [13], in which paraquat affected the morphology of sperm in mice. Another study has shown positive genotoxic effects in *Drosophila melanogaster*, in terms of increased sex-linked recessive lethality [14]. In conclusion, the results of this study indicate that paraquat is cytotoxic and genotoxic to male germ cells in the rat.

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Short communication

Genotoxic and cytotoxic effects in the bone marrow of rats exposed to a low dose of paraquat via the dermal route

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Abstract

The genotoxic effect of the herbicide paraquat was studied in rat bone-marrow by means of the micronucleus assay. Paraquat at dose levels of 6, 15 and 30 mg/kg body weight was given to rats in a single application via the dermal route. Marrow was collected at 24, 48 and 72 h after the application. The micronucleus assay was done as recommended by standard procedures. Paraquat gave rise to an increase in the number of micronuclei in a dose-dependent manner. The number of micronucleated polychromatic erythrocytes showed a maximum at 48 h and the toxicity was further prolonged, as there was no complete recovery at 72 h. These findings suggest a genotoxic effect of paraquat even after exposure via dermal application.
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Keywords: Genotoxicity; Paraquat; Micronucleus test; Dermal exposure

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride) is a non-selective contact herbicide. Its herbicidal and toxic properties are dependent on the ability of the parent cation to undergo a single electron addition to form free radical molecules. It is widely used in agriculture as a non-selective and useful herbicide, with a wide range of toxicity involving generation of reactive oxygen species (ROS) [1,2]. In vivo experiments with rats have demonstrated that paraquat-induced cell death is a consequence of the generation of ROS, which is reduced by pretreatment with a superoxide dismutase mimetic [2]. Numerous environmental and industrial chemicals are known to induce cytogenetic damage in human peripheral blood lymphocytes [3] and rat lung cells [4]. Paraquat is a widely used herbicide in agriculture since 1963 and freely available as a potential environmental pollutant. It is known to arrest the cell cycle

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of cultured Madin–Darby canine kidney (MDCK) cells in the S-phase [5]. Inhibition of cell growth and DNA synthesis were reported in R-66 human embryonic lung cells after exposure to paraquat [6]. Single and multiple injections of paraquat in mice resulted in a dose-dependent increase in mitotic index, the frequency of chromosomal aberrations indicating its mutagenic activity. In contrast to these genotoxic effects, there are studies in human peripheral lymphocyte cultures [7] and V79 Chinese hamster cells [8] that report no such effects. Paraquat exposure through the dermal route is common among the population, by means of direct contact during application or indirect exposure through the environment. However, studies highlighting its mutagenic potential after exposure via this route are scanty. In the current study we examined the genotoxic effects of dermal exposure to paraquat in rats by means of the micronucleus assay, which is widely used to detect the genotoxic effects of environmental mutagens.

2. Material and methods

2.1. Animals

Male Sprague–Dawley Albino rats (9–11 weeks old) were used in the present study. They were housed in polypropylene cages with wood dust bedding and given free access to food and water. Animals were segregated into different groups, i.e. a control group and an experimental group, each consisting of six rats.

2.2. Drug

Paraquat was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity commercially available. Paraquat was applied via the dermal route dissolved in distilled water (single exposure) at doses of 6, 15 and 30 mg/kg bw, equivalent to 1/15, 1/6 and 1/3 of the LD_{50}, respectively. Approximately 24 h prior to the application of paraquat, fur on the specified area (dorsal area of the trunk) was carefully removed by shaving and clipping and care was taken to avoid any abrasion on the skin. The test substance was applied uniformly over the skin with a porous gauze dressing and non-irritating tape for a period of 4 h. The area of application was further covered with a tape to retain the gauze intact. Restrainers were used to prevent the animal from reaching the area of drug application. At the end of the application period (4 h), the skin was cleaned with distilled water. The control group of rats was exposed under similar conditions with application of distilled water.

2.3. Bone marrow micronucleus assay

At 24, 48 and 72 h after application of paraquat (single application), animals were anaesthetized (sodium pentobarbitone, 45 mg/kg bw, i.p.) and killed by an overdose. The femora were dissected and the bone marrow was aspirated in a syringe with 1 ml of 5% fresh bovine albumin in phosphate-buffered saline (pH 7.2) as previously described [9,10]. Bone marrow was mixed thoroughly and the cell suspension was centrifuged at $180 \times g$ for 5 min. The pellet was then resuspended in fresh bovine albumin and smears were prepared on clean glass slides and dried. Smears were fixed in methanol for 5 min and stained with May–Gruenwald/Giemsa stain at pH 6.8 [10,11]. Stained slides were screened microscopically under oil-immersion objective after coding. The number of polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs), micronucleated PCEs (MNPCEs) and micronucleated NCEs (MNNCEs) were recorded until 2000 PCEs were scored per rat. For each rat, the percentages of MNPCE, MNNCE and PCE were computed.

2.4. Statistical analysis

For each group, the mean and S.D. were calculated and the data were analyzed by one-way ANOVA, post hoc Bonferroni test. $P < 0.05$ was considered as the level of significance.

3. Results

Dermal exposure of paraquat in rats resulted in the induction of micronuclei. The number of MNPCEs showed a dose-dependent increase ($P < 0.05$–0.001) in the bone marrow (Table 1). The maximal response was observed at 48 h after exposure. At 24 h after exposure to the drug the genetic damage was less compared with that at 48 h and the maximum toxicity was evident at 48 h at all dose levels studied. At 72 h after exposure, there was a dose-dependent increase in the number of

Table 1
Bone marrow micronucleus assay in paraquat-treated animals

Dose (mg/kg)	Post-sample time (h)	MNPCE (%)	MNNCE (%)	PCE (%)
0	–	0.15 ± 0.01	0.12 ± 0.01	50.12 ± 0.63
6	24	0.90 ± 0.04	0.32 ± 0.03	38.14 ± 1.24
	48	0.98 ± 0.06	0.30 ± 0.02	26.68 ± 1.86
	72	0.79 ± 0.07	0.62 ± 0.03	42.62 ± 1.47
15	24	1.09 ± 0.14	0.30 ± 0.05	23.11 ± 1.04
	48	1.28 ± 0.16	0.23 ± 0.04	36.13 ± 1.61
	72	0.71 ± 0.03	0.61 ± 0.03	41.56 ± 1.43
30	24	1.91 ± 0.14	0.33 ± 0.11	21.11 ± 1.17
	48	1.99 ± 0.04	0.22 ± 0.03	20.60 ± 1.06
	72	0.98 ± 0.03	0.53 ± 0.63	35.62 ± 1.54

Values are given as mean and S.D. from six rats per dose and sample time. All values are significantly different from the controls ($P < 0.05$–0.001).

MNPCE and MNNCE compared with the control, but it revealed a recovery compared with the 24 and 48 h post-exposure time point ($P < 0.05$). The recovery was significant and dose-dependent at 72 h compared with the 24 and 48 h samples ($P < 0.05$).

4. Discussion

Chromosomal damage was implicated as the major cause for the appearance of micronuclei at the last stage of mitosis and as an indicator of genotoxic insult to the nuclei [12]. There has been an enormous growth in the pesticide industry around the world, and herbicides like paraquat are targeted to destruction of noxious weeds. Both animals and human beings are frequently exposed to these chemicals either directly or indirectly. As exposure via the dermal route by means of skin contact is most of the time an unavoidable situation for the majority of the exposed population, it is important to evaluate any toxic effect of these chemicals affecting the health. Thus, the main aim of the present study was to evaluate the toxicogenetic effect of paraquat, administered by dermal exposure, on somatic cells of the bone marrow.

The results of our study revealed that paraquat induced the formation of micronuclei at the doses studied. Similarly Salam et al. [12] evaluated the genotoxic effects of paraquat in different living systems—*Allium cepa*, *Vicia faba*, yeast, *Drosophila melanogaster*—and in human lymphocytes. Most of these studies indicated that paraquat is mutagenic at the cytological level in a dose-dependent manner. Exposure to wastewater condensate from the paraquat industry also showed the clastogenic activity of paraquat [13] in V79 cells in the alkaline elution assay and the 6-thioguanine-resistance mutation assay. Intraperitoneal injection of paraquat in mice in single and multiple doses also revealed an increase in the frequency of chromosomal aberrations [14]. Ortiz et al. [15] observed an increase in the number of micronuclei at a dose of 2× 20 mg/kg bw (given intraperitoneally at 48 h interval) and could notice a high incidence of genotoxic insult up to 72 h after the last exposure. Since exposure via the dermal route through skin contact is a most relevant and common situation, our study further strengthens the evidence of genotoxic effects of paraquat in different doses and exposure times.

The present finding shows that exposure to paraquat significantly increases the frequency of micronuclei when administered via the dermal route. Furthermore, it has been suggested that the molecular mechanism of paraquat involves the generation of free radicals [16], which is known to cause extensive strand breakage and chromosomal damage [13]. This study concludes that paraquat exerts genotoxic and cytotoxic effects when administered to rats in a single dose via the dermal route. Herbicide applicators should take measures to avoid skin contact during their occupation.

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PERMOHONAN LANJUTAN TEMPOH KAJIAN DAN LANJUTAN KELULUSAN ETIKA (MANUSIA)
GERAN FRGS
(Request of Research Extension and Ethical Approval Extension [Human] FRGS Grant)
PUSAT PENGAJIAN SAINS PERUBATAN

1. Tajuk Penyelidikan: *(Research Title)* Evaluation of the Toxic effects of Paraquat and mechanism of its action on reproductive syst of male

2. Ketua Penyelidik: *(Principal Researcher)* Prof. Madya Dr. S. Sivachandra Raja

3. Penyelidik Bersama: (Co-Researcher) Che Norlah Othman & Prof. Madya M. Madhavan

4. a) Tarikh Mula diluluskan: *(Approval date of Commencement)* Nov-2002 b) Tarikh Tamat diluluskan.: *(Approval Date of Completion)* 14/11/2005

   c) Tempoh Penyelidikan: *(Research Duration)* 3 years

   d) Tarikh Mula Sebenar: *(Actual Commencement Date)* Nov-2002 e) Tarikh dijangka selesai: *(Completion Date)* 14/11/2005

5. Tempoh dipohon: *(Requested Period)* 3 months (Mulai *(starting)* 15/11/2005 Hingga *(until)* 14/2-2006

6. Sebab Lanjutan diperlukan
   (Reason for Request of Extension)

   Mile Stone-5 - Hormonal estimation is not yet finished. Hormonal kits just started arriving. We are in the middle of Hormonal estimation.

7. Peringkat (%) Kemajuan Projek
   (Progress of Project/Stage (%) 95%.

Diperakukan/Tidak Diperakukan

Tandatangan Pemohon *(Applicant's Signature)*

30/10/2005
Tarikh *(Date)*

Tandatangan Pengerusi Jawatankuasa
Penyelidikan & Etika PPSP/Dekan PPSP

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Date : 15 May 2002

Dr. Urban J. A. D. Souza
Department of Physiology
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KELANTAN

Dear Dr.,

APPLICATION FOR ETHICAL APPROVAL

Protocol Title : **Evaluation of the Toxic Effects of Paraquat and Mechanism of its Action on Reproductive System of Male Rat.**

I refer to your application of **14 April 2002.**

I am pleased to inform you that the Research & Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia has met on **14 May 2002** and has approved in principle the protocol study of the above title.

Title	: Evaluation of the Toxic Effects of Paraquat and Mechanism of its Action on Reproductive System of Male Rat.
Research Centre	: Universiti Sains Malaysia
Date Start	: May 2002
Duration	: 24 months
Number of Subjects	: 400 subjects
Name of Principal Researcher	: Dr. Urban J. A. D. Souza
Co-Investigator	: ⇨ Che Noriah Othman ⇨ Assoc. Prof. Madhavan M ⇨ Assoc. Prof. Sivachandra Raju
Master Students	: -
Financial Support	: Ministry of Education

Kampus Kesihatan, 16150 Kubang Kerian, Kelantan, Malaysia
Tel: 609-7663000/7663500 Fax: 609-7653370
e-mail: ppsp@kb.usm.my

The following item (✓) have been received and reviewed and in connection with the above study to be conducted by the above investigator.

(✓) Clinical Study Protocol (Dated : -)

( ) Patient Information Sheet
- ( ) English Version (Dated :)
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- ( ) Other Language (Dated :)

( ) Consent Form
- ( ) English Version (Dated :)
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( ) Investigator's Brochure (Dated :)
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Members of the Research & Ethic Committee who reviewed the above protocol/documents are as follows:

Member (Title and Name)	Occupation (Designation)	Male/Female (M/F)	Tick (✓) if present when above items, were reviewed
Chairperson : Assoc. Prof. Zabidi Azhar Mohd. Hussin	Dean, School of Medical Sciences	M	X
Secretary : Mr. Halim Othman	Chief Scientific Officer	M	✓
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1. Dato' Hj. Wan Mohamad Yusoff	Ex-State Secretary of Kelantan	M	✓
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5. Assoc. Prof. Jafri Malin Abdullah	Coordinator, Neuroscience Unit	M	X
6. Assoc. Prof. E. T. Larmie	Assoc. Prof., Physiology Department	M	X

7.	Madam Siti Hawa Ali	Lecturer, School of Health Sciences	F	✓
8.	Dr. Syed Hatim Noor	Coordinator, Biostatistic & Research Methodology Unit	M	✓
9.	Dr. Ab. Hamid Mat Sain	Head, Surgery Department	M	✓
10.	Dr. Ramli Saad	Director, Hospital U.S.M.	M	X
11.	Dr. Mary Abraham	Director, Hospital Kota Bharu, Kelantan	F	X
12.	Hj. Ismail Hassan	Ex-USM Linguistic Teacher	M	X

Please submit your **Progress Report** (6 months after commence) and a **Final Report** upon completion of the research to Chairman of Research & Ethics Committee, School of Medical Sciences, Health Campus.

Thank you.

"GLOBAL COMPETITIVENESS: OUR COMMITMENT"

Yours sincerely,

[redacted]

(ASSOC. PROF. ZABIDI AZHAR MOHD. HUSSIN)
Chairman of Research & Ethics Committee

c.c. ☛ **Secretary of Research & Ethics Committee**

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