DETECTION OF BIOMOLECULES INDUCED AT SUBLETHAL LEVELS OF PYRETHROIDS

THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

By
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December – 2006

DECLARATION

I hereby declare that the thesis entitled **"DETECTION OF BIOMOLECULES INDUCED AT SUBLETHAL LEVELS OF PYRETHROIDS"** submitted to the University of Mysore, for the award of the degree of Doctor of Philosophy in Biotechnology, is the result of the research work carried out by me in the Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore, under the guidance of Dr. B. E. Amitha Rani, during the period November, 2001 to November, 2006.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

Date:
Place: Mysore

Ritesh Narayanpur
Senior Research Fellow

CERTIFICATE

I hereby certify that this thesis entitled **"DETECTION OF BIOMOLECULES INDUCED AT SUBLETHAL LEVELS OF PYRETHROIDS"** submitted by Mr. Ritesh Narayanpur for the award of the degree of Doctor of Philosophy in Biotechnology, University of Mysore, is the result of the research work carried out by him in the Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore, under my guidance and supervision during the period November, 2001 to November, 2006.

Dr. B. E. Amitha Rani
Scientist
FPIC Department

Date:
Place: Mysore

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ABBREVIATIONS

~ - Nearly equal to

% - Percent

^{0}C – degree Celsius

2^{0} – Secondary

Ab – Antibody

Ag – Antigen

AGE – Agarose gel electrophoresis

Apaf1 - Apoptotic protease-activating factor 1

BSA - Bovine Serum Albumin

d.w. – distilled water

EDTA - Ethylene Diamine Tetraacetic acid

EGTA - Ethylenebis(oxyethylenenitrilo) tetraacetic acid

ELISA – Enzyme Linked Immunosorbent Assay

etc - et cetera

FADD - Fas-associated death domain protein

Fig – Figure

FIGE - Field Gel Electrophoresis

g – Centrifugal force

GC – Gas Chromatography

h – hours

H_2O_2 – Hydrogen Peroxide

Ha - Hectare

HRP – Horse Radish Peroxidase

i.e. – That is

K – thousand times

KD – kilo daltons

L – Litre

M – Molar

mA – milli amperes

M – Molar

mA – milli amperes

mg – milligram

mg/kg b.w. – milligram per kilogram body weight

min – minutes

ml – millilitre

mM – milli Molar

mV – milli volts

N – Normality

NC – Nitrocellulose

NaCl – Sodium Chloride

ng – nanograms

nm – nanometers

nmol – nano moles

nNOS/NOS – neuronal Nitric Oxide Synthase

No. - Number

OC - Organochlorine

OP - Organophosphate

PAGE – Polyacrylamide Gel Electrophoresis

pg – pictogram

ppm - parts per million

ppt – precipitate

rpm - revolutions per minute

RT – room temperature

SDS – Sodium Dodecyl Sulphate

TLC – Thin Layer Chromatography

TMB - (4,4',5,5') Tetramethyl Benzidine

TNF - Tumour necrosis factor

TRAIL - TNF-related apoptosis-inducing ligand

TTN buffer - Tris-Tween-NaCl (TTN) buffer

µg – micro gram

µl – microlitre

µmol – micro mole

viz - namely

vol - volume

w/v – weight/volume

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SYNOPSIS OF THE Ph.D THESIS

DETECTION OF BIOMOLECULES INDUCED AT SUBLETHAL LEVELS OF PYRETHROIDS

Pesticide residues are often reported in foods and known to gain entry into the human body. Pesticide body burden can induce cell mortality and thereby pose health threat. Synthetic pyrethroids like Deltamethrin, Cyhalothrin etc are the new generation pesticides used widely on crops and insect ectoparasites. According to a safety report of the WHO in 2005, pyrethroids are widely used in public health because of their relative safety for humans, high insecticidal potency at low dosages and rapid knock-down effects. Apart from their use in agriculture, pyrethroids play an important role in public health programmes. Globally, more than 520 tonnes of active ingredient of pyrethroids is annually used in vector control programmes alone. Hence, it becomes imperative to understand the safety mechanism of the pyrethroids and the reason behind their low toxicity in non-target species. In this study, we have tried to probe the possibility of programmed cell death or apoptosis being one of the possible safety mechanisms for the pyrethroids in non-target species.

Cell death is due to a programmed sequence of morphological, biochemical and molecular changes which are characteristic and often unique to this mode of cell death. Specific features of apoptic cells causing these changes serve as early markers to alert the mode of cell death and can be developed as a

tool to forecast the programmed cell death. The extent of apoptosis in cultures or in tissues can be measured qualitatively and quantitatively to assess cell death.

Many methods have been developed to identify these changes and are based on alterations in cell morphology, plasma membrane, molecular structure, and DNA stability to denaturation and endo-nucleolytic DNA degradation. Electron microscopy, agarose gel characterization, flow cytometry, immunoassays and trypan blue exclusion are techniques generally used to identify these changes. Release of ^{3}H thymidine by relabeled cells is another method used frequently. However, no indigenous immunoassays are available.

Presence of any stress factor is required in apoptosis to trigger the cascade of biochemical events described above. This could be starvation, pollutants, physical stress etc. A few pyrethroids like deltamethrin although claimed to be “safe” have been reported to cause apoptotic changes in male rats. Deltamethrin is known to cause cell death due to apoptosis which is a genetic phenomenon involving the DNA. In general, apoptosis involves a chain of events wherein the zymogen, Caspase 3, is activated which in turn triggers Caspase 6 leading to DNA fragmentation (DNA “Ladder” formation & Nucleosome formation-mediated by endonucleases)

In the present study, it was aimed to screen some pyrethroid pesticides for their apoptotic potential in CFT-Wistar rats and to study the biochemical deviations of early apoptotic biomarkers. Deltamethrin and Cyhalothrin were chosen as

triggers for apoptosis. In view of this, the following objectives were chosen for the present study:

OBJECTIVES:

1. To study the genotoxic potential of selected pyrethroid pesticides.
2. Biochemical and morphological deviations as a result of xenobiotic exposure using *in vivo* (rat) and *in vitro* (HeLa cell culture) model systems.
3. Measurement of Caspase 3 and NOS levels to indicate initiation of apoptosis.
4. Use of the markers to develop quick detection methods.

Chapter 1 contains a **brief introduction and review of literature** on the pyrethroids and their mechanism of action. It describes the various studies that have observed the effects of pyrethroids including their apoptotic potential in different animal models at different doses. The various methods used to detect apoptosis currently in vogue are also discussed.

Chapter 2 describes the study of the **apoptotic potential of the selected pyrethroids** (cyhalothrin and deltamethrin) at sublethal doses at both acute and sub-acute doses in adult and young Wistar rats. The pyrethroids were also tested in an *in vitro* model (HeLa cells) to screen their cytotoxicity potential. The rat tissues were used for isolation of genomic DNA and biomarkers of apoptosis. The tissues were also examined for histopathological and haematological aberrations. The DNA was analysed for typical apoptotic fragmentation. Cyhalothrin residue was analysed in all tissues of the cyhalothrin-treated rats.

In vitro studies showed that the two pesticides showed significant cytotoxicity at doses of 1 and 0.01 ppm compared to the control. *In vivo* studies revealed that deltamethrin was found to be apoptotic in the young rats (kidney tissue) at a dose of 200 mg/kg b.w. as seen by DNA laddering in the kidney, while the adults and other young rat groups did not show evidence of apoptosis. The apoptotic tissues were used to extract the biomarker – caspase-3 and the activity of this enzyme was found to be 3-times higher in the treated tissue extract than that of the control tissue extract. The activity of the commercial caspase-3 was found to be 4 times more than that of the control. There was no change in the haematological and histopathological profile in the pesticide treated rats when compared to the control rats.

Cyhalothrin was not found to be apoptotic at any of the age groups (Young or Adult), dose groups, treatment groups or routes of administration used. Cyhalothrin residue analysis by TLC and GC showed its presence in all the tissues at the 200 mg/kg b.w. group and only in the adipose and uterus in the other treatment groups. TLC showed the presence of the hydroxy metabolite in the reproductive organs and the adipose tissue. GC demonstrated that the Retention time (R_t) of cyhalothrin was 15.15 min, while it was 14.64 min and 14.98 min for the hydroxy and acid metabolite respectively. A preferential accumulation in the reproductive organs was observed.

Higher amount of the hydroxy metabolite was seen in the liver and the uterus

while the parent cyhalothrin was found in the adrenal and uterus at the highest level based on their lipid content. Lipid-rich organs like adipose and the reproductive organs (uterus and ovary) showed higher concentrations of cyhalothrin. Higher amount of hydroxy metabolite of cyhalothrin was seen in liver and uterus while the parent molecule was found at a higher concentration in adipose, uterus and ovary in that order.

Chapter 3 elucidates the **development of an immunoassay based on rabbit antibodies** for the detection of the 2 chosen apoptotic biomarkers namely, caspase-3 and neuronal Nitric Oxide Synthase (nNOS). Biomarkers of apoptosis were extracted from the deltamethrin-treated rat tissues (caspase-3 and rat nNOS) and untreated porcine brain (porcine nNOS). The purity of the biomarkers was estimated by using Native and SDS-PAGE followed by immunoblotting using commercial antibodies against nNOS and caspase-3. The purified biomarkers were used to raise antibodies in the rabbit and an ELISA method was developed and standardized for their detection.

Optimum conditions for the assay were: carbonate buffer as coating buffer, PBS as dilution buffer, antisera dilution of 1/6400 for caspase-3, antisera dilution of 1/1600 for nNOS from both rat and porcine and 1/10 K 2^0-Ab-HRP conjugate compared to 1/1000 for the commercial Ab dilution.

An IgG-based ELISA method was developed for detection of caspase-3 and nNOS. This method was very sensitive and could detect up to 1ng of caspase-3

from rat kidney and 3 ng of nNOS from both rat and porcine brain. This assay was comparable to the commercial antibody assay. This immunoassay can be developed as a sensitive tool for the detection of apoptotic biomarkers and has immense potential in health forecasting.

Chapter 4 deals with the **development of an immunoassay based on chicken egg-yolk antibodies for the detection of caspase-3 and nNOS**. This is the first report of an IgY-based method for the detection of biomarkers of apoptosis i.e. the caspase-3 and nNOS. Native PAGE was used for characterization of the IgY purified by all the 3 methods

Of the 3 purification methods for IgY tested, the M1 (CFTRI patented method) and M3 (Chloroform method) were found to be more efficient than the M2 method (PEG method). Both the CFTRI patented method (M1) and chloroform method (M3) gave high yield of the IgY antibody (65-75 mg/egg) over the entire experimental period while M2 gave a low yield of 15-25 mg/egg.

Assay development with the antibodies obtained from the 3 different purification methods revealed a sensitivity of 3 ng with M1, 10 ng with M2 and 3 ng with M3. IgY antibodies from the hen were produced in the range of 6-7 g/hen unlike rabbits which produce only 0.18-2 g/rabbit. Hence a large quantity of antibody obtained in hens spares the efforts of immunizing the animal repeatedly.

The assay was found to be as sensitive as the IgG-based assay (chapter 3) and could detect 3 ng of caspase-3 and nNOS. This IgY-based method is non-

invasive and inexpensive alternative to IgG-based methods used for detection of caspase-3 and nNOS.

Chapter 5 describes a **semi-quantitative method based on dot blotting for the detection of caspase-3 and nNOS** using commercial, rabbit and egg-yolk antibodies. The dot blots were also analysed using colour capture device (CCD) imaging.

Dot blots for the biomarkers could detect up to 30 ng of caspase-3 and 100 ng of nNOS by all the 3 antibodies used. The primary antibody and 2^0-Ab-HRP dilutions used for ELISA in chapter 3 and 4 were applicable for the dot blot methods also. Dot blots were found to be a comparable alternative to ELISA, but as a semi-quantitative method and as a quick yes/no test for caspase-3 and nNOS. However, cost wise, ELISA and dot blots were similar.

CCD imaging of the dot blots indicated the method to be as sensitive as visual analysis. However, it had the added advantage of being quantifiable and more accurate.

A bibliography of the various articles, books and journals referred to for the thesis is also included.

CONTRIBUTION OF THE THESIS

This is the first reported study on the:

- Apoptotic potential of cyhalothrin
- Use of IgY antibodies for detection of caspase-3 and nNOS
- Dot blots and CCD image analysis for caspase-3 and nNOS.

The other highlights of the thesis are:

- ✓ Among the two pyrethroids used, Deltamethrin was found to be apoptotic at 200 mg/kg b.w. in young female CFT-Wistar rats but Cyhalothrin did not show evidence of apoptotic changes in both adult and young rats.
- ✓ Apoptosis could be a safety mechanism resulting in low toxicity of the pyrethroids.
- ✓ Residues of Cyhalothrin and its metabolite were found to preferentially accumulate in the reproductive organs besides the liver and adipose.
- ✓ An ELISA method developed in this study using IgG from rabbits had a sensitivity of 3 ng for detection of both caspase-3 and nNOS, at a titre and sensitivity comparable to the commercial antibody.
- ✓ The ELISA method developed here using IgY had a similar sensitivity (as the IgG) of 3 ng for detection of both caspase-3 and nNOS.
- ✓ IgY antibodies could be a cheaper and non-invasive method of producing antibodies against caspase-3 and nNOS.

- ✓ A Dot blot method was developed as a quick and viable alternative to ELISA for semi-quantitative detection of the biomarkers and it had a sensitivity of 30 ng for caspase-3 and 100 ng for nNOS, as indicated visually and by CCD imaging.
- ✓ CCD imaging has a distinct advantage over virtual analysis of the dot blots as it is quantifiable and thus more accurate.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

INTRODUCTION

The use of pesticides in agricultural practices has been in vogue since hundreds of years. But only in the 20th century did their use include synthesized derivatives of natural pesticides. One of the first pesticides synthesized was DDT, by Muller in 1939, followed by Lindane in 1942 (Casida and Quistad, 1998). Based on their chemical structure, the pesticides may be classified into the organochlorines, the organophosphates, the carbamates and the pyrethroids. The organochlorine pesticides include DDT, Gammaxene, Hexachlorocyclohexane (HCH) etc and were developed in the early part of this century. However, their properties such as low volatility, chemical stability, lipid solubility and slow rate of biotransformation that made them ideal pesticides, also brought about their end because of their persistence in the environment, bioconcentration and biomagnification within various food chains (Ecobichon, 1996). Organophosphates (OP) are less persistent and easily biodegradable than the organochlorines, but are extremely toxic to mammals. Their use needs to be reduced to avoid the dangers to the agricultural workers since the OP compounds are known inhibitors of cholinesterase enzymes. Carbamates are similar in their action as the OP in inhibiting cholinesterase enzymes and are rapidly biotransformed *in vivo* but are as toxic as the OP pesticides for non-target species.

Pyrethroids were in use as naturally occurring botanical pesticides. Pyrethroids are derived from the naturally occurring pyrtherums, which in turn are the oleo-resin extracts of dried chrysanthemum flowers. These pyrethrins owe

their insecticidal activity to the ketoalcoholic esters of chrysanthemic and pyrethroic acids. A number of synthetic pyrethroids have been derived from the structures of the natural esters. They have a very specific effect against the insect pests and are relatively non-toxic to humans and other non-target species. They are metabolized rapidly and eliminated from mammalian systems. Their persistence in the environment is in the order of weeks, unlike organochlorines, which persist for years (Elliot, 1976). These have now gained widespread and popular use due to their properties vis-à-vis the OC and OP compounds.

The world market value of pyrethroids was 2.1 billion US $ in 1995, which is 23% of the total pesticide production in the world, second only to the organophosphorous pesticides (Casida and Quistad, 1998).

1.1 History of synthetic pyrethroids

The term ‘pyrethroid’ is commonly used to designate a synthetic insecticide that is derived structurally from the natural pyrethrins, the six insecticidal constituents of pyrethrum extract (Elliot, 1995). Decades of research and development by the agrochemical industry and by government and academic research laboratories have resulted in a wide range of pyrethroid structures and a multitude of uses in agriculture, veterinary, medical and household pest control (Soderlund *et al*, 2002). The first important synthetic pyrethroid still used today, is allethrin, developed by Schecter, Green and La Forge in 1949 (Schecter *et al*, 1949). Allethrin and other pyrethroids with a basic cyclopropane carboxylic ester structure are type I pyrethroids. Much of allethrin has found use in mosquito coils.

The insecticidal activity of these synthetic pyrethroids was further enhanced by the addition of a cyano group to give alpha-cyano or type II pyrethroids, such as cypermethrin, fenvalerate, deltamethrin etc (Bradberry *et al*, 2005). The critical stages of pyrethroid development are attributable to Elliot and colleagues of the Rothamsted experimental station in England and Sumimoto chemical company in Japan. Neopynamin or Tetramethrin was the next synthetic pyrethroid produced commercially in 1964 (Kato *et al*, 1964). 1966 saw the synthesis of resmethrin and bioresmethrin (Elliot *et al*, 1967). Deltamethrin was synthesized in 1974 (Elliot *et al*, 1974). Consequently came the development of cypermethrin, cyfluthrin, cyhalothrin (1977) and tralomethrin by 1980. The structure of some of the synthetic pyrethroids are illustrated in Fig 1.1.

In commercial formulations, the activity of pyrethroids is usually enhanced by the addition of a synergist such as piperonyl butoxide, which inhibits metabolic degradation of the active ingredient. Permethrin proved to be the first synthetic pyrethroid with sufficient photostability for agricultural use (Soderlund *et al*, 2002).

1.2 Advantages of using Synthetic pyrethroids:

Synthetic pyrethroids have an edge over the other classes of pesticides in several ways. Some of the major achievements in pyrethroid research are the following:

(a) Photostability without compromising biodegradability

(b) Selective toxicity conferred by target-specificity and metabolic degradation

(c) Modification of every part of the molecule with retention of activity

(d) Development of compounds effective as fumigants and soil insecticides (i.e. tefluthrin)

(e) Optimisation of potency to allow corresponding reduction in environmental contamination (Casida and Quistad, 1998).

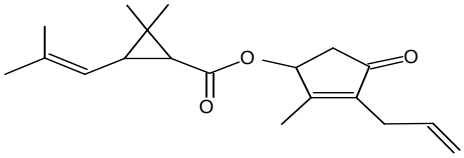
1.3 Epidemiology

In 1965, the world output of pyrethrum (the natural pyrethrin) was approximately 20,000 tons with Kenya alone producing some 10,000 tons (Cremlyn, 1978). Then came the synthetic pyrethroids in the late 1970s. In 1989-1990, the worldwide annual production of synthetic pyrethroids was 2000 tons (IPCS, 1989) including some 250 tons of deltamethrin (UKPID, 1990).

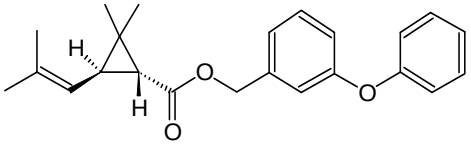
Pesticide consumption in India is 288 g/ha, which is low compared with a global average of 900 g/ha (Agnihotri, 2000). However, consumption has not been uniform in the country, and it varies with the intensity of pests and diseases, cropping patterns and agro-ecological regions. Pesticide use is high in regions with good irrigation facilities and in areas where commercial crops are grown. For instance, although cotton and paddy are grown in 5 per cent and 24 per cent of the total cropped area, they receive about 45 per cent and 20 per cent of total pesticides respectively. The use of pesticides is high in a few states such as Andhra Pradesh, Karnataka, Maharashtra, Gujarat and Punjab (Shetty, 2004). As seen in Table 1.1, Insecticides contribute the major share of pesticide use in India while fumigants usage is only 1% compared to 5% in the world.

Fig 1.1: Structure of some synthetic pyrethroid insecticides.

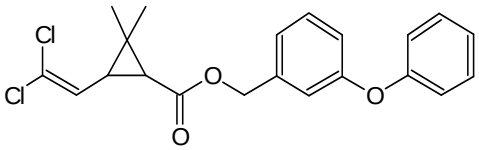
Type I Synthetic Pyrethroids



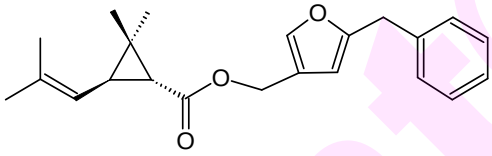
Allethrin, Bioallethrin



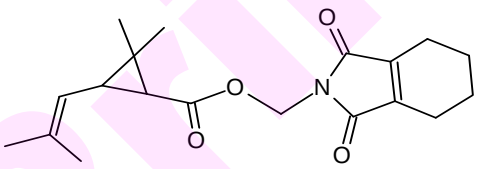
Phenothrin



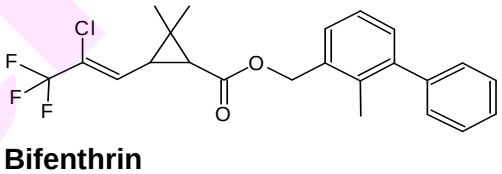
Permethrin



Resmethrin

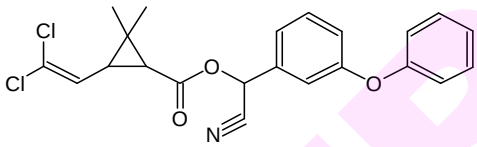


Tetramethrin

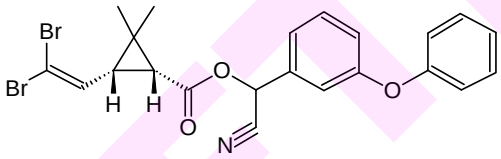


Bifenthrin

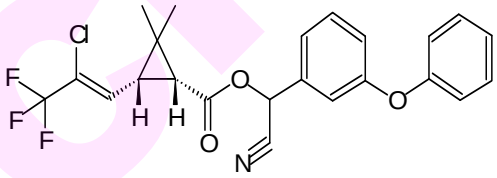
Type II Synthetic Pyrethroids



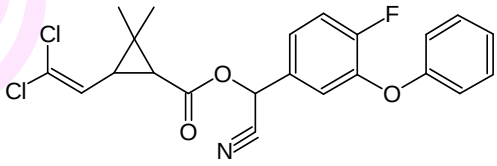
Cypermethrin



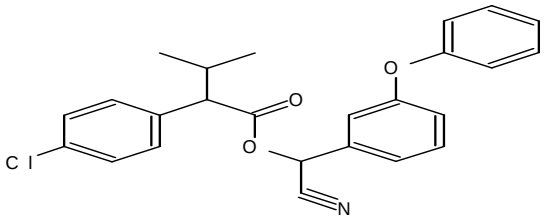
Deltamethrin



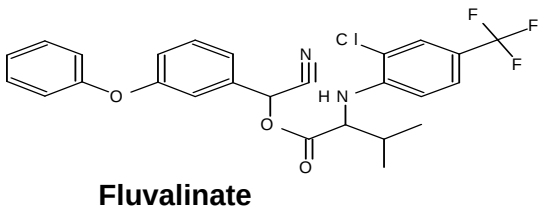
Lambda-Cyhalothrin



Cyfluthrin



Fenvalerate



Fluvalinate

Source: Lee *et al* (1998), J. Agri. Food Chem., 46, 2, 535-546

Table 1.1: Consumption Pattern of pesticides in India

Segment	Indian Share (%)	World Share (%)
Insecticides	76	44
Herbicides	10	30
Fungicides	13	21
Fumigants	1	5

Source: Pesticide Association of India (2001)

1.4 Mechanism of Insecticidal Action

Pyrethroids are known nerve poisons, like the organophosphate group of insecticides. Pyrethroids are known to alter the normal function of insect nerves by modifying the kinetics of voltage-sensitive sodium channels (VSSCs), which mediate the transient increase in the sodium permeability of the nerve membrane that underlines the nerve action potential (Soderlund and Bloomquist, 1989). Perturbations of sodium channel function by pyrethroids is stereospecific (Narahashi, 1996). Those stereoisomers that are the most potent disruptors of sodium channel function also have the most potent insecticidal or toxicological activity (Ray, 2001). Pyrethroids slow the activation or opening of VSSCs. In addition, they slow the rate of VSSC inactivation (or closing) and shift to more hyperpolarized potentials the membrane potential at which VSSCs activate. The result is that sodium channels are held open longer and allow more sodium ions to cross and depolarize the neuronal membrane.

1.5 Toxicology and Metabolism

The first systematic study of pyrethroid toxicity (Verschoyle and Barnes, 1972) compared the acute oral and intravenous toxicities to rats of pyrethrum, pyrethrin I, pyrethrin II, bioallethrin, isomers and isomer mixture of resmethrin. This study documented the modest oral toxicity of the tested compounds, the significant intravenous toxicity of the pyrethrins and bioallethrin, and the profound differences in the toxicity of resmethrin isomers by both routes of administration. Animal studies suggested that the two structural types of pyrethroids generally

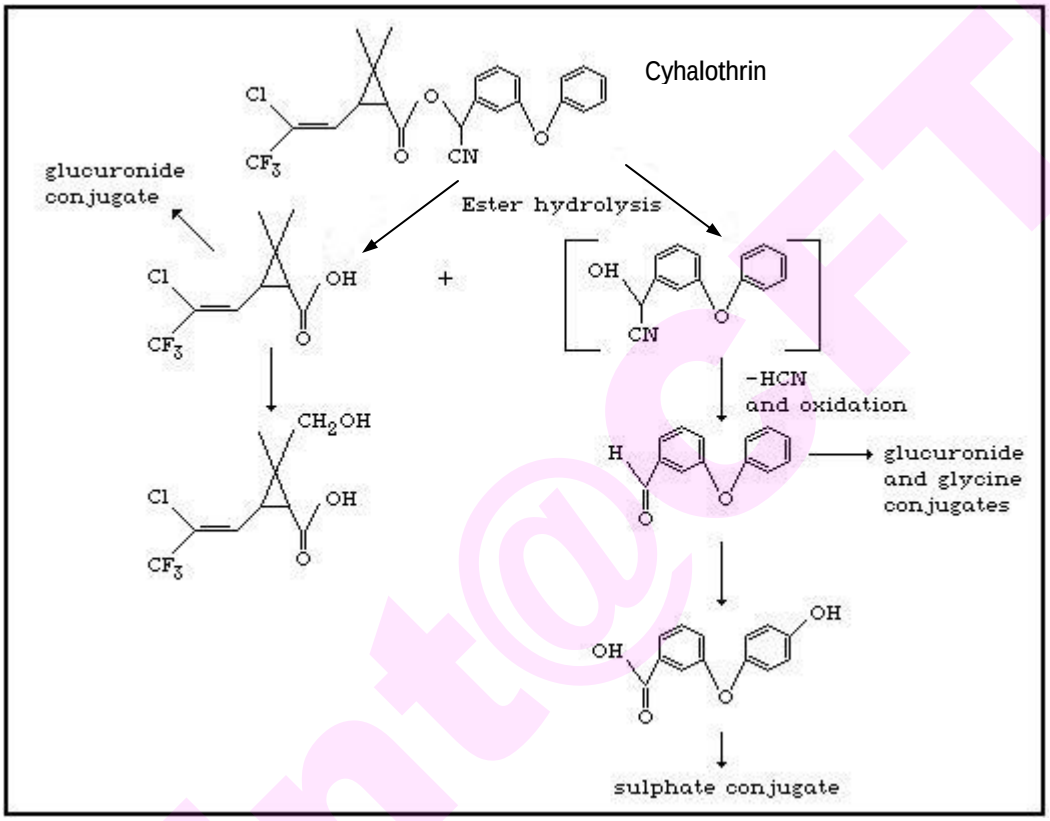
give rise to two distinct patterns of systemic toxic effects. Type I pyrethroids produce in animals the so-called “T (tremor) syndrome, characterized by tremors, prostration and altered “startle” reflexes. Type II pyrethroids produce the so-called “CS (choreosathetosis/salivation) syndrome” with ataxia, convulsions, hyperactivity, choreoathetosis and profuse salivation being observed in experimental studies (Cage *et al*, 1998). Despite differences in the symptoms, both types of pyrethroids have the same major target site: the sodium channel of the nerve membrane, i.e., the channel directly responsible for generating action potentials (He, 1994).

Pyrethroids are some 2250 times more toxic to insects than mammals because insects have increased sodium channel sensitivity, smaller body size and lower body temperature. In addition, mammals are protected due to poor dermal absorption and rapid metabolism to non-toxic metabolites (Bradberry *et al*, 2005). In spite of their long history of use, there are relatively few reports of pyrethroids toxicity and deaths from their usage. Comparing the oral toxicities to rats, the pyrethroids have acute oral LD_{50} values following administration in vegetable oils lying between 50 and 500 mg/kg (except few pyrethroids) and are therefore considered to be moderately toxic (EPA category II) (Soderlund *et al*, 2002). In contrast to the moderate oral toxicity of most pyrethroids, the pyrethroids as a class exhibit very low levels of systemic toxicity following dermal exposure (Clark, 1995). Acute regulatory neurotoxicity studies for the pyrethroids show that the effects of the pyrethroids were transient with peak effects observed

within hours of exposure and full recovery within 1-14 days after treatment (Verschoyle and Aldridge, 1980).

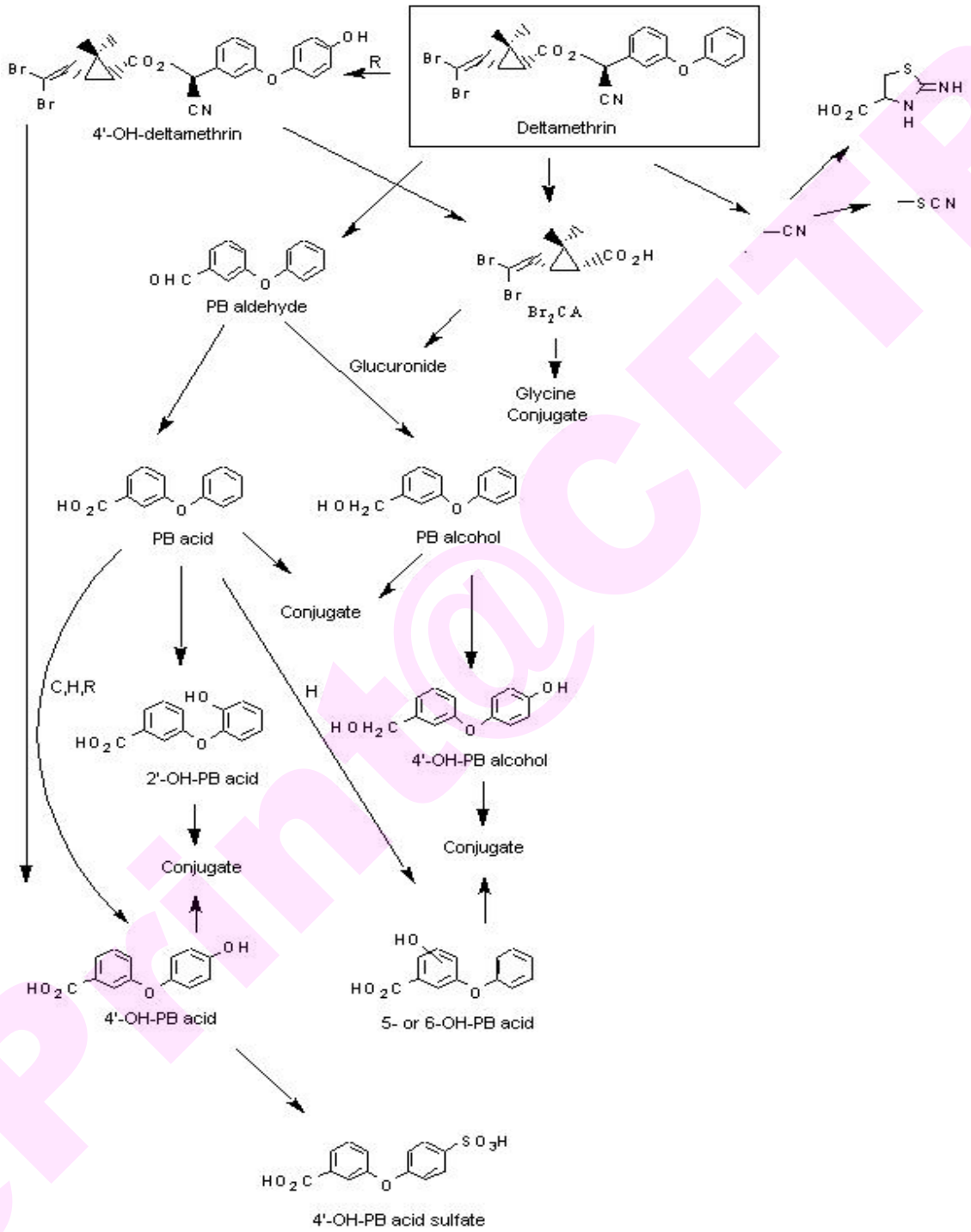
Synthetic pyrethroids undergo biotransformation in mammals both oxidatively (by P-450 monooxygenases) and hydrolytically (by esterases), and depending on the type of compound either of the pathways may predominate (Miyamoto, 1976 and Soderlund *et al*, 2002). Pyrethroids are rapidly hydrolyzed in the liver to their inactive acid and alcohol components. Then, further degradation and hydroxylation at the 4' position occurs and oxidation produces a wide range of metabolites (Hutson, 1979, Anadon *et al*, 1996). Fig 1.2 shows a proposed pathway of metabolism of cyhalothrin, wherein the parent compound undergoes glucuronide conjugation and ester hydrolysis by 2 different pathways. Similarly, the metabolic pathway of deltamethrin follows glucuronide conjugation, conjugation and hydrolysis to give various intermediates (Fig 1.3).

Fig 1.2: Proposed metabolic pathway of Cyhalothrin in mammals



Source: WHO, 1990. EHC 99.

Fig 1.3: Proposed metabolic pathway of deltamethrin in mammals.



Adapted from: WHO and IPCS, 2000. Deltamethrin JPMR report.

1.6 Deltamethrin

Deltamethrin was first synthesized in 1974 by Elliot *et al*. It is sold under many proprietary names such as decamethrin, butox, decis etc. It is a type II pyrethroid and its IUPAC name is (S)–alpha-cyano-3-phenoxybenzyl (1R)-cis-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropanecarboxylate ($C_{22}H_{19}Br_2NO_3$). It is in the form of colourless and odourless solid crystals with a molecular weight of 505.2 (e-pesticide manual, 1999).

The behavioral effects of low acute doses of deltamethrin results in decreased motor activity, operant response rates, acoustic startle amplitudes, loss of righting reflex and impaired performance on an inclined plane (Crofton *et al*, 1995). Vehicle and route of exposure are known to influence the absorption and kinetics of xenobiotics (Benet *et al*, 1990). Thus, the differential potency of deltamethrin may also be dependant on the route of administration and/or the vehicle used. Early work on the lethality of the pyrethroids demonstrated that when administered by the i.v. route, they were one or two orders of magnitude more potent than when administered orally (Barnes and Verschoyle, 1974 and Verschoyle and Barnes, 1972). Crofton *et al* (1995) also reported differences in the motor effects of deltamethrin treatment in rats by i.p. and oral routes and using different vehicles wherein the ED_{50} for deltamethrin was 5.1 mg/kg when administered orally in corn oil and 1000 mg/kg when given orally in methylcellulose.

Deltamethrin has many behavioral and neurological effects in treated animals. Adult male albino rats treated at 7 mg/kg b.w. in corn oil orally for 15 days showed decrease in body weight, increase in monoamine oxidase and acetylcholinesterase activity in different brain regions, increased spontaneous locomotor activity and aggressive behavior, and morphological changes in purkinje neurons (Husain *et al*, 1996). The reported oral LD_{50} in rats treated with deltamethrin dissolved in corn oil is reported to be 80-100 mg/kg b.w. as reported by Varsho (1996). In the acute neurotoxicity study of deltamethrin (Nemec, 1998a), male and female Sprague-Dawley rats were administered 5, 15 and 50 mg/kg b.w of deltamethrin dissolved in corn oil. After 3 h, the animals treated with 50 mg/kg of deltamethrin exhibited salivation, a flattened posture with limbs extended, clonic or tonic convulsions, tremor, biting or gnawing the cage, decreased reaction to removal or handling, hindlimbs splayed or dragging, decreased response to stimuli and mortality. At the next lower dose, the only evidence of toxicity was salivation and impaired mobility. No effects were observed at the lowest dose in both sexes. In the subchronic dietary study by the same author (Nemec, 1998b), deltamethrin was administered to male and female Sprague-Dawley rats at concentrations of 50, 200 and 800 ppm (4, 15 and 56 mg/kg per day, respectively). At the 800 ppm dietary level, deltamethrin caused impairment in mobility and gait, postural changes, hypersensitivity to noise, reduced grip strength, convulsions and mortality. No treatment-related neurobehavioral changes were observed at lower dietary concentrations.

1.7 Cyhalothrin

Cyhalothrin was developed in 1977. Its IUPAC name is alpha-cyano-3-phenoxybenzyl3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclo-propanecarboxylate. Technical grade cyhalothrin is a yellow-brown viscous liquid (melting point: approximately 10^0C) and contains more than 90% active material. It is composed of four cis isomers in the ratio of 1:1:1:1. Although it is insoluble in water, it is soluble in a range of organic solvents such as aliphatic and aromatic hydrocarbons. It is stable to light and heat and has a low vapour pressure.

Technical grade lambda-cyhalothrin is a beige solid (melting point: 49.2^0C) and contains more than 90% active material. The enantiomer ratio of the *(Z), (1R, 3R),* S-ester to the *(Z), (1S, 3S),* R-ester is 1:1. It is sparingly soluble in water but soluble in a range of organic solvents and has a low vapour pressure. Both cyhalothrin and lambda-cyhalothrin are rapidly hydrolysed under alkaline conditions but not in neutral or acidic media.

Cyhalothrin (a pyrethroid insecticide) is similar in structure to deltamethrin, but with F_3C and chlorine functional groups instead of bromine as in deltamethrin. Its trade names include karate and lambda-cyhalothrin. It has a reported LD_{50} of 55-80 mg/kg b.w. in rats treated with an oral dose of cyhalothrin in corn oil as reported by Southwood (1985). It has a high level of activity against a wide range of Lepidoptera, Hemiptera, Diptera and Coleoptera spp. of insects. It also has miticidal activity. The compound is a stomach, contact and residual

insecticide. Like other photostable synthetic pyrethroids, cyhalothrin is relatively stable to degradation in sunlight.

In the acute neurotoxicity study with lambda-cyhalothrin (Barmmer, 1999), Alderly Park rats were administered 2.5, 10 and 35 mg/kg b.w of cyhalothrin in corn oil. After 7h, the animals showed signs of decreased activity, ataxia, reduced stability, tiptoe gait, decreased landing foot splay and decreased tail flick response only in the 35 mg/kg treated group. Some signs were evident at the 10 mg/kg dose, but none at the lowest dose level.

1.8 Pyrethroids and Apoptosis

Apoptosis or programmed cell death (PCD) is an ordered and fundamental biological process designed for safe disposal of surplus, aged or damaged cells (Kerr *et al*, 1972; Wyllie e*t al*, 1980). Apoptotic cells are phagocytosed whole or as discrete fragments bound by an intact membrane, thus ensuring their disposal without release of their contents (Walsh *et al*, 1998). There have been several reports of pyrethroid pesticides triggering apoptosis in treated animal models and cell lines *in vitro*. Among them, several reports are available that link deltamethrin to apoptotic cell death in both *in vivo* and *in vitro* model systems. The toxic effects of pyrethroids can also be expressed in the form of apoptosis either in cases of treatment studies and in accidental pyrethroid poisoning cases as well. This may result from a sublethal dose of pyrethroids wherein any externally visible manifestation may not be there. Several reports have indicated the effect of pyrethroids in causing apoptosis. Deltamethrin was reported to cause

apoptotic cell death in rat testicular cells when treated with a dose of 1 mg/kg b.w. (in corn oil) (El-Gohary *et al*, 1999).

Abu-Qare and Abou-Donia (2003) studied the combined exposure of N,N-Diethyl-m-Toluamide (DEET) and a synthetic pyrethroid, permethrin in rats. Initiation of PCD is evidenced by biomarkers like release of mitochondrial cytochrome *c* activity, activation of caspases, elevation of 8-hydroxy-2 deoxyguanosine levels and alterations of p53 gene expression (Abu-Qare and Abou-Donia, 2001a). Significant effects were seen following combined exposure to both DEET and permethrin than by exposure to a single compound. Exposure of male rats to a single dermal dose of 400 mg/kg of DEET and 1.3 mg/kg of permethrin significantly increased urinary concentration of 8-hydroxy-2'-deoxyguanosine, a marker of DNA damage (Abu-Qare and Abou-Donia, 2000). Abu-Qare and Abou-Donia (2001b) showed that at similar combined dermal doses of DEET and permethrin, rat brain mitochondrial cytochrome *c* was significantly induced. Thus, cytochrome *c* is a known marker for apoptosis.

Niederer *et al* (2005) studied the hamster fibroblast cell line (tsBN7), which is temperature-sensitive and displays deficient N-linked glycosylation activity at the restrictive temperature and activates cellular apoptosis via the release of cytochrome *c* from the mitochondria. In this study, treatment of these cells with cypermethrin, known to perturb Ca (2+) signaling in neuronal cells, was sufficient to arrest apoptosis.

1.9 Deltamethrin and Cyhalothrin in Apoptosis

El-Gohary *et al* (1999) demonstrated induction of testicular apoptosis in male rats exposed to 1 mg/kg (daily for 21 days) with an i.p. dose of Deltamethrin in corn oil. Chararacteristic DNA laddering, apoptotic changes in tissue sections of testis basal germ cells, increased plasma levels of NO and lipid peroxides was seen, indicative of apoptosis. Administration of a NO synthase (NOS) inhibitor, N-monomethyl L-arginine hydrochloride (L-NMMA, 1 mg/kg) 2 h before the deltamethrin treatment reduced DNA fragmentation and histopathological changes showing involvement of NOS in apoptosis and deltamethrin as a trigger for apoptosis.

Wu and Liu (1999) reported that the activity of NOS and PARP {poly (ADP-ribose) polymerase} was enhanced in the cortex and hippocampus of male Sprague–Dawley (SD) rat brains. Since PARP is involved as a caspase substrate, this increase may result in increased apoptosis. Immunohistochemical analysis also revealed the increase in nNOS immunoreactive cells 24 after deltamethrin–treatment in the same brain regions, showing its involvement in apoptosis in these regions of the rat brain. Wu *et al* (2003) treated cultured cortical neuronal cells with different concentrations (10, 100 and 1000 nmol) of deltamethrin causing widespread apoptosis in these cells. As demonstrated by TUNEL staining, numerous apoptotic bodies were seen in treated cultures at 100 nm of deltamethrin at 24, 48 and 72 h after treatment. Western blot analysis showed p53 and Bax expression (pro-apoptotic proteins) increased significantly

at the same time points of 24, 48 and 72 h while Bcl-2, a know apoptotic inhibitor, decreased at the same time. NOS mediated deltamethrin-induced apoptosis and altered expression of p53, Bax and Bcl-2 in these cultured neurons.

Wu and Liu (2000b) treated Male Sprague–Dawley rats with 12.5 mg/kg (i.p.) deltamethrin In corn oil, producing numerous apoptotic cells as detected by *in situ* end labeling (ISEL) and flow cytometric analysis in the hippocampus and cortex of rat brain at 24 and 48 h. DNA fragmentation was also markedly induced in the hippocampus at 24, 48 and 72 h after deltamethrin treatment.

As such, there have been no reports regarding the apoptotic effect of cyhalothrin or lambda-cyhalothrin, except reports on its genotoxic and cytotoxic potential (Celik *et al*, 2005; Cavas and Ergene-Gozukara, 2003).

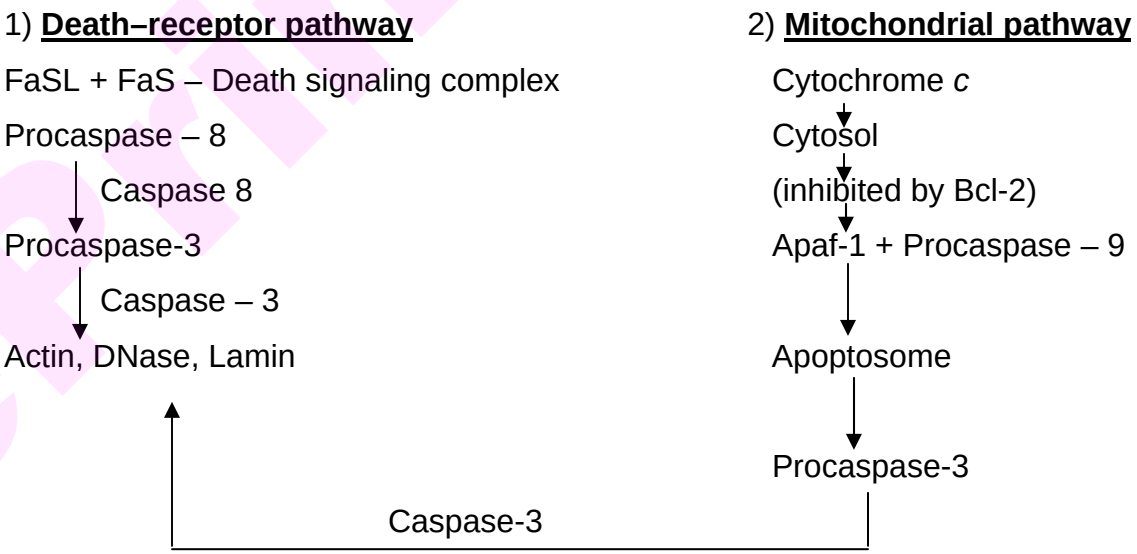
1.10 Apoptosis

Apoptosis is a gene-directed process and similar to other genetic processes, is one of many responses available to a cell when faced by any internal or external stimuli (Williams and Smith, 1993). This report also suggested involvement of p53 and Bax in inducing apoptosis and Bcl-2 in suppression of apoptosis. The ratio of Bax to Bcl-2 may be critical in determining the response of a cell faced by an external stimulus to undergo apoptosis.

The rapid disappearance of apoptotic cells *in vivo* shows that apoptotic lymphocytes may be phagocytosed before lysis, protecting the surrounding tissue from the damaging effects of released intracellular contents (Zhang *et al*, 1995, Henson and Johnston, 1987, Cohen and Duke, 1992).

Apoptosis is a physiological mode of cell death that affects many aspects of natural life from embryonic development through cellular homeostasis and disease. Various morphological and biochemical characteristics including loss of cell volume, internucleosomal DNA degradation and formation of apoptotic bodies sets apoptosis apart from the accidental cell death process known as necrosis. The loss of cell volume or cell shrinkage can be examined by flow cytometry using various fluorescent dyes or markers and also to study cell viability, DNA content, changes in mitochondrial membrane potential and caspase activity (Bortner and Cidlowski, 2001).

Otsuki *et al* (2001) reported that many studies recently implicated several proteins such as the Fas antigen (Fas) / tumor necrosis factor receptor (TNFR), the Fas Ligand (FaSL)/TNF, P53 and Myc, in addition to proteins of Bcl-2 family in the regulation of apoptosis. A recent study by Hengartner (2000) summarized 2 pathways for apoptosis as given below:



1.11 Nitric Oxide Synthase (NOS) & Nitric Oxide (NO) in Apoptosis

NOS is an enzyme involved in the production of NO, an important cell signaling molecule in the mammalian system. NO synthases constitute a family of isozymes that catalyse the oxidation of L-arginine to NO and citrulline (Nathan, 1992). Kitajima *et al* (1994) reported nitric oxide-mediated apoptosis in a mast cell line from the mouse due to action of inducible NOS (iNOS). Forstermann and Dun (1996) reported that NOS exists in 3 isoforms, two expressed constitutively in specific cell types – NOS I or neuronal NOS and NOS III or endothelial NOS and NOS II or inducible NOS which can be induced in many cell types with cytokines and other agents.

Zini *et al* (1996) reported a correlation between eNOS in human reproductive organs and apoptosis. Mishra and Delivoria–Papadopoulos (2006) studied the interaction between nNOS inhibition on caspase-9 in hypoxic brain from piglets. Caspase 9 is the initiator caspase and is seen at the beginning of apoptosis. It joins with the apoptotic protease activating factor-1 (Apaf-1) and cytochrome *c* to form a structure called apoptosome. This process can be inhibited by Bcl-2 family of proteins (Zou *et al*, 1997). In this study by Mishra and Delivoria-papadoupoulos, brain of new-born piglets exposed to hypoxic conditions were used to extract and assay tissue ATP, phosphocreatine, caspase-9 (cytosolic extract) by flourogenic substrate using amino-4-methyl-coumarin as standard; caspase 3 was assayed similarly as in caspase-9 with its own specific substrate. Caspase-9 activity increased in hypoxic group, but pretreatment with nNOS

inhibitor 7-nitroindazole-sodium salt (7-NINA) prevented the hypoxia-induced increase in caspase-9 activity. Consequently, caspase-3 activity also increased in only hypoxic group and pretreatment with nNOS inhibitor prevented the same. Thus, it shows that increase in caspase–9 activity is mediated by nNOS derived NO.

According to Guix *et al* (2005), there are 4 members of NOS family according to this report – nNOS, eNOS, iNOS and mitochondrial NOS (mtNOS). mtNOS is an isoform of nNOS present in inner mitochondrial membrane (Elfering *et al*, 2002). All NOS isoforms have four prosthetic groups: flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), iron protoporphyrin IX (heme) and tetrahydrobiopterin (BH4). mtNOS is associated with inner mitochondrial membrane found in rat liver, kidney, lung, testis, spleen, heart, muscle and brain (Elfering *et al*, 2002). The function of NO in the mitochondria could be related to the regulation of O_2 consumption by inhibiting the cytochrome *c*-oxidase (Brown and Cooper, 1994).

NO can also be produced by xanthine oxidase pathway or by H_2O_2 and L-Arginine in a non-enzymatic way (Nagase *et al*, 1997). NO production can both cause and prevent apoptosis. NO triggers apoptosis: by binding to cytochrome *c* oxidase and leading to free radical production, which damages cellular and mitochondrial structure. NO-induced apoptosis is related to increase in the Bax/Bcl-X_L rate, the release of cytochrome *c* and caspase activation (Kolb, 2000). Under physiological conditions, NO is an anti-apoptotic molecule. NO is

known to inhibit caspase-3 (Mannick *et al*, 1999), caspase–1 inhibition (Kim *et al*, 1998) inhibition of caspase-9 and release of Bax (Thippeswamy *et al*, 2001). NO also induces expression of cytoprotective genes such as HSP70 (Hao *et al*, 1999) which in turn binds to the Apaf-1 caspase domain preventing formation of the apoptosome (Beere *et al*, 2000). Mosser *et al* (1997) reported the direct inhibition of cytochrome *c* release by activation of HSP 70.

1.11.1 Detection methods for NOS

NOS are detected by immunostaining using Avidin/biotin peroxidase staining, NADPH diaphorase staining to histochemically localize nNOS and Immunofluoroscence. NADPH diaphorase staining involves ability of NOS to reduce soluble tetrazolium salts to an insoluble, dark formazan. NOS is responsible for production of NO. NO is known to regulate programmed cell death (Rosselli *et al*, 1998). Active induction of NOS in immune cells or certain epithelia causes apoptosis. The menstrual degradation of the endometrium also involves apoptosis mediated by NO (Tschugguel *et al*, 1998). Martin *et al* (2005) used 4 different antibodies for immunohistochemical analysis of nNOS. Here, monoclonal antibody was used for immunohistochemical detection of ssDNA as described by Frankturt *et al* (1996). It was seen that the absence of nNOS attenuated the accumulation of DNA damage in injured motor neurons. nNOS expressed production of $ONOO^-$ free radical from NO which in turn leads to DNA damage.

In the study by Zini *et al* (1996) endothelial nitric oxide synthase (eNOS) was extracted from cytosolic extracts of human testis, epididymis and vas deferens and this protein was localized using a monoclonal Ab against eNOS isoform in a steptavidin-biotin-amplified peroxidase technique and co-localized with NADPH diaphorase histochemistry. In this study, alkaline phosphatase–conjugated 2^0 Ab (1:500) and luminescence substrate, SPD (disodium 3-(4-methoxyspiro4-yl) phenyl phosphate: Boehringer – Mannheim) at 1:1600 dilution were used. eNOS protein at 130 KD was detected in human testis and epididymis by western blotting and in the same organs, pyknotic-nucleated cells were observed with visible staining for eNOS and NADPH diaphorase. This result shows the role of NOS in apoptotic pathway in these organs. DNA fragmentation *in situ* and eNOS staining in degenerating germ cells suggests role of NOS in germ cell apoptosis.

1.12 Detection Methods for Apoptosis

1.12.1 General methods

Various methods have been described for apoptosis. Immunoassays are also one among them utilizing antibodies to cell death markers. Varadachary *et al* (1999) used a sandwich ELISA based on the detection of cytoplasmic nucleosomal fragments released from the nucleus during the apoptotic process using an anti-histone monoclonal Ab (mAb) against extracted nucleosomes. A similar setup was used by the authors as above to detect apoptosis by sandwich ELISA using mouse mAb directed against DNA and histones of the nucleosomal fractions of the cell (Varadhachary *et al*, 1997). This study also employed the use

of immunofuoroscent labeling for FasR and FasL by directing antibodies against these 2 proteins (derived from T cell clones) and using a fluorescent chemical – tagged secondary antibody. Walsh *et al* (1998) described various methods to detect apoptosis and necrosis in eosinophils from humans. DNA degradation resulting in DNA ladder, cell viability by trypan blue exclusion and subsequent microscopic morphological assessment by Kimura staining and flow cytometry using FITC-labelled Annexin V and Propidium Iodide were the various methods used to detect apoptosis in these eosinophils.

Dolzhanskiy and Basch (1995) described a flow cytometric method for detection of free 3'-OH groups produced during endonucleolytic cleavage of DNA using the enzyme deoxyribonucleotidyl transferase and fluorochrome-coupled streptavidin. Immunofluorescence was used to identify the phenotype of apoptotic cells. Laxman *et al* (2002) described the development of a recombinant luciferase reporter molecule when expressed in mammalian cells, showed increased levels of this reporter. Since caspase-3 activation occurs in apoptosis, it was used to cleave the above reporter molecule, resulting in restoration of luciferase activity detected in living animal models by real-time bioluminescence imaging. Microscopy and agarose gel electrophoresis are reliable methods for detection of apoptosis but they are labour intensive and not sensitive.

Some other methods used for detection of apoptosis are described below:

a. Transmission Electron Microscopy (TEM):

Kerr *et al* (1972) proposed that apoptosis is a biological process involving

changes in cell such as chromatin condensation, cell shrinkage, budding and apoptotic bodies. Hence, TEM can easily identify these changes. Only disadvantage is that it detects only cells at terminal stage of apoptosis.

b. DNA agarose gel electrophoresis:

This method was first reported by Wyllie (1980). Agarose gel electrophoresis reveals low-molecular weight DNA fragments, which usually are in multiples of 180 bp, identical to nucleosomes, called as the 'DNA ladder". But it is now not considered to be always a necessary step in apoptosis (Oberhammer *et al*, 1993). Hence, according to these authors, the DNA may get cleaved to 50-kb fractions where pulsed-field gel electrophoresis (FIGE) revealed the presence of these fragments.

c. TDT-mediated dUTP-biotin Nick-End Labeling method (TUNEL method):

TUNEL assay is another method used for analysis of apoptotic DNA wherein DNA nicks are labeled using fluorochrome-tagged triphosphodeoxynucleotides (TDT) leading to TDT-mediated dUTP-biotin nick-end labeling or TUNEL assay. The TUNEL assay is of many types, one based on incorporation of Brd UTP is best in terms of ease of use, low cost and high sensitivity. Here, BrdU attached to double strand breaks (DSB's) is detected with an FITC-conjugated anti-BrdU antibody. The advantage with this method is that it can be used for detection of early stages of apoptosis.

*d. Bcl-2 expressio*n:

Bcl-2 expression can be studied by immunohistochemistry and immunoelectron microscopy of Bcl-2, a 26 KD protein, with specific antibodies against it.

e. Fas and FaSL detection:

These two important apoptotic proteins can be detected by immunohistochemistry and immunoelectron microscopy.

f. Caspase Activity

Otsuki (2001) and Jerome *et al* (2003) have shown the importance of caspases in apoptosis. Otsuki (2001) studied activity of caspase-3, - 8 and –9 in human endometrium and HHUA cell line (from endometrium carcinoma). The activities of caspase-3, -8, -9 were found to be highest in that order during late secretory phase of menstrual cycle. This was found to be similar to apoptotic HHUA cells induced to undergo apoptosis by treatment with anti-Fas IgM antibody. Jerome *et al* (2003) developed a flow-cytometry based assay for cytotoxic T-lymphocytes mediated cytotoxicity based on specific binding of antibody against caspase-3 in target cells.

1.12.2 Detection of apoptosis in tissue sections

The best studied anti-apoptotic proteins are members of the Bcl-2 protein family e.g. Bcl-2, Bcl-X_L , Mcl-1, the family of inhibitors of apoptosis protein (IAP) apoptosis suppressors, X-linked inhibitor of apoptosis protein (X-IAP), surviving, viral anti-apoptotic proteins such as crmA, IAP's and p35, heat shock protein 70

etc. (Stadelmann and Lassmann, 2000). These authors also have opined that the detection of activated caspase-3 is a valuable tool in identifying dying cells before manifestation of all the features of apoptosis (e.g. DNA fragmentation). No activation of the caspase cascade has been found in necrotic cell death (Armstrong *et al*, 1997; Dong *et al*, 1997).

1.12.3 Immunological Methods for detection of Apoptosis

Zurgil *et al* (2003) developed a real-time monitoring method to determine the radii of Jurkat cells which shrunk after apoptosis, by sequential measurements of the fluorescence intensity of the same individual cells. The only drawback with this method was the use of cell cultures, which may not be applicable to mammalian *in vivo* systems. Zhang *et al* (1995) described a method for simultaneous staining of cell surface markers with fluorescent monoclonal antibodies (FITC-conjugated anti-mouse Thy 1.2, CD4 and CD8 Ab's) and nuclear DNA breaks using *in situ* DNA nick translation detectable by fluorescence. This method was used for both *in vivo* (B6 mice Lymph Nodes) and *in vitro* (Lymphocytes) studies. DNA fragmentation was also seen in the same cells. In this study, clearance of nick positive cells (apoptotic cells) was very rapid *in vivo* since 13 h after gamma-ray irradiation, the nick-positive cells were at a level of 93% and reduced to 9% after 24 h, suggesting a dynamic mechanism for clearance of apoptotic cells *in vivo*, which was not seen *in vitro.*

1.12.4 Immunoassays and Markers for apoptosis

Frankfurt and Krishnan (2001) developed a solid-phase ELISA using a monoclonal Ab against single stranded DNA (ssDNA) to detect apoptosis from cell cultures. So ssDNA is also reported as a good marker for apoptosis. Salgame *et al* (1997) developed a sandwich ELISA against cytoplasmic nucleosomes using monoclonal Ab's. This method was reported to be 500 times more sensitive than detection of DNA ladder by agarose gel electrophoresis.

Holdenrieder *et al* (2001) reported that nucleosomes appear in elevated concentration in blood of patients with diseases such as malignant tumours, acute inflammation or autoimmune diseases. A commercial ELISA kit was used to detect nucleosomes from the serum of these patients by these authors. Thus, nucleosomes can serve as very good markers of apoptosis in patients with the above mentioned diseases like tumours and acute inflammation. Anti-DNA Ab's were used in this particular study.

Thus, based on the above information, many markers for PCD can be described ranging from DNA ladder, different caspases, nucleosomes, bcl-2 family of proteins, cytochrome *c*, ssDNA, Fas and FasL, morphological changes such as cell shrinkage, blebbing, apoptotic bodies seen by microscopy. The methods to detect apoptosis based on detection of these markers, has been summarized in Table 1.2.

Table 1.2 Techniques for detection of apoptotic cell death in tissues

TECHNIQUES	RESEARCHERS
Morphology of apoptotic cells	
Light microscopy	Kerr *et al*, 1972
Nuclear staining: hematoxylin, methyl green, nuclear red	
Fluorescence microscopy	
Nuclear stainings DAPI, propidium iodide, Toto-3	
Semi-thin sections stained with toluidine blue	Goldberg *et al*, 1990
Electron Microscopy	Goping *et al*, 1999
DNA fragmentation	
In situ hybridization for DNA strand breaks	Kishimoto *et al*, 1990
In situ nick translation	Gavrieli *et al*, 1992
Terminal transferase-mediated dUTP nick-end labeling (TUNEL)	Gorcyzyca *et al*, 1993
Antibodies against single-stranded DNA	Frankfurt *et al*, 1996
Poly (A) probes	Hilton *et al*, 1997
Hairpin oligonucleotides	Didenko *et al*, 1998
Membrane changes in apoptotic cells	
Annexin V in vivo labeling	van Engeland *et al*, 1998
Immunohistochemistry for apoptosis associated proteins	
Tissue transglutaminase	Piacentini *et al*, 1991
"Apoptosis specific protein"	Grand *et al*, 1995
Poly (ADP-ribose)	Negri *et al*, 1997
Activated caspase-3	Srinivasan *et al*, 1998
Caspase-cleavage products (fragments of actin or "fractin")	Yang *et al*, 1998
Cytokeratin 18	Leers *et al*, 1999
Poly (ADP-ribose) polymerase	Knaapen *et al*, 1999

Adapted from Stadelmann and Lassmann, 2000.

1.13 DNA damage and Apoptosis

Condensation of chromatin, internucleosomal DNA fragmentation, cell shrinkage and shedding of apoptotic bodies (blebbing) are hallmarks of apoptosis (Huang *et al*, 2005). Depending on the cation concentration, 3 distinct types of DNA fragmentation are known, which are mediated by different enzymes (Arends *et al*, 1990):

a. Presence of Mg^{2+} (2mM, DNA – fragmentation to 0.05 - 1mb size, type I, high molecular weight DNA fragmentation).

b. At low (nmol) Ca^{2+} conc. (~300 Kb fractions, type-II, intermediate fragmentation).

c. At μmole Ca^{2+} - (Type – III) – mono and oligo–nucleosomes, forming a ladder.

There are many nucleases involved in DNA fragmentation during apoptosis. The CAD (caspase-activated DNAse) and its inhibitor ICAD in mice (Arends *et al*, 1990) and its human homologue DFF40/DFF 45 (Enari *et al*, 1998). Other nuclease enzymes involved in apoptosis are DNAse – I, DNAse – II (Arends *et al*, 1990).

1.13.1 Methods of DNA analysis

In apoptotic cells, DNA loss leads to sub-G_1 peak on DNA content frequency histograms which can be quantified by specific software using flow cytometry (Nicolleti *et al*, 1991). Acridine orange staining differentially stains double stranded DNA versus denatured DNA (Traganos *et al*, 1977). Frankfurt (1999)

used antibodies reactive with single-stranded DNA (ssDNA) to detect denatured DNA immunocytochemically.

1.13.2 Detection of DNA damage by Genotoxic agents

DNA damage induced by genotoxic agents is more difficult to detect since it is less extensive than that of apoptosis. One of the methods used to detect DNA damage by genotoxic agents is the Comet assay. This technique is basically single-cell-DNA gel electrophoresis technique. Individual cells with damaged DNA embedded in agarose gels, when subjected to electric field, generates a characteristic pattern of DNA distribution which, after staining with a flourochrome, results in an image resembling a comet. The extent and length of the comet's tail indicates the severity of the DNA damage (Ostling and Johanson, 1984).

1.13.3 Immunocytochemical detection of DNA adducts

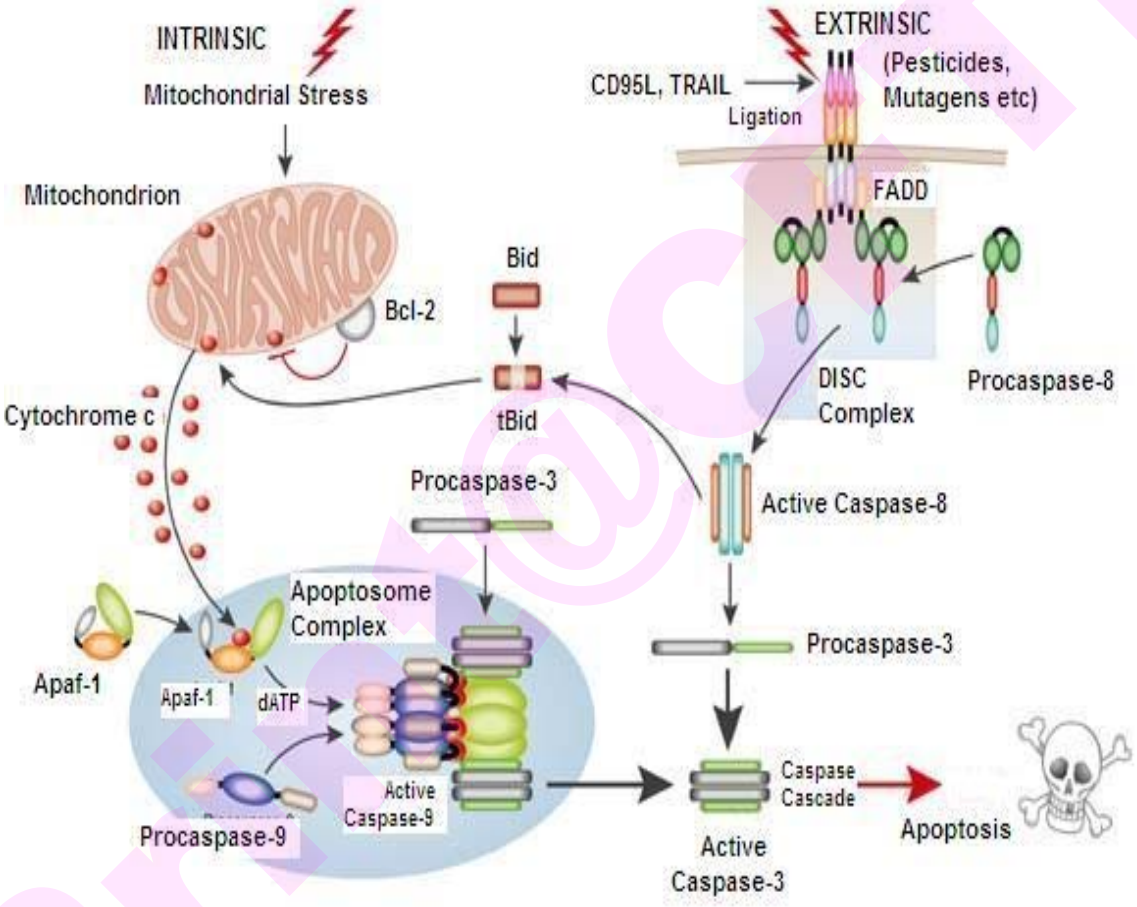
Antibodies have been successfully developed to detect a variety of DNA adducts induced by different genotoxic agents e.g. DNA adducts visualized in mouse tissues and human blood cells following treatment with benz[a]pyrene or its diolepoxide (Van Schooten *et al,* 1991).

1.14 NO, Caspase-3 and DNA fragmentation

Parikh *et al* (2003) reported induction of caspase-3 and DNA fragmentation by NO. They reported an increase in caspase–3 activity and caspase-3 immunoreactivity in hypoxic brain of piglets. This increase of caspase-3 came back to normalcy when treated with nNOS inhibitor, 7-nitroindazole sodium salt

(7-NINA). DNA fragmentation, seen in hypoxia – induced piglets, reduced to normalcy when treated with the nNOS inhibitor. 7-NINA even reduced NOS activity by 85% in pretreated groups when compared to hypoxic controls. This study demonstrates that 7-NINA, a specific inhibitor of nNOS, prevented hypoxia induced increase in caspase-3 activation, the enzyme activity as well as genomic DNA fragmentation, implicating nNOS as key mediator of these determinants of cell death in the newborn brain. Caspase-3 plays a central role in the process of apoptosis and it is a key biomarker protease in the process of apoptosis (Fig 1.4), leading further to the activation of the cellular cell death machinery through the caspase cascade and activation of cytosolic endonucleases.

Fig 1.4: Apoptosis: the 'extrinsic' and 'intrinsic' pathways to caspase activation.



Adapted from: MacFarlane and Williams. 2004. EMBO reports 5(7), 674–678.

1.15 Immunoassays and Apoptosis

Immunoassays are based on the specific antigen-antibody interaction and are used for detection of several different target molecules. Immunoassays are divided into direct and indirect assays. The direct methods are used to directly estimate the concentration of the analyte using specific antibodies conjugated to an enzyme, against the analyte. In the indirect methods, a secondary antibody linked to an enzyme against the primary antibody is used to estimate the concentration of the analyte. Both these methods are termed enzyme-linked immunosorbent assay or ELISA. There have been very few reports on the use of ELISA to detect apoptosis resulting from synthetic pyrethroids action on mammalian systems.

1.16 Need for the present research

The synthetic pyrethroids are used widely in agriculture today due to their low toxicity to non-target species and effectiveness to control various pests. But there are certain lacunae in:

1. Information on the safety mechanism of the synthetic pyrethroids.
2. The relation between pyrethroids and apoptosis.
3. Detection methods for apoptosis induced by pyrethroids.

Also, the reason for their low toxicity and rapid degradation in mammalian systems has not been completely understood. In the present study, we have attempted to evaluate the process of apoptosis as a probable safety mechanism for these pyrethroids in protecting the mammalian system from the deleterious

effects of synthetic pyrethroids. Two synthetic pyrethroids, deltamethrin and cyhalothrin were used as triggers for apoptosis in rats at sub-lethal doses and to study any apoptotic changes in the animals. Apoptotic biomarkers such as DNA laddering, caspase-3 enzyme activation and induction of nNOS were evaluated using the Wistar rat *in vivo* model, since these 3 biomarkers are interrelated in the mammalian system. Cytotoxicity of these two pesticides was also tested using HeLa cells as an *in vitro* model system. Finally, a quick detection method based on two different antibodies – rabbit antibody (IgG) and hen antibodies (IgY), raised in this study, was developed and compared with the commercially available antibodies. The detection method included an ELISA method and a yes/no test using dot blotting.

There are several methods available for the detection of biomarkers of apoptosis. DNA fragmentation, a hallmark of apoptosis, can be detected by agarose gel electrophoresis, terminal deoxynucleotidyl tranferase-mediated in situ-end labeling (TUNEL) assay and flow cytometry. Biochemical assays are commercially available for detection of all the caspase group of enzymes and the different isoforms of NOS. Commercial immunoassay kits and antibodies are also available for detection of apoptotic biomarkers. But most of these assays are for detection of these biomarkers from animal cell lines, not produced in India and are thus expensive when compared to indigenous methods of detection. Thus this study is an attempt to develop such a method for detection of apoptotic biomarkers indigenously and at a much affordable cost.

CHAPTER 2

APOPTOTIC POTENTIAL OF PYRETHROIDS AT SUBLETHAL DOSES

INTRODUCTION

Pesticides are 'chemical shields' to ward off ravaging pests, which can damage agricultural produce and processed food. Many pesticides are highly toxic to humans and other non-target organisms. Their use has been steadily on the rise over the years. The last three decades have seen the emergence and popular use of new generation pesticides - the synthetic pyrethroids. Synthetic pyrethroids are known to have high toxicity to pests and very low toxicity to non-target species. They are degraded very easily in the environment as well as in the mammalian system, leading to their label as 'safe' pesticides. However, as per our knowledge, no information is available on the 'safety' mechanism of the pyrethroids in non-target species.

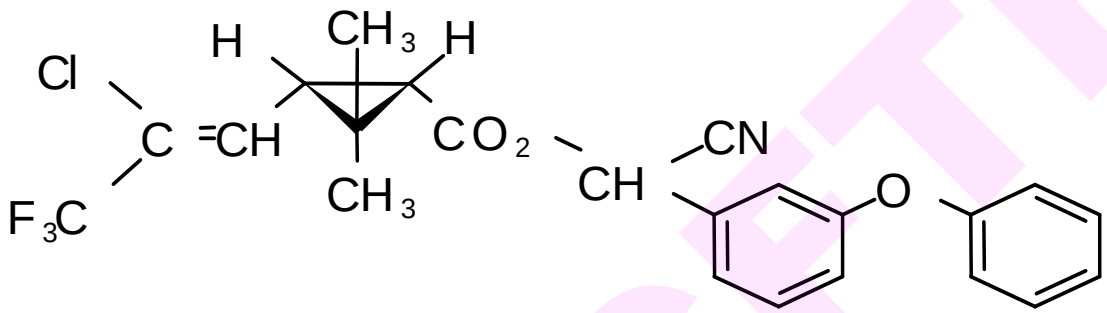
Reports on the apoptotic potential of pesticides, especially pyrethroids, are scanty. Several reports (El-Gohary *et al*, 1999, Wu *et al*, 2000a) are available on apoptotic potential of deltamethrin while there are very few reports on cyhalothrin. But none of them have explored the role of apoptosis as a safety mechanism in non-target species.

Apoptosis is a safety/regulatory mechanism in both plants and animals. It is an active mode of cell death characterized by a sequence of morphological, biochemical and molecular changes, specific to this mode of cell death i.e. apoptosis (Darzynkiewicz *et al*, 1998). These changes include chromatin condensation, cell blebbing, and DNA fragmentation (Darzynkiewicz *et al*, 1998). Apoptotic changes may be brought about by a variety of external stimuli, both chemical and physical. Chemical stimuli can be pesticides, topoisomerase

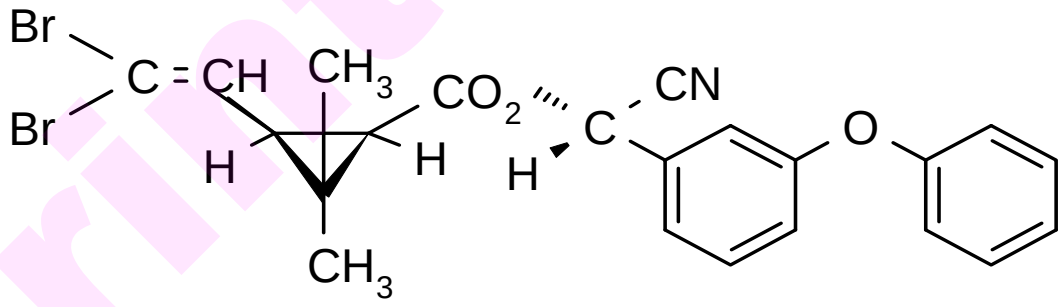
inhibitors like etoposide etc and physical stimuli can be starving, UV rays etc. Various factors such as the age of the animals, duration of exposure and route of exposure could also influence the apoptotic pathway (Darzynkiewicz *et al*, 1998). Apoptotic changes are characterized by the induction of caspases and endonucleases – group of enzymes leading to DNA "laddering" or cleavage into oligonucleosomal fragments (multiples of 180 bp). Caspases are a group of enzymes triggered off first in the apoptotic pathway, which in turn trigger off the activation of caspase activated endonucleases (CAD) leading to DNA fragmentation. DNA laddering is considered to be one of the markers for apoptosis (Wyllie *et al*, 1980). Caspases and DNA fragmentation are early events in apoptosis and are known markers in the apoptotic process. Hence, caspases were chosen in the present study as biomarkers of apoptosis to study the effects of the synthetic pyrethroids.

The present study was undertaken to explore the possible role of apoptosis in the safety of these pyrethroids since little information is available on the safety mechanism in non-target organisms against the synthetic pyrethroids. Two widely used synthetic pyrethroids, Cyhalothrin and Deltamethrin (Fig 2.1 and 2.2) were screened for their ability to cause apoptosis.

Fig 2.1: Structure of pyrethroids selected for the study



a: Cyhalothrin



b: Deltamethrin

1. Screening of pyrethroids for their apoptotic potential

1.1 MATERIALS AND METHODS:

a. Pesticides and chemicals:

Technical grade lambda-cyhalothrin (97% purity) was obtained from Astra-Zeneca India Ltd, Bangalore, while deltamethrin was purified in the laboratory using 95% ethyl alcohol from 25% tablet formulations of deltamethrin (Aventis Crop Science India Ltd, Gujarat). Molecular biology grade phenol, Ethidium bromide, Low-melting agarose, Tris base, Ethylene Diamine Tetracetic acid (EDTA-sodium salt), Coomassie brilliant blue dye (R250 and G250 grades), Sodium acetate, SDS, RNAse, Bromophenol blue, Xylene cyanole FF, Drabkin's solution, Trisodium citrate, glacial Acetic acid, groundnut oil and organic solvents were purchased locally. Ethylenebis(oxyethylenenitrilo) tetracetic acid (EGTA), Dulbecco's modified eagle medium (DMEM), Fetal calf serum (FCS), Leupeptin, Pepstatin, Penicillin-streptomycin mix, PhenylMethyl Sulphonyl Fluoride (PMSF), β-Mercaptoethanol and Florisil were from Sigma-Aldrich chemicals, USA. Other materials include pre-coated silica TLC plates (Merck Chemicals), o-tolidine (Fluka chemicals, Bangalore), 24-well culture plates (Greiner, Germany).

b. Purification of Deltamethrin from Commercial Tablets

The Deltamethrin tablets (2g/25ml) were dissolved in a beaker with (i.) Acetone and (ii.) Ethyl alcohol. The binding material was allowed to settle down and the upper layer, containing deltamethrin, was decanted into another beaker and diluted further at 1:1 ratio with the respective solvents (i) and (ii). The contents were then concentrated in a flash evaporator. The concentrate was allowed to

crystallise at room temperature. The crystals of deltamethrin were collected and analyzed for their purity using thin layer chromatography (TLC) along with technical deltamethrin standard.

c. Thin-Layer Chromatography (TLC):

TLC was carried out according to the method of Pasha *et al* (1993). The pesticide samples were dissolved in acetone and spotted on a silica-gel coated aluminium TLC plate (300 micron thickness) at a known concentration. Petroleum ether and diethyl ether in the ratio of 9:1, was used as the mobile phase and deltamethrin was detected by spraying the developed plate with o-tolidine and exposure to UV light.

d. Experimental models:

The dose for the animal studies was fixed based on sublethal levels of the LD_{50} values and tested using both the *in vitro* and *in vivo* model systems. The HeLa cell line was a gift from the Indian Institute of Science, Bangalore. Female CFT-Wistar albino rats were obtained from the rat colony maintained at CFTRI, Mysore, after appropriate ethical committee clearance.

1.2 *In Vitro* Studies

HeLa Cells were used for the *in vitro* studies and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, in 24-well culture plates at a seeding density of 1500 cells/cm^2. Antibiotic mix of penicillin and streptomycin were used to prevent microbial growth. An incubation temperature of 37^0C with 5% CO_2 was used to maintain the cell growth. The cell density was recorded

daily till the cells reached the confluence stage. A growth curve was plotted and used to determine the different phases of the cell growth for cytotoxicity studies.

1.2.1 Cytotoxicity studies

Six to seven day old HeLa cell cultures (10^4 to 10^5 cells – 70% confluent) were used to test cytotoxicity of deltamethrin and cyhalothrin. Different concentrations of these pesticides with methanol as the vehicle were tested at 0, 1 and 0.01 ppm for exposure periods of 2 and 6 h. The percent cell death was analysed with 2% trypan blue using exclusion method under a light microscope.

1.3 *In Vivo* Studies

Animals and treatment

Three-week old (weanling) and three-month old (adult) female CFT-Wistar rats (*Rattus norvegicus*, CFT strain) were obtained from the rat colony at CFTRI after appropriate ethical committee clearance. The animals were kept in polypropylene cages (5 animals/cage) covered with metallic grid and sterilized saw-dust bedding. The rats were maintained on commercial rat pellet diet and water *ad libitum* with a 12 h light/dark cycle.

1.3.1 Experimental Design

The apoptotic potential of the pesticides was studied based on different factors, which could affect apoptosis. The factors are listed below:

i. Age of animals: Young and Adult rats

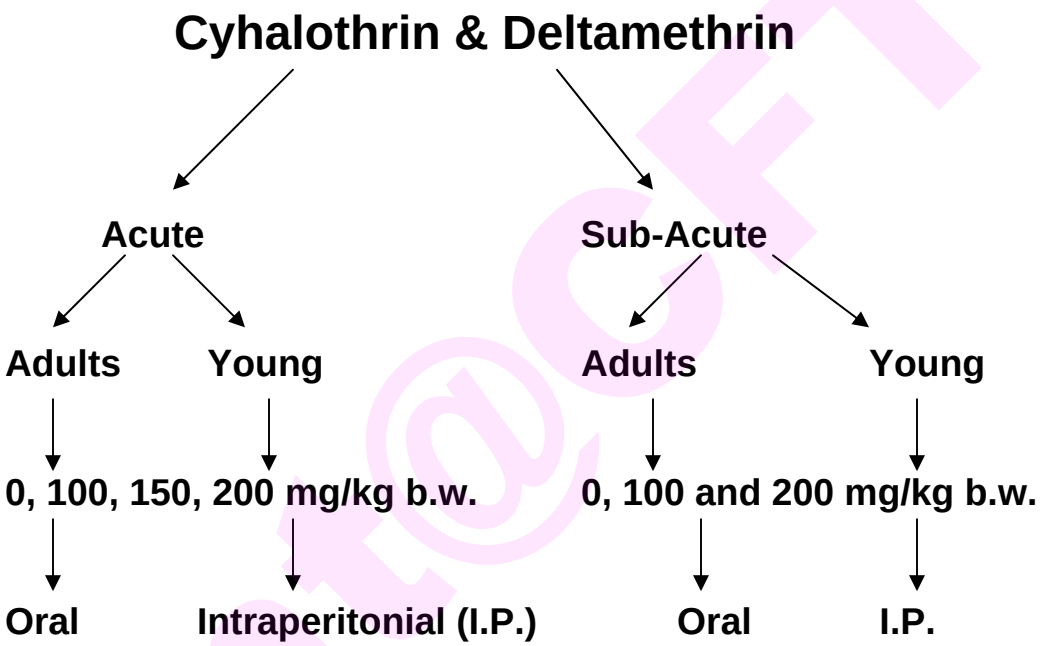
ii. Dosing regime: 15 min, 24 h, 72 h and 21 days

iii. Route of exposure: Gavage and Intraperitonial

iv. Doses: Various sublethal dose fractions were chosen for both acute and sub-acute studies. Based on the above parameters, the studies were conducted for

the pyrethroids, cyhalothrin and deltamethrin on both young and adult Wistar rats as per the experimental design illustrated in Fig 2.2:

Fig 2.2: Diagrammatic representation of the experimental design.



1.3.2 Acute Toxicity Studies:

Adult (3 month old) and weanling (3 weeks old) female CFT-Wistar albino rats (*Rattus norvegicus*) were grouped by the random block design of 5 rats per dose group. The average weight range of each group was approximately 180 ± 5 g. The weight range for the young rats was approximately 80 ± 5 g. The dose groups for the two pesticides were 0 (vehicle alone) 100, 150 and 200 mg/kg b.w. Technical grade λ-cyhalothrin and deltamethrin were suspended in groundnut oil

as the vehicle. The animals in all the above dose groups were given a single dose of the pesticides by oral gavage and intraperitonial route (maximum volume of 2 ml of the vehicle). The rats were necropsied under ether anaesthesia after 15 min, 24 h and 72 h of treatment.

1.3.3 Sub-Acute Toxicity studies

Female adult (3-months old with weight range of 180-200g) and young (3-weeks old with weight range of 80-100g) CFT-Wistar rats were used for the sub-acute toxicity studies of deltamethrin and cyhalothrin. The grouping was done by the random block design as under 1.3.2 with dose groups of 0, 100 and 200 mg/kg b.w. The animals were dosed by gavage and intraperitonial route. The total dose was divided over 21 days based on body weight recorded every 3 days. At the end of the 21-day period all the rats were necropsied under ether anaesthesia

Blood was collected from each of the groups of the two toxicity experiments (Acute and sub-acute toxicity) by heart venipuncture for haematology. The following tissues were collected – Liver, Kidney, Brain, Adipose, Adrenals, Uterus and Ovary and the following analysis carried out.

1.3.4 Haematology

The blood was collected directly from the heart in tubes coated with an anti-coagulating agent, recrystallised EDTA (1-2mg/ml blood) and the following parameters were studied

a. Haemoglobin (Hb) content – Hb content was estimated by Cyanmeth method (Bharucha *et al*, 1976). 20 μl of blood was diluted with 5 ml of commercial Drabkin's solution (containing 1 g sodium bicarbonate, 50 mg

potassium cyanide, 200 mg potassium ferricyanide in 1 L distilled water). The diluted samples were directly read after 10 min in the Ame's blood analyser (Miles India Ltd., Baroda) for Hb content

b. Total White Blood Corpuscles (WBC) count – Total WBC's were counted by bulk dilution method (Bharucha *et al*, 1976). 1:20 dilution of the blood was made with the diluting fluid (2 ml of 1% glacial acetic acid, 1 ml of 1% aqueous gentian violet and 100 ml distilled water). Total WBC's were counted using a haemocytometer.

c. Total Red Blood Corpuscles (RBC) count – Bulk dilution method as per Bharucha *et al*, 1976 was performed. 20 µl of the blood was mixed well with 3.98 ml of the RBC diluting fluid (3 g trisodium citrate and 1% formalin in d.w.). RBC's were counted using a haemocytometer.

d. Packed cell volume (PCV) – It was measured using a microhaematocrit (International equipment co., Boston). The blood was filled in a 75 mm capillary tube and sealed with plasticine. The sealed tubes were centrifuged at 11,500 rpm for 5 min and PCV was measured with the help of the reading device supplied along with the microhaematocrit.

1.3.5 Histopathology

Immediately after the rats were necropsied, known weights of the organs (Liver, Kidney, Adrenal, Adipose, Brain, Uterus, Ovary, Spleen, Thymus) were fixed in Bouin's fluid. After 20 h, these were transferred to 70% alcohol and 6 µm paraffin sections taken on a glass slide, stained with haematoxylin-eosin and observed

under a light microscope for histopathological changes. The rest of the tissues were stored at –25^{0}C for DNA analysis and biomarker purification.

1.3.6 Isolation of Genomic DNA

Genomic DNA from all the tissues of all the dose groups and treatments were isolated by the method of Sambrook *et al* (1989) and modifications described in Xu *et al*, (1996). In brief, 100 mg of each tissue was snap frozen using liquid nitrogen and dissolved in extraction buffer (10 mM Tris-NaCl - 1.2 ml/100 mg tissue). The samples were incubated at 50^{0}C for 2 h. The protein was precipitated using equilibrated phenol and chloroform mixture in a ratio of 1:1. Equilibrated phenol was prepared by the method of Sambrook *et al* (1989). The DNA was then precipitated by ½ volume ethanol and 2 volumes of 3 M sodium acetate. The DNA was dissolved in 1 ml of Tris-EDTA buffer. DNA yield was calculated spectrophotometrically at 260 nm (Kamalay *et al*, 1990).

1.3.7 Analysis of DNA laddering in rat tissues

The DNA from the various tissues was analysed by agarose gel electrophoresis as described by Sambrook *et al*, (1989). In brief, a 2% agarose gel was prepared by dissolving 800 mg of electrophoretic grade agarose in 40 ml of 1X Tris-Acetic Acid-EDTA (TAE) Buffer. The TAE buffer (pH 8.0) was prepared as a 50X stock solution by dissolving 24.2 g of Tris, 5.71 ml of glacial acetic acid and 10 ml of 0.5 M EDTA and made up to 100 ml with distilled water. The agarose was dissolved in 1X TAE buffer by boiling in a water bath. Equal concentration of DNA was loaded in a 1:1 ratio with the loading dye (0.25% Bromophenol blue, 0.25% Xylene Cyanole FF, 30% Glycerol) into the wells. The gel was run at 100

mV for 2 h. The DNA was then stained with ethidium bromide and the gel was visualized using a HeroLab gel documentation unit.

1.3.8 Isolation and purification of caspase-3 from rat tissues as biomarker of apoptosis

Caspases present in the cytosol of the cells were extracted by the method of Srinivasula *et al* (2001). The tissues from the young rats treated with an acute dose (200 mg/kg b.w. given i.p.) of deltamethrin were used for this extraction since they showed DNA fragmentation. In brief, 1 g of of all the tissues were homogenized in 250 mM ice cold Phosphate Buffered Saline (pH 7.4). The homogenate was centrifuged at 600 g for 3 min and the supernatant was aspirated and the cell pellet was resuspended in 4 vol. of cold HEPES buffer (pH 7.4) with protease inhibitors (PMSF, Leupeptin, Pepstatin). The cells were lysed by passing through a syringe and centrifuged at 10,000 g for 15 min at 4^0C. The supernatant was collected & recentrifuged at 100,000 g at 4^0C for 60 min. The supernatant (S-100 extract) was collected and protein concentration estimated by the method of Bradford *et al* (1956). The S-100 extract was aliquoted & stored at -80^0C.

This extract was further purified using an affinity column with commercial caspase 3 antibody as the ligand. A sepharose column was prepared by activation with cyanogen bromide as described by March *et al* (1974). Coupling of protein (IgG antibody) on antigen was done as per Hermanson *et al* (1992). To the activated gel, commercial caspase 3 antibody in 5 ml of 0.1 M sodium bicarbonate containing 0.5 M NaCl (pH 9.0) was added and shaken gently at 4^0C

for 10-12 h. 0.25 ml of ethanolamine was added to the above mixture and shaken for 2 h. The gel was packed in a plastic column and washed with 10 bed volumes of 0.1 M sodium bicarbonate containing 0.5 M NaCl (pH 9) followed by 10 mM PBS (pH 7.4). The S-100 extract obtained from the treated tissues (kidney of young rats) was passed through this gel and 2 ml fractions of the eluate were collected. The protein concentration for all the fractions was estimated, the peak fractions pooled and used for the biochemical assay, dot blotting and western blotting, raising antibodies and ELISA (described in chapter 3 and 4).

1.3.9 Biochemical Assay for Caspase – 3

Caspase-3 purified from the tissues of treated animals was analysed using a commercial fluorometric kit. The results were compared with the control and commercial caspase-3 activity. The Caspase-3 fluorometric assay is based on the following reaction:

Ac-DEVD-AMC—---**Caspase 3-**----→ Ac-DEVD + AMC where

Ac-DEVD-AMC: Acetyl-Asp-Glu-Cal-Asp- 7-amido-4-methylcoumarin (substrate)

AMC: 7-amino-4-methylcoumarin (Fluorophore)

Ac-DEVD-CHO was used as the inhibitor in this reaction as reported earlier (Nicholson *et al*, 1995). Ac-DEVD-AMC is the ideal substrate for caspase-3 based on the cleavage site of Poly (ADP-ribose) polymerase (PARP) at Asp-Glu-Val-Asp (DEVD) (Cohen, 1997). This substrate was first reported by Nicholson *et al* (1995) and it was designed based on the tetrapeptide-AMC motif using the PARP cleavage site P_1-P_4 tetrapeptide. Thus, the AMC linked to the

DEVD tetrapeptide is released and it is a direct measure of the activity of the enzyme.

The fluorescent AMC moiety was detected at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The caspase activity was measured using a standard curve prepared using different concentrations (25-250 nmol) of AMC. The caspase activity in nmol of AMC released per min per ml of tissue homogenate or positive control was calculated based on the following formula:

$$\text{Activity, nmol/AMC/min/ml} = \frac{\text{nmol AMC x d}}{\text{t x v}}$$

Where: v = volume of sample in ml

d = dilution factor

t = reaction time in minutes

1.4 Statistical analysis

Microsoft excel software was used for the data analysis. The haematological, cytotoxicity, caspase enzyme activity, pesticide residue and lipid estimation data was analysed by the t-test. Values were considered significant when $p < 0.05$.

2. RESULTS

2.1 Thin Layer Chromatography (TLC) of purified Deltamethrin

Both acetone and ethyl alcohol extracts showed presence of deltamethrin crystals. However, ethyl alcohol produced pure white crystals as compared to acetone, which produced brownish crystals, indicating presence of the binding material in the extract. The purified material was comparable with the technical deltamethrin standard with an R_f value of 0.41 (Fig 2.3). Fig 2.3 shows spots from both the extractants had the same R_f as technical deltamethrin. In the case of acetone crystals, no spot was seen at 10 µg (lane 3) whereas a faint spot was visible at 20 µg (lane 2). While at 10 µg the crystals from ethyl alcohol gave a very faint spot (lane 4), at 20 µg the ethyl alcohol crystals gave a larger spot (lane 5) identical to technical deltamethrin standard. Thus, ethyl alcohol extracted deltamethrin crystals were used for all further studies. These crystals were dissolved in methanol for cytotoxicity studies *in vitro* and suspended in groundnut oil for *in vivo* studies

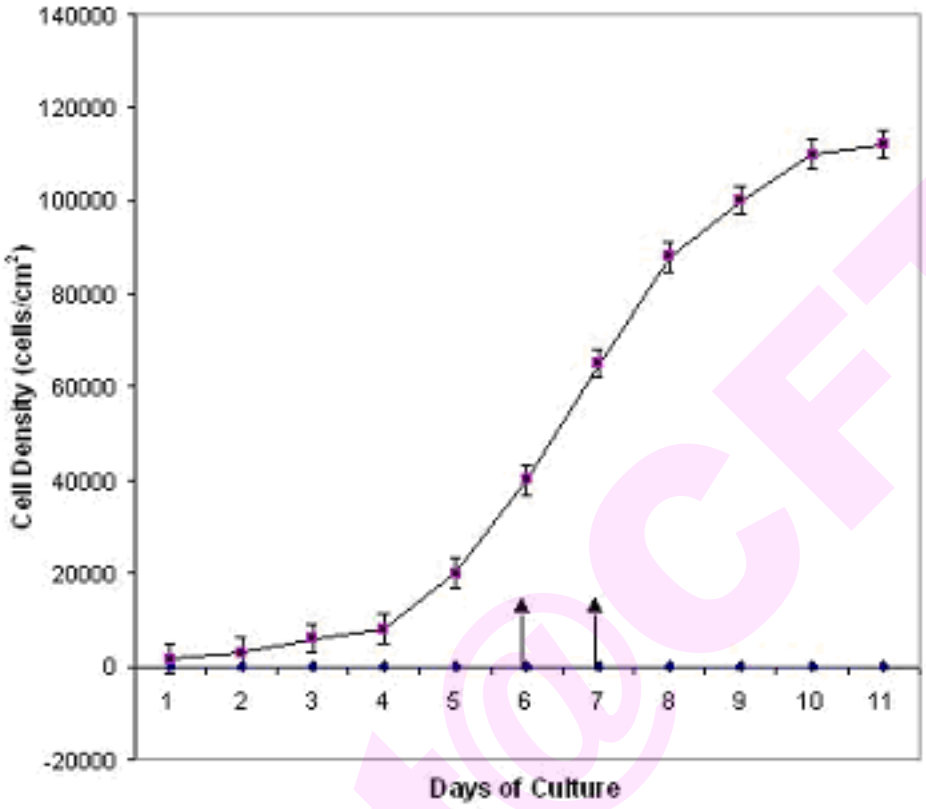
2.2 *In Vitro* studies

The growth of the HeLa cells in the DMEM medium supplemented with 10% FCS at 37^0C and 5% CO_2 was optimum. The HeLa cells reached confluent growth at 8-10 days (Fig 2.4). At confluency, the cell population was approximately 100,000 viable cells/cm^2 and reached a plateau at 10-11 days. The cells at 70% confluency (6-7 days culture) were used for the cytotoxicity studies.

Fig 2.3: TLC of Deltamethrin purified from a commercial formulation

Lane - Sample
1 - Tech. Deltamethrin - 20 µg
2 - Acetone extr. - 20 µg
3 - Acetone extr. - 10 µg
4 - Alcohol extr. - 10 µg
5 - Alcohol extr. - 20 µg

Fig 2.4: Growth curve of HeLa cells



Values are mean of 3 values ± S.E.
(Arrows indicate 70% confluent cells between 6-7 days after culture. The cells were harvested at this stage for cytotoxicity studies.)

2.3 Cytotoxicity studies with HeLa cells

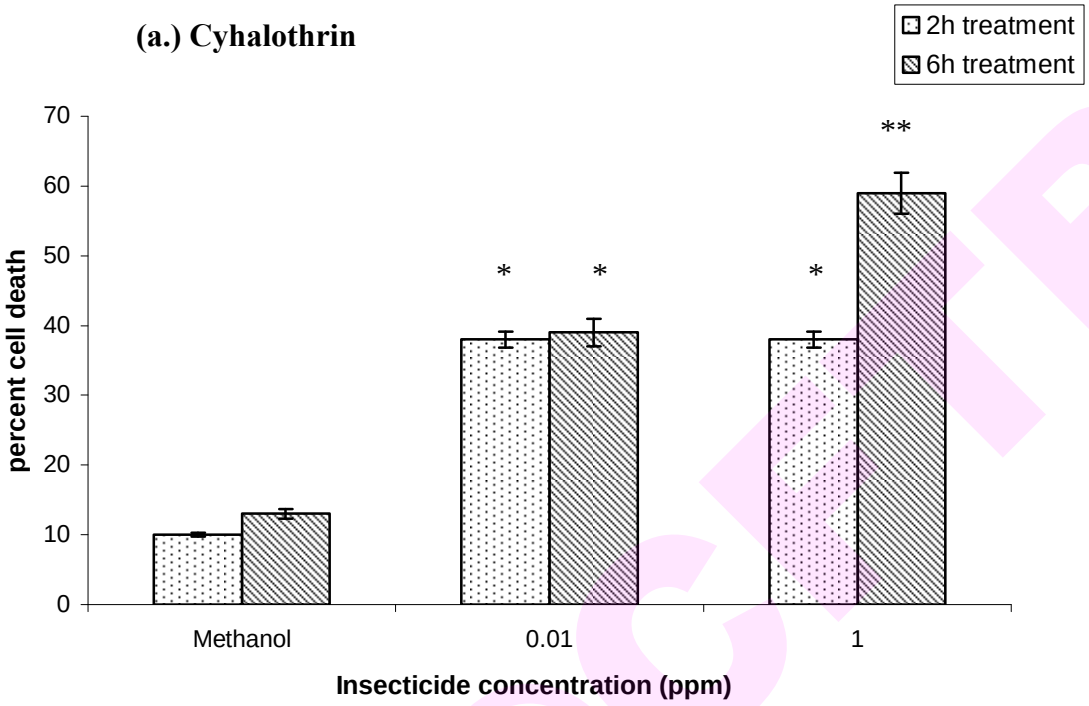
The cytotoxicity of cyhalothrin indicated the toxicity to be both time and dose dependant (Fig 2.6 a). Compared to the methanol control (10-15% cell death), 40% cell death was observed at 2 h at both the doses of 0.01 ppm and 1 ppm, while at 6 h, the cell death increased to 60% ($p<0.01$) at 1 ppm compared to 40% at 0.01 ppm.

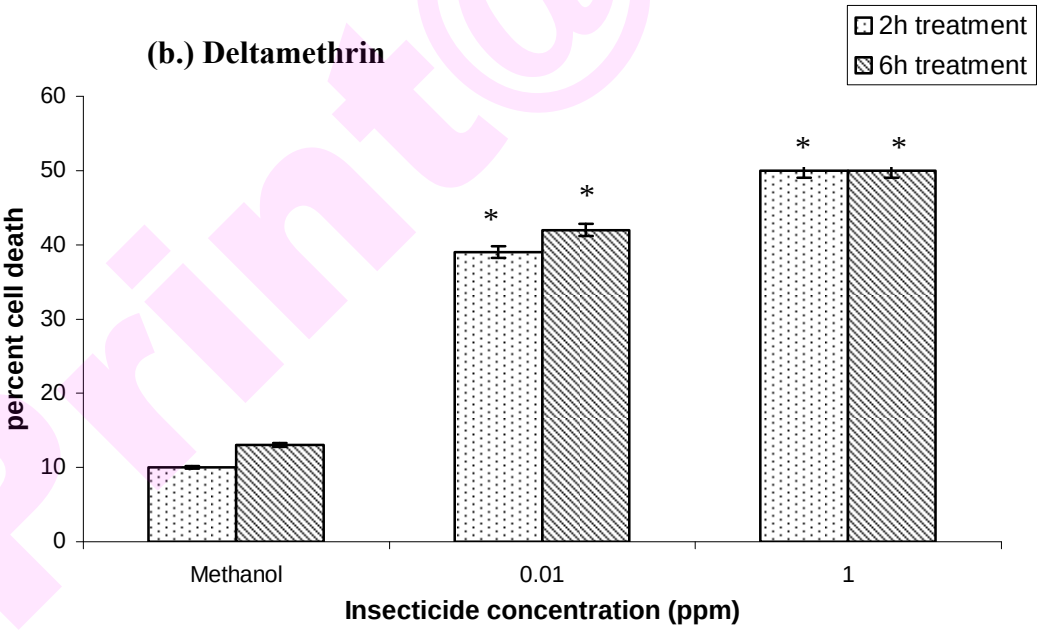
The cytotoxicity of deltamethrin showed that the toxicity was only dose dependant (Fig 2.6 b). At 0.01 ppm the percentage of cell death was 40% at 2h and 6h while at 1 ppm the cell death at both the time periods increased to 50% as that of the control. The cell death due to the vehicle was very minimal at 10% and 15% at 2h and 6h respectively. Hence, both cyhalothrin and deltamethrin treatment showed significant cytotoxicity ($p < 0.05$) at both the time intervals and concentrations when compared to the vehicle treatment alone.

2.4 Acute Toxicity studies

No significant change in the body weight of the animals and their food intake was observed with any of the treatments. The organ weights were also on par with the control tissues. It was also observed that the symptoms of pyrethroid toxicity such as salivation, ataxia, decreased response to stimuli, hyperexcitation, convulsions and paralysis reverted back in the treated animals after 24 h and the animals regained their normal motor functions.

Fig 2.5: Cytotoxicity studies using HeLa Cells.





Values are mean ± S.E.

*** - Statistically significant from control (methanol treated) p<0.05.**

**** - p<0.01.**

2.5 Haematology and Histopathology

Haematological studies with both cyhalothrin and deltamethrin showed no significant deviation from the control group in the profile of total RBC, total WBC, hemoglobin content and PCV (Table 2.1). The histopathological profile of the organs of all the treated groups was not significantly different from the control group and showed a normal histological picture.

2.6 Analysis of DNA laddering

DNA fragmentation by agarose gel electrophoresis was observed in young rats treated with an acute i.p. dose of deltamethrin (Fig 2.6) A typical “laddering” was observed in the DNA from the kidney of the deltamethrin-treated young rats at an acute dose, after 24 h. However, deltamethrin acute toxicity studies in adult rats did not show any DNA fragmentation in any of the tissues (Fig 2.7 a).

Cyhalothrin treatment did not produce any DNA fragmentation in the tissues in both young and adult rats treated at an acute dose of cyhalothrin (Fig 2.7 b). Even the different routes of treatment by cyhalothrin did not produce DNA fragmentation in the rat tissues.

The results are summarized in Table 2.2.

2.8 Sub-Acute Toxicity studies

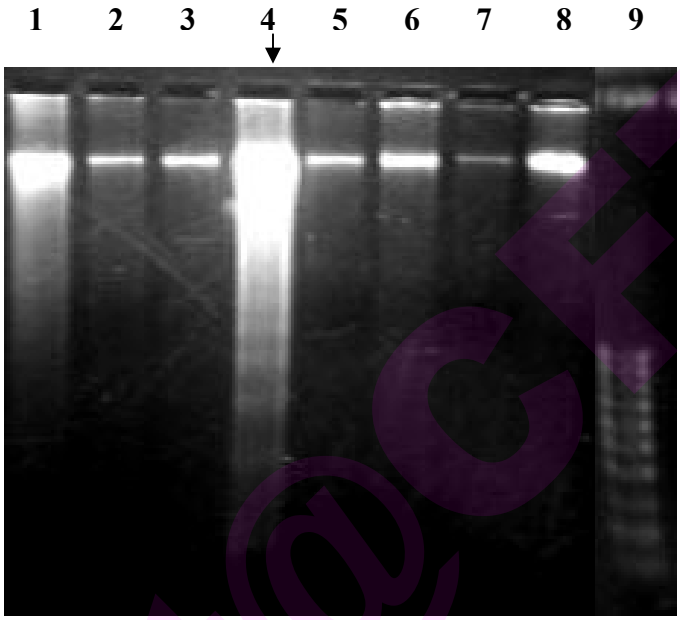
No significant changes were observed in the body weight and food intake of treated animals when compared to the control animals. The organ weights also did not show any changes in the treated animals. Haematology (Table 2.1) and histopathology of the treated animals showed a normal profile. Similar results

Table 2.1: Haematological profile of rats treated with cyhalothrin and deltamethrin

Toxicity study and agent used	Hemoglobin (g/dl)	Total RBC (10^6/µl)	Total WBC (/µl)	PCV (%)
Cyhalothrin (mg/kg) Acute Toxicity				
1. *Control*	14.4 ± 0.38	8.8 ± 0.6	7200 ± 300	33 ± 0.6
2. *100*	15.0 ± 0.30	9.2 ± 0.4	7700 ± 300	30.5 ± 0.5
3. *150*	14.3 ± 0.30	11.6 ± 0.4	7800 ± 600	27 ± 1.0
4. *200*	13.5 ± 0.45	12.4 ± 0.4	8700 ± 500	30 ± 2.0
Sub-Acute Toxicity				
1. Control	14 ± 0.2	9.6 ± 0.8	11375 ± 575	34 ± 0.0
2. 100	14.6 ± 0.0	10 ± 0.4	13100 ± 700	40 ± 2.0
3. 200	13.7 ± 0.9	10.8 ± 0.3	13400 ± 800	39 ± 1.0
Deltamethrin (mg/kg) Acute Toxicity				
1. *Control*	14.6 ± 0.15	10.1 ± 0.3	8800 ± 200	43 ± 2.0
2. *100*	13.6 ± 0.05	9.3 ± 0.2	9000 ± 305	40 ± 1.7
3. *150*	14.2 ± 0.40	9.8 ± 0.2	9066 ± 468	40 ± 1.1
4. *200*	13.4 ± 0.40	10 ± 1.2	9800 ± 600	37 ± 1.0
Sub-Acute Toxicity				
Control	14.0 ± 0.2	9.6 ± 0.8	11375 ± 575	34 ± 0.0
100	14.6 ± 0.4	10.0 ± 0.4	14000 ± 800	36.5 ± 0.5
200	14.0 ± 0.5	11.3 ± 0.05	8725 ± 975	36 ± 1.0

Values are mean of 3 samples ± S.E.

Fig 2.6: DNA Analysis of young rats treated at 200 mg/kg b.w. of Deltamethrin.

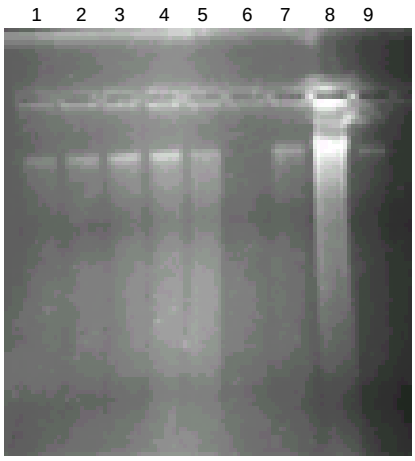


Lanes – Organs

1 Uterus
2 Ovary
3 Liver
4 Kidney
5 Adrenal
6 Brain
7 Adipose
8 Spleen
9 100 bp molecular marker

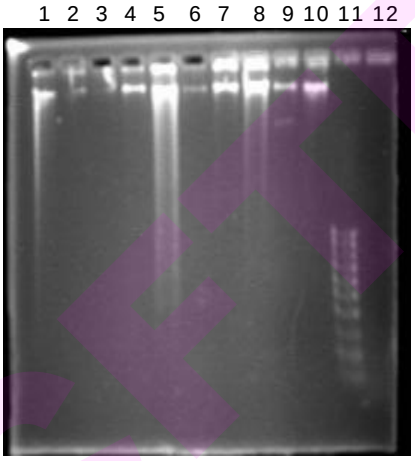
Fig 2.7: DNA analysis from adult rats treated with an acute dose of 200 mg/kg b.w:

a - Deltamethrin



Lanes - Organs
1 Uterus
2 Ovary
3 Adrenal
4 Liver
5 Kidney
6 Adipose
7 Brain
8 Uterus (Control)
9 Ovary (Control)

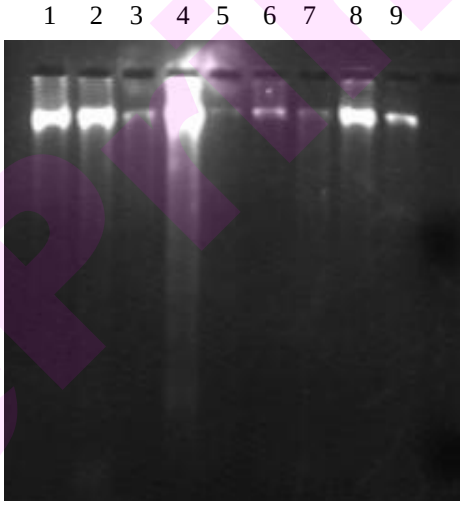
b - Cyhalothrin



Lanes - Organs
1 Uterus
2 Ovary
3 Adrenal
4 Liver
5 Kidney
6 Adipose
7 Brain
8 Uterus(control)
9 Ovary(control)
10 Adrenal (control)
11 100 bp marker
12 Blank

Fig 2.8: DNA analysis from adult rats treated with sub-acute doses of 200 mg/kg b.w.:

a – Deltamethrin



Lanes - organs

1 Uterus
2 Ovary
3 Liver
4 Kidney
5 Adipose
6 Brain
7 Adrenal
8 Thymus
9 Spleen

b– Cyhalothrin

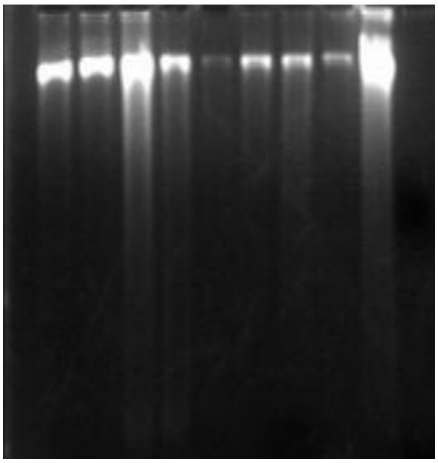


Table 2.2: Summary of *in vivo* apoptotic studies

Agent	Age	Duration of Experiment	Route	Dose (mg/kg b.w.)	DNA Fragmentation
Deltamethrin	Young	Acute	Oral	200	**YES**
				150	
			Intraperitonial	100	ND*
	Adult	Acute	Oral	200	ND
				150	
			Intraperitonial	100	ND
	Adult and Young	Sub-acute (21 d)	Oral	200	ND
			Intraperitonial	100	
Cyhalothrin	Young	Acute	Oral	200	ND
				150	
			Intraperitonial	100	ND
	Adult	Acute	Oral	200	ND
				150	
			Intraperitonial	100	ND
	Adult and Young	Sub-acute (21 d)	Oral	200	ND
			Intraperitonial	100	

*- ND - Not Detectable

were observed with DNA analysis of rats treated with a sub-acute dose of deltamethrin (Fig 2.8 a). The DNA analysis did not reveal any evidence of fragmentation in both the adults and the young rats. Even with the sub-acute toxicity studies of cyhalothrin, no DNA fragmentation was observed in both adult and young rats in all the dose groups (Fig 2.8 b). The summary of the sub-acute study is shown in Table 2.2.

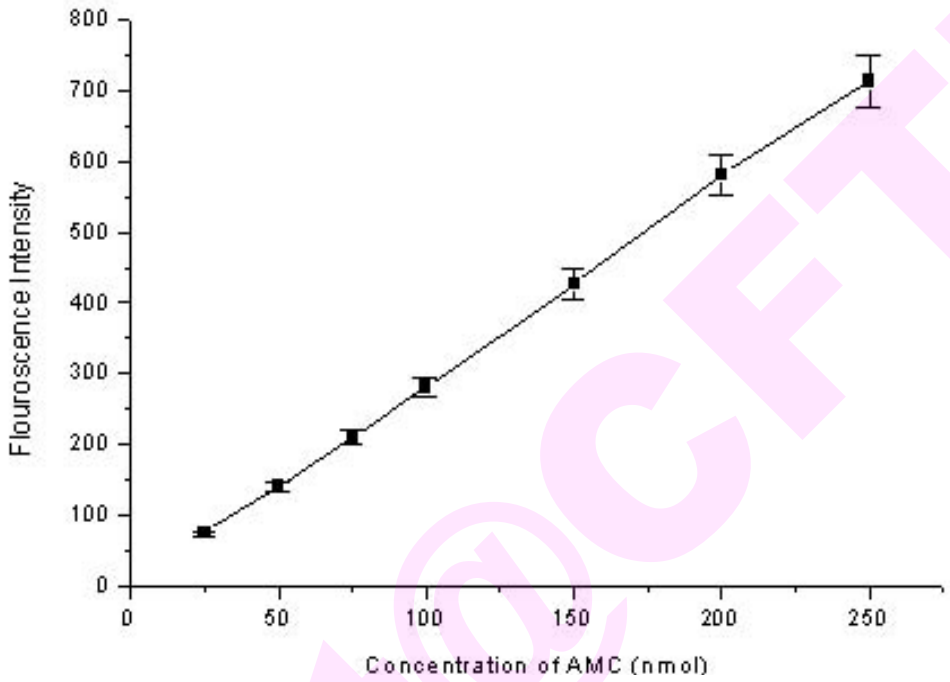
2.7 Biochemical assay for Caspase-3

The caspase-3 activity was calculated from the standard curve of AMC (Fig 2.9). The minimum detection limit of the assay was 25 nmol/AMC/min/ml. The activity was seen to be comparable in the kidney from the 200 mg/kg deltamethrin treated group to the caspase-3 positive control. The maximum activity was seen at 30 min after the initiation of the reaction. The caspase activity was 3 times more pronounced as that of the caspase-3 from the treated group (CFTRI Casp-3) when compared to the vehicle-treated (control) group. In the case of the positive control (commercial caspase-3), the activity was 4-fold more higher than the control. The caspase activity was 1666.6 nmol/AMC/min/ml for the deltamethrin-treated group and 2166 nmol/AMC for commercial caspase-3, compared to 500 for the control group (Table 2.3).

The activity of caspase-3 from both control and treated samples was reduced by the inhibitor (Ac-DEVD-CHO) to the extent of 26-46% at all the time intervals (Fig 2.10 a and b). For the control caspase-3, the fluorescence intensity was 211.25 at 30 min compared to 112.35 at the same time interval with the

inhibitor. Caspase-3 sample from the treated samples showed a fluorescence of 356.5 without Inhibitor compared to 264.9 with the inhibitor after 30 min. At 30 min, the commercial caspase-3 activity was 362.5 without inhibitor and 240.73 with the inhibitor. The activity was less than the commercial caspase-3 activity by one-fold. Thus, it was observed that deltamethrin treatment at 200 mg/kg b.w. induced caspase-3 activity markedly in the kidney tissue when compared to the vehicle control.

Fig 2.9: AMC Standard Curve



Values are mean of 3 separate experiments ± S.E.

Table 2.3: Caspase-3 activity in deltamethrin treated rats at 30 min

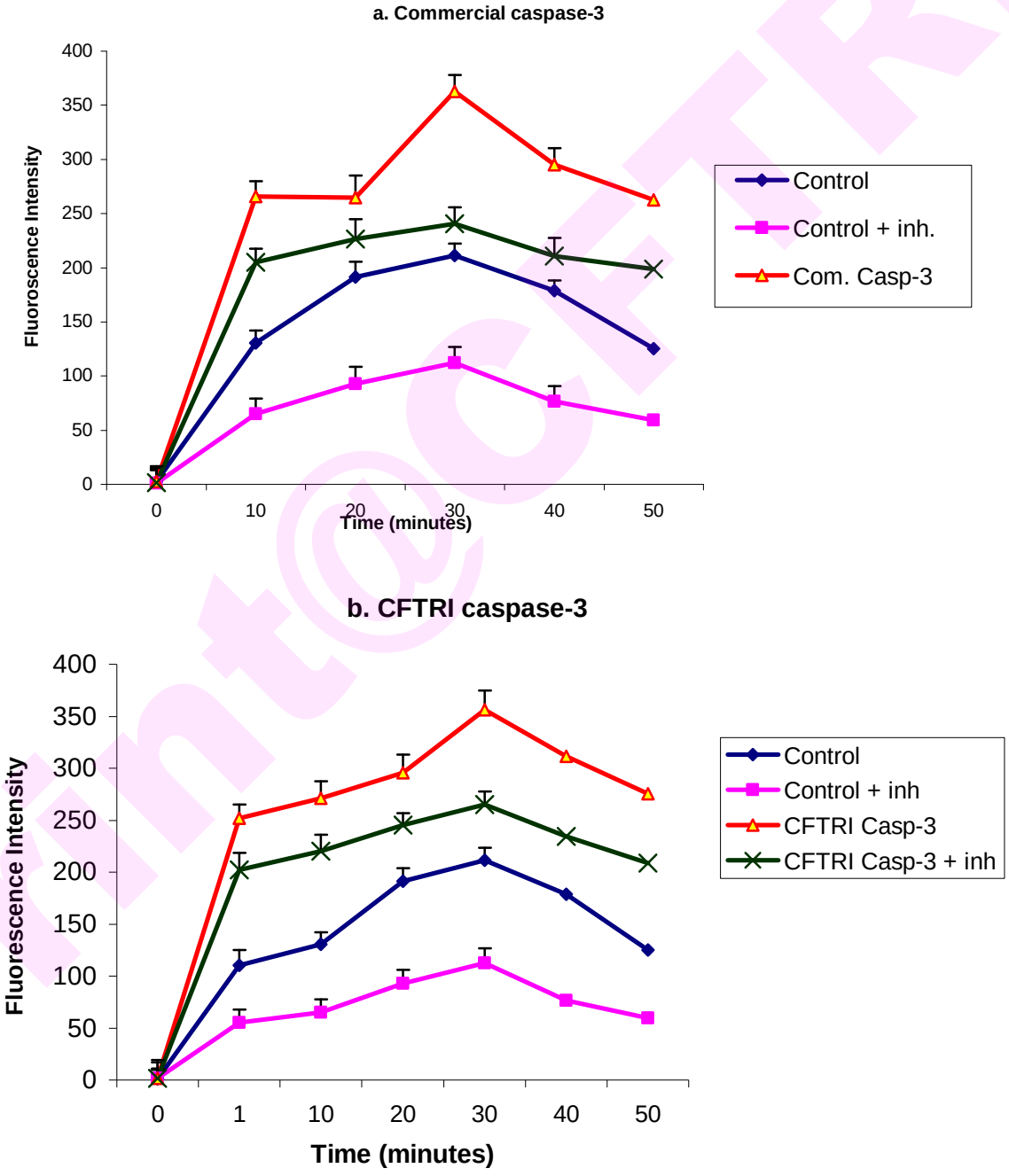
Parameter	Control	Commercial Caspase-3	CFTRI Caspase-3
AMC value	75	130	125
Volume (ml) (v)	1	0.4	0.5
Dilution (d)	200	200	200
Activity (nmol AMC/min/ml) #	500 ± 30.4	2166 ± 48.7 *	1666.6 ± 51.5 *

* - $p<0.05$

- Activity calculated from the formula: $\text{nmol/AMC/min/ml} = \frac{\text{nmol AMC} \times d}{t \times v}$

Values are mean of 3 separate experiments ± S.E.

Fig 2.10: Kinetics of Caspase-3 activity in the treated samples



Values are means of 3 separate experiments ± S.E.

3. DISCUSSION

Pyrethroids are synthetic derivatives of naturally occurring pyrethrins from pyrethrum, the oleo-resin extract of dried chrysanthemum flowers. The insecticidal properties of pyrethrins are due to ketoalcoholic esters of chrysanthemic and pyrethroic acids. These acids are strongly lipophilic and rapidly penetrate many insects and paralyse their nervous system (Beyond Pesticides, **www.beyondpesticides.org**, Washington DC, 2003). They are less effective in non-target species like mammals and have a high LD_{50} in mammals of 100-1000 mg/kg (WHO, 1999) and hence are considered safe to humans. But little light has been shed on their safety mechanism in mammals. One such mechanism that can have an influence on the safety of pyrethroids could be apoptosis and this study is a step in that direction.

In the present study, cyhalothrin and deltamethrin were administered to rats of different ages. The results indicated DNA laddering in the kidney of young rats treated with an acute dose of 200 mg/kg b.w. of deltamethrin after 24 h as observed by agarose gel electrophoresis. However, DNA laddering was not observed in the young and adult rats treated at sub-acute doses. Sheets *et al* (1994) has reported that the LD_{50} dose for treated weanling rats (11 and 21-day old) was 5.1 and 11 mg/kg b.w. when compared to 81 mg/kg b.w. for the adult rats (72 days old). Thus, the study by Sheets *et al* showed that weanling rats are more susceptible to deltamethrin than adult rats. Similar studies in young mice using another synthetic pyrethroid permethrin showed apoptosis in the thymocytes population (Prater *et al*, 2002). DNA laddering was observed by Wu

et al (2000b) in adult Sprague-Dawley rats 24-72 h after administration of deltamethrin by i.p. route at 12.5 mg/kg b.w. Dayal *et al* (1999), Aziz *et al* (2001) and El-Gohary *et al*, (1999) have reported other behavioural and biochemical changes in deltamethrin treated rats. Dayal *et al* (1999) reported a dose- and time-dependant increase in the activity of microsomal cytochrome P450 enzymes in the brain and liver of Wistar rats treated with different doses of deltamethrin. The same study also confirmed this increase with an immunoblot study against the enzymes P450 1A1/1A2 and 2B1/2B2. Aziz *et al* (2001) reported an increase in acetylcholinesterase enzyme activity and Growth Associated Protein-43 (GAP-43, a neuron specific protein) in the hippocampal brain region of Wistar rats treated with deltamethrin. This study also showed a significant decrease in learning and memory of the deltamethrin treated rats. El-Gohary *et al* (1999) reported DNA fragmentation and increase in apoptotic cells in testes of rats treated with deltamethrin and its modulation by a Nitric Oxide synthase (NOS) inhibitor. Since these studies were carried out in different species or strains of rats and the difference in the effect of their treatment regimes could be the reason for the differences in the effects observed in the present study. In our study, it was seen that DNA fragmentation was seen in kidney of young rats and caspase-3 was also triggered off in the same tissue. Thus, this result reveals that caspase-3 may have a role in causing DNA fragmentation in mammalian tissues by activating the endonucleases. It is possible that the mammalian system metabolizes pyrethroids very quickly if they are within a certain dose range. This

lends further credence to the safety of pyrethroids used in agriculture (Goodman and Gilman, 1996).

Biochemical analysis of caspase-3 extracted from kidney of deltamethrin treated young animals showed that the activity of the CFTRI caspase-3 was comparable to the activity of commercial caspase-3 (Table 2.3). The control extract also exhibited a detectable caspase activity but much lower at 500 nmol/AMC/min/ml than the extracted caspase-3 and commercial caspase-3. Thus, this activity of the caspase-3 from the kidney extract may be one of the reasons for the appearance of DNA fragmentation in the kidney since caspase-3 is known to activate cellular endonucleases which cleave the DNA (Koester and Bolton, 2001). The activity of caspase-3 peaked at 30 min after which the reaction slowed down and showed a decreasing trend at the end of 50 min (Fig 2.10).

Cyhalothrin in the present study did not affect both young and adult rats at any of the doses tested. Reports by Deshmukh (1992) on reproductive studies in mice and genotoxicity and cytotoxicity of cyhalothrin by Celik *et al* (2005) are the only studies on cyhalothrin. Deshmukh *et al* (1992) conducted a 3-generation study in mice and showed no significant change in the body weight gain in all 3 generations. Celik *et al* (2005) reported that cyhalothrin treatment caused significant dose-related increase in the micronucleus formation in bone marrow and colonic crypt epithelial cells. In both bone marrow and gut epithelial cells, an interaction was found between all the doses of cyhalothrin and micronucleus frequency. Cyhalothrin also caused an increase in the frequency of binucleated

cells in gut epithelial cells (Celik *et al*, 2005). Hence, Cyhalothrin treated rats were used for analysis of pesticide residue to check if the lack/low levels of pesticide in the tissues was responsible for the absence of any apoptotic changes observed in the present study.

Our findings of apoptotic changes in deltamethrin treated young rats (at 200 mg/kg b.w.) demonstrate that apoptosis may play a role in weeding out cells affected by deltamethrin toxicity. It may thus be a probable safety mechanism in preventing deltamethrin toxicity in mammals.

SUMMARY:

- Deltamethrin and Cyhalothrin were tested in the *in vitro* model (HeLa cells) for cytotoxicity and in the *in vivo* model (CFT-Wistar rats) for their apoptotic potential.
- *In vitro* studies showed that the two pesticides showed significant cytotoxicity at doses of 1 and 0.01 ppm compared to the control.
- *In vivo* studies revealed that deltamethrin was found to be apoptotic in the young rats (kidney tissue) at a dose of 200 mg/kg b.w. as seen by DNA laddering in the kidney, while the adults and other young rat groups did not show evidence of apoptosis.
- The apoptotic tissues were used to extract the biomarker – caspase-3
- The biochemical analysis of caspase-3 indicated the activity of this enzyme to be 3-times higher in the treated tissue extract than that of the control tissue extract.
- The activity of the commercial caspase-3 was found to be 4 times more than that of the control and 1-fold greater than the treated rats.
- Cyhalothrin was not found to be apoptotic at any of the age groups(Young or Adult), dose groups, treatment groups or routes of administration used as indicated by DNA laddering.

INTRODUCTION

Pesticide residues are known to accumulate in the environment and also in living organisms. Among these, the organochlorine group of pesticides are among the most persistent. The pesticides may enter the organism through the food chain and are found at higher levels in certain tissues and organs whenever the animal is exposed to them. Some pesticides have more affinity towards certain organs due to various factors such as lipid content of the organs/tissues (Lee *et al*, 2003), functional groups of the pesticides (Marei *et al*, 1982) etc. This affinity and the metabolism of pesticides determines their level of persistence in affected organs. But pyrethroids, on the other hand are known for their low persistence. They have short half-lives in mammals ranging from 4-10 days due to the metabolism of each of the pyrethroids by ester cleavage and phenoxy hydroxylation (Marei *et al*, 1982).

Since 1970, there has been a variety of approaches to quantify pyrethroids in biological samples (Kim *et al*, 2006). These approaches can be classified as biological, immunological and chemical. Biological assays such as the LC_{50}, were carried out by entomologists (Vale *et al*, 2004). Immunoassays were developed to rapidly detect trace levels of pyrethroids in environmental and food samples (Lee *et al*, 2003, Lentza-Rizos *et al*, 2001, Lee *et al*, 1998). Chemical analysis include gas chromatography (Nakamura *et al*, 1994), Thin-layer chromatography (Pasha *et al*, 1993, Akhtar *et al*, 1986) and HPLC methods (Kim *et al*, 2006, Anadon *et al*, 1996).

In the present study, both young and adult animals were treated with 0, 100, 150 and 200 mg/kg b.w of cyhalothrin by both gavage and intraperitonial routes. Cyhalothrin did not produce any apoptotic changes such as DNA laddering in the tissues. This could be due to two reasons:

1. Cyhalothrin is not apoptotic or

2. The pesticide has not reached the target organ to trigger the apoptotic action.

To test which of these hypotheses are correct, residue analysis was carried out in the cyhalothrin treated rats using TLC and GC. There are no reports available about the residue data for cyhalothrin in treated animals. Hence, this study was essential to relate the non-apoptotic changes and to understand the residue pattern of cyhalothrin in treated rats.

1. MATERIALS AND METHODS:

1.1 Chemicals:

Florisil, pre-coated silica TLC plates were from Sigma Chemicals, o-tolidine was from Fluka chemicals. Sodium chloride, sodium acetate, magnesium sulphate, sodium hydroxide and all solvents were purchased locally.

1.2 Synthesis of Cyhalothrin metabolites:

Hydroxy metabolite: 1 g cyhalothrin was added to a mixture of 10% sodium hydroxide dissolved in ethanol and was heated at 80^0C for 90 min with continuous stirring. The pH was adjusted to 5-6 by the addition of 20% HCl. The upper organic layer and the lower aqueous layer were separated. The organic layer was washed 3 times with distilled water. The organic layer was separated and the aqueous layer discarded. The organic layer was passed through magnesium sulphate and the eluate evaporated in a rotary evaporator. The residue thus obtained is the dry hydroxy metabolite.

Acid Metabolite: The aqueous layer after the adjustment of pH with 20% HCl, was extracted with dichloromethane (2X) and the solvent was allowed to evaporate to dryness at room temperature. Aqueous saturated sodium bicarbonate solution was added to the residue and the mixture was again extracted with dichloromethane (2X). The organic layer was separated and discarded, while the aqueous layer containing the acid moiety of cyhalothrin was retained. Its pH was adjusted between 5-6 by the addition of 20% HCl and

extracted twice with dichloromethane (2X). Finally, the dichloromethane layer was separated and evaporated to obtain the free acid metabolite as a residue. The purity of the metabolites was qualitatively determined by the TLC method of Pasha *et al* (1993) described earlier under the sub-heading 1.1, c in Section A.

1.3 Pesticide Residue Extraction from treated rat tissues

The pesticide residues were extracted from different organs by the method of Dale *et al* (1970) and analysed. The pesticide residue was extracted with acetonitrile and n-hexane and then partitioned with sodium chloride and ethyl acetate. The samples were subjected to clean-up using florisil and analysed by Thin Layer Chromatography (Pasha *et al*, 1993) as described in Section A and gas Chromatography (Nakamura *et al*, 1994).

1.4 Gas Chromatography of pesticide residues.

The tissue extracts were analysed by Gas Chromatography for pesticide residues using the method of Nakamura *et al* (1994). A 5% phenyl polysilphenylene siloxane (BP X5) capillary column with nitrogen as the carrier gas was used. Temperature programming was used with an initial column temperature of 60^0C with an increase of 20^0C per minute till 280^0C. An Electron Capture Detector (ECD) was used with an injection temperature of 240^0C and a detector temperature of 280^0C. Acetone was used as the solvent.

1.5 Lipid Estimation

Lipid was estimated gravimetrically in the different dose groups using a Rafatec II 1050 extractor by the method of Folch *et al* (1957). The extractant used was

chloroform and methanol in a ratio of 20:1. Approximately 100 mg of each tissue was taken for this analysis.

2 RESULTS:

2.1 Cyhalothrin Residue Analysis using TLC

The residue analysis of cyhalothrin in tissues of treated animals using TLC showed that technical cyhalothrin, acid and hydroxy metabolites were separable in the mobile phase used (petroleum ether and diethyl ether at 9:1) (Fig 2.11). The R_f value for the parent molecule was 0.5, acid metabolite was 0.46 and hydroxy metabolite was 0.26 (Table 2.4). This method had a minimum detectability of 10 μg. Bluish spots corresponding to cyhalothrin metabolites were observed on the TLC plate at a concentration of 50 μg per lane. Residue analysis revealed the presence of cyhalothrin residues in the reproductive tissues i.e. uterus and ovary besides the adrenal and adipose (Fig 2.11). These spots corresponded to the hydroxy metabolite. The rest of the tissues did not show any cyhalothrin residues.

2.2 Cyhalothrin Residue Analysis in treated rat tissues using GC

The concentration of cyhalothrin and metabolites were determined using the standard graph (Fig 2.12 and Fig 2.13) The Retention time (R_t) of the parent molecule was 15.15 min while the R_t of the metabolite was 14.64 min for the hydroxy metabolite and 14.98 min for the acid metabolite (Fig 2.14). Fig 2.13 a depicts the standard graph of hydroxy metabolite while Fig 2.13 b shows the standard graph of the acid metabolite of cyhalothrin. Both the metabolites showed a concentration dependant increase in the area under the curve.

Fig 2.11: **Residue profile of Cyhalothrin and its metabolites from tissues.**

1 2 3 4 5 6 7

Lane – Sample
1 – Std Cyhalothrin
2 – Hydroxy Metabolite
3 – Acid Metabolite
4 – Adrenal
5 – Uterus
6 – Ovary
7 – Adipose

Table 2.4 - Thin Layer Chromatography (TLC) for Cyhalothrin and its metabolites

S. No.	*Sample*	*Retention factor (R_f)*
1	Std Cyhalothrin	0.5
2	Acid moiety	0.26
3	Hydroxy metabolite	0.46

Fig 2.12: Standard graph of cyhalothrin (GC)

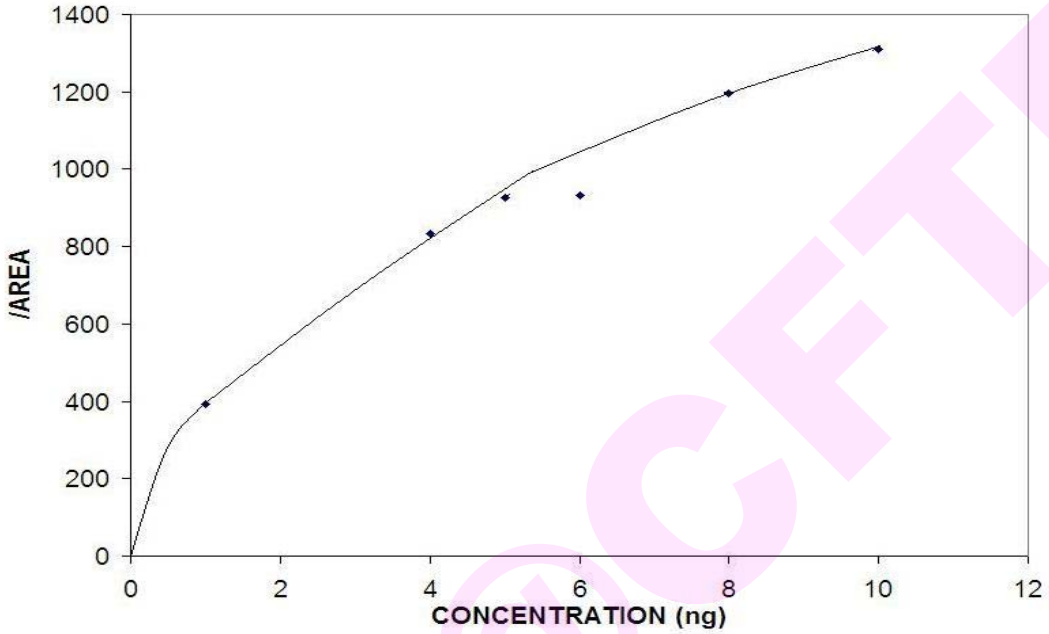
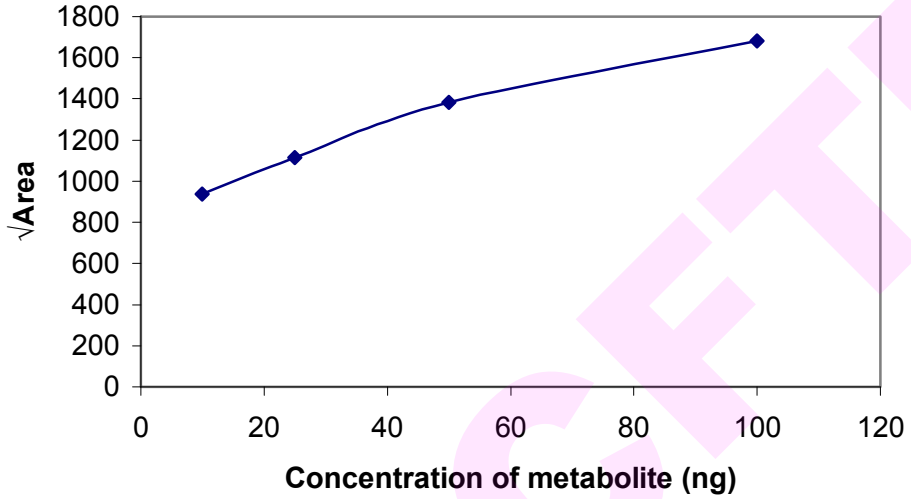


Fig 2.13: Standard curve for cyhalothrin metabolites.

a. Hydroxy Metabolite



b. Acid Metabolite

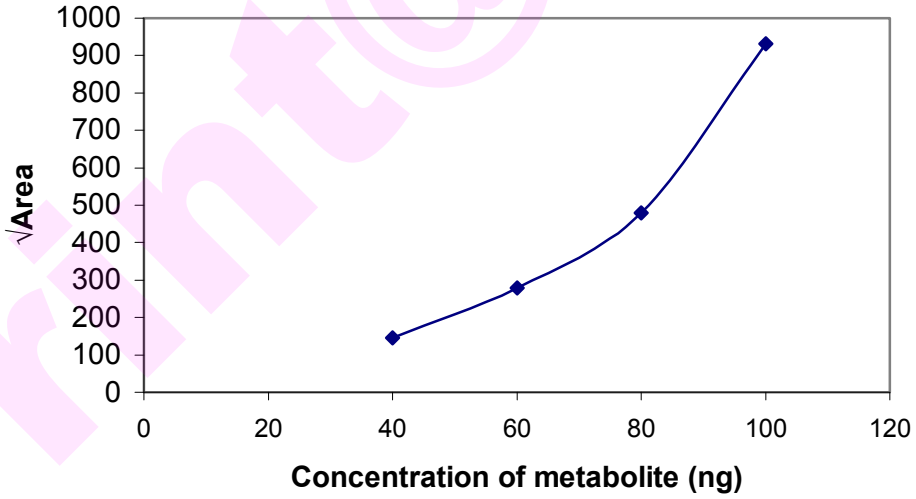
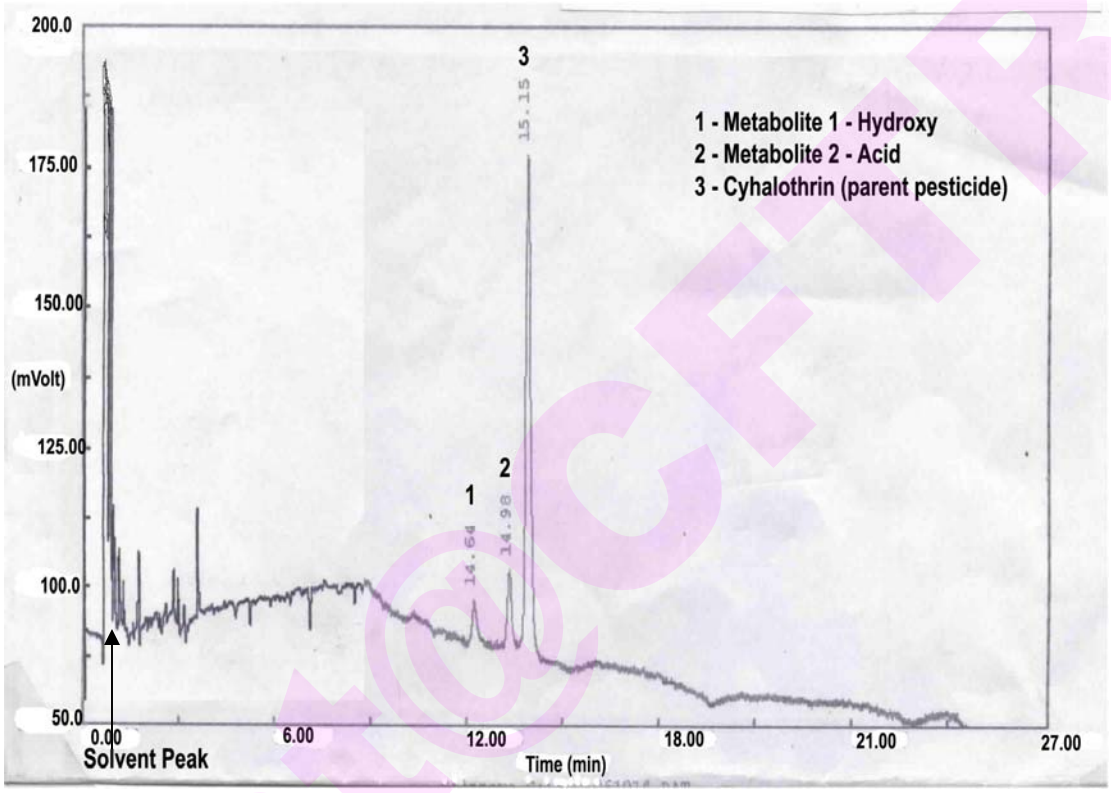


Fig 2.14: Chromatogram showing the separation of cyhalothrin and its metabolites.



The distribution of cyhalothrin in the rat tissues was as follows: **Adipose>Uterus>Ovary>Brain>Adrenal >Kidney> Liver** at 200 mg/kg b.w. (Fig 2.15). It can also be seen that concentration of cyhalothrin was dose dependant. Lower concentrations were found in the 100 and 150 mg/kg dose groups, while the 200 mg/kg dose group had the maximum cyhalothrin concentration. Only uterus and adipose showed cyhalothrin residue at 100 and 150 mg/kg dose groups. In the treated animals, maximum cyhalothrin residue was found in the reproductive organs of the rats (200 mg/kg b.w. treated group) besides the adipose. Adipose had 104 ng/g of cyhalothrin, uterus and the ovary had 75.8 ng/g and 60.5 ng/g respectively of cyhalothrin. In the liver and kidney, it was 2.7 and 8.1 ng/g of tissue respectively. It was interesting to note the preferential accumulation of cyhalothrin in the reproductive organs.

The profile of the cyhalothrin hydroxy metabolite in different rat tissues showed that liver had the highest concentration of this metabolite followed by the Uterus (Fig 2.16). The rest of the tissues showed lower quantities of this metabolite.

Fig 2.17 shows the metabolic profile of cyhalothrin in the treated rats. As expected, metabolism in the liver was found to be the highest as indicated by the lower level of the parent and higher hydroxy metabolite levels, while kidney had lower levels of both the parent and the metabolite. Adipose, due to the high lipid content, had a greater accumulation of the parent and lower levels of the hydroxy metabolite. In the reproductive organs, high metabolic turnover of cyhalothrin was seen in the uterus followed by adrenal and ovary.

2.3 Relationship between Lipid and Pesticide load of treated rats

The Lipid content/tissue was found to be highest in the Adipose tissue (656 mg/g tissue) followed by Ovary >Adrenal >Uterus >Kidney and Liver (Fig 2.18). However, when the pesticide load was calculated per mg of lipid, it was found to be highest in the Adrenal at 80 ng of cyhalothrin per mg of lipid compared to the adipose, which had 3.5 ng of cyhalothrin per mg lipid. This was followed by Uterus at 15 ng/mg lipid and then Adipose > Ovary > Kidney and Liver (Fig 2.19).

Fig 2.15: Profile of cyhalothrin residue in treated female rats (200 mg/kg b.w)

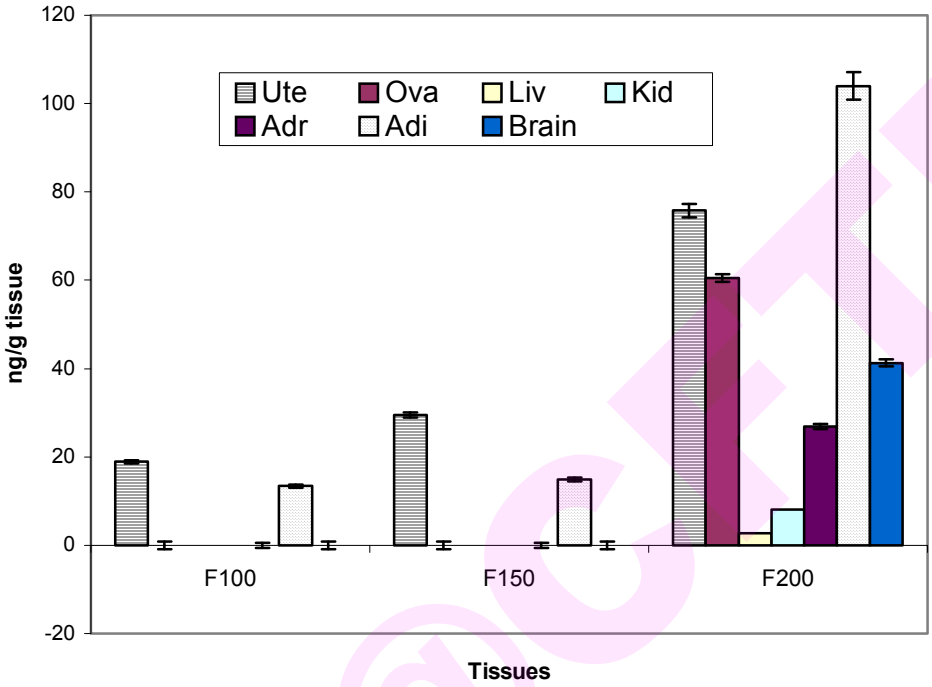


Fig 2.16: Profile of cyhalothrin hydroxy metabolite in rat tissues (200mg/kg b.w.)

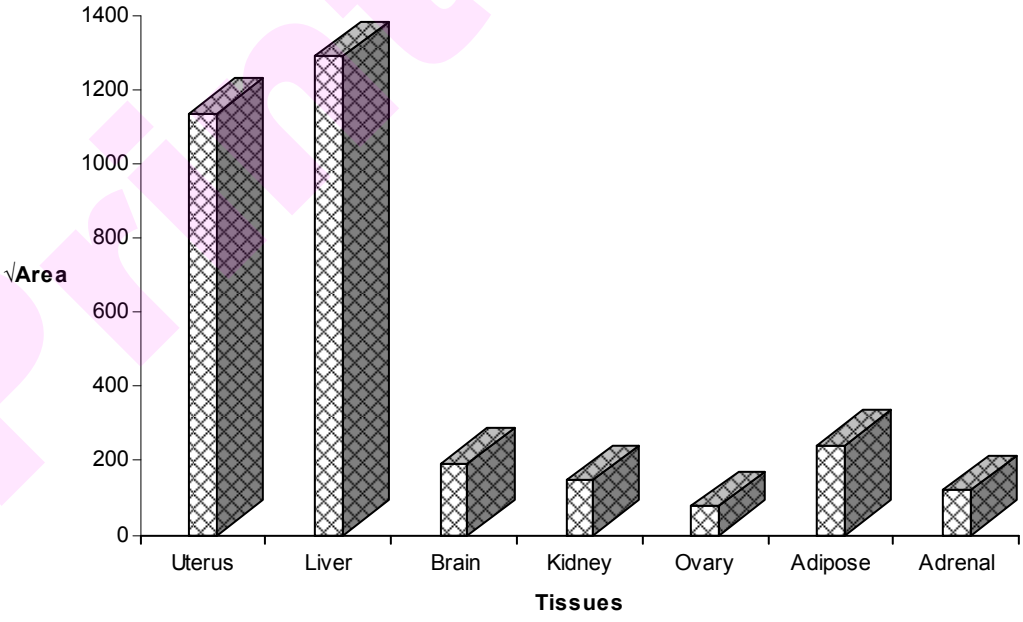


Fig 2.17: Profile of Cyhalothrin Metabolism in Female Rats

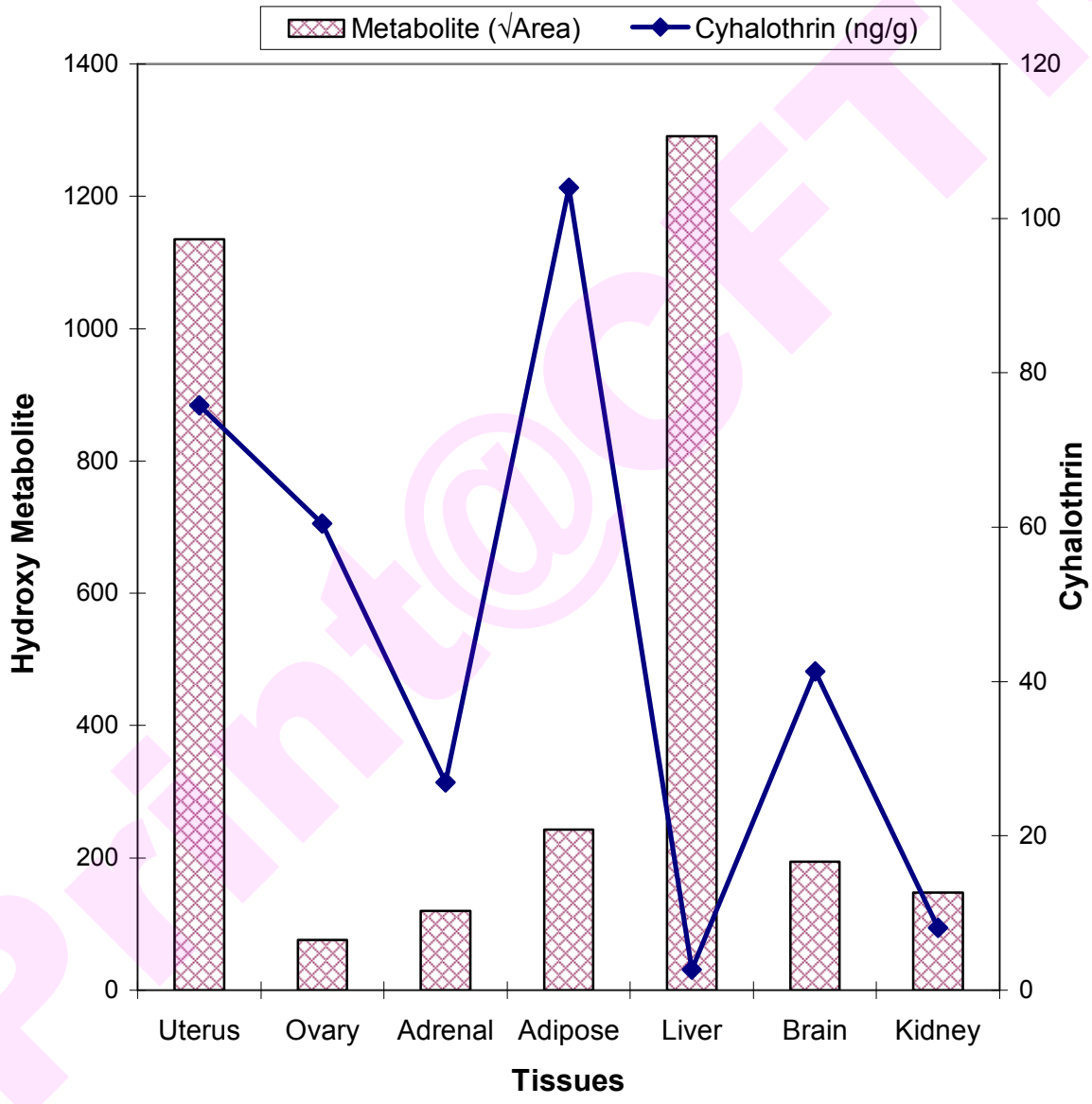


Fig 2.18: Lipid Content of rat tissues.

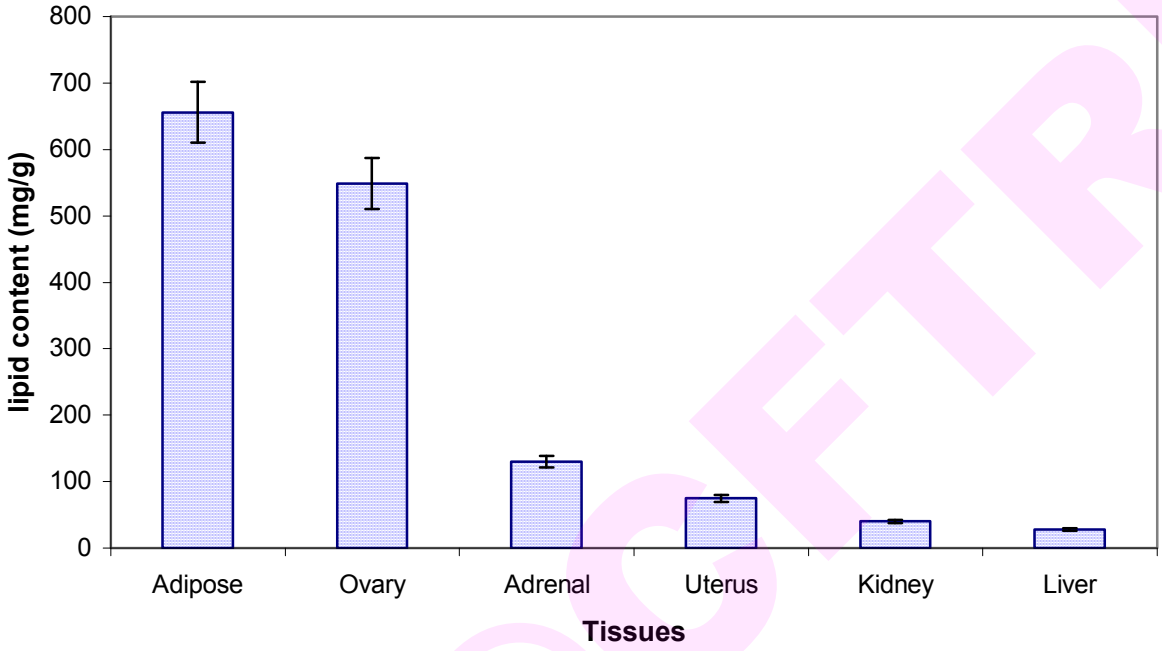
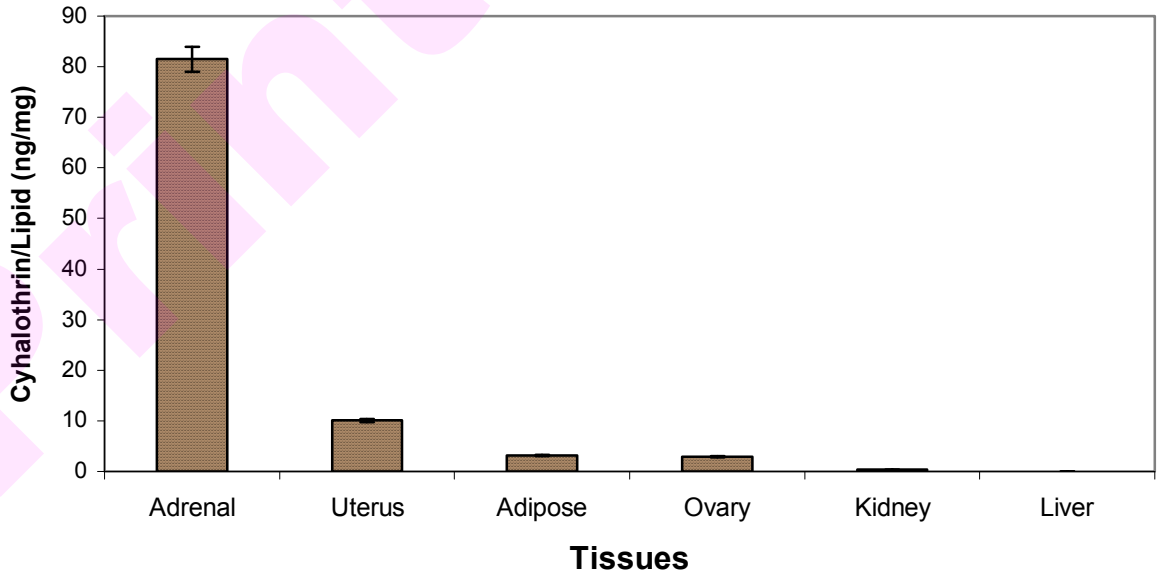


Fig 2.19: Relationship between lipid & pesticide load



3. DISCUSSION

For any xenobiotic to cause toxicity, it needs to reach and be available in the target organ at a specific dose (Anadon *et al*, 1996). Usually the pyrethroids are known to be metabolized rapidly in the mammalian system. After oral administration to male rats, deltamethrin and its metabolites are almost completely eliminated from the body within 2-4 days (Ruzo *et al*, 1978). According to one report (Anadon *et al*, 1996), deltamethrin was eliminated from the blood after 38.5 h and 33 h of oral and intravenous administration of deltamethrin.

In the present study, apoptotic changes were not observed in the cyhalothrin treated rats. As hypothesized earlier, cyhalothrin may not have produced apoptosis in this study either due to its lack of apoptotic nature or absence of the toxic cyhalothrin in the target organ. Residue analysis done in the tissues revealed that the cyhalothrin residue was found in all tissues and thus it had reached all the organs. Cyhalothrin was found in lower concentrations (20 - 30 ng) in the lower dose groups and found between 30-104 ng at the highest dose group. Thus, although the residue reached the target organ, it was not apoptotic probably due to low levels of the pesticide. Presence of hydroxy metabolites indicates that the animal was able to metabolise the parent compound to the less toxic hydroxy metabolites. The presence of the hydroxy metabolite in the liver and uterus suggests the detoxification of the parent cyhalothrin by hydroxylation in these two organs. In this study, cyhalothrin reached the tissues and was found at 20-100 ng level in the tissues. It was

observed that this level was almost 0.0005 % of the dose actually given to the rats i.e. 200 mg/kg. Hence, it appears that cyhalothrin was rapidly metabolized and excreted by the animals in this study as the metabolites were found at a high level in both the liver and the uterus (Fig 2.16). This may explain the reason for the non-induction of apoptotic changes, like non-induction of the caspase-3 enzyme, by cyhalothrin and also for the apparent safe nature of this pesticide as well.

Higher concentrations of the pyrethroid tested were found in the lipid rich tissues like adipose and in the reproductive organs like uterus and ovary. Since these tissues are lipid-rich and had more cyhalothrin per mg of lipid compared to other tissues (except adrenal), this may explain the preferential accumulation in the uterus and ovary.

Lipid content of the tissues is known to play a role in accumulation of the pesticides and this accumulation of the toxic molecule may lead the animal to resort to apoptosis to weed out the affected cells at the earliest. No reports are available on the residue profile of cyhalothrin in rat tissues. The neurotoxicity of the pyrethroids like deltamethrin (Wu *et al*, 2000a) may be due to its accumulation as Marei *et al* (1982) reported the rat brain to have persistence of the pyrethroids pesticides such as deltamethrin, permethrin and cypermethrin. In another study by Anadon *et al* (1996), peak concentration of deltamethrin and its metabolite in the brain regions was reached within 4-8 h after treatment of rats with an oral dose of deltamethrin. That may be the cause of caspase-3

induction in the deltamethrin treated rats rather than in the cyhalothrin treated rats as observed in the present study.

In our study, it can be observed that since the 200 mg/kg treated animals had the maximum pyrethroid residue, the induction of DNA fragmentation could be due to the result of the pyrethroid action as described in other similar studies (Wu *et al*, 2000b). Usually the pyrethroids are known to be metabolized rapidly in the mammalian system. Rats hydroxylate deltamethrin predominantly to form 4'-HO-deltamethrin, which appeared rapidly in plasma after oral and i.v. administration (Anadon *et al*, 1996). This rapid clearance of deltamethrin from the mammalian system coupled with its ability to cause apoptosis in the mammalian system is suggested to be one of the reasons for the "safe" tag of this pesticide.

SUMMARY:

- Cyhalothrin residue analysis by TLC and GC showed its presence in all the tissues at the 200 mg/kg b.w. group and only in the adipose and uterus in the other treatment groups.
- The presence of the hydroxy metabolite in the reproductive organs and the adipose tissue was detected by TLC and GC.
- GC demonstrated that the Retention time (R_t) of cyhalothrin was 15.15 min, while it was 14.64 min and 14.98 min for the hydroxy and acid metabolite respectively.
- Higher amount of hydroxy metabolite of cyhalothrin was seen in liver and Uterus while the parent molecule was found at a higher concentration in adipose, uterus and ovary in that order.
- Lipid-rich organs like adipose and the reproductive organs (uterus and ovary) showed higher concentrations of cyhalothrin.
- Elevated level of the hydroxy metabolite was seen in the liver and the uterus while the parent cyhalothrin was found in the adrenal and uterus at the highest level based on their lipid content.

CHAPTER 3

DEVELOPMENT OF AN IMMUNOASSAY BASED ON RABBIT ANTIBODIES TO DETECT CASPASE-3 AND NEURONAL NITRIC OXIDE SYNTHASE (nNOS)

INTRODUCTION

A major biochemical change during apoptosis is the activation of a class of cysteine protease enzymes known as caspases, which are responsible for activation of cytokines and endonucleases. This in turn leads to DNA fragmentation and a host of other apoptotic changes like alterations to cell membrane and compaction of cytoplasmic organelles (Cohen *et al*, 1997). Caspases are induced in response to any internal (aging and cell damage) or external stimuli (UV rays, starvation). Caspases are synthesized as inactive proenzymes which are activated by cleavage at specific Asp residues to active enzymes containing both large (17-20 KD) and small subunits (8-10 KD)(Cohen *et al*, 1997). The caspases are divided into 3 types based on their functional analysis during the process of apoptosis: Initiators, Executioners and Cytokine Activators, which activate the release of cytokines into the blood stream and to the apoptotic cells. (Srinivasula *et al*, 2001). Caspase 3 is one of the key executioners of apoptosis, responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which are cleaved in many different systems during apoptosis (Nicholson *et al*, 1995). Caspase-3 exists as an inactive 32 KD zymogen in the cytosol and upon activation leads to the activation of another group of enzymes called as the endonucleases. These endonucleases cleave the DNA into the characteristic “ladder” patterns (Darzynkiewicz *et al*, 1998).

Caspase activation occurs as a result of growth factor withdrawal, exposure to radiation or chemotherapeutic agents, or initiation of the

Fas/Apo-1 receptor-mediated cell death process. Active caspases participate in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic disassembly of dying cells. The biological substrates of caspases include poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), lamins, topoisomerases, Gas2, protein kinase C d (PKC d), sterol regulatory element binding proteins (SREBP), U1-70kDa protein and Huntingtin protein (Vaux *et al*, 1996, Kumar *et al*, 1996, Nicholson *et al*, 1997, Rosen, 1996). Three subfamilies have been identified based on amino acid sequence and substrate and inhibitor specificities (Thornberry, 1997). Of these, caspase-3 plays a central role in the apoptotic cascade. Caspase-3 specifically cleaves at the C-terminal side of the aspartate residue of the amino acid sequence DEVD (Asp-Glu-Val-Asp). Thus, caspase-3 is a known marker of apoptosis and was chosen to serve as a biomarker of apoptosis in our study.

Nitric Oxide (NO) is another important signaling molecule that has been demonstrated to be involved in apoptosis (Stuehr *et al*, 1992, Zini *et al*, 1996). Nitric Oxide Synthase (NOS) is an enzyme responsible for the production of Nitric Oxide (NO), which plays a crucial role in apoptosis. The excessive production of NO may also damage DNA, leading to the activation of poly (ADP ribose) polymerase-1, which in turn, induces apoptotic cell death through the activation of caspase-3 in the injured rat spinal cord (Bao and Liu,

2003). NOS exists in 3 known isoforms namely inducible macrophage NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). Endothelial NOS, as its name implies, is present in vascular endothelial cells (Uttenhall *et al*, 1998). Inducible NOS has been purified from the cytosol of activated murine and rat macrophages (Hevel *et al*, 1991) as well as in hepatocytes (Moncada *et al*, 1991). The neuronal NOS is present in the central and autonomic nervous systems and has been cloned from rat (Bredt *et al*, 1990) and human brain (Nakane *et al*, 1993). The nNOS in our study was obtained from two sources – Rat and Porcine.

Enzyme-Linked ImmunoSorbent Assay (ELISA) in microtiter plate format is widely used for high-throughput screening of chemicals, drugs, biomolecules etc. These assays are very specific, allow for rapid evaluation of large number of samples and can be readily adapted for automatic manipulations (Kenny *et al*, 1998; Sundberg, 2000). It has also been used for detection of various apoptotic markers such as for caspases, nucleases, microsomes etc (Saunders *et al*, 2000). However, the immunoassay kits for the detection of caspase-3 are expensive and need to be imported. The average cost of an apoptotic kit is US $ 250/- and the antibodies available against caspase-3 or nNOS cost US $ 200/- and above. Hence, in the present study, caspase-3 and nNOS were isolated from the pyrethroid treated tissues, used to raise antibodies and an indigenous ELISA method was developed for the detection of these biomarkers.

1. MATERIALS AND METHODS

1.1 Chemicals and Buffers

Ammonium Sulphate, Acrylamide, Bis-Acrylamide, Ethylene Diamine Tetraacetic acid (EDTA-sodium salt), HEPES, Tris base, Sodium Chloride, Disodium Hydrogen orthophosphate, Sodium dihydrogen orthophosphate, and all other salts were purchased locally. Goat anti-rabbit Horse Radish Peroxidase (HRP) conjugate, rabbit anti-chicken HRP conjugate, Tetramethyl Benzidine/Hydrogen Peroxide (TMB-H_2O_2) (10X) were purchased from M/S Bangalore Genei, Bangalore, India. Ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA), Bovine Serum Albumin (BSA), TEMED, Leupeptin, Pepstatin, Aprotinin, Dithiothreitol, Freund's complete adjuvant, Freund's incomplete adjuvant, (4,4',5,5') Tetramethyl Benzidine (TMB), Sodium Dodecyl Sulphate (SDS), Dimethyl Sulfoxide (DMSO), Tween-20 (Polyoxyethylene-sorbitan monolaurate), commercial caspase-3, commercial polyclonal anti-caspase 3, commercial monoclonal anti-nitric oxide synthase, commercial molecular weight markers for native and SDS-PAGE, Nitrocellulose membrane (0.45 µm pore size) and β-Mercaptoethanol were purchased from Sigma Chemicals, USA.

1.2 Equipments

SDS-PAGE electrophoresis chamber, wet immunoblotting apparataus, electrophoresis power pack were supplied by Bangalore Genei, Bangalore. ELISA plates (Maxisorp immunoplates) were obtained from Nalge Nunc Inc,

Roskilde, Denmark. Other instruments included ELISA reader (Thermo Electron Corporation, China, Multiskan ex mode), High speed refrigerated centrifuge (Sorvall, Germany) and Ultracentrifuge (Beckman Instruments, Denmark).

1.3 Isolation and purification of Caspase-3 from rat tissues as biomarker of apoptosis

Caspases are present in the cytosol of the cells and were extracted by the method of Srinivasula *et al* (2001). Tissues from the young rats treated with deltamethrin (200 mg/kg b.w. i.p.) were extracted by the method as described in chapter 2, Section A. The S-100 caspase extract was purified using an antibody-affinity column as described in Chapter 2, Section A.

1.4 Native and SDS - PAGE of Caspase 3

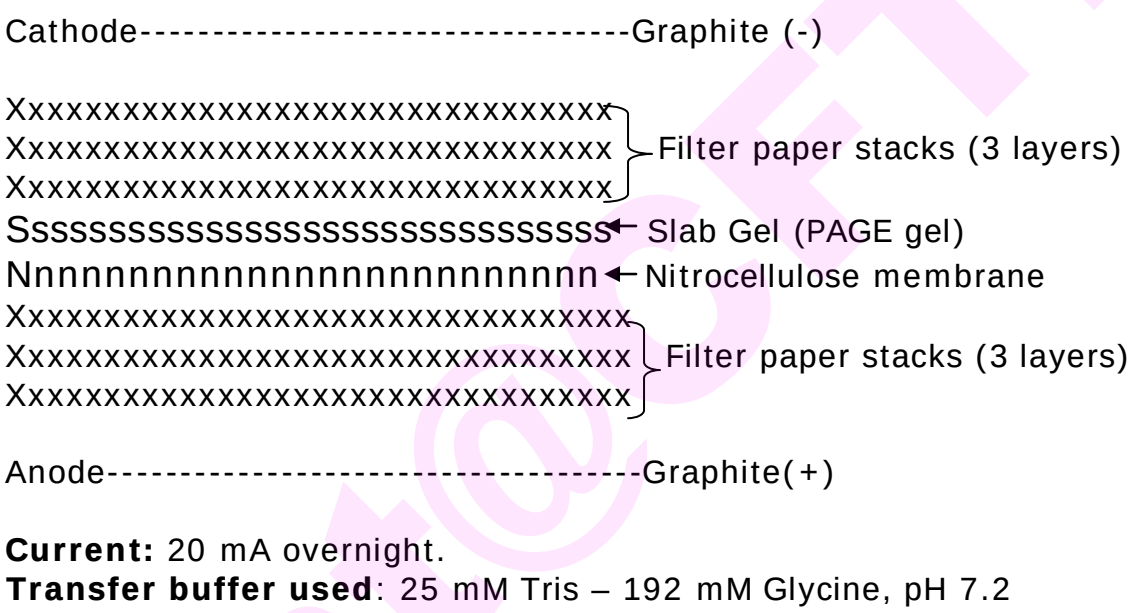
Purity of the isolated caspase-3 was identified by a Native PAGE and Western Blotting after SDS-PAGE. Samples of casapse-3 extracts from control, 100, 150 and 200 mg/kg b.w. were analysed on both native and SDS-PAGE. Native and SDS-PAGE was performed with a resolving gel of 12%, stacking gel of 5% with 20 µg protein/lane, run at 75 mV for 2 h in a vertical electrophoresis unit. The molecular weight of caspase-3 is 32 KD. Consequent to SDS-PAGE, caspase-3 was further identified by an immunoblot using commercial anti-caspase-3.

1.5 Western Blotting for Caspase-3

After SDS-PAGE, western blotting was performed to determine the specificity of the isolated caspase-3, as described by Towbin *et al* (1974). Briefly, a 12%

SDS-PAGE was carried out on a vertical slab gel apparatus according to Laemmli (1970). Each lane was loaded with 20 µg of the caspase-3 protein extracts from all the deltamethrin-treated groups. Commercial caspase-3 was applied as a positive control along with corresponding SDS molecular weight markers. Electrophoresis was performed at 75 mV for 2 h. The protein transfer to nitrocellulose membranes (NC membrane, 0.45 µm pore size) followed the procedure of Towbin *et al* (1974) using 25 mM Tris - 192 mM Glycine buffer (pH 7.4) with 20% methanol (transfer buffer) overnight at 20 mA at 4^0C in a wet blotting apparatus. This procedure is represented diagrammatically in Fig 3.1. The transfer of the proteins to the NC membrane was confirmed with Ponceau S staining, which was removed by washing the membrane with distilled water and drying the membrane. After drying, the membranes were blocked in 2% BSA in Tris-NaCl (TTN) buffer for 1 h at 37^0C. After washing the blots with TTN buffer (3 x 5 min), they were incubated with the primary antibody, commercial anti-caspase-3 (1:1000), for 2 h at 37^0C. Blots were washed with TTN buffer (3 x 5 min) and incubated with commercial HRP-conjugated secondary antibody (Goat anti-rabbit, 1:5000) for 2 h at 37^0C. They were once again washed with TTN buffer and examined by developing in insoluble TMB/H_2O_2 solution for 10 min. The appearance of blue/violet bands signaled the end of the reaction.

Fig 3.1: Diagrammatic representation of immunoblotting-wet transfer system.



Current: 20 mA overnight.
Transfer buffer used: 25 mM Tris – 192 mM Glycine, pH 7.2

1.6 Isolation of nNOS from treated rat brains and control porcine brain

Neuronal NOS (nNOS) was isolated from 2 sources – rat and porcine.

a. Rat brain: Rat neuronal NOS was extracted by the procedure adapted from Bredt *et al* (1990). In brief, rat cerebellum were homogenized in 50 mM Tris-HCl buffer pH 7.4, with protease inhibitors. The homogenate was centrifuged at 20,000 g for 15 min in a sorvall centrifuge at 4^0C. The resultant supernatant was divided into 2 parts: one part was stored at -25^0C as 20K fraction and the other part centrifuged at 100,000 g for 45 min in a Beckman ultracentrifuge. This supernatant was stored as 100K fraction at -25^0C for PAGE and all further assays.

b. Porcine brain: Porcine neuronal NOS was extracted by the method of Mayer *et al* (1990). Briefly, 600 g of porcine cerebellum was washed in isotonic NaCl (0.9%) and homogenised in 3 vol of a 50 mM Tris buffer, pH 7.5. The homogenate was centrifuged at 10,000 g for 30 min. The proteins were precipitated at 4^0C with 10 % ammonium sulfate and the suspension was divided into two parts. One half of suspension was aliquoted & stored at -25^0C as 10 % ppt. Another half of suspension was centrifuged at 10,000 g for 15 min. The pellet was washed with 50 mM Tris buffer & the pellet resuspended in 20 mM of Tris buffer and the protein was precipitated with 30% Ammonium sulfate. The pellet was suspended in 20 mM Tris buffer and this suspension was recentrifuged at 10,000 g for 40 min. The supernatant was aliquoted and stored at –25^0C as 30% ppt. The 10% and 30%

precipitates were used for SDS-PAGE to check the yield and purity of the enzyme after each precipitation.

1.7 Native and SDS - PAGE of nNOS

The nNOS extract samples from both porcine (10% and 30% precipitate fractions) and rat (20K and 100K fractions) were subjected to native and SDS-PAGE along with corresponding molecular weight markers. Aliquots of tissue samples corresponding to 20 µg of total protein were run on 8% resolving gel at a voltage of 100 mV for 2 h. The molecular weight of nNOS is 155 KD. SDS-PAGE for the above samples had the same conditions as the native PAGE. After SDS-PAGE, a western blot was performed using commercial anti-nNOS antibody for identification of nNOS in the sample.

1.8 Western Blotting for nNOS

After SDS-PAGE for nNOS (as described above in section 1.7), the protein samples were blotted onto a NC membrane (0.45 µm pore size) as described in section 1.5 above. The membranes were blocked using 2% BSA for 1 h at 37^0C. Immunoblotting of blotted proteins was carried out using a mouse monoclonal anti-NOS antibody (1:1000 dilution) and a HRP-labeled Goat anti-mouse secondary antibody (1:5000 dilution). The blots were developed using a commercial TMB/H_2O_2 substrate-chromogen solution.

1.9 Production of antibodies against the biomarkers:

a. Animals

Female 6-month old New Zealand albino rabbits were obtained from the CFTRI animal colony after appropriate ethical committee clearance. The average weight of the animals was 3 ± 0.2 Kg. The rabbits were kept in stainless steel cages (1 animal/cage) with water and commercial rabbit chow *ad libitum* and maintained on a 12 h light/dark cycle.

b. Immunization

The rabbits were immunized with the biomarkers extracted from the following sources:

I. Purified Caspase-3 from the kidney of young rats treated with 200 mg/kg deltamethrin

II. Brain NOS extracted from

a. Rats treated with 200 mg/kg b.w. of deltamethrin.

b. Porcine – control pigs

Initially, a primary dose of the affinity purified antigen was given to each of the rabbits. 1 mg of each antigen was suspended in complete freund's adjuvant and saline and was injected sub-epidermally at 40 different sites on the rabbit's nape. Booster doses were given after every 30 days interval with 0.5 mg antigen emulsified in incomplete freund's adjuvant and saline given intra-muscularly. Bleeds were collected from the marginal ear vein on 9^{th} day after each booster. The blood was allowed to clot, the serum collected and

incubated at 56^0C for 1 h to deactivate the serum proteases. The sera were then stored at -20^0C for further development of ELISA. Working aliquots were stored at 4^0C.

1.10 Antibody Capture Assay (ACA):

The specificity and sensitivity of polyclonal antibodies raised above were analysed using the antibody capture assay. In this method, the antigen was coated on a 96-well microtitre plate and probed with specific antibodies against it. Unbound molecules were removed by washing and a secondary enzyme-labeled antibody used to indirectly determine the concentration of the antigen in the sample.

The antigen (Ag) (caspase-3 or nNOS) was coated on 96-well ELISA plates at different concentrations (ranging from 1 µg to 1 pg) dissolved in 50 mM carbonate buffer (pH 9.6) at 100 µl/well. The plate was incubated at room temperature (RT) overnight. After the coated plate was washed 3 times with washing buffer (0.05% Tween-20 in 50 mM PBS), the plate was then blocked with 150 µl of 1% BSA in 50 mM PBS (for blocking the non-specific sites of the antigen) and the plate was incubated at RT for 1 h. After tipping off the BSA, 50 µl of the primary antibody (Ab) at different antisera dilutions in 50 mM PBS was added to the wells and the plate was incubated for 1 h at RT. After washing the plate with wash buffer, 100 µl of anti-rabbit horseradish peroxidase-conjugated secondary Ab (1:5000 for nNOS and 1:10,000 dilution for caspase-3) diluted in 50 mM PBS was added to the wells. The plate was

then incubated for 30 min at RT. After washing off the excess antibody, 150 µl of substrate solution was added to each of the wells. The colour development was stopped after 20-30 min with 50 µl/well of 2.5 N H_2SO_4. The absorbances of the wells were read at 450 nm in an ELISA reader.

The above assay was performed with both the commercial antibodies and the antibodies raised in this study, as in 1.8 above. Control kidney and brain homogenates were used as the negative controls for caspase-3 and nNOS respectively. The methodology to prepare the buffers for ACA is given below.

1.11 Preparation of Buffers for ACA and ELISA:

a. **PBS (250 mM, pH 7.4):** 33.79 g of disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) + 9.0 g of Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) + 45 g of NaCl dissolved in 1 L of d.w. and pH was adjusted to 7.4 with Sodium Hydroxide (NaOH) pellets.

b. **Wash Buffer (50 mM PBS-0.05% Tween-20):** 250 mM PBS was diluted 5 times with d.w. and 5 ml of 10% Tween-20 was added per litre.

c. **Carbonate Buffer (50 mM, pH 9.6):** 1.6 g of sodium carbonate (Na_2CO_3) + 2.9 g sodium bicarbonate ($NaHCO_3$) dissolved in 1 L of d.w.

d. **Chromogen:** 10 mg of 4',4',5',5'Tetramethyl Benzidine (TMB) in 1 ml of Dimethyl Sulfoxide (DMSO).

e. **Substrate (pH 5.0):** 4.1 g Sodium acetate + 1.25 g β-cyclodextrin + 1.25 g citric acid + 75 mg urea H_2O_2 dissolved in 500 ml d.w.

The substrate and chromogen were mixed in the ratio 97:3 just before use.

f. **Stop Solution:** (2.5 N). 34.72 ml of conc. H_2SO_4 made up in 500 ml d.w.

1.12 Formatting of the Assay

The indirect non-competitive ELISA format was used for the assay of both the antigens. The assay was formatted by checker board analysis with different coating buffers, diluants, antigen concentrations, antibody concentrations and HRP-conjugated secondary Ab. The coating buffers used were 50 mM carbonate buffer (pH 9.6) and 50 mM PBS (pH 7.4) while the diluants used were PBS and PBS-FG (0.5% Fish Gelatin). Antigen concentrations used were in the range of 1 µg to 1 pg while primary antibody dilution used were from 1/400 to 1/64 lakhs. HRP-conjugated secondary antibody dilutions were 1/5K to 1/10K.

2. RESULTS

2.1 Purification and identification of Caspase – 3

Protein Content:

The extracts from the treated rat tissues were analysed for the protein content using the method of Bradford *et al* (1976). The protein values per gram tissue are shown in Table 3.1. Maximum protein concentration was seen in the ovary at 17.2 mg/g tissue in the 150 mg/kg b.w. group. Next was the liver extract at 16 mg/g tissue and 15.7 mg/g tissue in the 100 mg/kg and 200 mg/kg groups respectively. Lowest protein concentration was observed in the uterus (3.3-4.4 mg/g) and the brain (4.3-6.2 mg/g).

Native and SDS PAGE:

Native PAGE showed a band at 32 KD, the molecular weight of caspase-3, in kidney extracts from the control, 100, 150 and 200 mg/kg b.w. treatment groups, (Fig 3.2). The SDS-PAGE and immunoblotting for all unpurified samples (Fig 3.3) showed that the 32 KD caspase-3 was prominent in the kidney extract from 200 mg/kg group while in the other treatment groups, the band was quite faint. At all doses, the 32 KD band represented the precursor of caspase-3 while the active sub-units of caspase-3 at 17 KD and 12 KD were not visible. Affinity purification of the S-100 extract helped in purifying the caspase-3 further (though not to homogeneity) as shown in the western blotting in Fig. 3.4 a. The detected signal at 55 KD may represent the known dimer of caspase-3 (Fig 3.4 a). Fig 3.4 b shows the specific recognition of the

affinity-purified caspase-3 by the commercial anti-caspase-3 and the antibody raised in this study.

2.2 Western blot analysis for Caspase-3

The optimum antibody concentration for the western blotting of affinity-purifed caspase-3 extract was 1/1000 of commercial primary antibody and 1/5 K of secondary antibody-HRP dilution. The caspase-3 purified from the treated rat tissues were challenged with the commercial antibody. The commercial anti-caspase reacted with the band at 32 KD with the purified caspase-3 (CFTRI Ag) from the kidney of 200 mg/kg deltamethrin-treated rats (Fig 3.4 b). As seen in the figure, the purified caspase-3 in lane 3 (from kidney of 200 mg/kg b.w. deltamethrin-treated rats) was recognized by the commercial anti-caspase 3 as was the commercial caspase-3 antigen in lane 4. Lane 1 showed the recognition of CFTRI antigen by the CFTRI antibody. Thus, this purified caspase-3 was used for raising antibodies in the rabbits.

Table 3.1: Protein Concentration in treated rats (Caspase 3).

Groups (mg/kg b.w)	Uterus	Ovary	Adrenal	Liver	Kidney	Adipose	Brain
	(mg/g tissue)						
100	4.4	5.60	8.0	16.1	16.2	8.0	4.32
150	3.3	17.2	7.1	12.6	6.8	1.6	6.20
200	3.5	6.0	--	15.7	3.3	2.9	4.40

Fig 3.2 : Native PAGE for Caspase - 3

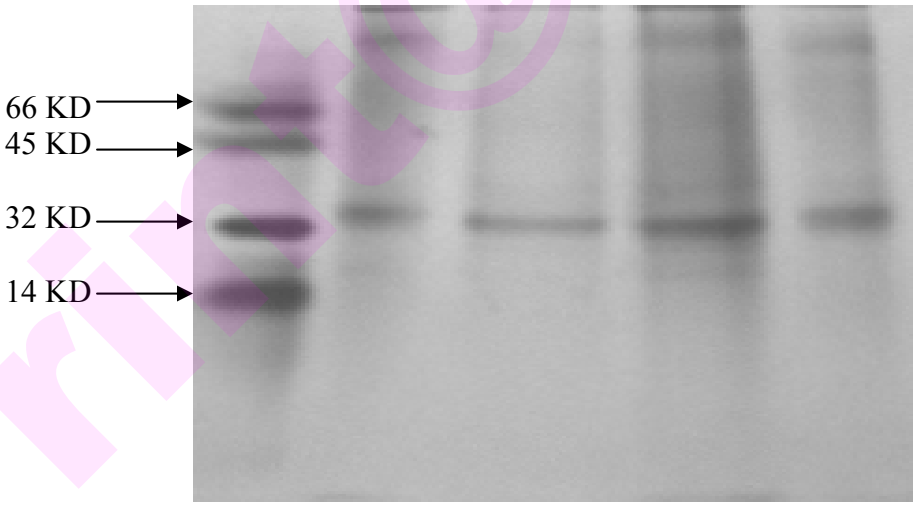
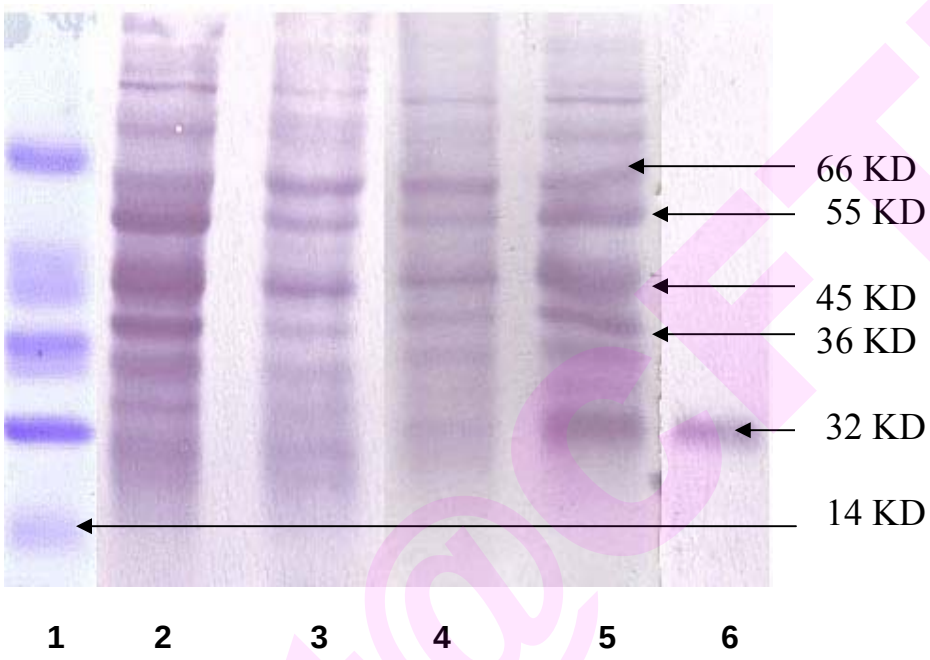


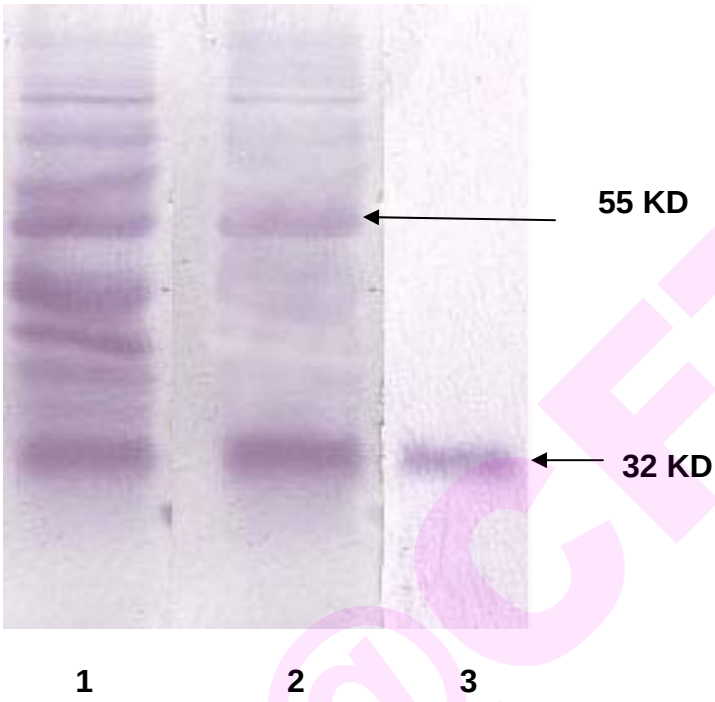
Fig 3.3: Western Blotting for Caspase-3 samples from kidney of all treated groups (unpurified samples)



Lanes

1. Marker; 2. Control; 3. 100 mg/kg extract; 4. 150 mg/kg extract; 5. 200 mg/kg extract; 6. Commercial Caspase-3

Fig 3.4 a: Comparison between affinity purified and unpurified caspase-3 by western blotting



Lanes
1. Caspase-3 – S-100 extract; 2. Affinity-purified; 3. Commercial caspase-3

Fig 3.4 b: Western Blotting for Caspase – 3 from 200 mg/kg treated young rats

Lanes
1 CFTRI Casp-3 from 200 mg/kg treated rats kidney (Vs CFTRI Ab); 2. Control; 3. CFTRI Casp-3; 4. Commercial Caspase-3 (2,3,4 Vs Commercial anti-caspase 3)

2.3 Purification and identification of Rat and Porcine NOS

Protein content:

The protein content of the NOS extracted from the rat and porcine cerebella was estimated by the method of Bradford *et al* (1976). The protein yield is shown in Table 3.2. For the 10% porcine extract, the protein yield was 0.3 mg/g tissue while it was 5.4 mg/g tissue for the 30% extract. The yield for rat nNOS in the 20 K and 100 K fractions was almost the same at 6.2 and 6.5 mg/g of the tissue respectively. Thus, the 30% porcine NOS extract and 20 K rat NOS extract were used for all further studies.

Native and SDS-PAGE:

The native PAGE for the nNOS samples showed the presence of a protein band at 155 KD (Fig 3.5) in all the nNOS samples except the 10% porcine NOS extract. The SDS-PAGE for nNOS showed the presence of a band at 155 KD (Fig 3.6) in both 20 K and 100 K rat nNOS and 30% porcine nNOS, which is the molecular weight of neuronal NOS.

2.4 Western Blot for nNOS samples

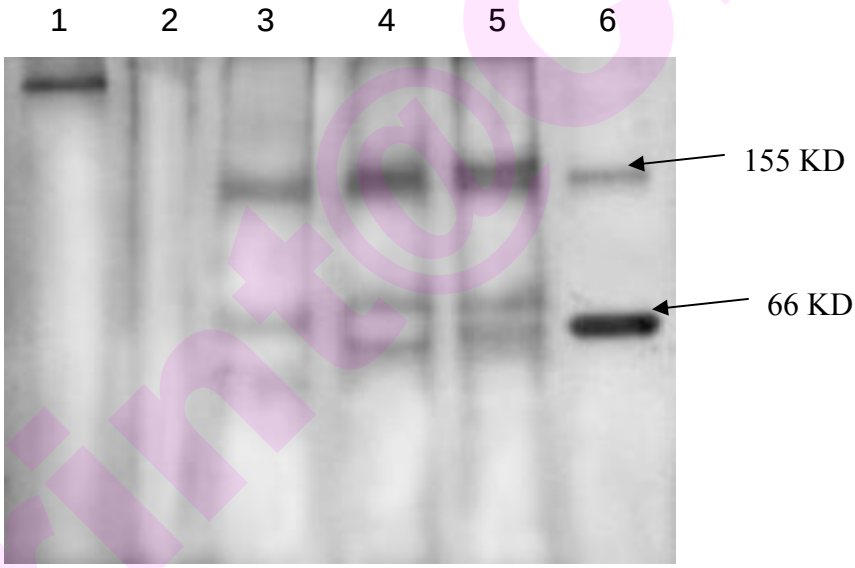
Optimum conditions for western blotting of nNOS samples were found to be - primary commercial antibody dilution of 1/1000 for both Porcine and Rat NOS and secondary Ab-HRP dilution of 1/4000. Both porcine and rat nNOS reacted with the commercial anti-nNOS with a band at 155 KD (Fig 3.7). This indicates the presence and purity of nNOS in the purified extracts. Hence,

these purified extracts from both porcine and rat nNOS were eluted from the gel and used in the present study to raise antibodies in the rabbit.

TABLE 3.2: Protein concentration in Rat & Porcine extracts (mg/g tissue)

Rat extract (mg/g tissue)		Porcine extract (mg/g tissue)	
20,000 g	*100,000 g*	*10% AS*	*30% AS*
6.2	6.5	0.3	5.4

Fig 3.5: Native Gel for NOS



1 - 345 KD marker.
2 - 10% Porcine nNOS
3 - 30% Porcine
4 - Rat nNOS 20,000g
5 - Rat nNOS 100,00 g
6 - BSA marker (Monomer & Dimer)

Fig 3.6: SDS-PAGE for nNOS.

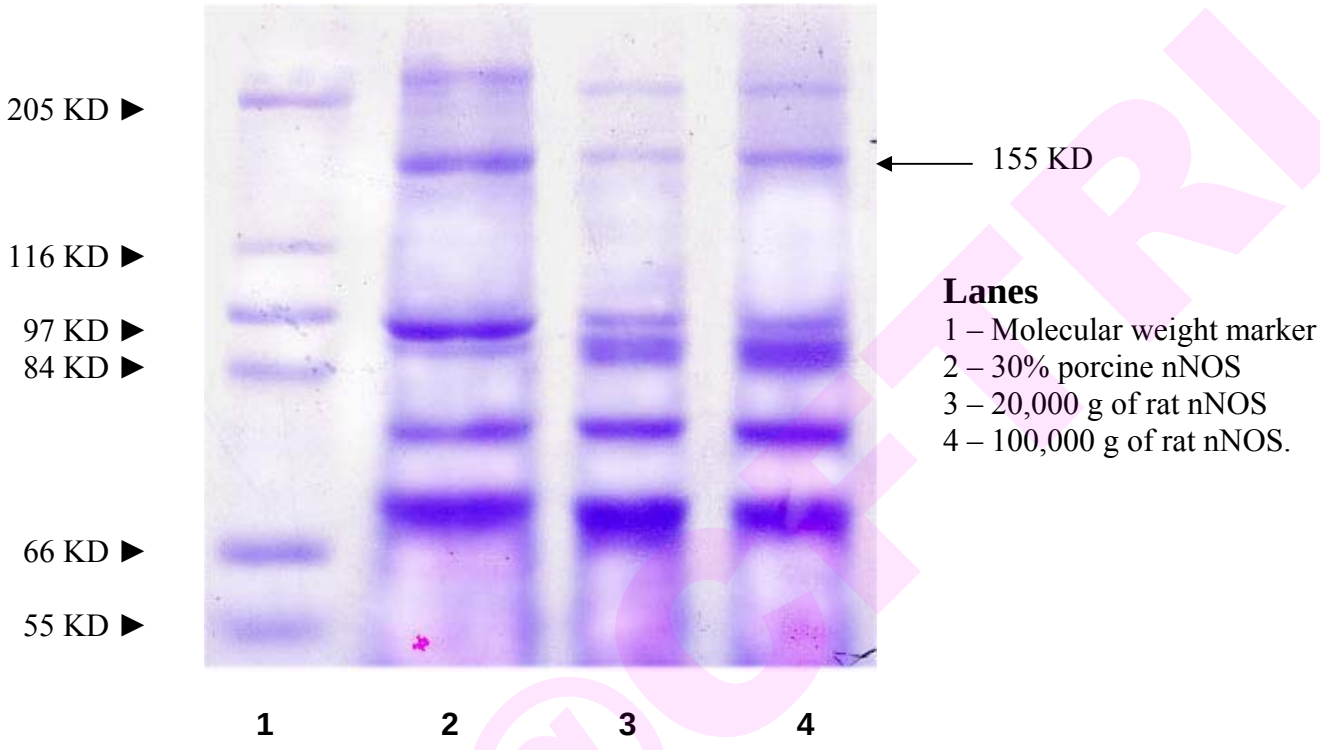
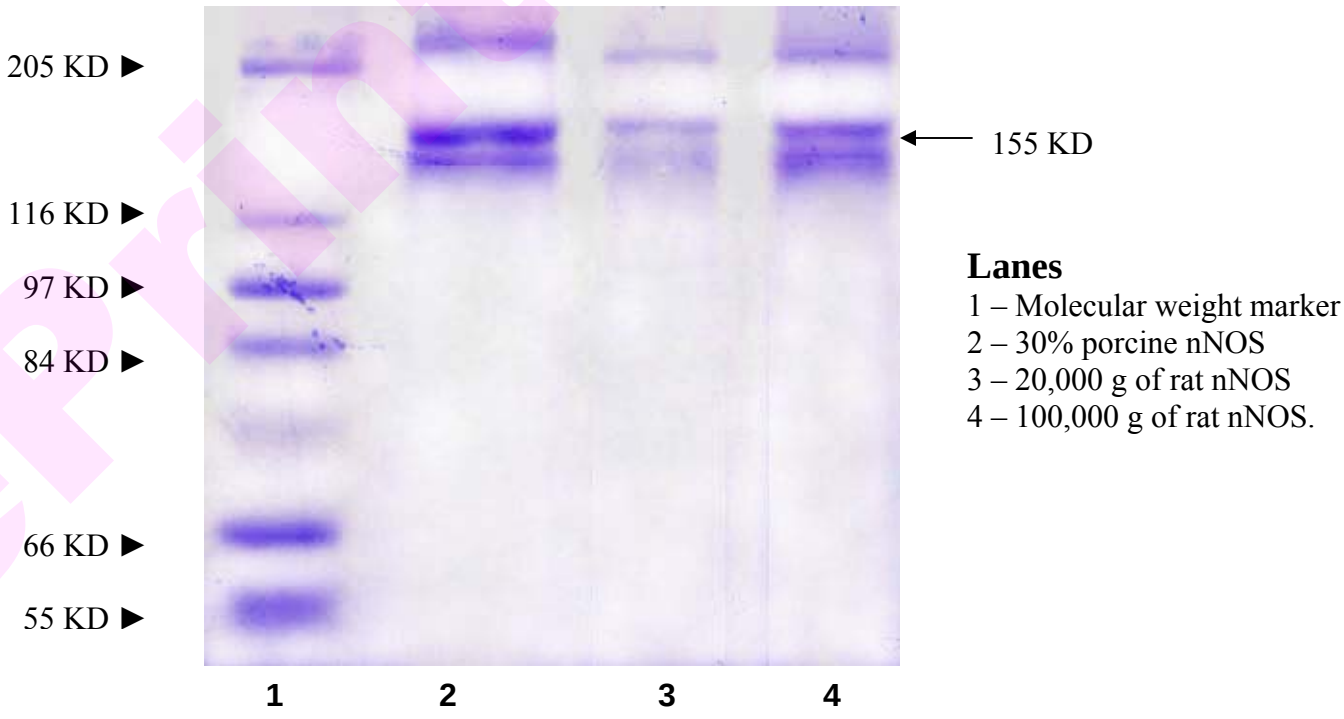


Fig 3.7: Western Blots for nNOS samples vs Commercial anti-nNOS antibody



2.5 ACA for Caspase 3

The results of the ACA showed that both the commercial anti-caspase Ab (Fig 3.8 a) and the CFTRI antibody (Fig 3.8 b) raised in this study were able to recognize the purified caspase-3 antigen at three different concentrations of 1.25, 0.62 and 0.31 µg. The negative control (rat kidney homogenate) did not produce any reaction with both the antibodies. The commercial antibody titre was 1/1600 at all the antigen concentrations, while for the antibody raised at CFTRI it was 1/1600 at Ag concentration of 0.62 µg and 0.31 µg/well and was 1/400 at 1.25 µg (Fig 3.8 a and b).

2.6 Assay Formatting for CFTRI caspase-3

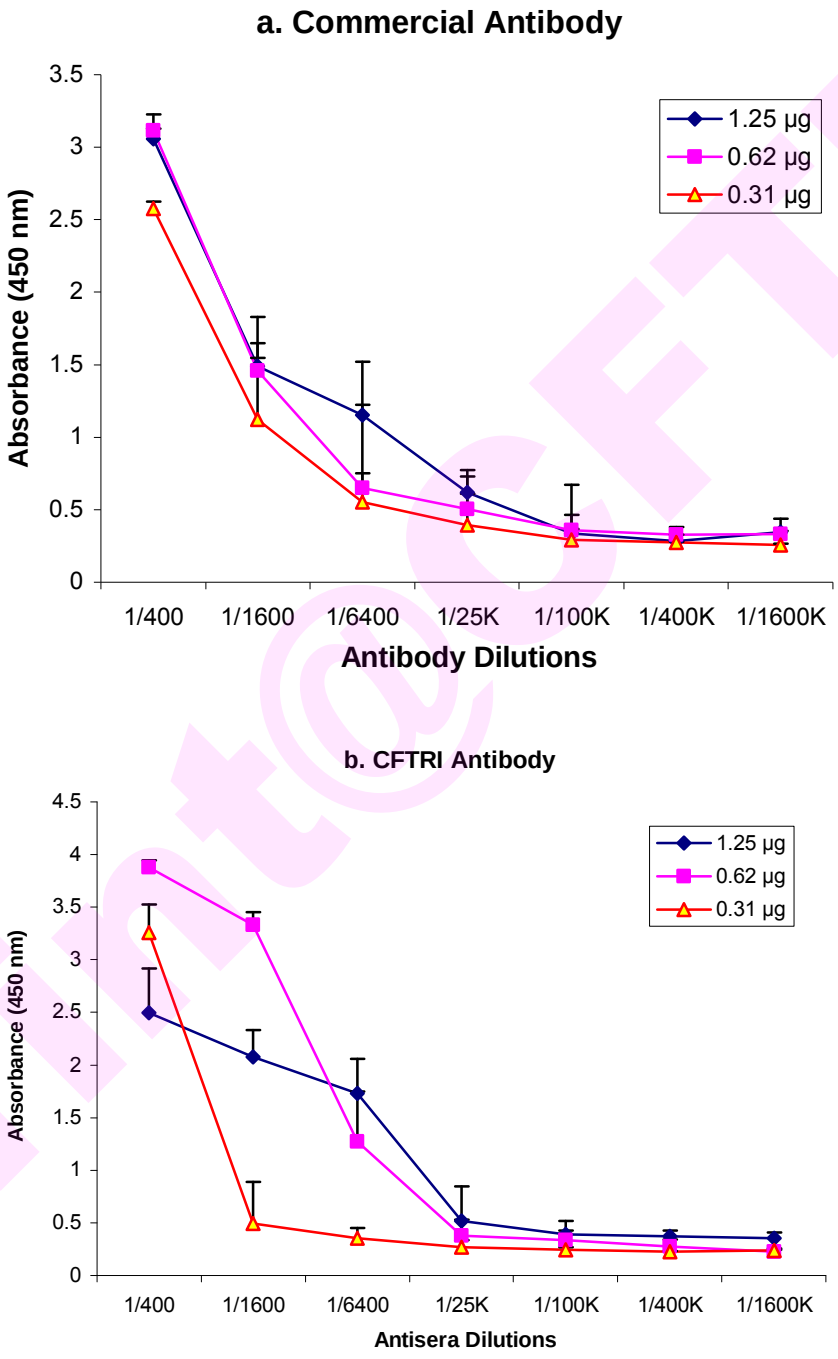
The assay formatting of the ELISA was carried out using different coating buffers, diluants, antigen concentrations, and antisera dilutions. Both the coating buffers-PBS and carbonate buffer, were identical with respect to the sensitivity of the assay and antisera dilutions (Fig 3.9 a). Both responded well to antisera dilution of up to 1/6400. Carbonate buffer was used in all further assays as it is the buffer commonly used in ELISA's. The two diluants, PBS and PBS-FG, gave a good signal (Fig 3.9 b). PBS gave a better signal at higher concentrations and was thus preferred to PBS-FG as the diluant.

Antigen concentrations of purified caspase-3 ranged from 1 ug to 1 ng antigen/well. The anti-serum dilution used was 1/6400. All the 3 bleeds were tested against the antigen to evaluate the sensitivity of the bleeds. The sensitivity of the assay showed that the 1st and 2nd bleed had a sensitivity of 1 ng while the 3rd bleed was able to detect 3 ng of the antigen (Fig 3.10). Thus, the assay

revealed that the antibodies raised in this study were very sensitive. All the 3 bleeds showed a linear range from 10 to 300 ng of antigen. The regression value was close to 1 for all three bleeds, demonstrating the high sensitivity of the antibodies obtained. With the commercial antibody, the sensitivity was 3 ng at 1/1600 dilution against both the commercial and purified caspase-3 (Fig 3.11). The negative control (liver extract) did not react with the commercial antibody, thus showing the specificity of the antibody. Hence, it could be concluded that the antibody raised in the present study was comparable to the commercial antibody in terms of sensitivity and it worked at a much lower dilution than the commercial antibody.

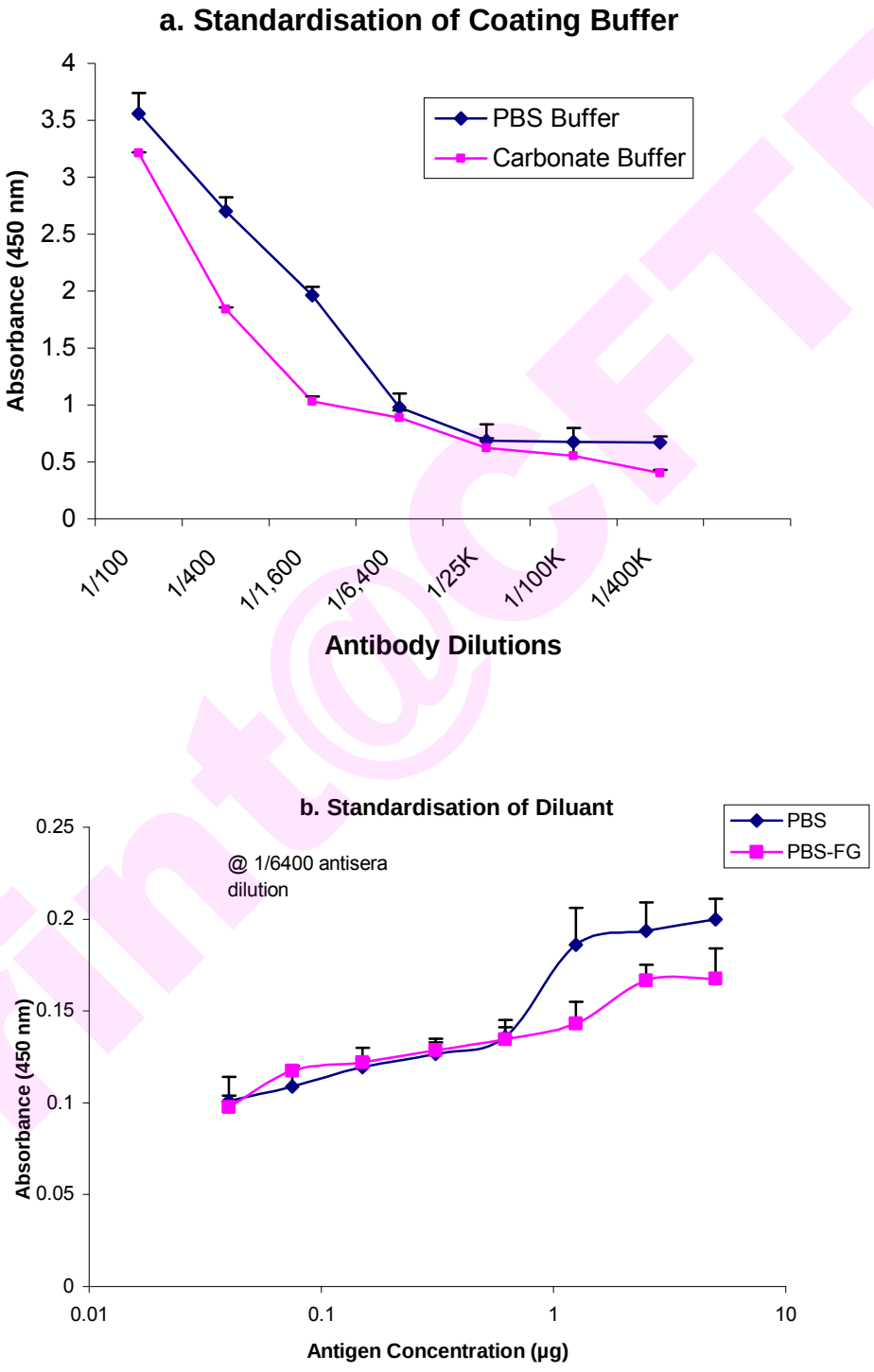
The caspase-3 purified from the 3 different dose groups was tested against the commercial antibody (Fig 3.12). The results indicated that the antibody recognized the caspase-3 antigen from the 200 mg/kg b.w. treated group the best. The caspase-3 from the 100 and 150 mg/kg b.w. showed very weak binding.

Fig 3.8: Antibody Capture Assay of Caspase-3



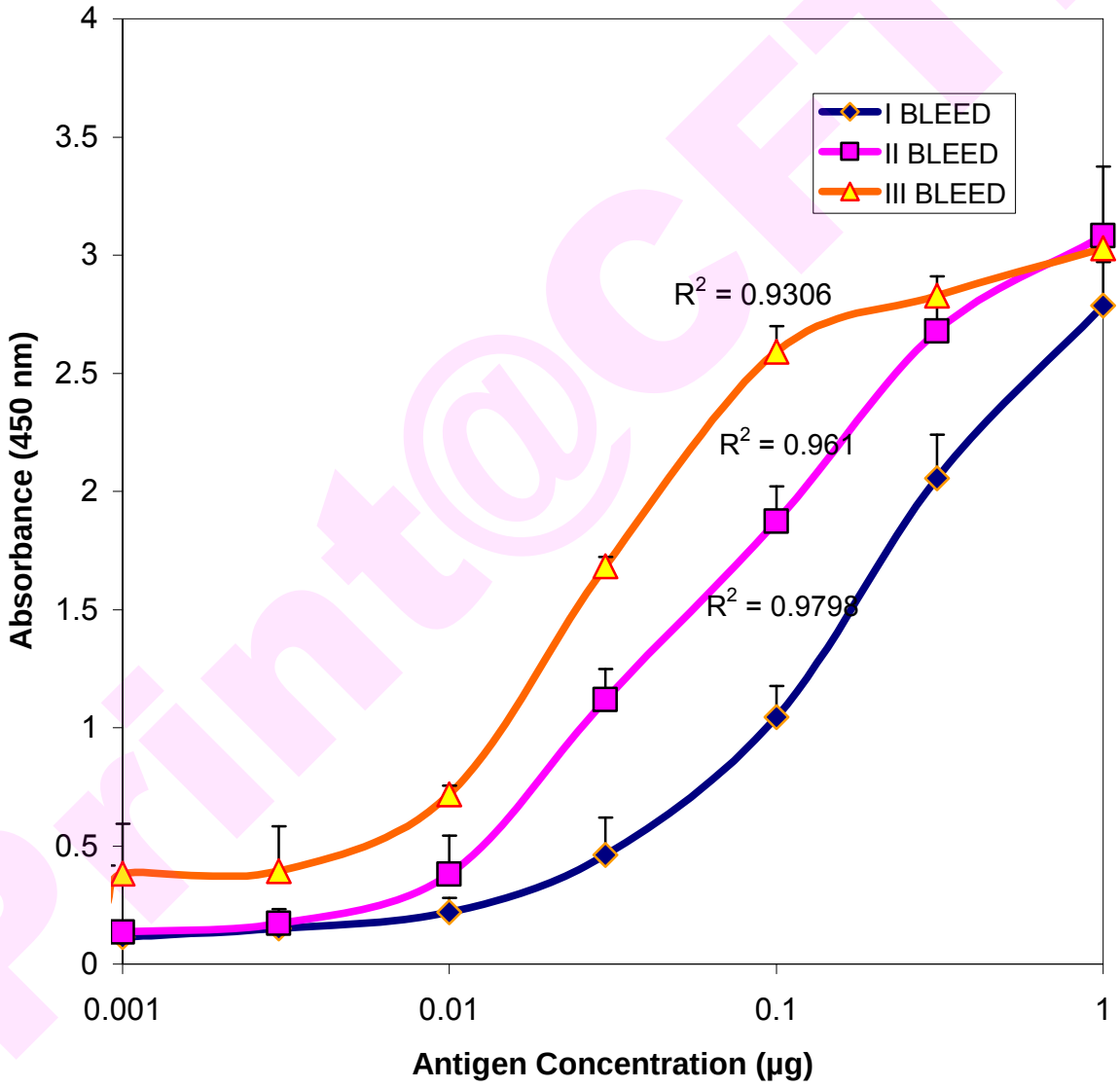
Values are mean of 3 separate experiments ± S.E.

Fig 3.9: Assay formatting for caspase-3 (commercial antibody)



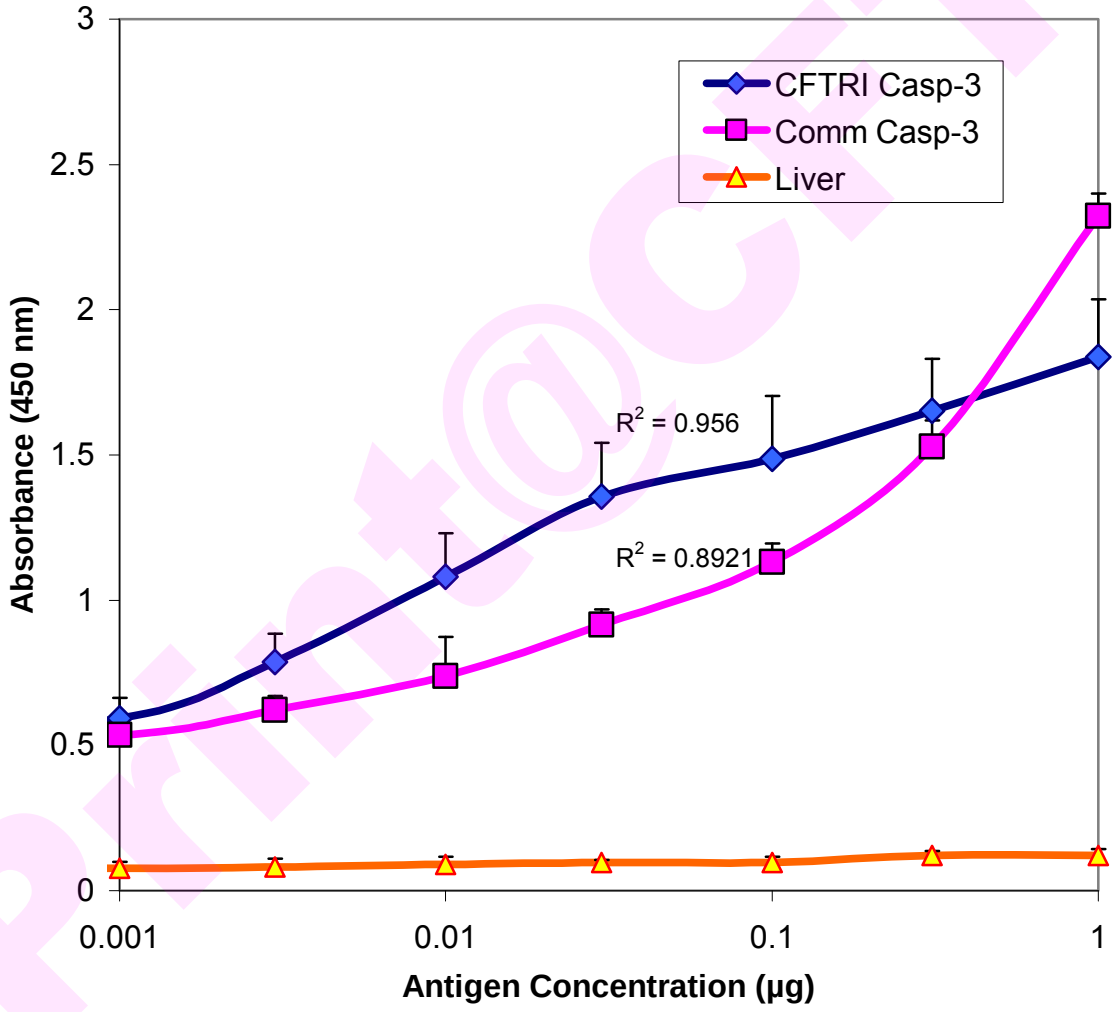
Values are mean of 3 separate experiments ± S.E.

Fig 3.10: Assay Formatting for Caspase-3 using CFTRI antibody



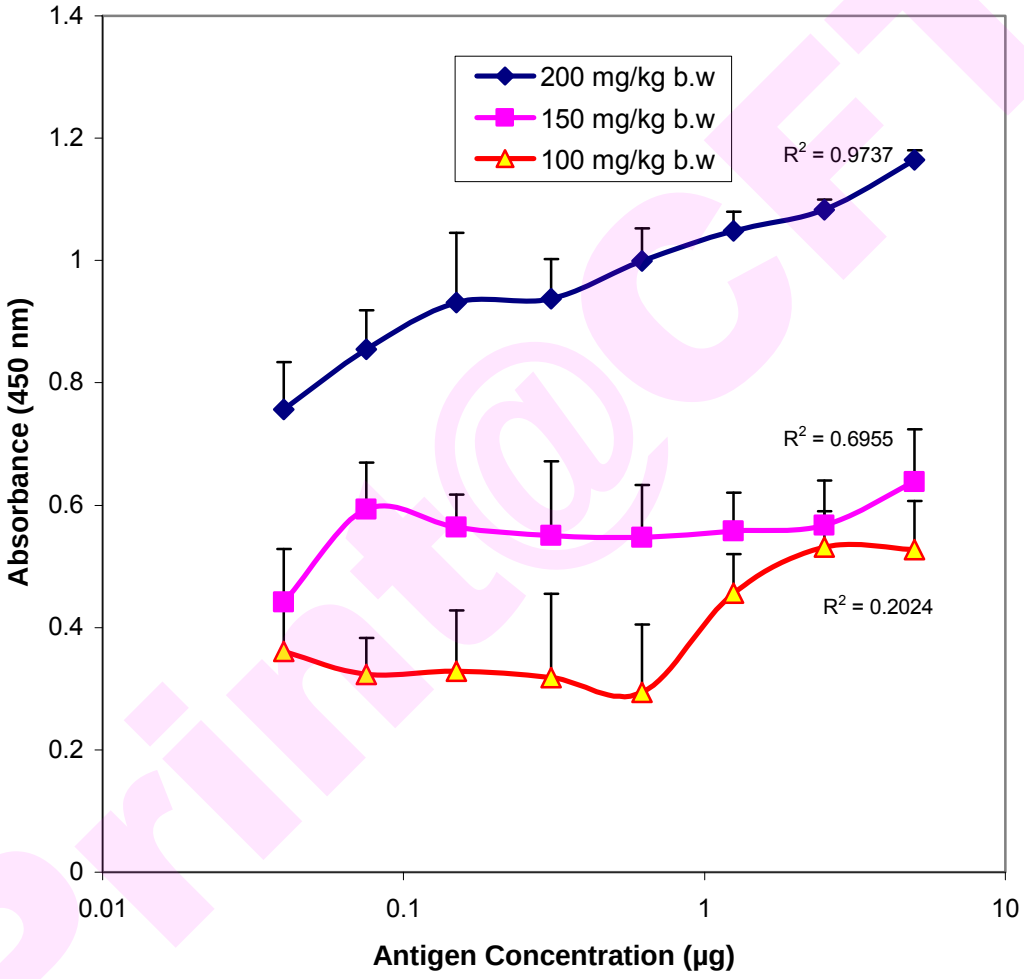
Values are mean of 3 separate experiments ± S.E.

Fig 3.11: Sensitivity of the assay to commercial antibodies



Values are mean of 3 separate experiments ± S.E.

Fig 3.12: ELISA standard curve for Caspase-3



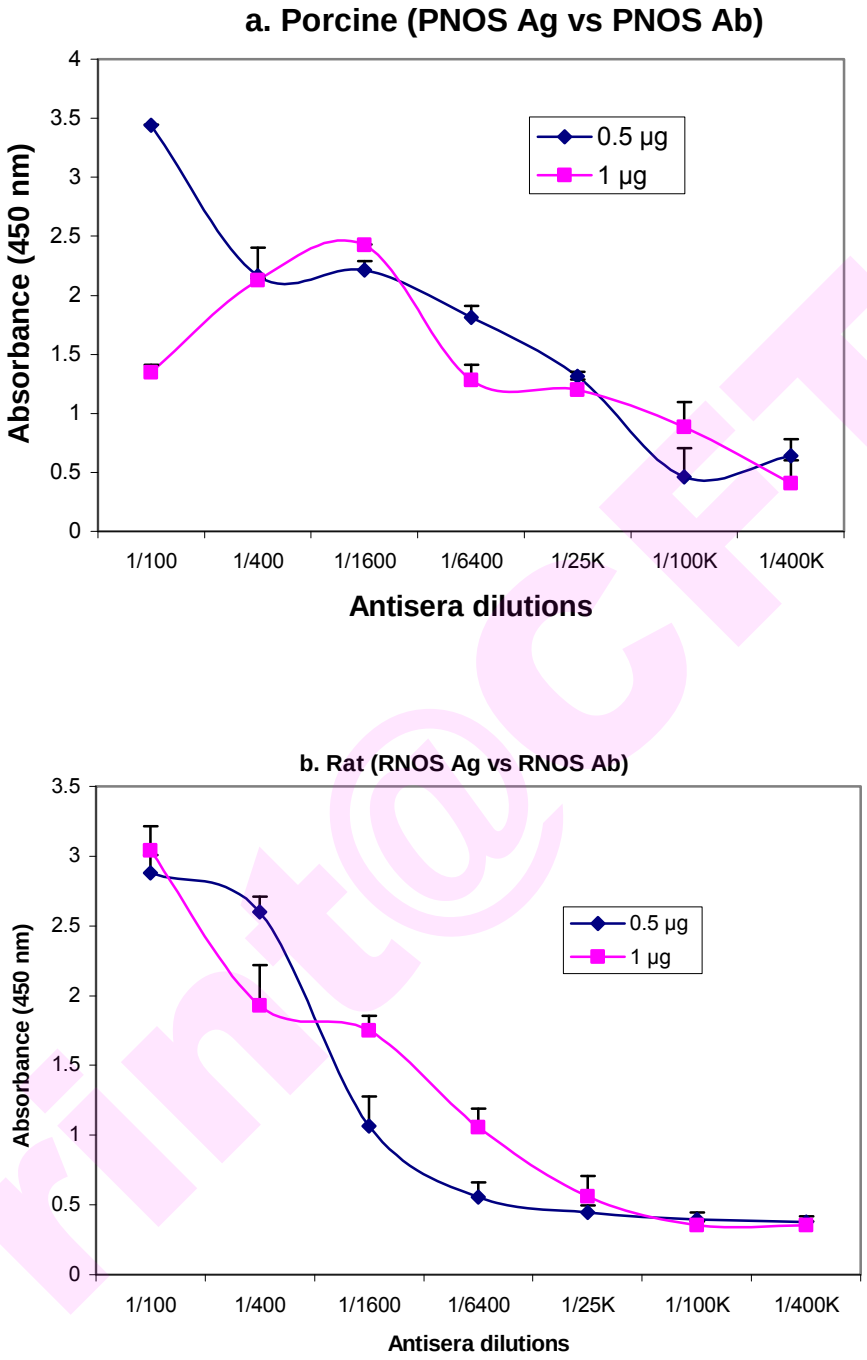
Values are mean of 3 separate experiments ± S.E.

2.7 ACA for nNOS samples:

The results of the ACA showed that the antibodies raised against both the purified rat and porcine NOS were able to recognise the antigens at 1 and 0.5 µg of the antigen (Fig 3.13 a and b). Antisera dilutions from 1/100 to 1/400K were used to determine the titre. Fig 3.13 a shows the assay of porcine NOS and Fig 3.13 b shows the assay of rat nNOS. Antibodies of both porcine and rat recognized the antigens at 0.5 and 1 µg. However, at 1 µg of porcine NOS, there was a slight shift in the Ab titre from 1/100 to 1/1600. The antisera titre for porcine NOS was 1/1600 at 1 µg antigen concentration and 1/25K at 0.5 µg of the antigen. The antisera titre for rat NOS was 1/6400 for 1 µg and 1/1600 for 0.5 µg. It was seen that with increasing antisera dilution, the absorbance values came down as expected.

Fig 3.14 a and b show the assay for cross-reactivity of rat and porcine antigens with cross-antisera. Both rat and porcine antibodies cross-reacted with each other's antigens at 0.5 and 1 µg. The antisera dilution for the rat NOS was 1/1600 at both 0.5 and 1 µg of porcine NOS antigen (Fig 3.14 a). The antisera dilution for porcine NOS antibody was 1/25 K at both 0.5 and 1 µg of the rat NOS antigen (Fig 3.14 b). Thus, porcine antibody was more sensitive as it had a better titre compared to rat antibody in cross-reactivity studies.

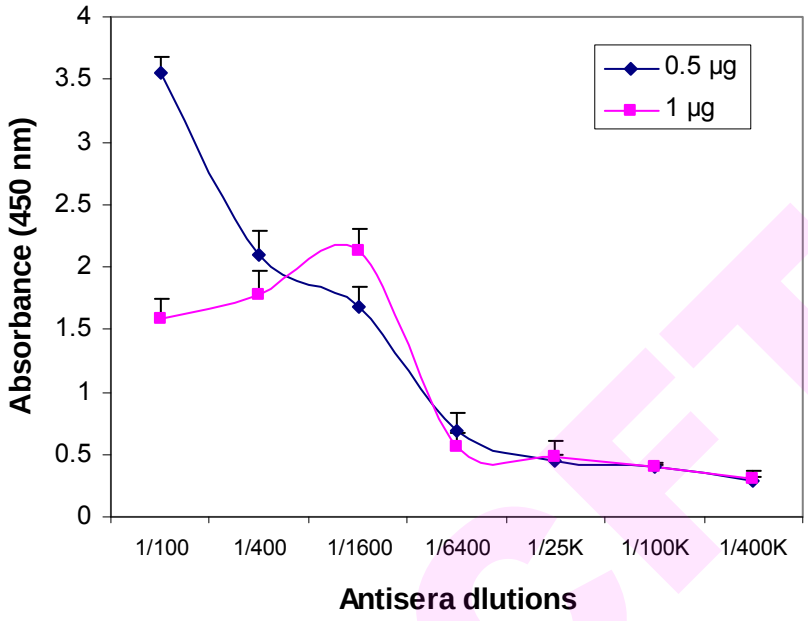
Fig 3.13: Checker Board Analysis – NOS antigens



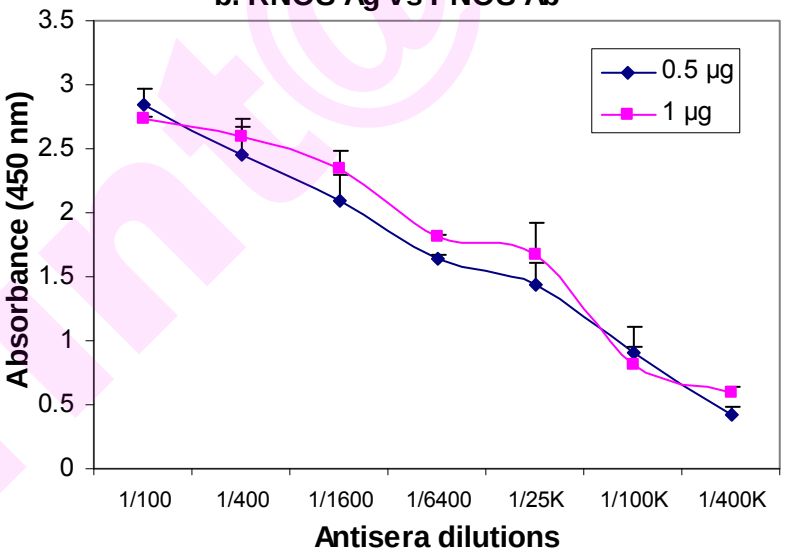
Values are mean of 3 separate experiments ± S.E.

Fig 3.14: Cross-reactivity studies of NOS antigen

a. PNOS Ag vs RNOS Ab



b. RNOS Ag vs PNOS Ab



Values are mean of 3 separate experiments ± S.E.

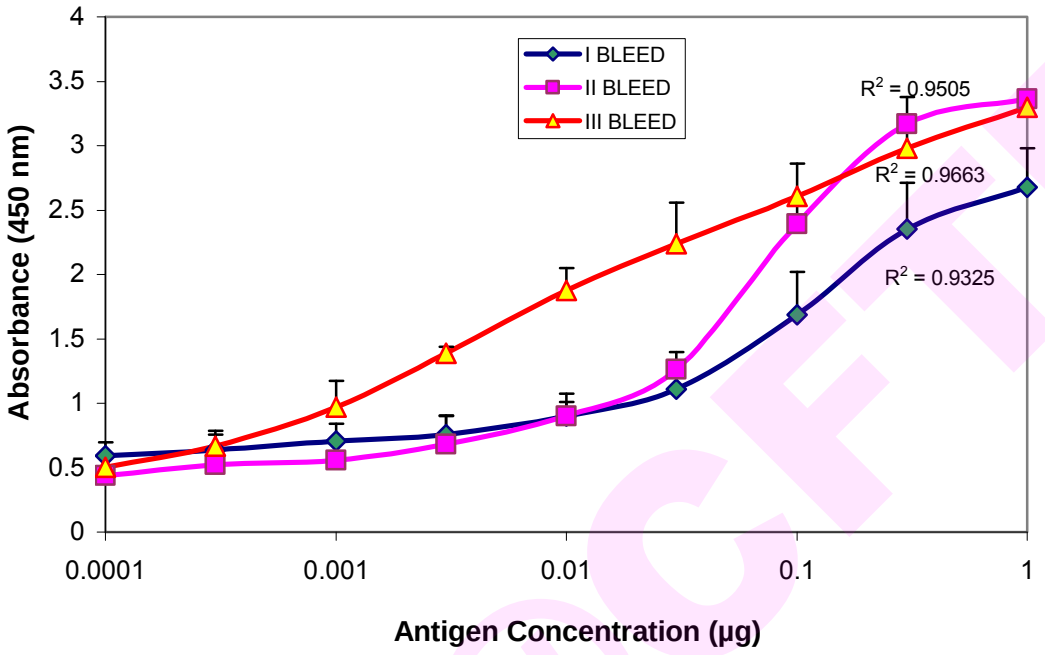
2.8 Assay Formatting

Indirect non-competitive ELISA was formatted for the NOS assay using the 1st, 2nd and 3rd bleeds (Fig. 3.15). Different concentrations of the rat and porcine nNOS antigens were tested to determine the assay sensitivity. The sensitivity increased with the bleeds and the 3rd bleed had the greatest sensitivity at 1 ng of both Rat nNOS and Porcine nNOS (Fig 3.15 a and b). The anti-serum dilution used was 1/1600. The curve was found to be linear between 500 ng and 10 ng of antigen for the rat nNOS curve while the curve for porcine nNOS was linear between 100 ng to 3 ng of the antigen. All the 3 bleeds against the rat NOS had regression values greater than 0.9, demonstrating the high sensitivity of the bleeds (Fig 3.15 a). Among the porcine NOS bleeds, only the 3rd bleed had a slightly low regression value of 0.87 while the other two bleeds had regression values close to 1 (Fig 3.15 b).

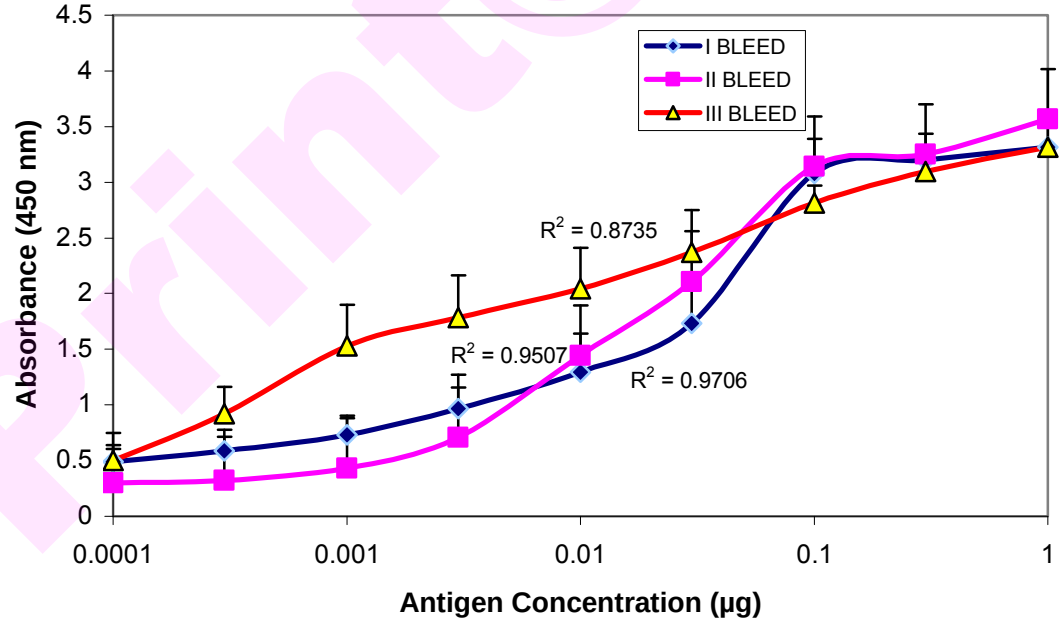
The standard curve of nNOS using the negative control (brain homogenates from untreated rats) did not elicit any reaction with the antibodies as is seen in Fig 3.16 at an Ag concentration range of 5 µg to 1 ng and at an antisera dilution of 1/1600. It did not show any change in absorbance values as seen with the rat and porcine NOS antigens. This illustrates the specificity of the assay developed to nNOS.

Fig 3.15: Assay formatting for nNOS

a. Rat NOS Antigen

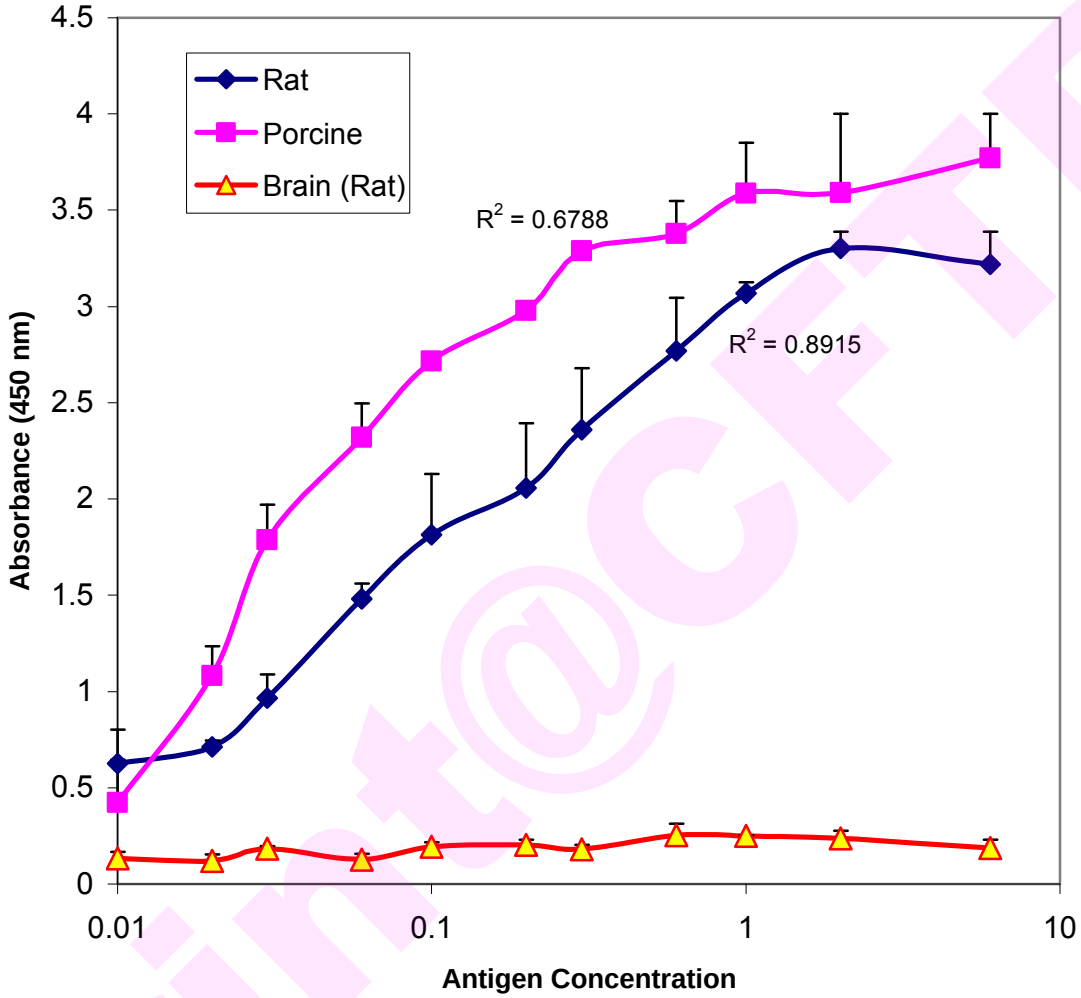


b. Porcine NOS Antigen



Values are mean of 3 separate experiments ± S.E.

Fig 3.16: Standard Graph of NOS assay using antibodies from different sources



Values are mean of 3 separate experiments ± S.E.

3. DISCUSSION

There have been several attempts to develop an ELISA based-method to detect markers of apoptosis, notably the nucleosomes. In one study (Salgame *et al*, 1997), nucleosomes from jurkat cells were used to raise antibodies and an ELISA method was developed to detect nucleosomes and was reported to be 500 times sensitive than apoptotic detection by DNA laddering. Another study by Frankfurt *et al* (2001) used ELISA to develop an immunoassay for the detection of single strand DNA (ssDNA) which are produced during apoptotic DNA degradation. Frankfurt *et al* also used cell cultures to study apoptotic DNA degradation. In our study, antibodies were raised in the rabbit against caspase-3 and nNOS - the biomarkers of apoptosis. An indirect ELISA format was used for the assay of these two biomarkers. The purity of the extracted antigens was confirmed by immunoblotting with commercial anti-caspase 3 and anti-nNOS. Studies by Samali *et al* (1998) indicated the presence of pro-caspase 3 in different tissues. Pro-caspase-3 from rat liver, thymus, heart, kidney, brain and spleen was found to be localized in both the cytosolic and mitochondrial fractions. The studies indicated that the ratio of cytosolic to mitochondrial pro-caspase-3 varied between different tissues. Mitochondrial pro-caspase-3 content in thymus and spleen was higher than that of heart and kidney. Based on this report, cytosolic fraction of kidney was used in all our studies on caspase-3.Most of the studies to detect these biomarkers were based on *in vitro* cell-culture using animal cell lines. But there are very few reports that have used ELISA to detect nNOS or caspase-3 from the *in vivo* system.

Currently, the caspase family consists of 14 members that exist in the cytoplasm in the zymogen form (Wundrich *et al*, 2006). The implementation of the apoptotic program requires the participation of the initiator caspases which activate the executioner caspases, that in turn cleave the targeted intracellular substrates (Nicholson et al, 1995, Cohen, 1997). The actual activation of the executioner caspases, including caspase-3 requires formation of Apaf-1-Casp-9-cyt c apoptosome complex which facilitates their recruitment to Apaf-1 (Koester and Bolton, 2001).

Our immunoblotting results revealed the presence of bands at 32 KD only at an antibody dilution of 1/1000. However, the active form of caspase-3 at 17 and 12 KD was not visible at all dose groups. This finding correlates with the non-induction of DNA fragmentation at the dose groups 100and 150 mg/kg b.w. Absence of the zymogen and active caspase-3 at 100 and 150 mg/kg b.w. correlates with the absence of DNA ladder and ELISA signal as shown in Fig 3.12. At 200 mg/kg b.w. the sensitive ELISA signal correlates with the observed DNA laddering, an indicator of apoptosis. The absence of the band at 17 KD/12 KD at 200 mg/kg b.w. inspite of DNA fragmentation may be due to the low levels of the sample. Western blot was also used to monitor caspase-3 Ab epitopes (Fig 3.3). Active caspase-3 was not observed at 200 mg/kg b.w. while the inactive zymogen at 32 KD was observed at all the 3 dose groups of 100, 150 and 200 mg/kg b.w. This result correlates with the observation of DNA fragmentation and biochemical assay and ELISA. Since SDS sample buffer used in western blot

abolishes enzymatic activity, it is highly unlikely that caspases are activated during sample preparation for gel electrophoresis. The correlation between the western blot and the active caspase-3 ELISA indicates that caspase activation during sample preparation for the ELISA is minimal. It appears that the ELISA measures the amount of active caspase-3 present in cells at the time of harvest (Saunders *et al*, 2000).

Immunoblotting methods used earlier for identification of caspase-3 used 1/100 (Wundrich *et al*, 2006) and 1/500 (Bernath *et al*, 2006) dilutions. As seen in the western blotting, the zymogen of caspase-3 was seen at 32 KD at all dose groups. Salgame *et al* (1997) also reported the detection of apoptosis by ELISA not accompanied by detection of DNA fragmentation. The ELISA for nucleosomes had almost 500 times greater sensitivity as compared to DNA ladder fragmentation although release of nucleosomes is also an early event with DNA fragmentation. Although apoptotic cell death can be disconnected from DNA breakdown, this event is probably beneficial for the efficient removal of toxic cell debris from the organism.

The ELISA developed in our study at CFTRI was sensitive and could detect up to 1 ng of purified caspase-3. It was interesting to note that the Ab titre dilution increased with Ag reduction with CFTRI Ab (Fig 3.8 b). At an Ag concentration of 0.31 µg, the Ab titre was 1/1600 (Fig 3.8 b) while at an Ag concentration of 1 µg-1 ng, it was 1/6400 (Fig 3.10). However, with the commercial Ab it was 1/1600 at all Ag concentrations. This indicates the

sensitivity and specificity of the CFTRI anti-caspase-3. An ELISA to detect active caspase-3 has been described previously (Saunders *et al*, 2000) from caspase-3 extracted from jurkat cells. Our studies revealed that the sensitivity of the ELISA for caspase-3 increased with the bleeds, pointing out to an increase in specificity of the immune reaction to the antigen with time and development of more specific and sensitive Ab's. In the report by Salgame *et al* (1997), ELISA was found to be 500 times more sensitive in detecting apoptosis when compared to DNA ladder fragmentation. Laxman *et al* (2002) have used non-invasive imaging of apoptosis using caspase-3 as the marker. In cells undergoing apoptosis, a caspase-3 specific cleavage of the recombinant product occurs, resulting in the restoration of luciferase activity that can be detected in living animals with bioluminescence imaging. Frankfurt and Krishan (2001) induced apoptosis by etoposide, H_2O_2 and staurosporine and analysed it by ELISA. A near linear increase in signal was seen as the number of apoptotic cells increased from 500 to 5000. The method could detect as few as 1000 apoptotic cells. Saunders *et al* (2000) used an ELISA format which was linear between 0.03 and 4 ng. This report uses a biotinylated inhibitor to label active caspases, a caspase-3-specific antibody to capture caspase-3. Specificity of the assay for caspase-3 was shown by the absence of signal with control tissues which lack caspase-3.

Neuronal NOS (nNOS) is a crucial enzyme responsible for production of NO, which is an important molecule in cell signaling and also in apoptosis in both the central and peripheral nervous systems (Zini *et al*, 1996). nNOS is localized

to neurons throughout the peripheral and central nervous system (Bredt *et al*, 1990; Dawson *et al*, 1991). NO is an important messenger molecule and plays a role under both physiologic and pathological conditions (Moncada *et al*, 1991). NOS catalyses the oxidation of L-arginine to form nitric oxide (NO) and citrulline and has a close structural homology with cytochrome P-450 reductase (Bredt *et al*, 1991). Biochemical tests (Castro-Blanco *et al*, 2003), immunocytochemical localization (Kim *et al*, 1999), immunoassays, immunoblotting along with densitometry (Diaz-Ruiz *et al*, 2005) and commercial ELISA's (Boehringer Manheim ELISA kit) are available to detect NOS. The biochemical method is widely used and is a standard protocol (El-Gohary *et al*, 1999). But it requires radioactively-labeled arginine which makes it hazardous and also expensive. Immunocytochemical localization is also a much used method but has low sensitivity compared to the other methods.

Our study is one of the first to describe an immunoassay for nNOS from animal tissues. Three isoforms of NOS exist, including Ca^{2+}- dependent constitutive NOS (cNOS/nNOS in neurons), endothelial NOS (eNOS) and Ca^{2+}-independent inducible NOS (iNOS). In the present study, ELISA with antibodies raised against nNOS revealed that the antibody could detect up to 3 ng of antigen at a dilution of 1/1600, comparable to titre of commercially available antibodies. Porcine NOS Ab's were more sensitive than rat NOS Ab's as observed by the Ab titre. It was also interesting to note that the assay was more sensitive at lower Ag concentration (0.5 µg) than at 1 µg of the antigen. Similar

results were obtained with the cross-reactivity studies wherein the porcine NOS Ab was more sensitive at 0.5 µg of rat NOS Ag than at 1 µg of the Ag. In addition, immunoblotting confirmed the presence of the nNOS in the protein extract from both rat and porcine cerebella. Western blotting also showed the presence of the antigen at 155 KD, the same as the molecular weight of nNOS and it was detected by the commercial Ab at 1/1000 dilution. The Native PAGE indicated the presence of NOS by a band at 155 KD in both procine and rat NOS. However, the concentration of the RNOS was higher in the rats than in the porcine. This could be due to the induction by the pyrethroid deltamethrin. Interestingly, the western blot indicated the recognition of the NOS from both porcine and rat by the commercial monoclonal NOS monoclonal antibody. Many immunoblotting studies using anti-nNOS antibodies have been conducted using polyclonal rabbit antibodies at 1/2500 (Cai *et al*, 1998), 1/3000 (Kim *et al*, 1999), 1/7000 (Castro-Blanco *et al*, 2003), 1:2000 (Uttenhall *et al*, 1998) dilutions whereas monoclonal antibodies against nNOS have been used at 1/750 (Lajoix *et al*, 2001), 1/2500 (Diaz-ruiz *et al*, 2005), 1/1000 (Zinck *et al*, 2005) dilutions. Increased nNOS expression and NO synthase was seen in studies by Castro-Blanco *et al* (2003), Zinck *et al* (2005) and Uttenhall *et al* (1998) in Wistar rats demonstrated by western blotting and enzymatic activity procedures. On the other hand, a study by Diaz-Ruiz *et al* (2005), using western blot and enzymatic assay, showed that Cyclosporin-A, an immunosuppressant, inhibits the NOS activity and nNOS expression after spinal cord injury in rats. Most methods

described for immunoassays of nNOS have been from animal cell-culture studies. There is now sufficient evidence to dispel the notion that DNA fragmentation is an essential step in apoptosis. Some cells do not exhibit DNA fragmentation during death (Collins and Duke, 1992).

Hence, an ELISA for caspase-3 and nNOS is a vital and essential tool to detect the advent of apoptosis in tissue samples and other biological material. It could have applications in cancer research, clinical diagnosis and therapeutic intervention in case of a positive response for apoptosis from the biological test material. The ELISA developed in the present study was sensitive and could detect 1 ng and 3 ng of the chosen biomarkers - caspase-3 and nNOS, respectively.

SUMMARY:

- Biomarkers of apoptosis were extracted from the deltamethrin-treated rat tissues (caspase-3 and rat nNOS) and untreated porcine brain (porcine nNOS).
- Optimum conditions for the assay were: carbonate buffer as coating buffer, PBS as dilution buffer, antisera dilution of 1/6400 for caspase-3, antisera dilution of 1/1600 for nNOS from both rat and porcine and 1/10 K 2^0-Ab-HRP conjugate compared to 1/1000 for the commercial Ab dilution.
- An IgG-based ELISA method was developed for detection of caspase-3 and nNOS.
- The ELISA method developed was very sensitive and could detect up to 1ng of caspase-3 from rat kidney and 3 ng of nNOS from both rat and porcine brain.
- The assay was comparable to the commercial antibody assay.
- The immunoassay developed has immense potential and could serve as a sensitive tool for the detection of apoptotic biomarkers.

CHAPTER 4

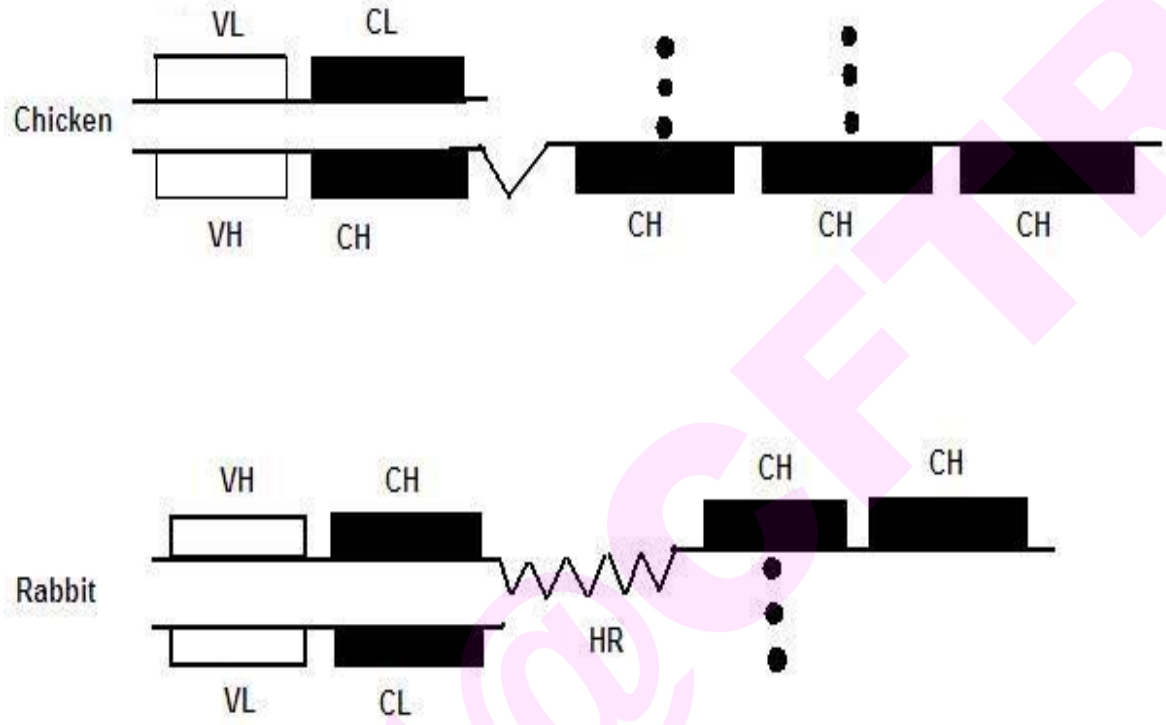
DEVELOPMENT OF AN IMMUNOASSAY BASED ON EGG-YOLK ANTIBODY (IgY) TO DETECT CASPASE–3 AND nNOS

INTRODUCTION

The enzymes caspase-3 and nNOS have an important role to play in the process of apoptosis. In chapter 3, development of an immunoassay based on IgG antibodies from rabbits was described for the detection of these biomarkers of apoptosis. Antibodies from rabbit are obtained in a limited amount and often require repeated experimentation to obtain larger quantities of antibody of reproducible/constant sensitivity. Reproducibility of the test method is an essential requirement for any routine analysis. Thus, there is a need to develop an alternate system to raise Ab's and to develop a method for detection of these biomarkers. In this chapter, an ELISA method based on egg-yolk antibodies or "IgY" obtained from the hen against the biomarkers has been described. These antibodies were used for detection of caspase-3 and nNOS samples. The advantages of IgY are many, most notably the large quantity of antibody (g/hen) that can be obtained from hen eggs and their non-homology with mammals conferring IgY greater specificity in detecting mammalian antigens of interest. Another unique feature is that there is no need to immunize the animals repeatedly as large quantity and sensitive Ab's are obtained with one time experiments.

Structurally there is a clear difference between hen IgG and mammalian IgG (Fig 4.1). The heavy chain of hens' Ab has an additional constant domain and no hinge region as compared to only 2 carbohydrate moieties and a hinge in mammalian IgG. So the molecular weight of hen IgG (about 190 KD) is greater

Fig 4.1: Structures of chicken IgY and rabbit IgG.



HR = Hinge region, C = constant domain, L = light chain, H = Heavy chain, V = Variable domain, CH = constant domains of heavy chains

than that of mammalian IgG (150 KD). The IgY concentration obtained in the yolk is around 70-100 mg/egg with a purity of 98% (Hatta *et al*, 1990). There are many advantages of avian antibodies over mammalian antibodies. Since there is a large phylogenetic distance between hens and mammals, the antibodies from hens can recognize more epitopes on mammalian antigens (Erhard and Schade, 2001). This evolutionary spread also means that there is no immunological cross-reactivity between chicken IgY and mammalian IgG (Hadge and Ambrosius, 1984). As a result, chicken is a better choice than e.g. rabbits for the production of antibodies against conserved mammalian proteins (Horton *et al*, 1984). Due to this evolutionary difference, chicken antibodies may bind to more epitopes on a mammalian protein than the corresponding mammalian antibody. It has been shown that 3-5 times more chicken antibody than swine antibody will bind to rabbit IgG which will amplify the signal in an immunological assay (Horton *et al*, 1984; Olovsson and Larsson, 1993). Chicken antibodies also recognize other epitopes than mammalian antibodies (Song *et al*, 1985). This gives access to a different antibody repertoire than the traditional mammalian antibodies.

The amount of avian antibodies produced is almost 10-20 times more than IgG from mammals (2-5 g in a hen compared to 140-200 mg for rabbit). The non-invasive nature of obtaining IgY from egg-yolk compared to the bleeding required to obtain antibodies from rabbit serum also makes IgY an ethical and favourable method to minimize pain to laboratory animals (Erhard and Schade, 2001). The

IgY antibodies are also equally or sometimes more sensitive than rabbit Ab's (Schade *et al*, 1996).

The IgY is transported from the hen's circulatory system to the egg-yolk at the time of the egg formation. The amount of IgY transported is independent of egg size and known to be proportional to the maternal serum IgY concentration (Loeken and Roth, 1983). A delay of three to four days is observed between the appearance of IgY in serum until it is found in the yolk. The ratio of the concentration of IgY in the yolk to the serum IgY concentration is reported to be 1.23:1 (Woolley and Landon, 1995) . The density of yolk is about 1.1 g/ml. About 50 % of the yolk is non-aqueous material.

1. MATERIALS AND METHODS

1.1 Chemicals:

Acrylamide, Bis-Acrylamide, Ethylene Diamine Tetraacetic acid (EDTA-sodium salt), Tris base, Glycerol, Sodium Chloride, PEG 8000, sodium azide, Disodium Hydrogen orthophosphate, Sodium dihydrogen orthophosphate, all other salts and solvents were purchased locally. Rabbit anti-chicken-Horse Radish Peroxidase (IgY-HRP) was from Bangalore Genei, Bangalore, India. Bovine Serum Albumin (BSA), TEMED, Freund's complete adjuvant, Freund's incomplete adjuvant, (4,4',5,5') Tetramethyl Benzidine (TMB), Sodium Dodecyl Sulphate (SDS) and β-Mercaptoethanol were purchased from Sigma Chemicals.

1.2 Development of egg-yolk (IgY) antibodies against the biomarkers:

a. Animals

Healthy 22-week old white Leghorn hens with a proven laying ability and having an average weight of 2 ± 0.2 kg were housed in stainless steel poultry cages and provided with commercial poultry feed and water available *ad libitum*. The water was changed daily and the hens were maintained on a 12 h light/dark cycle.

b. Immunization

The hens were immunized with:

i. Purified caspase-3 from kidney extract of 200 mg/kg deltamethrin treated rats.

ii. nNOS- extracted from brain of healthy porcine.

Initially, the hens were immunized with a primary dose of 1 mg antigen emulsified in complete Freund's adjuvant and saline at 8-10 different intra-muscular sites in the hen's breast muscle. Booster doses were given at 30 days interval with 0.5

mg antigen suspended in Freund's incomplete adjuvant and saline given intra-muscularly. Eggs were collected from the 7th day after first booster till the end of all boosters. The eggs were stored at 4^{0}C until purification. The yolk was used to obtain the antibodies after purification.

1.3 Purification of the egg-yolk antibody (IgY)

The egg-yolk was separated from the albumin prior to purification. The egg-yolk antibody was purified by 3 different methods from week 1 to week 7 and the most sensitive method with high antibody yield was chosen for IgY purification for 12 weeks. Yolk from a single egg was divided into 3 equal parts (by volume) and used for purification by the following 3 methods.

a. **CFTRI patented method (M1)**
b. **PEG precipitation (M2)**
c. **Chloroform extraction (M3)**

a. CFTRI patented method (M1)

This method used a gum, carrageenan, for the precipitation of the lipoprotein fraction and further IgY purification. 10 ml of the yolk was mixed with 10 ml of d.w. This mixture was homogenized for 30 seconds and filtered through a cheese cloth to remove any remaining yolk sac or albumin. Carrageenan was added along with 40 ml of d.w, mixed well and incubated at room temperature for 30 min. This solution was centrifuged at 10,000 g (7800 rpm) for 15 min. The resultant precipitate was discarded and the supernatant, containing the Water-Soluble Protein Fraction (WSPF), was filtered through filter paper no. 2. The filtrate was precipitated once with 14% (w/v) PEG 8000. The mixture was stirred

on a magnetic stirrer to dissolve the PEG and then centrifuged at 7500 rpm for 25 min. The IgY precipitate was reconstituted in 10 mM PBS containing 0.005 % sodium azide and 5% glycerol. The IgY was stored at 4^0C for routine usage after ultrafiltration to approximately 5-10 mg/ml. For long term storage, the IgY was kept frozen at -20^0C.

b. PEG precipitation (M2)

The yolk was mixed well with equal volume of d.w. The pH of the mixture was adjusted to 7.4 with dilute hydrochloric acid (HCl), mixed well, 3.5% (w/v) PEG 8000 added and centrifuged at 7800 rpm for 15 min. WSPF obtained after centrifugation was carefully decanted out and its pH was adjusted to 5 with dilute HCl. 14% (w/v) PEG 8000 was added to the WSPF and the mixture was incubated at room temperature for 10 min. This mix was centrifuged at 7500 rpm for 25 min, the precipitate thus obtained was dissolved in 10 ml of 10 mM PBS and 10 ml of pre-cooled ethanol and centrifuged at 10,500 rpm for 25 min at 4^0C. The sediment obtained was diluted in 10 ml of 10 mM PBS. The above mixture was dialyzed against 10 mM PBS for 24 h at 4^0C with change in the buffer every 8 h. The IgY sample thus obtained was aliquoted and stored at -20^0C.

c. Chloroform extraction (M3)

One volume of yolk was mixed with 4 volumes of 0.1 M PBS and 1 volume of chloroform. This mix was shaken well and centrifuged at 10,000 rpm for 30 min. The top aqueous layer containing IgY was precipitated with 14% (w/v) PEG

8000, mixed well and centrifuged at 5000 g (7500 rpm) for 25 min at 4^0C. The precipitated IgY was reconstituted in 10 mM PBS containing 0.005 % sodium azide and 5 % glycerol. For routine use, the IgY was stored at 4^0C and at -20^0C for long term storage.

1.4 Native PAGE for IgY

To check the purity of the IgY obtained by different methods, a native PAGE was run. The conditions for PAGE were 5 % stacking gel and 6 % resolving gel run at 50 mV for 3 h with 20 µg of protein/lane. The samples used were IgY purified by all the 3 methods (M1, M2 and M3), unpurified egg-yolk, affinity purified IgG along with molecular weight markers.

1.5 Antibody capture assay (ACA) for caspase-3.

The ACA methodology used for IgY was as described in Chapter 3, section 1.10 to determine the sensitivity and specificity of the Ab. Checker board analysis was used for optimisation of the Ag and Ab concentrations with different coating and dilution buffers. Different antibody dilutions ranging from 1/20 till 1/1 lakh were used for caspase-3 concentration of 1 µg.

1.6 Assay formatting for caspase-3

The assay for caspase-3 was formatted using an indirect ELISA method to establish the optimum assay conditions. Different caspase-3 concentrations (Ag) ranging from 1 µg till 1 ng were analysed while the antibody concentration used was 1/20 and 1/100. The HRP-conjugated secondary antibody dilution was 1/1000 to 1/10K. The ELISA was formatted using the IgY obtained from the 3

purification methods. Antibodies from 7 weeks were used for the assay formatting to choose the antigen working range and to check the sensitivity of the assay.

1.7 ACA for nNOS

The checker board analysis was used for optimisation of the assay conditions. The ACA was performed as described in chapter 3, 1.10 and intended to determine the Ab specificity and sensitivity. The antibody dilutions ranged from 1/20 till 1/1lakh at 1 µg/well of the nNOS antigen.

1.8 Assay formatting for nNOS

The antigen concentrations ranged from 1 µg/well to 1 ng/well, while the egg-yolk antibody dilution used was 1/20 and 1/100. HRP-conjugated secondary antibody dilution used was 1/5K. The ELISA was formatted by using antibodies obtained through 7 weeks from all 3 purification methods.

2. RESULTS:

2.1 Native PAGE for IgY

The antibody was found to be pure when it was extracted by either M1 or M3 as they gave a single band as seen in the native PAGE (Fig 4.2). The approximate molecular weight was 190 KD, the known molecular weight of IgY. M2 did not give a band corresponding to IgY but revealed a lipoprotein band at 90 KD. Native PAGE of antibodies obtained by M3 showed the presence of lipoprotein bands (molecular weight of 66 KD) along with IgY (190 KD). The rabbit antibody in Lane 5 (IgG) was seen to have a molecular weight of 150 KD and was used as a marker for this gel.

2.2. Yield of Caspase-3 IgY Ab:

Egg-yolk antibody production began from the 1st week after the 1st booster and the average yield was:

a. 55 mg IgY/egg for M1

b. 8 mg IgY/egg with M2

c. 67 mg IgY/egg with M3

Comparison of the caspase-3 egg-yolk antibody (IgY) concentration across 7 weeks for all 3 methods is shown in Fig 4.3 a. The results indicated that CFTRI patented method gave an average yield of 55 mg IgY/egg for all the weeks except week III, and the yield in case of M3 was 67 mg IgY/egg. The yield from M2 was the poorest at 8 mg/egg. The CFTRI patented method was used for the purification of IgY from all the rest of the eggs for an additional 5 weeks to give a profile for 12 weeks (Fig 4.3 b). The average yield up to 12 weeks was

55 mg/egg, which was the same as the average yield over 7 weeks. IgY from weeks 3, 7 and 9 had a slightly lower concentration of 40 mg/egg compared to above 55 mg/egg for all the other weeks.

2.3 ACA for Caspase-3

The indirect ELISA format was used for the ACA. The ACA showed that the optimum antibody dilution for detection of caspase-3 was 1/20. Even at 1/100 dilution, the antibody was sensitive enough to detect the antigen. At both these dilutions, the antibody was able to detect caspase-3 at 3 ng and above.

2.4 Assay formatting for Caspase-3

Assay formatting for caspase-3 revealed that carbonate buffer was ideal as a coating buffer and PBS was optimum as a dilution buffer (as in chapter 3). The antibodies from all the 3 methods were used for detection of caspase-3 enzyme. The sensitivity for caspase-3 IgY antibodies from different weeks is shown in Fig 4.4. Antibodies obtained by M1 purification method showed a sensitivity of 3 ng and its linear range was from 300 ng to 10 ng (Fig 4.4 a). The IgY dilution best suited for the detection of caspase-3 by M1 was 1/100 and secondary-antibody HRP dilution of 1/5000.

Antibodies obtained by M3 method showed a sensitivity of 3 ng and had a linear range between 300 ng to 10 ng as in M1 (Fig 4.4 c). The optimum IgY dilution for M3 was also same as M1 at 1/100 and 1/5000 secondary-Ab HRP dilution. M2 IgY showed low absorbance values and had a linear range of 300 to 100 ng. The IgY dilution best suited for the detection of caspase-3 was 1/20 for M2 and

1/5000 of secondary-Ab HRP dilution. M2 gave a lower sensitivity of 10 ng (Fig 4.4 b). The IgY was found to work best at a dilution of 1/20.

2.5 Yield vs Sensitivity for caspase-3

The sensitivity of M1, M2 and M3 methods along with the yield are shown in Figures 4.5 a, b and c respectively. The sensitivity across the weeks showed that M1 had an initial sensitivity of 30 ng in the first week along with a yield of 60 mg/egg (Fig 4.5 a). The sensitivity improved to 10 ng from the 2nd to 4th week even though the yield came down to 40 mg/egg in the 3rd week. The sensitivity was lower in the 5th week at 30 ng and again it improved to 3 ng by the 7th week, although the IgY yield remained constant (Fig 4.5 a). The average sensitivity of M1 IgY for the 7-week period was found to be 10 ng.

M3 was comparable with M1 and had an initial sensitivity of 30 ng in the 1st week, reached a plateau of 10 ng in the 2nd to 5th weeks and reached 3 ng by the 6th and 7th weeks (Fig 4.5 c). Thus, M3 IgY sensitivity increased with the weeks. The average sensitivity for M3 was 10 ng over the 7 weeks.

M2 on the other hand, had a constant sensitivity of 300-100 ng across the weeks except the 4th and 5th weeks where it was 10 ng (Fig 4.5 b). The yield and the absorbance values of M2 IgY were also quite low compared to the other two methods. The overall sensitivity of M2 was 100 ng for all the 7 weeks.

Fig. 4.2: Native PAGE of IgY samples purified by different methods

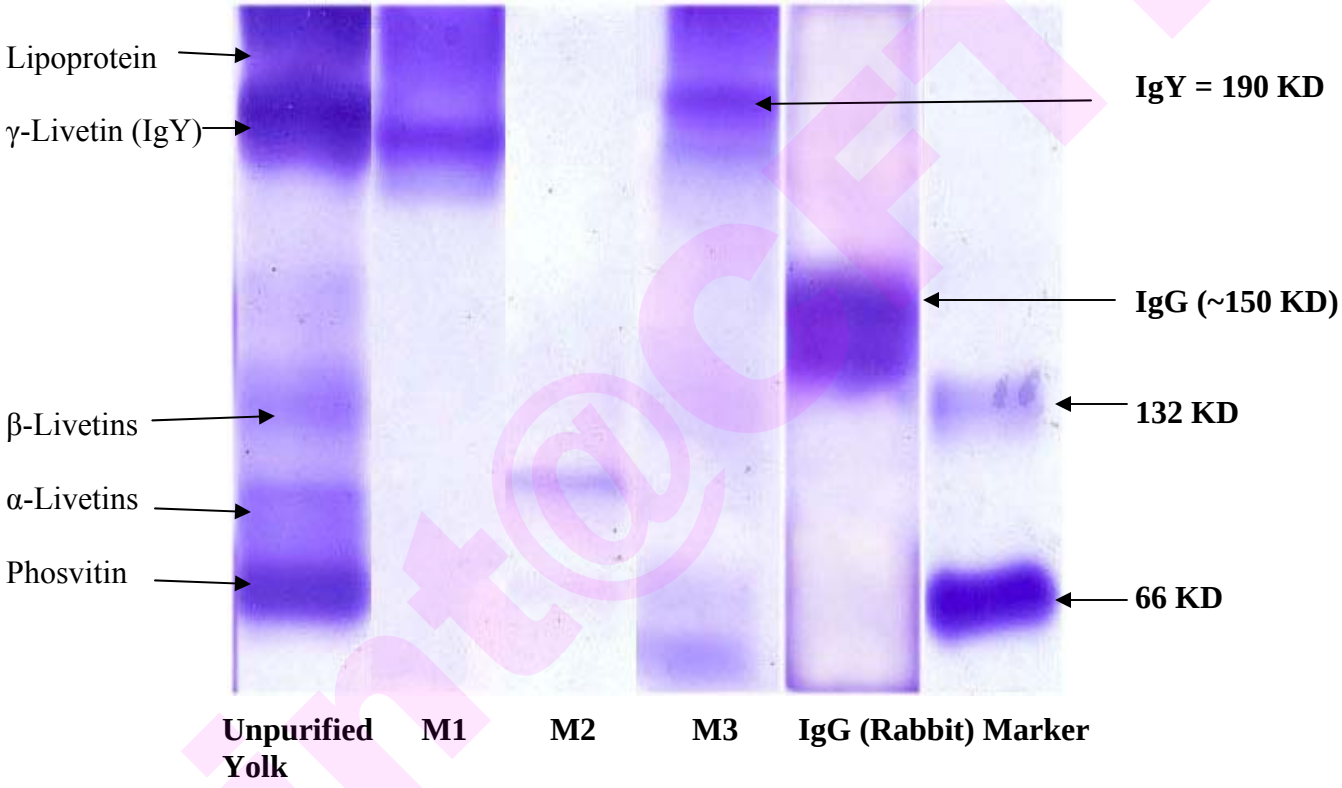
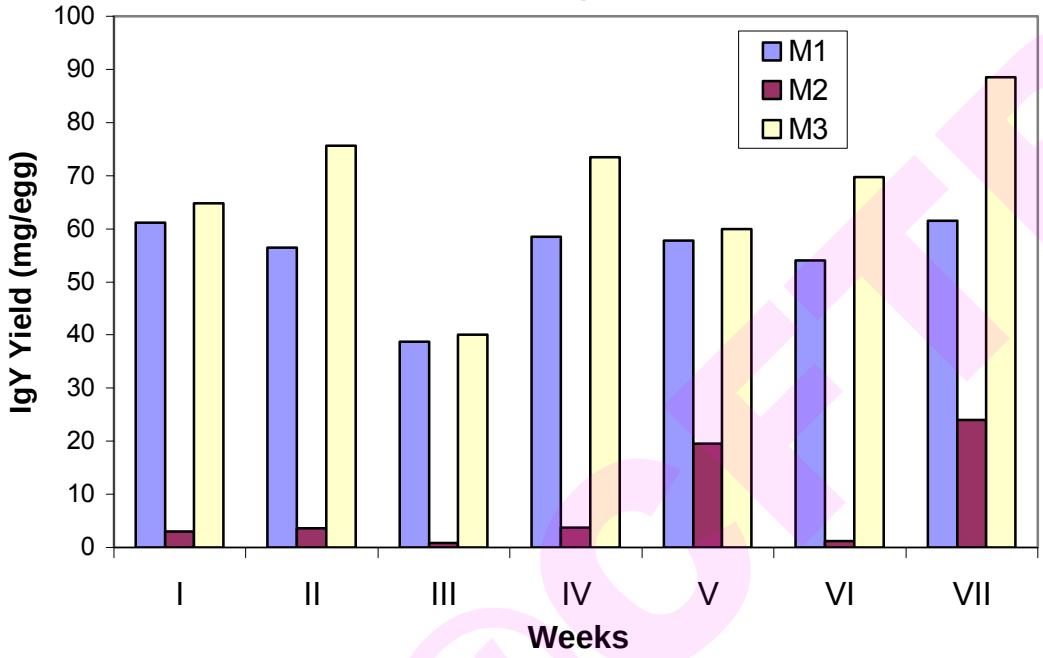


Fig 4.3: Weekly Yield of Caspase-3 IgY

a. Yield of Caspase-3



b. Yield of Caspase-3 IgY by M1

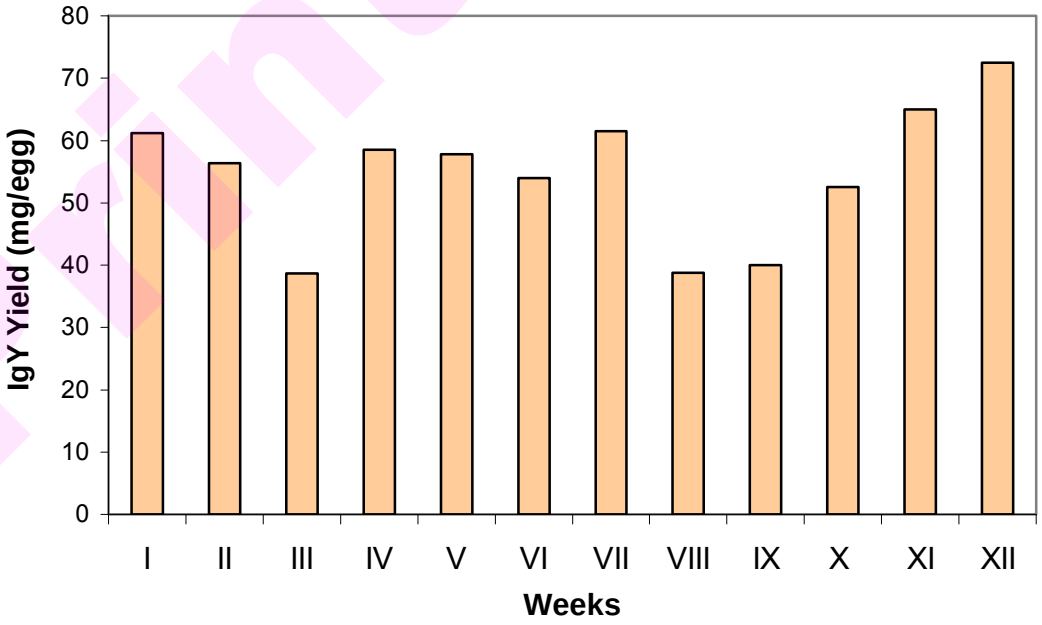
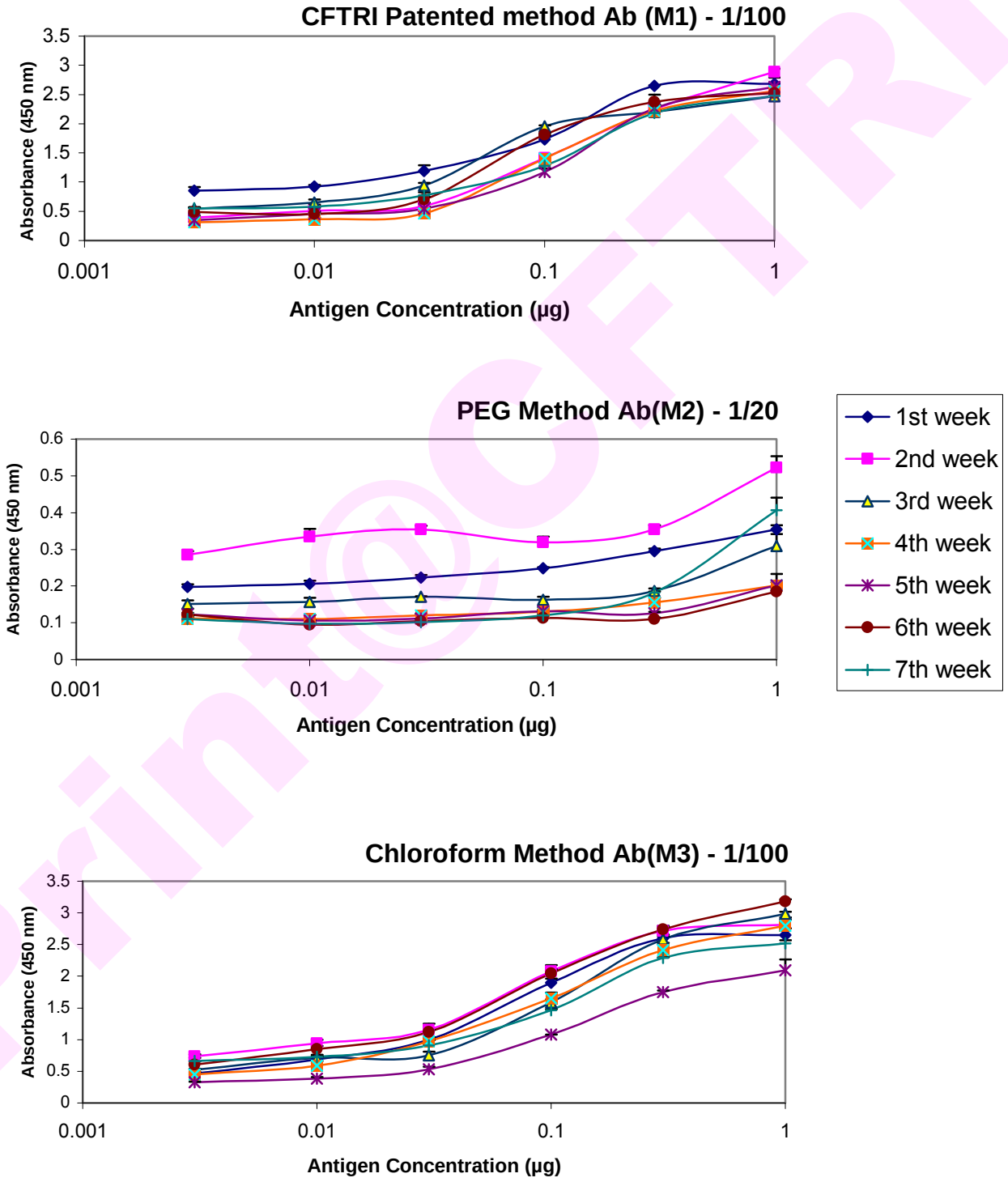
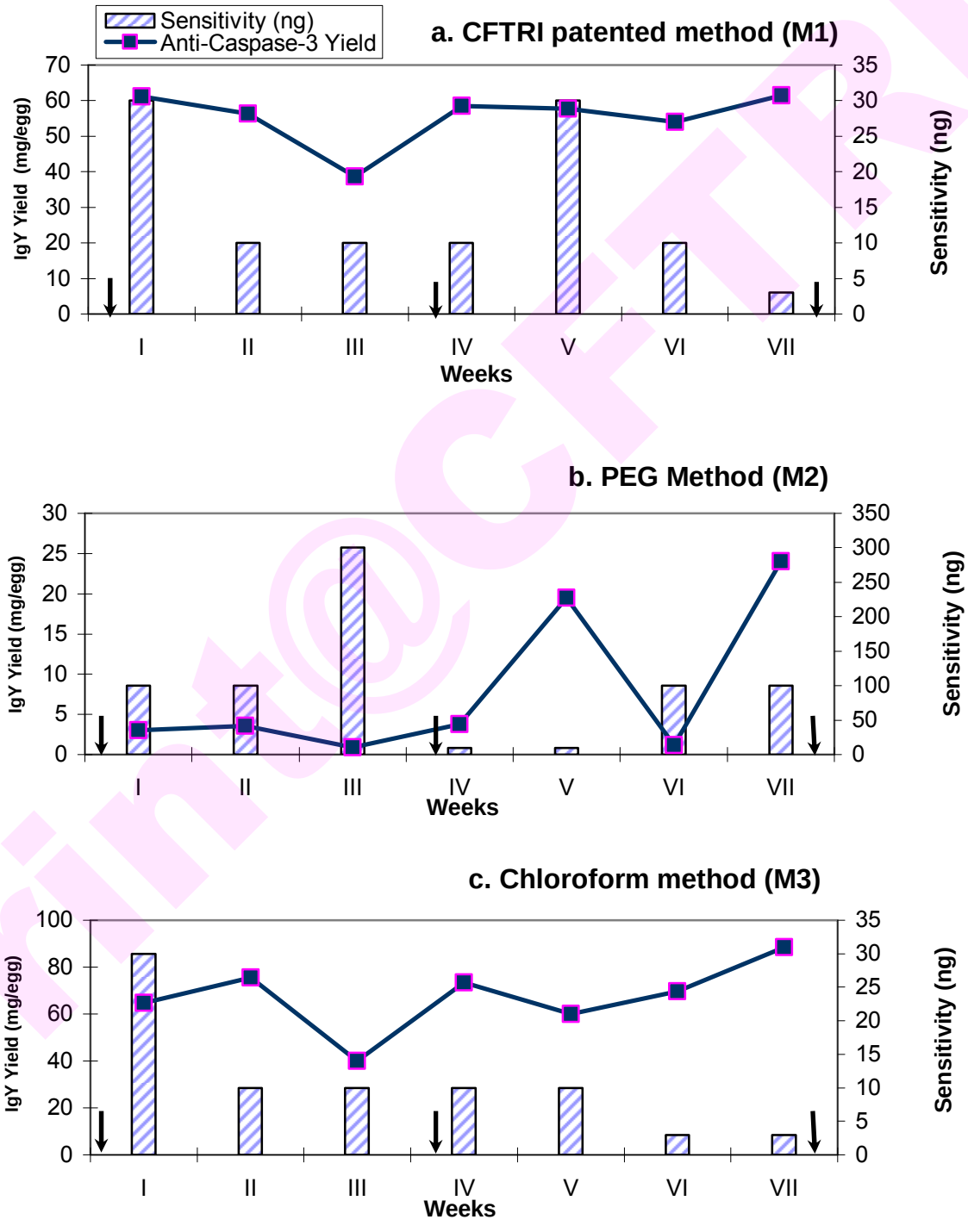


Fig. 4.4: Assay Formatting Using Caspase-3 Antibodies Obtained by Various Purification Methods



Values are mean of 5 separate experiments ± S.E.

Fig 4.5: Sensitivity vs Yield for Caspase-3 Antibodies



Arrows indicate the booster doses.

2.6 Yield of IgY against nNOS

The egg-yolk antibody production against nNOS gave the following yields:

a. 70 mg/egg for M1

b. 15 mg/egg for M2

c. 77 mg/egg for M3

In the case of porcine nNOS, M1 had an average yield of 70 mg/egg for the 7 weeks. Purification by M3 gave 77 mg/egg while a yield of 15 mg/egg was obtained by the M2 method (Fig 4.6 a). Thus, in both caspase-3 and nNOS, M3 was found to be better in terms of antibody yield. A slightly higher average yield was found in case of IgY against porcine nNOS compared to caspase-3 IgY. The yield of porcine IgY across 12 weeks showed that the average yield was 60 mg/egg, which was slightly lower than the average yield for the 7 weeks by M1 (Fig 4.6 b). This may be due to low IgY yield from weeks 7, 11 and 12 of 30 mg/egg in case of M1.

2.7 ACA for nNOS

The ACA for NOS revealed that the ideal IgY dilution was 1/100 for both M1 and M3 while it was 1/20 for the assay by M2 antibodies. All the 3 antibodies worked best at an antigen concentration from 3 ng till 1 µg. Hence an antibody dilution of 1/100 (M1 and M3) and 1/20 (M2) was used for the assay formatting.

2.8 Assay Formatting for nNOS

The assay was formatted using IgY extracted from 3 different methods (Fig 4.7) as discussed earlier in section 2.2. The assay based on antibodies obtained from M1 was able to detect up to 3 ng of the antigen and the linear range was from 300 ng to 3 ng (Fig 4.7 a). The IgY antibody titre used for antibodies obtained from M1 was 1/100 and 1/5000 of IgY-HRP.

Antibodies from M3 method were very similar to M1 in detecting a minimum of 3 ng of porcine NOS (Fig 4.7 c). An antibody titre of 1/100 was used for M3 IgY antibodies and the linear range was found to be 300 ng to 3 ng.

A higher dilution of 1/20 was required for M2. The sensitivity and titre of M2 was poorer compared to the other two methods (Fig 4.7 b). M2 was able to detect 10 ng of porcine nNOS antigen at 1/20 antibody dilution for the 1st and 2nd weeks while IgY from the rest of the weeks obtained by M2 could detect only 30 ng of the antigen (Fig 4.7 b).

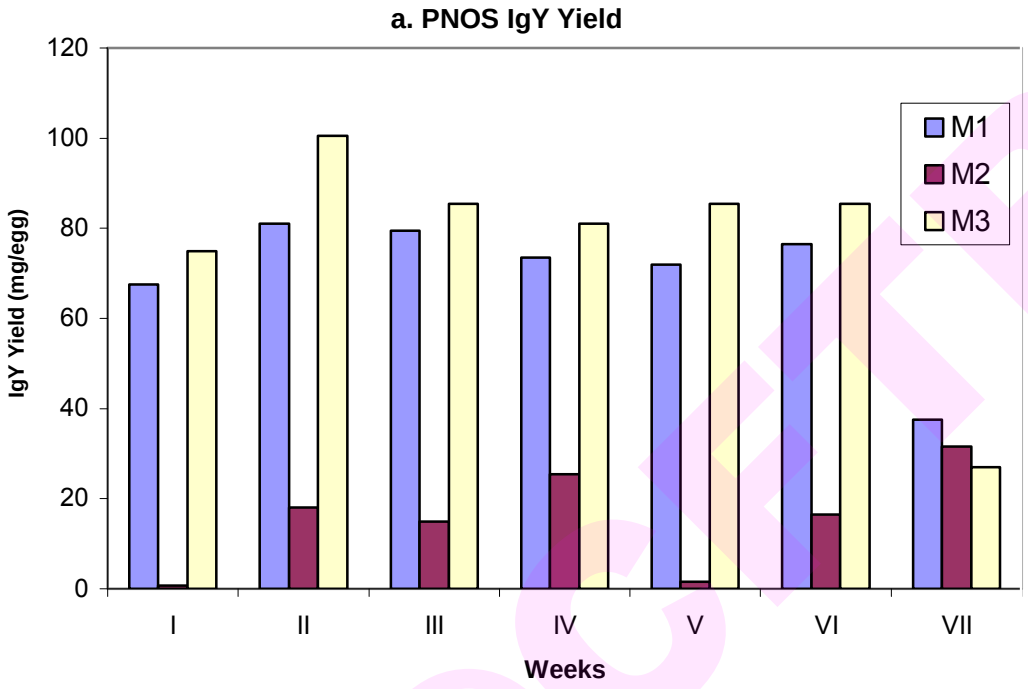
2.9 Yield vs Sensitivity for nNOS:

The sensitivity of the nNOS IgY and its relationship to the yield for all the 3 methods is shown in Fig 4.8 a, b and c. It reveals that M1 had a constant yield of 75 mg IgY/egg over the 7-week period. In the 7th week its yield was low at 45 mg/egg. M1 had an initial sensitivity of 30 ng in the first week but the sensitivity increased to 1 ng in the 2nd week. The M1 IgY had an overall sensitivity of 10 ng for all the weeks.

M3 IgY yield for porcine NOS was maintained at an average of 80 mg/egg for all the 7 weeks and only in the 7th week, it decreased to 40 mg/egg. M3 IgY sensitivity showed that it had an overall sensitivity of 8 ng for all the weeks but only in the 4th week, the sensitivity dropped to 30 ng (Fig 4.8 c).

M2 IgY yield was around 15 mg/egg for all the weeks with marginal increase in the 4th and 7th weeks yield. M2 IgY had an initial sensitivity of 10 ng for the 1st and 2nd weeks but it decreased to 30 ng by the 3rd week and remained at 30 ng level for the remaining weeks (Fig 4.8 b).

Fig 4.6 Weekly Yield of Porcine nNOS IgY



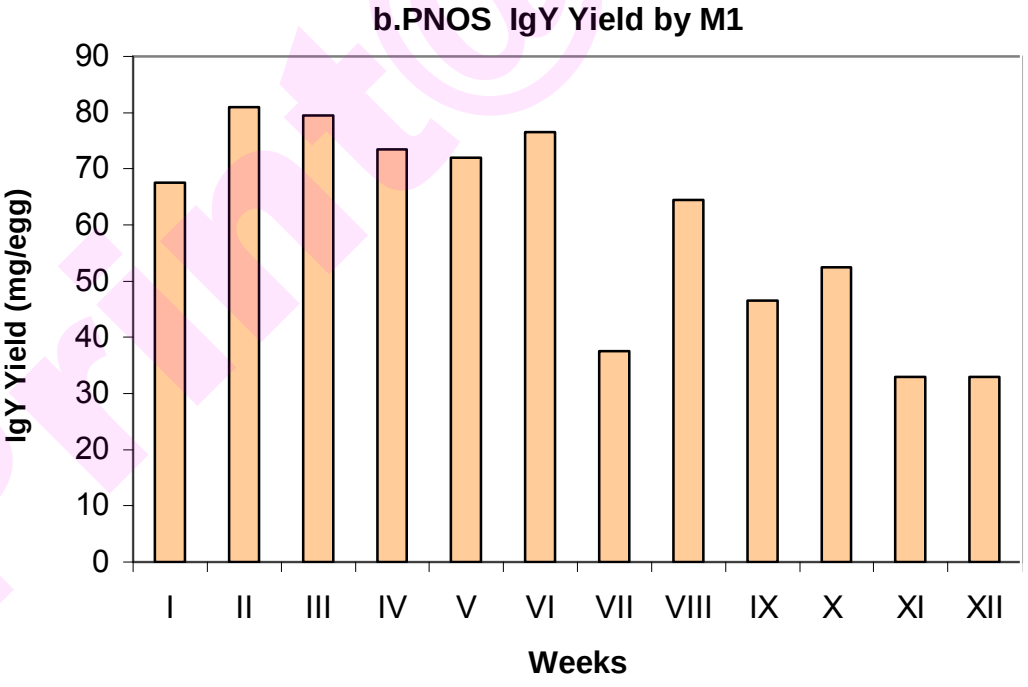
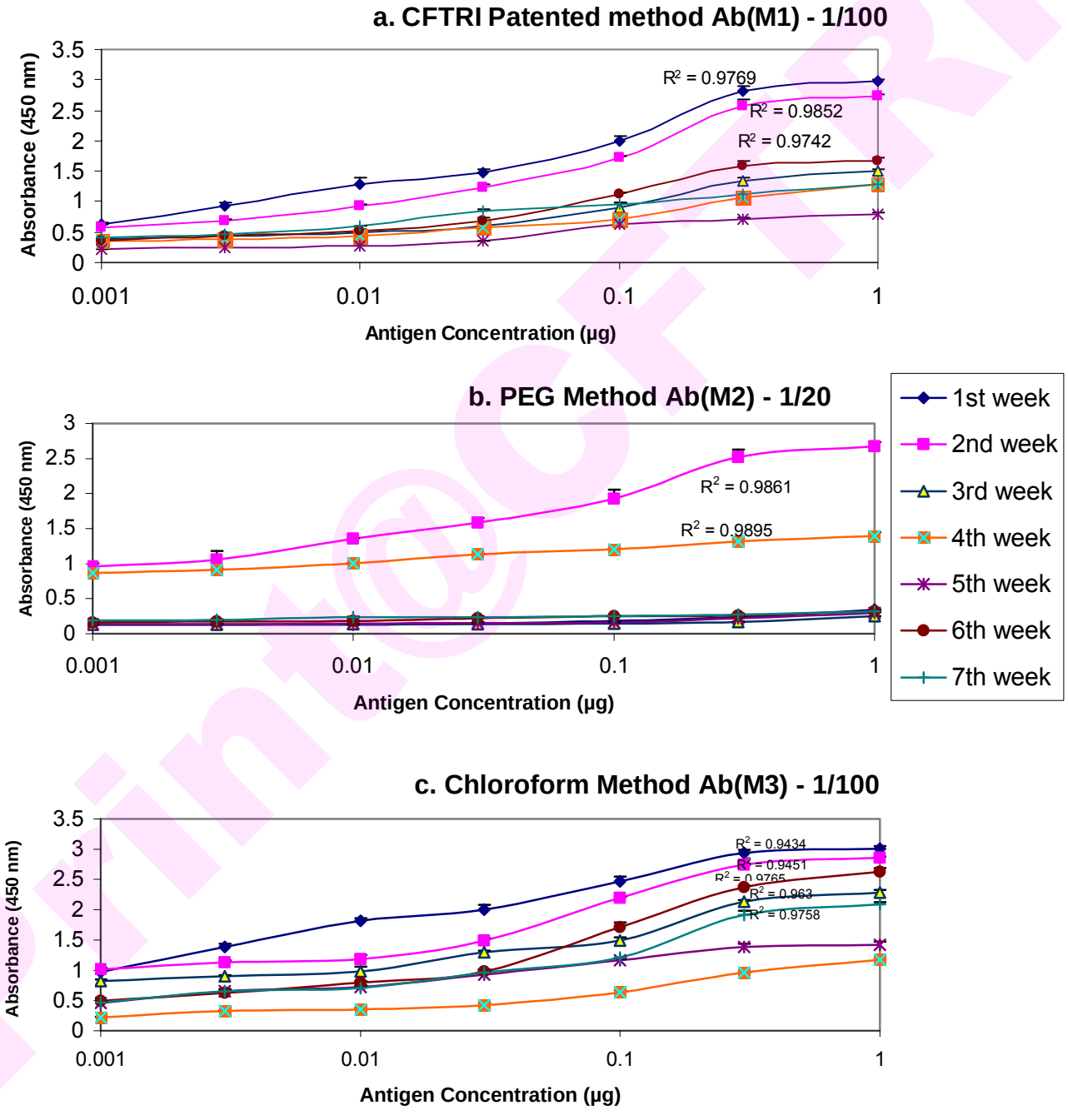
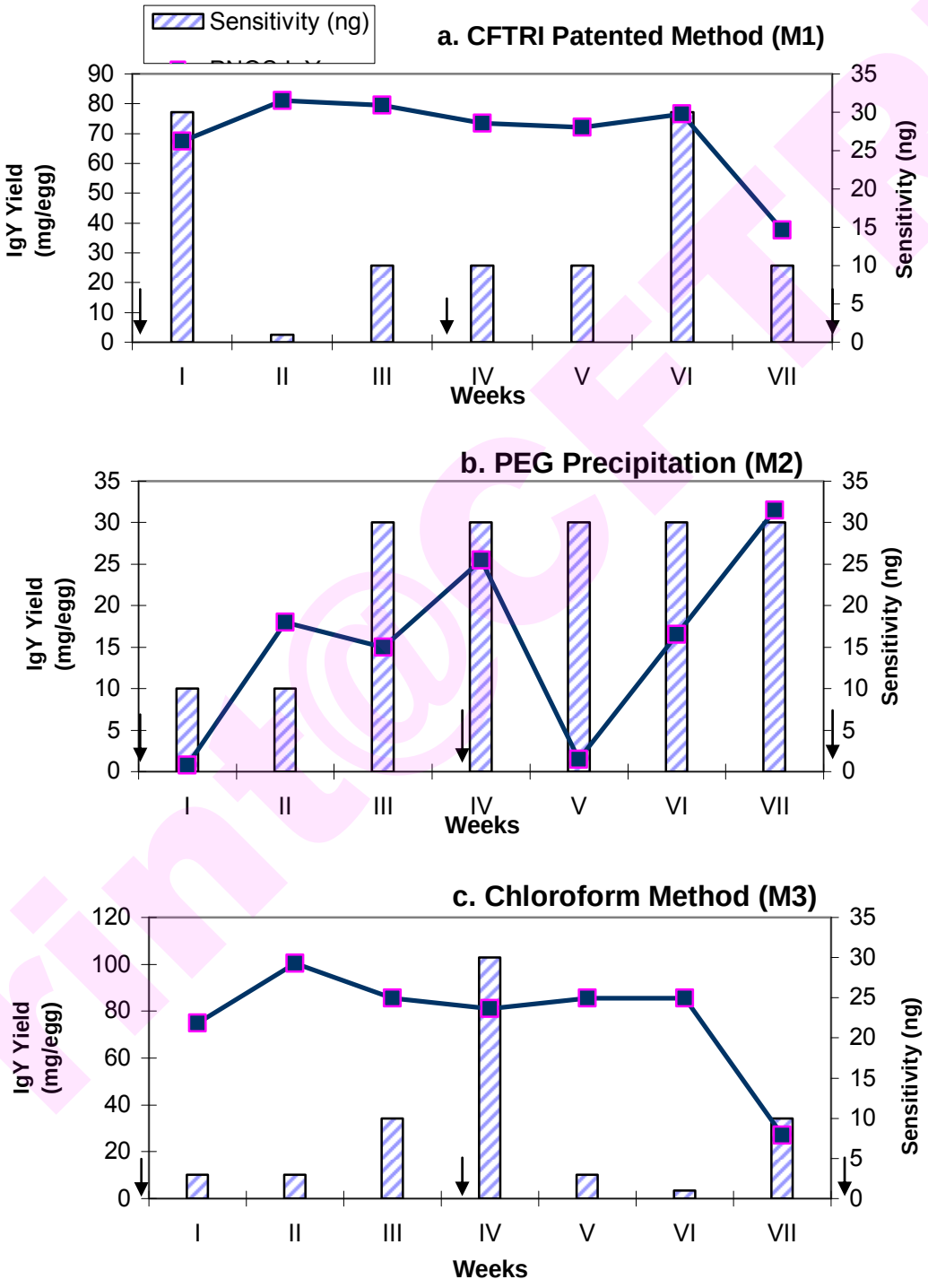


Fig. 4.7: Assay Formatting Using neuronal NOS Antibodies Obtained by 3 purification Methods.



Values are mean of 5 separate experiments ± S.E.

Fig 4.8: Sensitivity vs Yield for porcine NOS IgY Antibodies



Arrows indicate the Booster doses.

2.10 Comparison between IgY and IgG-based immunoassay for detection of the biomarkers

a. Yield:

On comparing the yield of the antibodies, IgY yield ranged from 60-70 mg/egg or 5-6 g/hen in both Casp-3 IgY and nNOS IgY against 60 mg/bleed or 180 mg/rabbit. Hence, the yield of IgY antibody was almost 30 times more than the yield of IgG from rabbits. Since the IgY yield is much higher than the yield of rabbit antibody, the number of assays that can be performed with IgY is more than 180 times higher than IgG (Table 4.1). This means that once the IgY is produced, it can be stored at -20^{0}C without loss of activity for a longer time and there is no need to go back to the animal for further immunization.

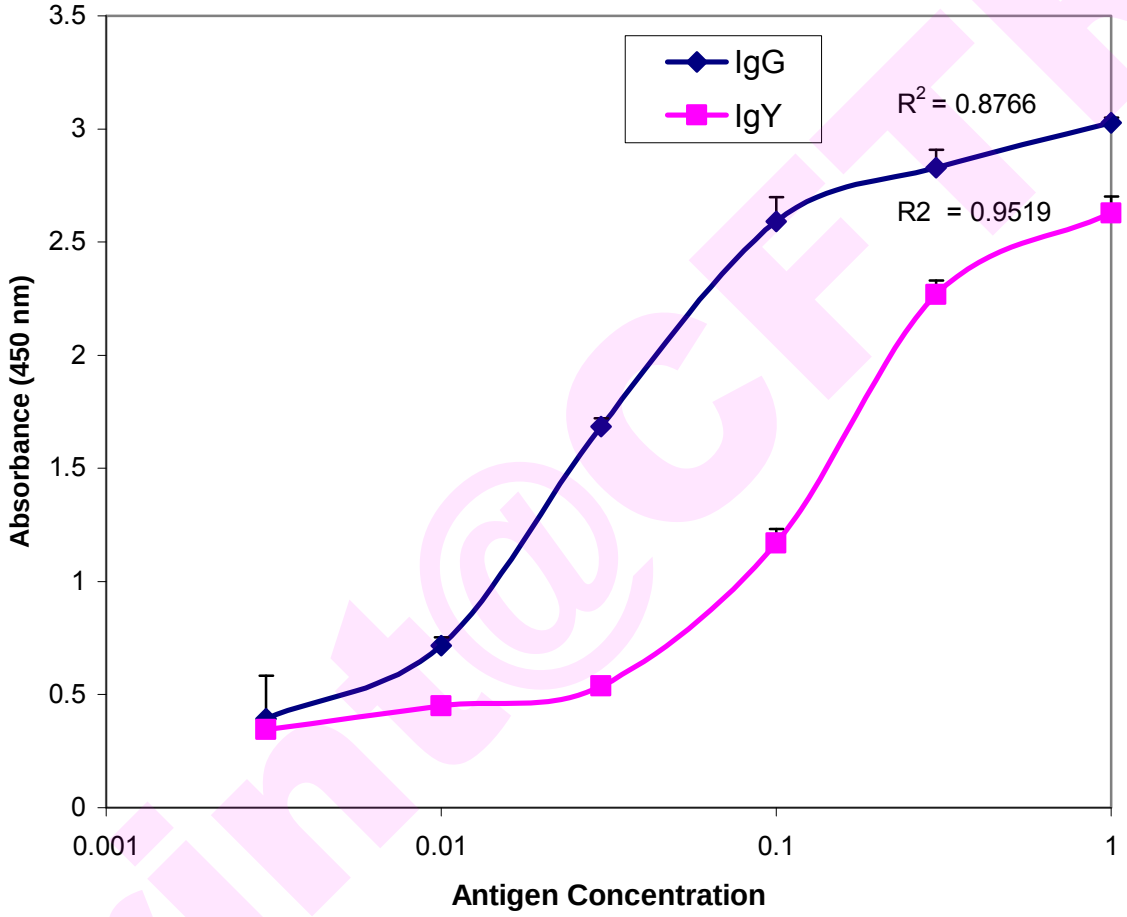
b. Sensitivity:

Comparison of the results of IgY and IgG-based assays for caspases and nNOS samples indicates that both the antibodies were comparable in their sensitivities. Both IgG and IgY antibodies could detect up to 3 ng of the antigen (Fig 4.9) The linear range was also the same for both the antibodies from 300 ng to 3 ng of the antigen. Thus, both IgY and IgG worked at a wide range of antigen concentrations. The purification methods of IgY using M1 and M3 holds equally good for IgG. These observations imply that the sensitivity of the antibodies is similar irrespective of their origin whether it is hen egg or rabbit blood in this particular study.

c. Cost:

The costing of IgY antibodies reveals them to be very economical compared to mammalian antibodies. The cost of one assay kit for IgY works out to 25 paise compared to Rs 47 and Rs 25 for rabbit IgG and monoclonal antibodies respectively (Table 4.1). Hence, IgY antibody is 200 times more cheaper than IgG antibodies.

Fig 4.9: Comparison Between IgG and IgY based ELISA



Values are mean of 5 separate experiments ± S.E.

Table 4.1: Costing of Antibodies from different sources

	Rabbit	**Mouse**	**Hen**	
Cost of animal	75	60	100	
Cost of antibody production – immunisation , purification etc	1000	1000	1000	
Cost of rearing for 6 months	600	300	600	
Cost of assay performance	1000	1000	1000	
Total cost rounded off	2675	2360	2700	A
Infrastructure cost	2675	50,000	2700	B
Total A+B	5350	52,360	5400	C
	Rabbit	Mouse	Hen	
Overhead charges @ 15% of C	802.5	7854	810	D
Total C+ D	6152	60,214	6210	E
Corporate expenditure @ 100% of E	6152	60,214	6210	F
Total E+F	12,304	1,20,428	12,420	G
Profit @ 100% of G	12,304	1,20,428	12,420	H
Total cost of G+H rounded to	25,000	2,50,000	25,000	I
	Rabbit	Mouse	Hen	
Antibody obtained in one cycle of immunization	52.68 mg	1000mg	9630.5 mg	
Ab required for one assay	1ug	1ug	1ug	
Total assays possible (nos.)	52,680	10,00,000	96,30,500	J
Cost of one assay (I/J)	25,000/ 52,690 = 0.47	2,50,000/ 10,00,000 = 0.25	25,000/ 96,30,500 = 0.00259	
Cost of Ab required for one plate	100 X .47= Rs 47	100 X .25= Rs 25	100 X .00259 = 25 paise	
Cost of one lab kit containing one Ab coated plate	**Rs 47= 1$**	**Rs 25 =0.5$**	**25 paise = 0.5319 cents.**	

Source: Amitha rani *et al*, 2006.

3. DISCUSSION

Antibodies have been used for the quantitation and detection of a whole range of substances. The specific recognition between antigens and antibodies has led to many different methods to detect various substances of human interest like viruses, bacteria, chemicals, pesticides etc. Specific antibodies can be obtained by collecting serum from vertebrates immunized with specific doses of antigens. Traditionally, the species chosen for antibody production are need based and have been taken from mammals like mice, rats, rabbits, sheep, goat, horses etc. Larger animals are generally chosen to get a higher volume of polyclonal antisera for large-scale production of antibodies. Mammalian antibodies are considered to be of high quality and have less chance of causing hypersensitive reactions. But recently there has been a growing use of hens for antibody production (Behn *et al*, 2001).

Avian antibodies differ from mammalian antibodies in various parameters. The IgY are heavier than mammalian IgG due to an additional constant domain. The IgY antibody is taken from the egg-yolk of the hens and it has applications not only in diagnostics but also for therapeutic purposes since eggs are an edible source of proteins for humans. Eggs are being used as therapeutic sources for treatment of diseases such as dental caries (Michalek *et al*, 1987), rotavirus diarrhea (Hatta *et al*, 1993), Heliobacter pylori infection (Horie *et al*, 2004), Escherichia coli infection (Girard *et al*, 2006) etc. The egg yolk contains 3 different classes of antibodies: the IgG/IgY, IgM and IgA. IgY is the major

constituent making up 6 mg/ml while IgM and IgA make up 1.3 and 0.6 mg/ml respectively (Rose *et al*, 1974). Due to the high quantity of IgY in egg-yolk, some researchers recommend little or no yolk IgY extractions (Erhard *et al*, 1996). But in the present study to develop an ELISA, there was a need to remove the interfering substances and reduce non-specific binding to eliminate false positive results.

The IgY purification method developed was comparable with the IgG method described in chapter 3, Section A for both caspase-3 and nNOS antigens. The antibodies from mammalian serum have been in popular use for a long time in immunoassays but currently IgY technology is gaining popularity due to its distinct advantages. Earlier, IgY methods were developed as a means to passive immunization therapy in humans (Hatta *et al*, 1990) but now they are being used for detection of chemicals and biologicals. IgG based assays for detection of caspase-3 and nNOS are already commercialized and readily available. IgY antibodies to these biomarkers of apoptosis have not been described earlier. This is the first report on the development of ELISA based on IgY to detect caspase-3 and neuronal NOS.

In the present study, 3 different methods were used to purify the IgY from the yolk to compare their efficiency in recognizing the antigens – caspase-3 and nNOS. Both the CFTRI patented method (M1) and chloroform method (M3) were almost similar in their binding ability and sensitivities (10 ng) and yield of IgY (60-75 mg/egg). In the case of caspase-3, the sensitivity was 3 ng for M1 IgY from

the 7th week but as the 5th week and 1st week IgY had a lower sensitivity of 30 ng, the average sensitivity for all 7 weeks reduced to 15 ng. The sensitivity of the 1st week IgY from M1 could be lower since it was just after the 1st booster but the reason for reduced sensitivity of the 5th week IgY could not be deduced. If the sensitivity of M1 IgY was calculated by excluding the 5th and 1st week, the average sensitivity was found to be 8.5 ng. Similarly 1st week caspase-3 IgY from M3 showed a lowered sensitivity of 30 ng decreasing the average sensitivity of M3 IgY to 11 ng from 8 ng, whereas the sensitivity of the 6th and 7th week M3 IgY was 3 ng. As seen in Fig 4.5 a and 4.5 c, IgY purified by both M1 and M3 recognized caspase-3 at ~ 8 ng level, which showed that both were comparable in their sensitivities. The yield of IgY was also high by both the methods at 65-75 mg/egg for caspase-3 IgY antibodies. The yield of caspase-3 IgY did not change due to the boosters in the case of M1 and M3 (~70 mg/egg) and only a marginal decrease was seen in the 3rd week (40 mg/egg). The sensitivity was found to decrease with the boosters but overall it increased over the entire 7 weeks period in both M1 and M3 IgY antibodies.

On the other hand, yield of caspase-3 IgY purified by M2 increased in the 5th and 7th weeks to 25 mg/egg. This may have been due to the 2nd booster dose. But its sensitivity did not improve with the boosters and was 100 ng over the 7-week period (Fig 4.5 b). There was no observable trend of either the yield or sensitivity of antibodies purified by M2 due to the boosters. In general, it was interesting to note that the yield of Ab was more or less constant throughout the study period.

This can be attributed to nature's way of safeguarding/protecting the developing young one in the yolk against foreign substances

Porcine nNOS was recognized at a level of 3 ng by both M1 and M3 IgY (Fig 4.7 a and 4.7 c) and both had a good titre of 1/100. The average sensitivity of M1 IgY for nNOS was found to be 15 ng and after removing the deviant 1st and 6th weeks sensitivity, the sensitivity was found to be 8.5 ng. Similarly, average sensitivity of M3 IgY was 10 ng but on excluding the 4th week Ab, the average sensitivity for remaining weeks was found to be 7 ng.

The PEG (M2) method used for porcine nNOS IgY extraction did not give a good yield of IgY (average of 25 mg/egg) and hence its sensitivity was also low at an average of 30 ng for detection of nNOS and had a low titre of 1/20 (Fig 4.8 b).

The yield of M1 and M3 was unaffected by the boosters in the case of porcine NOS IgY, decreasing only in the 7th week. This decrease in the 7th week yield may have been due to reduction in the immune reaction since 3 weeks elapsed between the 2nd booster and the 7th week IgY. No observable trend was seen in the sensitivity of M1 and M3 due to the boosters and was at an average of 10 ng and 8 ng for the 7-weeks respectively (Fig 4.8 a and c). The yield of M2 decreased at the 5th week (2 mg/egg), one week after the 2nd booster but regained its average level of 30 mg/egg at the 7th week (Fig 4.8 b). The sensitivity due to the boosters remained unaffected for M2.

Since both the CFTRI patented method (using carrageenan) and chloroform method gave good yield of IgY with comparable sensitivities, the safer of the two

methods i.e. the gum method could be preferred as chloroform vapours can be deleterious to the workers. IgY fractions have been stored in 0.9% NaCl, 0,02% NaN_3 at $+4^0C$ for over 10 years without any significant loss of antibody titre (Olovsson and Larsson, 1993). Affinity-purified and biotinylated antibodies have after 5 years of storage at $+4^0C$ retained high activity (Olovsson and Larsson, 1993). The purified antibodies also retained their antigen binding capacity after 6 months at 20^0C or 1 month at 37^0C (Carlander, 2002). An egg can be stored in 4^0C, with just a small loss of IgY activity for at least six months (Ricke *et al*, 1988). Since IgY can be produced in abundance and has a very good shelf life (at -20^0C) when compared to IgG, it can be stored and used for a longer time (couple of years) without the need for raising antibodies repeatedly. Another advantage of IgY is the production of a single batch of antibodies with uniform sensitivity unlike rabbit antibodies where sensitivity could vary from animal to animal. Additionally, the antibodies from hens do not bind with mammalian Fc-receptors or rheumatoid factors nor with proteins A and G. Thus, the use of IgY antibodies rarely produces false positive reactions in immunochemical assays (Erhard and Schade, 2001).

Costing of the IgY vis-à-vis IgG also reveals the big cost benefit for these antibodies (Table 4.1). The cost of maintenance of a hen is almost the same as that of the rabbit, but it gives 30 times (5000 mg) more antibody compared to a rabbit (180 mg) for the same cost. Thus development of an IgY based ELISA (25

paise/assay) is more economical when compared to IgG based assay development (Rs 47/assay).

The non-invasive nature of IgY antibodies is another advantage since the hens do not need to be bled for extracting the antibodies as in the case of rabbits. Thus, the IgY method deserves a closer look as an alternative over the conventionally used IgG method and holds promise as a good diagnostic tool for the future.

SUMMARY:

- This is the first report of an IgY-based method for the detection of biomarkers of apoptosis i.e. the caspase-3 and nNOS.
- Of the 3 purification methods tested, the M1 (CFTRI patented method) and M3 (Chloroform method) were found to be more efficient than the M2 method (PEG method).
- Both the CFTRI patented method (M1) and chloroform method (M3) gave high yield of the IgY antibody (65-75 mg/egg) over the entire experimental period while M2 gave a low yield of 15-25 mg/egg.
- Assay development with the antibodies obtained from the 3 different purification methods revealed a sensitivity of 3 ng with M1, 10 ng with M2 and 3 ng with M3.
- IgY antibodies from the hen were produced in the range of 6-7 g/hen unlike rabbits which produce only 0.18-0.2 g/rabbit. Hence a large quantity of antibody obtained in hens spares the efforts of immunizing the animal repeatedly.
- The assay was found to be as sensitive as the IgG-based assay (chapter 3) and could detect 3 ng of caspase-3 and nNOS.
- The IgY-based method developed in the present study is non-invasive and an inexpensive alternate to IgG-based methods used for detection of caspase-3 and nNOS
- The costing per assay indicated 25 paise for IgY compared to Rs 47-IgG and Rs 25-monoclonal antibodies.

CHAPTER 5

A QUICK SEMI-QUANTITATIVE METHOD TO DETECT APOPTOTIC BIOMARKERS

INTRODUCTION

Immunodiagnostic methods need to be sensitive, cost effective, and in addition must have a high throughput, essential to screen large number of samples. Various immuno-based assays such as dipstick, tube assays and Ab-based biosensors are available for the detection of pesticides, aflatoxins etc. Dot blot is one such method. Dot blot is a simple but quick method of detection of any sample for which a preliminary, quantitative/semi-quantitative, quick analysis and screening is the requirement. They are very similar to the immunoblots but differ from them in the non-requirement of any equipment such as electrophoresis chambers and subsequent transfer by electroblotting. Here, the sample is applied directly to the blotting membrane, with subsequent probing done using antibodies specific to the antigen. Dot blots can be applied either under vacuum or directly to various membranes such as nitrocellulose or PVDF.

Dot blots are used as preliminary tests for detection and identification of an array of substances such as Plasmid DNA (Khanna *et al*, 2000), chemicals, and proteins involved in various enzymatic and non-enzymatic processes. Apoptotic proteins can also be detected by means of dot blotting. Various methods are available for the detection of apoptosis biomarkers. One report by Antonsson *et al* (2000) reports the detection of the Bax protein, a pro-apoptotic molecule and a member of the Bcl-2 family of proteins, by using a dot blotting technique. Sueur *et al* (2005) also used dot blotting for the detection of Bcl-2 and Bax proteins using specific antibodies directed against these two apoptotic

molecules. They used 100 µg of Bcl-2 and Bax protein extracts for dot blotting with an antibody dilution of 1/400.

In the present study, we had adapted dot blots as a semi-quantitative method to detect the apoptosis biomarkers of caspase-3 and nNOS at different concentrations of the biomarkers, using both commercial and CFTRI antibodies. The hue and intensity values of the dot blot images were analysed using a colour capture device (CCD) and computed using the software Matlab, Version 6.5. The CFTRI antibodies used were from two sources, namely rabbit serum (IgG) and hen antibodies (IgY). This method was developed as a quick method for detection of the biomarkers and was compared with the plate immunoassay method described in chapter 3 and 4.

1. MATERIALS AND METHODS

1.1 Chemicals:

Commercial polyclonal anti-caspase-3 from rabbit and anti-nNOS monoclonal antibody, BSA and nitrocellulose membrane were from Sigma Chemicals. HRP-labeled secondary antibodies and TMB-H_2O_2 (10x) were from Bangalore Genei, Bangalore, India. CFTRI antibodies were from two sources – rabbit and hen. They were produced as described under section 1.9 and section 1.2 in chapter 3 and 4 respectively.

1.2 Dot Blots for Caspase-3

Different concentrations of both the biomarkers, caspase-3 and porcine NOS, ranging from 10 µg to 0.03 were manually blotted onto nitrocellulose membranes (NC membrane, 3cm x 3cm, 0.45 µm pore size). The volume of the sample blotted was maintained at 5 µl by appropriate dilution of the enzyme with the respective extraction buffers (caspase-3 and nNOS extraction buffers). After air drying, the blots were incubated in 2% BSA in 10 mM Tris--NaCl (TN) buffer (pH 7.4) for 1 h at 37^0 C. The Blots were then washed 3 x with TN buffer containing 0.05% Tween-20 (TTN Buffer for 5 min). For standardization of the antibody dilution, the blots were incubated with the primary antibody at 1:1000, 1:2000 and 1:3000 dilutions, for 2 h at 37^0 C, with constant shaking. Blots were again washed 3x with TTN buffer (5 min) and incubated with HRP-labeled secondary antibody at 1:1000 and 1:5000 dilutions for 2 h at 37^0 C. A commercially available rabbit polyclonal anti-caspase-3 and the antibodies raised in chapter 3 (IgG) and chapter 4 (IgY) were used as the primary antibody.

Commercial goat anti-rabbit IgG-HRP conjugate and rabbit anti-chicken IgY-HRP conjugate were used as the secondary antibodies for IgG and IgY dot blots respectively. The blots were once again washed 3x with TTN Buffer (5 min) and developed in insoluble TMB/H_2O_2 solution for 10 min. The colour developed was imaged on a colour capture device (CCD) linked to a digital camera and the intensity values were compared to the concentration of antigen used.

1.3 Dot Blots for nNOS

Dot blots for nNOS samples were carried out as described above in section 1.2. Different antigen concentrations ranging from 9 to 0.03 µg were blotted onto the NC membrane. Commercial anti-NOS antibody (from mouse ascites fluid) and CFTRI antibody at different dilutions (1:20 to 1:2000) were used to determine optimum conditions for the dot blots. CFTRI antibodies were from two sources: rabbit (IgG) and hen (IgY) which were obtained as described under section 1.9 and 1.2 in chapter 3 and 4 respectively. The NOS obtained from experimental rats (treated with deltamethrin) was tested against the IgG. The dot blots were probed using commercial anti-mouse and anti-rabbit HRP-labeled antibody (1:1000 and 1:4000 dilutions). The blots were developed using a commercial TMB/H_2O_2 solution. The colour developed was captured using a digital camera and analysed for hue and intensity values.

1.4 Computation of CCD Imaging of Dot blots

The dot blot images were captured using a digital camera and scanner, converted to JPEG images and analyzed. Matlab from Mathworks USA, R13

version 6.5 was used for Image processing. The high level description of the steps followed in the image processing operation are listed below:

1. Acquisition of Image (direct acquisition using cams, opening an existing file, images acquired from mathematical operations).

2. Preprocessing of the image:

a. Format Conversion.

b. Normalization based on histograms.

c. Reduction of background noise levels.

d. Selection of Interested area of the Image.

3. Processing on the Image:

a. Extraction of Edge, Contour and other properties.

b. Extraction of Features from the Image (Area, Centroid, Extent, ConvexHull, etc)

c. Performing Specific operations like BLOB Analysis, Pattern Matching, Filtering, etc.

4. Computation of Results based on the processed details.

a. Observing the trend of a particular Parameter.

b. Predicting the value or extent or boundary based on the observed trend.

c. Classification of the Image or only the ROI.

d. Writing the processed image in to a new file.

e. Generation of the Report based on the Processing and Computations.

The hue and intensity values of the images were analysed.

2. RESULTS

2.1 Dot Blots for Caspase-3

Dot blots for caspase-3 using the commercial anti-caspase-3 showed that the optimum antibody concentration for blotting of caspase 3 was 1:1000 of primary antibody and 1:5000 of secondary antibody-HRP dilution (Fig 5.1 a). At higher secondary-antibody HRP dilutions (1:1000), there was lot of background colour and hence the spots merged with the background colour. The assay was able to recognize up to 30 ng of the protein. It was visually observable that the colour of the dot blot decreased in intensity with the decrease in concentration of the antigen used.

The dot blots with the CFTRI rabbit and hen antibodies gave similar results as the commercial antibody. As seen in Fig 5.1 b, the CFTRI IgG was equally sensitive in the detection of caspase-3 and was able to detect upto 30 ng of the antigen at an antisera dilution of 1/500. Studies with CFTRI IgY at a dilution of 1/20 were able to detect caspase-3 up to 30 ng (Fig 5.1 c). Thus, both IgY and IgG obtained in the present study were as sensitive as the commercial antibody and comparable.

2.2 Dot blots for nNOS samples

Dot blots for the porcine nNOS samples with the commercial anti-nNOS antibody revealed that the commercial antibody was able to recognize up to 100 ng of the antigen blotted on the NC membrane at a primary antibody dilution of 1/1000 for both porcine (Fig 5.2 a) and rat NOS (Fig 5.3 a) and secondary Ab-HRP dilution

of 1/4000. Here too, a concentration-dependant decrease in colour intensity was observed with decrease in concentration of nNOS used.

The commercial antibody against the rat nNOS was able to recognize 100 ng of the antigen at a dilution of 1/1000 at an HRP dilution of 1:5000 (Fig 5.3 a). IgG against rat NOS had a sensitivity of 100 ng as seen in Fig 5.3 b which was identical to the sensitivity of the commercial antibody. IgG against rat NOS was used at the same antisera dilution of 1/1600 as the antisera against porcine NOS.

The dot blots for NOS using CFTRI IgG and IgY gave similar results. IgG and IgY against porcine NOS were able to detect up to 100 ng of the antigen (Fig 5.2 b and c), comparable to the commercial antibody. IgG was used at an antisera dilution of 1/1600, while IgY antibody was used at a dilution of 1/20. It was observed that with IgY there was minimal background.

Fig 5.1: Dot Blots for Caspase-3

a. Commercial Antibodv.

Ag Conc. (ug): 10 5 3 1 0.3 0.1 0.03

Primary Ab Conc.: 1/1000 commercial Ab

b. Rabbit Antibodies (IgG)

Ag Conc. (ug): 3 1 0.3 0.1 0.03 0.01

Primary Ab Conc.: 1/500 IgG Antisera

c. Hen Antibodies (IgY)

Ag Conc. (ug): 3 1 0.3 0.1 0.03 0.01

Primary Ab Conc.: 1/20 IgY Antibody

All blots are at dilution of 1/5 K secondary Ab-HRP.

Fig 5.2 - Dot Blots for porcine nNOS

a. Commercial Antibody

Ag Conc. (µg): 9 3 1 0.3 0.1

Primary Ab Conc.: 1/1000 commercial Ab

b. Rabbit Antibodies (IgG)

Ag Conc. (µg): 9 3 1 0.3 0.1 0.03

Primary Ab Conc.: 1/1600 IgG Antisera

c. Hen Antibodies (IgY)

Ag Conc. (µg): 9 3 1 0.3 0.1 0.03

Primary Ab Conc.: 1/20 IgY Antibody

All blots are at a secondary Ab-HRP dilution of 1/4 K

Fig 5.3: Dot Blots for Rat nNOS samples

a. Commercial Antibody

Ag Conc. (µg): 9 3 1 0.3 0.1

Primary Ab Conc.: 1/1000 commercial Ab

Secondary Ab-HRP dilution: 1/5 K

b. Rabbit Antibodies (IgG)

Ag Conc. (µg): 9 3 1 0.3 0.1 0.03

Primary Ab Conc.: 1/1600 IgG Antisera

Secondary Ab-HRP dilution: 1/4 K

2.3 CCD Imaging of Dot Blots

The results of the CCD imaging are shown as Fig 5.4, 5.5 and 5.6. In general, it was seen that evaluating the image using intensity as a parameter was better than considering hue. Imaging of caspase-3 dot blots based on IgG and IgY showed IgY to be more sensitive. With increasing concentration of caspase-3, there was a distinct decrease in the intensity using IgY (Fig 5.4 b). However, with IgG, a drop in the intensity was seen only at 3 µg (Fig 5.4 a). Dot blot analysis of porcine nNOS by CCD imaging showed that CFTRI antibodies were better than commercial antibodies (Fig 5.5). However, as shown in Fig 5.6, commercial antibodies are better than CFTRI rat nNOS antibodies.

These results correlate with the ELISA cross-reactivity studies of rat and porcine NOS, which indicated that porcine antibodies were more sensitive than rat antibodies.

Dot blots were comparable both visually and by CCD imaging. However, the advantage of CCD imaging is that it is quantifiable unlike dot blots which are semi-quantitative.

2.3 Comparison of Dot Blot and ELISA for the biomarkers

Table 5.1 shows the comparison between the sensitivity of ELISA and dot blots in detecting the biomarkers. ELISA was about 10 times more sensitive than dot blots in detection of caspase-3. ELISA had a sensitivity of 3 ng for caspase-3 using commercial and CFTRI antibodies. The dot blots on the other hand, were able to detect 30 ng of caspase-3 using both commercial and CFTRI antibodies.

The detection limit was 100 ng in the case of porcine and rat NOS using the dot blots but ELISA had a greater sensitivity of 3 ng. Thus, ELISA for nNOS was about 33 times more sensitive than the dot blots. The advantage of dot blots was that the same antibody and HRP dilution used for ELISA could be put to use in dot blots too. Since this was a colour based system, a fluorescence technique could be more sensitive in the detection,

Table 5.2 shows the costing comparison of dot blots versus ELISA using various parameters. ELISA requires costly equipment and infrastructure but dot blots have no such requirement. The number of assays possible per hour are 200 for both dot blots and ELISA. Costing of the membrane and plates are not very different. Dot blots can be performed by untrained personnel while ELISA requires a semi-trained person to do it. ELISA plates are not easily biodegradable since they are made of plastic polystyrene while NC membranes used in dot blots are eco-friendly and biodegradable.

Fig 5.4: CCD Analysis of Caspase-3 dot blots.

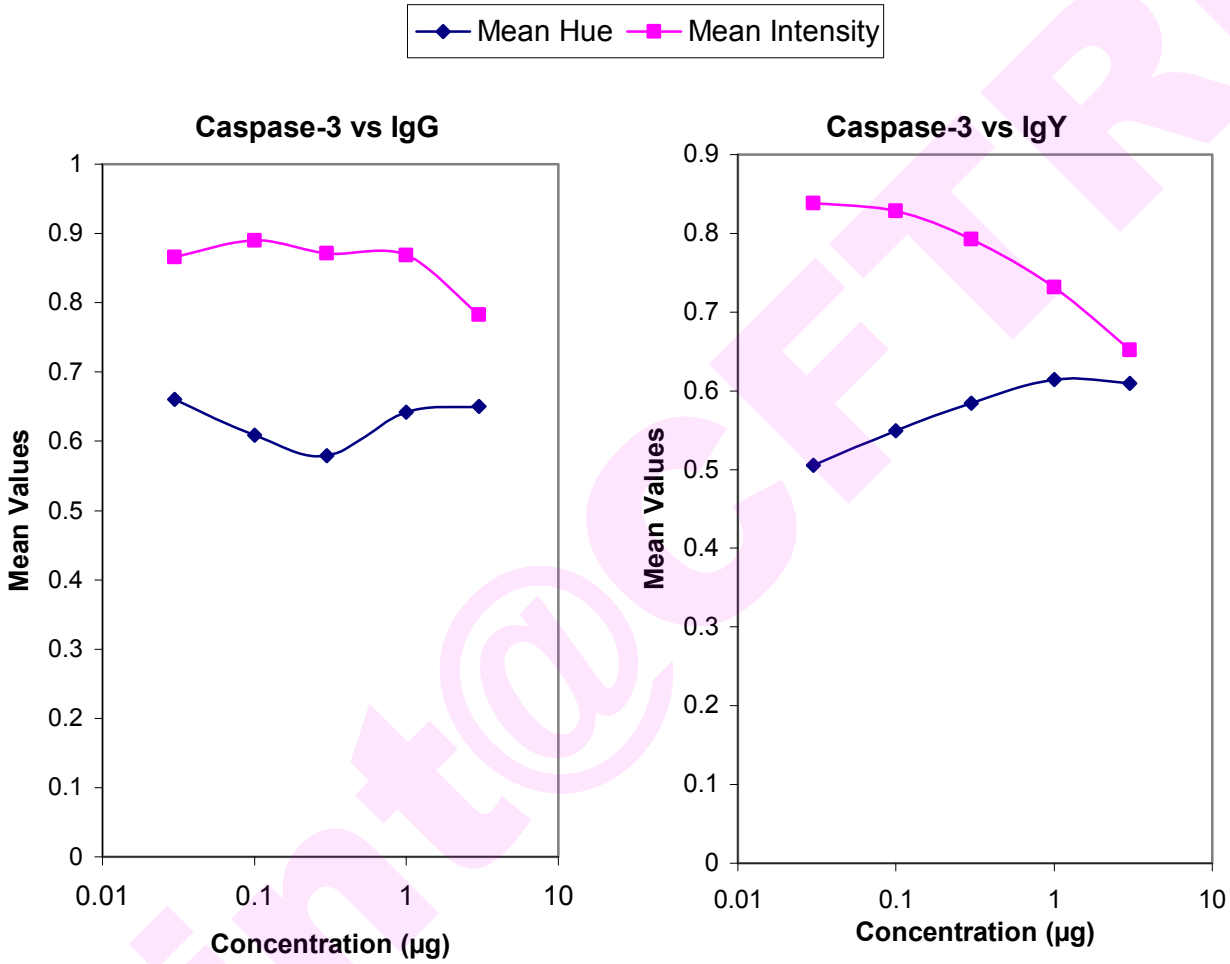


Fig 5.5: CCD Analysis of PNOS dot blots.

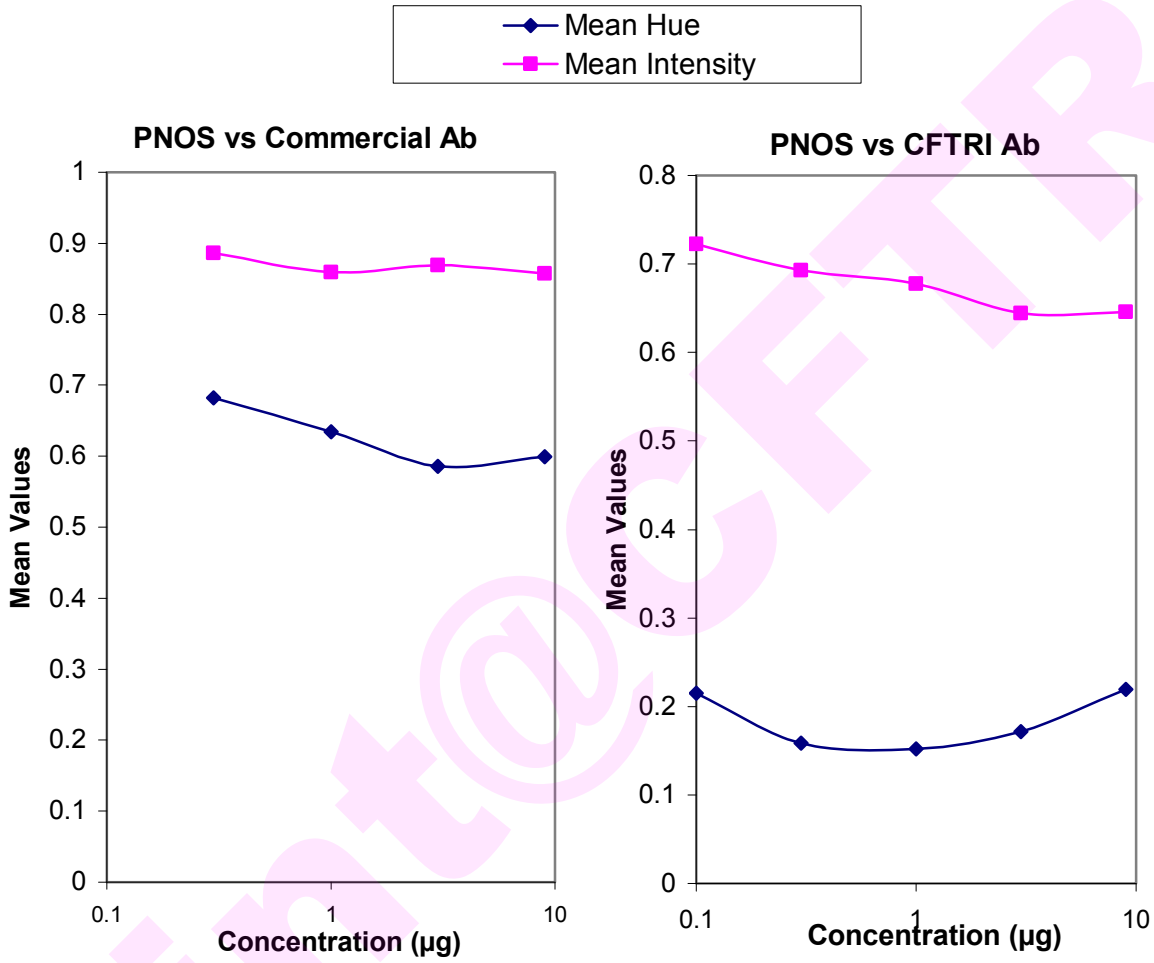


Fig 5.6: CCD Analysis of RNOS dot blots.

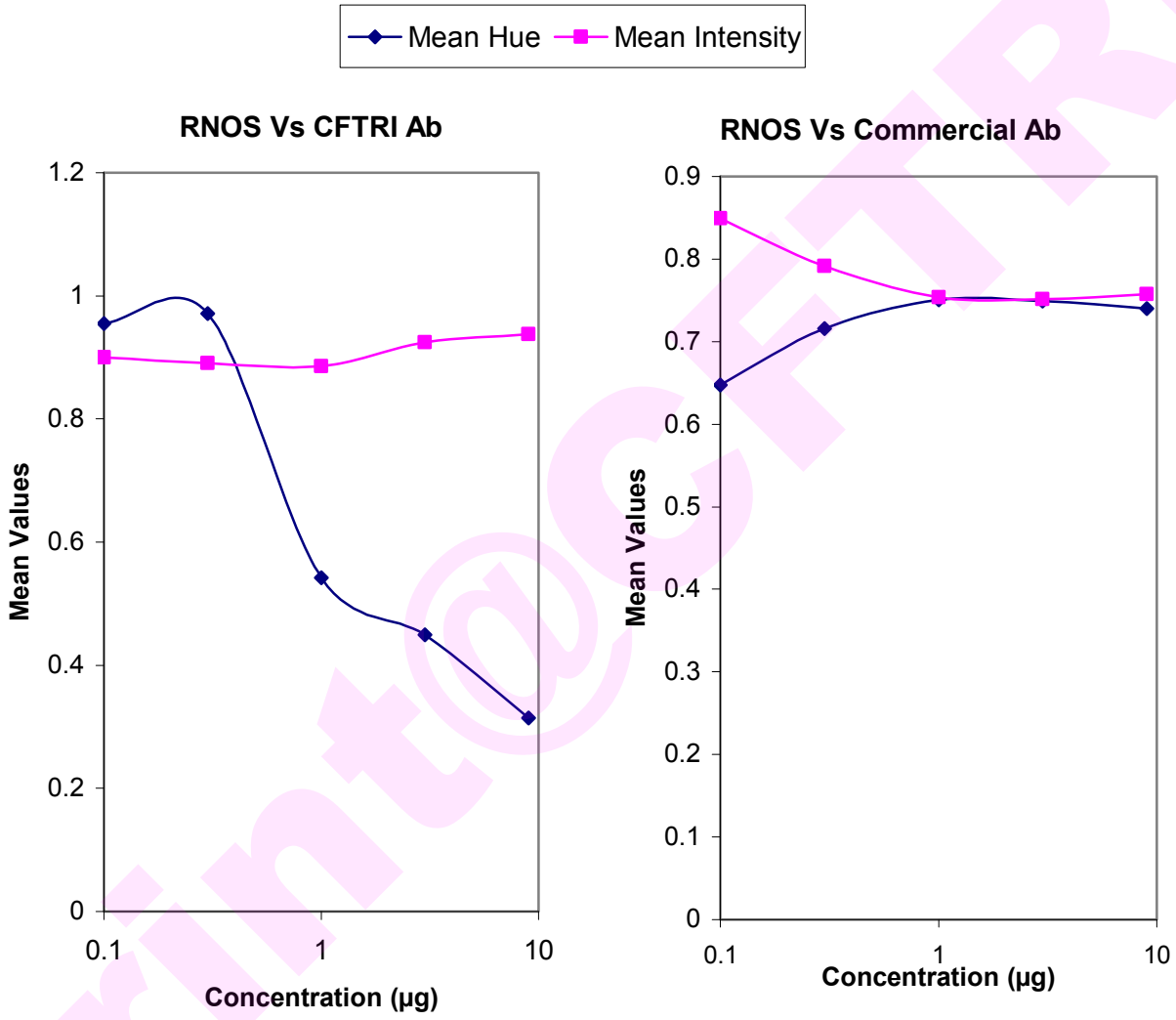


Table 5.1: Comparison between sensitivity of ELISA and Dot Blots

ASSAY	ELISA (ng)	DOT BLOT (ng)
Caspase-3		
1. Commercial Ab	3	30
2. IgG (Rabbit)	3	30
3. IgY (Hen)	3	10
Porcine nNOS		
1. Commercial Ab	3	100
2. IgG (Rabbit)	3	100
3. IgY (Hen)	3	100
Rat nNOS		
1. Commercial Ab	3	100
2. IgG (Rabbit)	3	100

Table 5.2: Comparison of dot blots and ELISA

PARAMETER	ELISA	DOT BLOT
Equipment	**+**	**-**
Sensitivity	Quantitative	Semi-Quantitative
No. of assays/h	200	200
Cost	Plate - Rs100/-	NC Memb- Rs 300/- (per strip)
Reagent cost	200/-	200/-
Technical training	Semi-trained	untrained
Power	**+**	**--**
Disposable	plastic	Paper (biodegradable)

3. DISCUSSION

Dot blots are used in conjunction with western blotting for detection of various substances of biological or chemical origin. Dot blots are relatively simpler compared to ELISA due to its ease of use and visual comparison of the results. Dot blots also need much lower level of skill and expertise when compared to other immunoassays and have no requirement for costly equipment. It can be used as a screening method to quickly assay the working range of an antibody reaction and also as a Yes/No test to determine the presence of any antigen. Dot blots have been used for detection of various intermediates in apoptosis as well. One report by Khanna *et al* (2000) used dot blotting for confirmation of cDNA clones involved in apoptosis in rat thymocytes. There are various immunoblot studies for detection of apoptotic proteins such as caspases-3, 8 and 9, Bcl-2, Bax and poly-(ADP-ribose)-polymerase (PARP) as described by Park *et al* (2000) and Wundrich *et al* (2006). Most of them used polyclonal antibodies in dilutions ranging from 1/500 to 1/7000. In our study, we have used 1/1000 of the antibody dilution to obtain a good detection system for both caspase-3 and nNOS using dot blotting.

The advantages of dot blotting are many. Firstly, a very small quantity of antibody is required for the detection. Secondly, many samples utilizing the same antibody can be analysed at the same time in a single assay. This makes dot blotting a quicker method of analysis with high throughput, economical and comparable to ELISA in this aspect. Thirdly, costly equipments, as in ELISA, are

not required and hence dot blotting can be performed with a minimal infrastructure.

As seen in the present study, up to 30 ng of the caspase-3 biomarker antigen can be detected by this method, which makes it less sensitive than the immunoassay developed in chapter 3, Section A and chapter 4. Based on the concentration of the antigen, the intensity of the colour in the dot blot varies. Hence, this change in colour intensity can be utilized to develop a chart or visual aid for easy visual comparison and determination of the antigen concentration present in the sample. It can also be linked up to a colour-based sensor, which will allow for accurate determination of the antigen concentration.

In this study, it was seen that the dot blot method was comparable to the ELISA method developed in chapter 3 and 4. IgG antisera dilution (1/6400) and IgY dilution (1/20) were the same dilutions as used in the immunoassays in chapter 3 and 4. In the case of porcine and rat NOS, dot blotting could detect 100 ng of the antigen, which is lesser compared to 3 ng by ELISA (Table 5.1). But the IgG dilution at 1/1600 and IgY dilution at 1/20 was the same as used in ELISA. Since dot blotting has been used here as a semi-quantitative method, its similarity to ELISA in sensitivity and antibody dilutions makes it a quick, easy and reliable alternative to ELISA.

This is the first report on CCD imaging for caspase-3 and nNOS. The dot blots were comparable with both the methods of analysis, viz. visually and by

CCD imaging. CCD imaging had the added advantage of being a quantitative method.

Hence, it is seen that dot blotting for caspase-3 and nNOS can be a quick, reliable and inexpensive alternative screening method for these two biomarkers. It is also comparable to the immunoassays developed in chapter 3 and 4 and can serve as an important semi-quantitative diagnostic tool for the detection of these biomarkers of apoptosis.

SUMMARY:

- Dot blots for the biomarkers could detect up to 30 ng of caspase-3 and 100 ng of nNOS.
- The primary antibody and 2^0-Ab-HRP dilutions used for ELISA in chapter 3 and 4 were applicable for the dot blot methods also.
- Dot blots were found to be a comparable alternative to ELISA, but as a semi-quantitative method and as a quick yes/no test for caspase-3 and nNOS.
- However, cost wise, ELISA and dot blots were similar.
- This is the first report of a CCD imaging method for analysis of caspase-3 and nNOS.
- CCD imaging enabled quantification of the dot blots and the sensitivity was comparable to dot blots and ELISA.

CONTRIBUTION OF THE THESIS

This is the first reported study on the:

- Apoptotic potential of cyhalothrin
- Use of IgY antibodies for detection of caspase-3 and nNOS
- Dot blots and CCD image analysis for caspase-3 and nNOS.

The other highlights of the thesis are:

- ✓ Among the two pyrethroids used, Deltamethrin was found to be apoptotic at 200 mg/kg b.w. in young female CFT-Wistar rats but Cyhalothrin did not show evidence of apoptotic changes in both adult and young rats.
- ✓ Apoptosis could be a safety mechanism resulting in low toxicity of the pyrethroids.
- ✓ Residues of Cyhalothrin and its metabolite were found to preferentially accumulate in the reproductive organs besides the liver and adipose.
- ✓ An ELISA method developed in this study using IgG from rabbits had a sensitivity of 3 ng for detection of both caspase-3 and nNOS, at a titre and sensitivity comparable to the commercial antibody.
- ✓ The ELISA method developed here using IgY had a similar sensitivity (as the IgG) of 3 ng for detection of both caspase-3 and nNOS.
- ✓ IgY antibodies could be a cheaper and non-invasive method of producing antibodies against caspase-3 and nNOS.
- ✓ A Dot blot method was developed as a quick and viable alternative to ELISA for semi-quantitative detection of the biomarkers and it had a sensitivity of 30 ng for caspase-3 and 100 ng for nNOS, as indicated visually and by CCD imaging.
- ✓ CCD imaging has a distinct advantage over virtual analysis of the dot blots as it is quantifiable and thus more accurate.

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LIST OF PUBLICATIONS

1. Toxicity and apoptotic potential of two synthetic pyrethroid pesticides - Under preparation for Food and Chemical Toxicology.

2. Development of an ELISA method to detect enzyme biomarkers for apoptosis – Under preparation for Journal of Immunological Methods.

3. A quick semi-quantitative method for the detection of biomarkers of apoptosis - Under preparation for Analytica Chimica Acta.