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Effects of atrazine on the histology and expression of NOS and IP3 receptor in cardiac and cerebellar Purkinje cells of juvenile and adult Xenopus frog

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Effects of atrazine on the histology and expression of NOS and IP_3 receptor in cardiac and cerebellar Purkinje cells of juvenile and adult *Xenopus* Frog

By

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A dissertation submitted to School of Anatomical Sciences Faculty of Health Sciences University of the Witwatersrand, in fulfilment of the requirement for the degree of Master of Science in Medicine

Johannesburg

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DECLARATION

I, the undersigned hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirely or in part submitted it at any university for a degree.

Signature

Name in full

_____/_____/_____

Date

DEDICATION

To my loving husband Mr Stanley Johnson, I could not have done it without you.

PRESENTATIONS FROM THIS STUDY

Oral presentations titled:

1. "The effects of atrazine on the histology of cardiac myocytes and Purkinje fibres of *Xenopus* frogs" presented at the Anatomical Society of Southern Africa conference, May 2016, Bloemfontein (Award winning presentation).

2. "Neurotoxicity of atrazine in juvenile and adult *Xenopus* frog; a study on cerebellar Purkinje cell population" accepted for presentation at the Anatomical Society of Southern Africa conference, April 2017, Cape Town.

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ABSTRACT

Atrazine is a synthetic pesticide, which is extensively used in agriculture and horticulture to boost crop quality and meet food demands of the growing human population. Atrazine's physical properties enable it to be transferred from point of application to non-targeted areas, gain access to water sources and persist in environment following application. Atrazine has been shown to be embryotoxic but the effects reported in adult species have been mostly on endocrine disruption and gonadal morphology. It is hypothesized that atrazine might affect cardiac contractility and cerebellar synaptic transmission by disrupting the expression of homeostatic proteins.

Ten- days old juvenile and 270 days old adult *Xenopus* frogs divided into 4 groups; one control group and three experimental groups were exposed to atrazine at concentrations of (0.01 µg/L, 200 µg/L and 500 µg/L) for 90 days. Harvested hearts and brains were processed and the morphological, histological investigations made (Mallory one step connective tissue stain for cardiac and cresyl violet stain for cerebellum). Additionally, immunohistochemical and immunofluorescence expression of key homeostatic calcium gated channel proteins; NOS and IP_3R in cardiac and cerebellar tissues of the juvenile and adult frogs were measured.

Mallory one step connective tissue stain revealed increased dilated cardiomyopathy effects such as thin wavy myocytes and infiltration of connective tissue in 0.01 µg/L and 200 µg/L treated frog groups, whereas hypertrophied cardiomyopathy effects; thickened darkly stained myofibrils were observed in sections of the heart from 500 µg/L groups in both juvenile and adult frogs. Immunohistochemistry and immunofluorescence cardiac eNOS expression was decreased in 200 µg/L and 500 µg/L treated frog groups in both juvenile and adult frog groups. The IP_3R expression in adult frog's heart insignificantly ($p > 0.05$) increased and decreased in 0.01 µg/L and 500 µg/L groups respectively, and significantly decreased ($p<0.03$) in the 200 µg/L group. These changes are suggestive features of cardiac arrhythmias.

Immunohistochemistry and histology (Cresyl violet stain) of the cerebellar cortex (in juvenile and adult frog groups) reveal reduction in the number of Purkinje cell expressing IP_3Rs relative to atrazine doses/concentrations, suggesting visual and/or motor function effect. In addition IP_3Rs

expressions were significantly decreased ($p < 0.037$) in all the treated juvenile groups, indicating greater cerebellar toxicity of atrazine on juvenile Xenopus species.

In conclusion atrazine exposure (low dose to high doses) showed some alterations on Purkinje cells immunochemical expression in the heart and cerebellum and these effects were more severe in the juvenile than the adult frog. In addition, the high juvenile mortality and distinct histological alterations characteristic of cardiomyopathy, arrhythmia and disruptive motor effects reported in both juvenile and adult groups indicate the potential toxicity of atrazine at high concentrations in marine habitats and the ecosystem at large.

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ABBREVIATIONS

eNOS – endothelial nitric oxide synthase

nNOS- neuronal nitric oxide synthase

EE- endocardial endothelium

IP_3R- 1, 4, 5-inositol trisphosphate receptor

IP_3Rs- 1, 4, 5-inositol trisphosphate receptors

NO- nitric oxide

NOS- nitric oxide synthase

ASD- autism spectrum disorders

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

1.1.1 Atrazine

Atrazine is a well-known, affordable and reportedly efficient pesticide/herbicide used extensively in the agriculture, residential lawns and sports field's the world over (Syngenta Crop Protection, 2003). Atrazine application has been successful in controlling pests and weeds to enhance productivity with improved quality of food and livestock. Consequently human and other vertebrates are constantly inevitably exposed to atrazine through their interaction with the environment. Additionally, the increase in world population (currently around 1.11% per year (2017) an average increase in population change (increase) of about 80 million per year) (www.worldometers.info/world-population), imply increased demand on agricultural production (food & livestock).

Therefore, the need to increase agricultural productivity and the quality of its products to meet the ever increasing global demand for food supply, have led to atrazine and other agro-chemicals being increasingly used for agricultural activities or processes. Consequently, humans and other non-targeted organisms (aquatic and terrestrial) and therefore the ecosystem are incessantly exposed to atrazine.

The above facts underscore the need to study and document the effects of atrazine on various organ tissues for possible adverse effects that may be detrimental to normal body homeostasis. Also, the knowledge of atrazine adverse effects will be useful clinically in time of atrazine poison or spillage. Available literature indicates dearth of information available on effects of atrazine on some organ tissues (such as the heart and brain) and this study was designed against this backdrop.

1.1.2 Atrazine exposure and contamination

According to the South African department of Water Affairs and Forestry, atrazine is the second most common contaminant found in ground water (London *et al* .,2000). The Environmental Protection Agency (EPA) of the United States regulates the use of atrazine and classified it as Restricted Use Pesticide (RUP) (Food Chemical News Inc 1990). Other countries like New Zealand have completely banned the use of atrazine, however in most developing countries and South Africa, atrazine is used freely and indiscriminately (United Nations environmental programme, 2001).

Atrazine gains access to the environment (from the site of application to non-targeted sites) through run-off water after heavy rains to surrounding water bodies and also penetrates into underground water bodies especially due to its very slow degradation in the soil (Dabrowski *et al.,* 2002). Atrazine is a volatile compound and can evaporate into the air and be inhaled by terrestrial animals and humans. Curwin *et al* (2007) reports that atrazine has been detected in food of farming and non-farming families in rural areas. Since some farming facilities are located within close proximity of some urban cities, the possibility of atrazine contamination in homes and in food of city dwellers are high and may not be totally ruled out. Atrazine contamination has been implicated in the disruption of reproductive potential (Hayes *et al*., 2010), biochemical changes (Abdali *et al.*, 2011; Santos and Martinez, 2012), normal development including organogenesis (Murphy *et al.*, 2006; Lenkowski *et al*., 2008) and neurochemical disruptions (Lin *et al.,* 2013). However the effects on cardiac and cerebellar tissue have not received much attention. It is therefore imperative that the bio-effects of atrazine be documented. This study was designed to determine the effects of atrazine on NOS and IP_3 receptor expression in cardiac and cerebellar Purkinje cells of the *Xenopus* frog.

1.1.3 *Xenopus* as a model for studies on environmental pollution

Xenopus belong to genus of African frogs commonly known as the African clawed frogs. *Xenopus* laveis are routinely utilized by researchers, due to the ease with which they can be spawned to produce large quantities of eggs.

The *Xenopus* as an amphibian are phylogenetically suitable for correlation to different vertebrates, having evolved from amniotes linage (mammals, birds and reptiles) (Hellsten *et al.*, 2010). Furthermore the *Xenopu*s metamorphosis has many similarities with post-embryonic development in mammals; from several months before birth to several months after birth in humans (Tata, 2003). *Xenopus* metamorphosis has also been compared to the physical, physiological and hormone dependent changes in human puberty (Gilbert and Krebs, 1991). In addition the sequencing of *Xenopus laveis* genomes displays remarkable structural similarity with the human genome (Hellsten *et al.*, 2010); implying that discoveries, if any, of atrazine effects on *Xenopus laveis* could give insight into atrazine contributions to conditions of illnesses in different vertebrates and mammals.

Finally the *Xenopus laveis* is an amphibian that has a dual habitat (aquatic and terrestrial), and therefore ideal for toxicological studies on environmental pollution. Hence, *Xenopus* presents a suitable model to study the impact of atrazine on some mammalian tissues through its agricultural application and consequently contamination of available water resources and the ecosystem in general.

1.2 RESEARCH OUTLINE

1.2.1 Problem statement

Amphibian species are on the brink of extinction (Ballie *et al*., 2004; Stuart *et al*., 2004) due to uncontrolled used of agro-chemicals in the agricultural sector (especially in developing countries) to boost food production and enhance quality of food products (Ballie *et al*., 2004). Amongst these chemicals, atrazine use has been on the increase the world over, but more especially in Southern African region (Dinham, 1993). Furthermore, the *Xenopus* tadpole is often used as bait for fishing and the resultant decrease in number could adversely affect the ecological equilibrium by disrupting the herbivorous tadpole and carnivorous role of the adult frog in the environment (Naiman and Turner, 2000; Heimeire *et al.*, 2010).

Additionally, the increased use of atrazine in agriculture may constitute occupational health hazard (including but not limited to) to the farm workers (who carry out the actual application) and their households who live on the farm, livestock's in habitats closely associated with the

farms as well as communities in close proximity of the areas of atrazine application. Therefore, there is need to study and document the adverse effects of atrazine use on some mammalian tissues.

1.2.2 Hypothesis

Researchers have widely accepted the contributions of genetics (Neale *et al*., 2012; Sanders *et al.*, 2012) and heritability (Rosenberg *et al*., 2009) to autism. But a study on twins using prenatal insecticide exposure reported that a variable combination of environmental and genetic factors can be associated with Autism Spectrum Disorders (ASD) (Hallmayer *et al.*, 2011). Furthermore, defects in Purkinje cell morphology and electrophysiology has been linked to behaviour deficits associated to ASD (Sudarov, 2013). However, there has been no study on the effect of atrazine exposure on the histology and immunohistochemistry of Purkinje cells/fibres in the brain and cardiac tissue. Such a study may possibly help correlate the defects in Purkinje cell morphology (due to atrazine) to the behavioural deficits seen in ASD as previously reported (Rosenberg *et al*., 2009; Hallmayer *et al.,* 2011) and also on morphology and histoarchitecture of the heart relative to production of eNOS and IP_3Rs expression. It is envisaged that effects of atrazine on Purkinje cells and the expression of Purkinje cell IP_3R will attempt to highlight possible links between Purkinje cell morphology and ASD as well as cardiac arrhythmias and atrial fibrillation. The results will illuminate further possible research areas for contributions of atrazine exposure to ASD and possible cardiac effects.

1.2.3 AIM

To investigate the effects of atrazine on the expression of NOS in the heart and cerebellar cortex and 1, 4, 5- inositol trisphosphate (IP_3R) expression in cardiac and cerebellar tissues of pro-metamorphic tadpoles and adult frogs.

1.2.4 OBJECTIVES

The objectives of this study were to determine the:

- Effect of atrazine on the morphology (gross) of the heart in juvenile and adult *Xenopus* frogs.
- Acute/chronic effects of atrazine exposure on the histoarchitecture of the cardiac tissue and cerebellum cortex in juvenile and adult *Xenopus* frogs.
- Effects of atrazine exposure on collagen fibre deposition in pro-metamorphic juvenile and adult *Xenopus* frogs using Mallory one step staining.
- Developmental effects (comparing pro-metamorphic juvenile and adult frogs) of atrazine exposure (in dose related manner) on the level of expression of eNOS in cardiac, nNOS in cerebellar and IP_3R in cardiac and cerebellar tissues using immunohistochemistry and immunofluorescence techniques.

CHAPTER TWO

LITERATURE REVIEW

2.1.1 Chemical properties of atrazine

Atrazine (6-chloroN-(1-methl\lethyl)-1,3,5-trazine-2,4-diamine) is a white crystalline solid, used to kill broad leaf weeds in corn and sugar cane farms. However atrazine is also used for the maintenance of weeds in golf courses, sports fields and residential lawns.

According to the World Health Organization's (WHO) water sanitation and health unit, atrazine has a half-life of 20-50 days at 20-25 ºC (WHO, 2011). Atrazine is soluble in acetone, chloroform and methanol at 298K (Tomlin *et al.*, 1994). Studies have shown that atrazine is resistant to degradation, degrading as slow as 1-2 years when in water column (Ribaudo and Bouzaher, 1994; Diana *et al.*, 2000). This slow rate of degradation of atrazine in the soil and its possible persistence in the soil has led to increasing concern about its potential to infiltrate and contaminate groundwater (Orme and Kegley, 2004; Dabrowski, 2014). These physical and chemical properties contribute to the potential risk posed by the ability of atrazine to move from the point of application to non-target areas (Dabrowski, 2013).

2.1.2 Atrazine use in South Africa

Dabrowoski (2015) summarises estimates of total atrazine application on different crops per province (Fig 1.1). Although geographical and physiochemical factors determine the migration of atrazine into surface water, the amount and rate of use of pesticide in a region is a critical marker of the level of contamination of non-target environment.

In 1990, South Africa spent 500 million dollars on pesticides (Rother and London 1998). In the Western Cape and Eastern Cape provinces, fruit farming has created a great demand for millions of tons of atrazine (London and Myer, 1995). Yet there remains little or conclusive research on its effects and appropriate regulations on its use in South Africa.

Recent studies have documented the widespread contamination by pesticides including atrazine in surface and ground water at low concentration in South Africa (Mentjies *et al*., 2000; London *et al*., 2002; and Sereda and Meinhardt, 2003). A previous report had remarked that South Africa is the major marketer for atrazine in sub-Saharan Africa (Dinham, 1993) and atrazine is freely used by farmers in South Africa (Dabrowski, 2015). This suggests the existence of a possible contamination of some water sources, food amongst others, which may contribute to public health problems. This potential environmental hazard can often go unnoticed, especially with inadequate facilities to monitor and continuously investigate the effects of atrazine in several species at different age groups. In order to avoid sudden extinction of some susceptible and endangered aquatic species in our environment and in addition, the consequent health effects on human population, it is important for research on atrazine to be carried out.

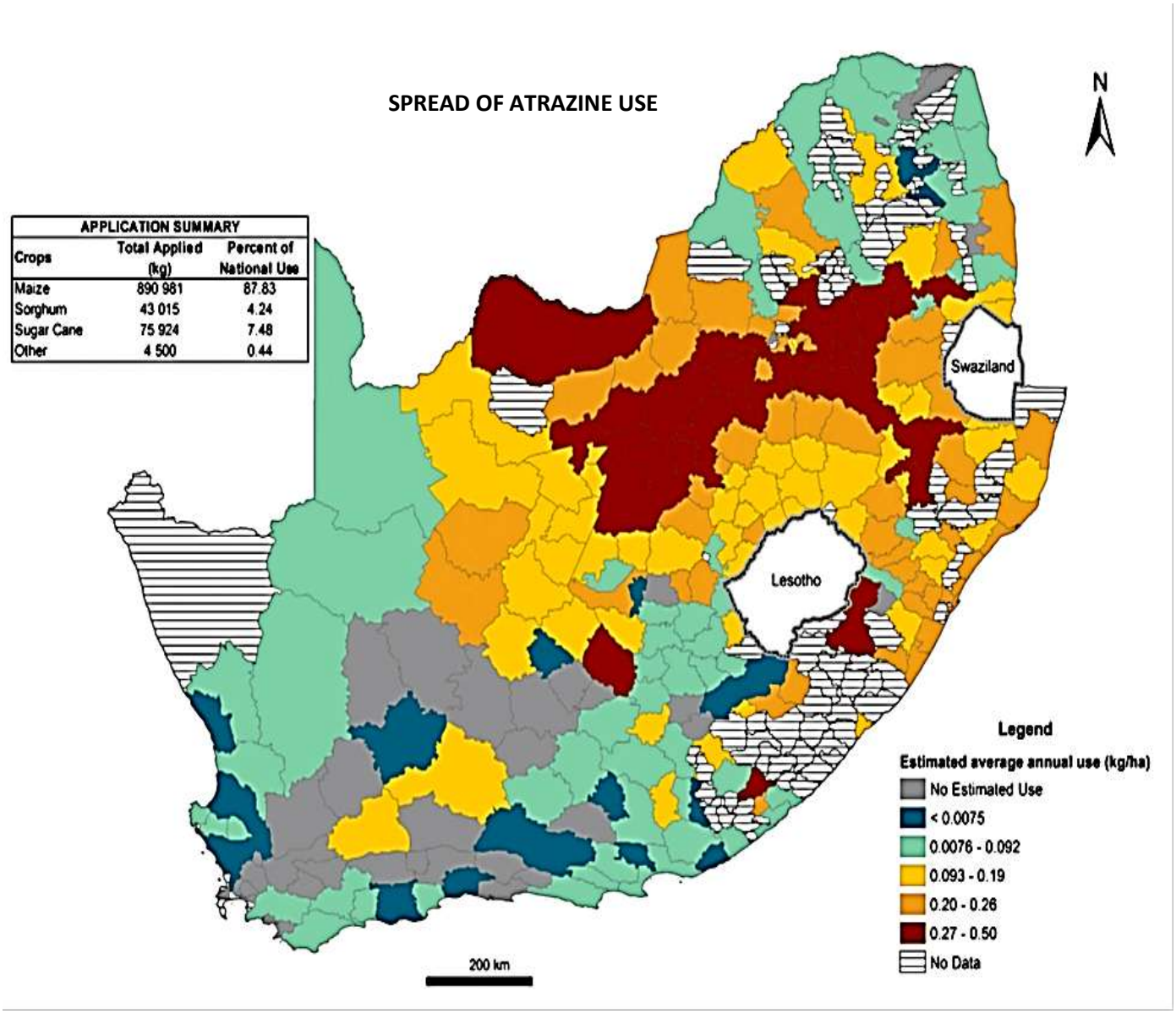


Figure 2.1 Map showing the average annual use of atrazine per hectare of agricultural land in magisterial districts of South Africa for the year 2009 [Estimated from pesticide sales and agricultural crop census data, (Dabrowoski 2015)]

2.1.3 Effects of atrazine at embryonic and adult stages of development

Atrazine has been observed at various concentrations in the environment, and in an attempt to determine its effect on aquatic life, embryonic studies in several species at varying concentrations have been carried out and the results are indicative of the teratogenicity associated with atrazine exposure 24-96 hours post fertilization (Ton *et al.,* 2006; Lenkowski *et al.,* 2008; Scahill, 2008; and Lin *et al.,* 2013). This implies that sudden and even short spikes of atrazine concentration in surface soils, ground waters and nearby water bodies following application and aided by storms, can be especially harmful to embryonic life. Additionally researchers have reported that atrazine has no significant effects an adult anuran (frogs and toads) at different concentrations (Allran and Karasov, 2000; Sifkarovski *et al.,* 2014). However these reports on adult frogs have been limited to gonadal morphology and endocrine disrupting potential of atrazine (Hayees *et al.,* 2006; Carr *et al.,* 2006; Jooste *et al*., 2005, Tavera-Mendoza *et al*., 2002; and Wirbisky, 2016). Thus reports are lacking in literature on possible effects of atrazine on the histology and/or histopathology of some organ tissues (heart and brain) of adult and juvenile frogs which the present study seeks to address.

2.1.4 Atrazine as an endocrine disrupting chemical

In 2004, Atrazine was on the list of environmental pollutants described as Endocrine Disrupting Chemicals (EDC) published by the endocrine society (EDSTAC 1998). EDC's were selected on the basis of their potential to disrupt the normal functioning of all hormone-secreting glands (EDSTAC 1998).

The thyroid gland and its hormones are vital in the proper development and function of many organs (including the brain and heart). Thyroid hormones have been reported to contribute in dendritic and or axonal growth and maturation of Purkinje cells of the cerebellum (Durst and Flamant, 2012). The level of thyroid hormones in rat has been implicated in delayed and poor myelination, lack of cell migration and establishment of neurocortical layers (Berber *et al.,* 2001). In addition thyroid dysfunction has also been linked with the potential for development of cardiovascular diseases (Biondi and Cooper, 2008). Therefore, atrazine as an EDC can potentially interfere with the thyroid hormone homeostasis and thereby result in organ toxicity of the heart and brain (cerebellum). This amongst others has led to the need to investigate the effects

of atrazine on cardiac histology as well as the cardiac and cerebellum Purkinje cells in the juvenile and adult frogs.

2.1.5 Neurotoxicity of atrazine

Atrazine exposure has been implicated disrupting the antioxidant enzyme activities including malondialdehyde MDA content in the brain and kidney of common carp fish (Xing *et al*., 2012). In addition atrazine has been reported to disrupt the hypothalamus-pituitary gonadal axis in mammals, fish and anuran species (Coady *et al.,* 2004; Wirbisky and Freeman, 2015). While the above confirms the neurochemical toxicity of the atrazine in aquatic species, its effect on other regions of the brain like the cerebellum has received little attention.

In the cerebellum of mammals reside Purkinje neurons, which are major motor neurons of the central nervous system. The cerebellum is the center for sensory input from the sensory systems, spinal cord, and rest parts of the brain. It regulates motor (voluntary) movements such as posture, balance, speech, resulting in smooth and balanced muscular activity. Consequently, cerebellar toxicity would entail disruption of coordinated movements. Oral atrazine administration in rats is known to induce a significant decrease in the spontaneous firing of Purkinje cells and cerebellar activity (Podda *et al.,* 1997). The disruption of behaviour, motor coordination and equilibrium has also been recorded in rats treated with atrazine (Castano *et al.*, 1992; Lin *et al*., 2013) indicating that atrazine may affect Purkinje cell motor function. This creates the need to study atrazine effects on the histology and immunohistochemistry of Purkinje neuron.

2.1.5.1 The *Xenopus* cerebellar tissue: history of Purkinje cells

Similar to the mammalian cerebellum, the cerebellum of the *Xenopus* is located on the ventral surface of the brain, and also consists of 3 layers, outer molecular, middle Purkinje layer and inner granular layer (Gless et al., 1958; and Manzano et al., 2017).

Johannes Evangelist Purkinje, a Czech physiologist first depicted these large equally proportional neurons in the cerebellum in 1837. He described the cerebellar Purkinje layer as being characterised by typical flask-like cells, whose curved dendrites can be followed into the first

quarter of the molecular layer, the cerebellar cortex's only output pathway and a key connection in the complicated motor control of muscle tone and movement. Defects in Purkinje cell and decreased Purkinje cell volume have been implicated in people with autism spectrum disorders (Duong *et al*., 1986; Fatemi *et al.,* 2002). Autism is on the increase globally and especially in the United States (Herze-Piccioto and Delwiche, 2009). Therefore the effects of atrazine on the histology and immunohistochemical expression of calcium gated receptors utilized by Purkinje cells for synaptic transmissions need to be studied and documented.

2.1.5.2 Role of Purkinje cells: expression of IP_3R

Cerebellar Purkinje cells use changes in the intracellular free calcium concentration to transmit information and modulate synaptic transmissions (Eilers *et al*., 1996). This calcium influx into the cell is achieved through 1,4,5 inositol -trisphosphate (IP_3), an intracellular messenger which binds to IP_3 receptors (IP_3Rs) located on the endoplasmic reticulum (ER), and triggers the release of calcium from the endoplasmic reticulum(ER) into the cytosol, initiating several intracellular signaling processes (Ehrlich *et al*., 1994).

It is not known whether, atrazine disturbs IP_3R protein expression in the Purkinje cells and consequently synaptic transmission. It is therefore essential to study the effects of atrazine on the IP_3R expression in cerebellar Purkinje cells to be studied, considering that motor development in children can be seen as the nexus for development, out of which other developmental skills can spring. Additionally the cerebellum is an important component for motor control and learning of coordinated movement (Glickstein, 1998).

2.1.5.3 Nitric oxide synthase

Furchgott *et al* (1998) received a Nobel Prize for physiology and Medicine for their discovery that nitric oxide produced endogenously signal molecule in the cardiovascular system. Nitric oxide (NO) is a free radical reactive gas which serves as a major messenger molecule with different functions. Nitric oxide (NO) functions primarily as a vasodilator or signalling molecule (Tousoulis *et al.*, 2012), a local blood pressure regulator (Hanafy *et al*., 2001) and in neuronal development (Champlin and Truman, 2000). NO has a short half-life and therefore cannot be

stored, implying that signalling for its production is very concise and specific. Nitric oxide synthase (NOS) are highly regulated enzymes that signal for the production of NO. There are three forms of nitric oxide synthase: endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) (Föstermann and Sessa WC., 2012)

2.1.5.4 Chemical injury; role of neuronal NOS

nNOS is expressed in specific neurons in the central nervous system (CNS). nNOS has been reported to play a role in synaptic plasticity (learning and memory formation), central control of blood pressure, relaxation of penile erection, vasodilatation and gut peristalsis (Forsterman and Sessa, 2011). Furthermore abnormal signalling of nitric oxide can be linked to a variety of degenerating neurological conditions such as stroke, multiple sclerosis, Alzheimer, Parkinson disease and excitotoxicity (Steinert *et al.,* 2010). These effects indicate neuro-protective effects of nNOS at certain concentrations, but nitric oxide can become neurotoxic if concentration continues to increase, causing oxidative stress in neurons, that impede neuronal function and ultimately causing cell death.

Cerebellar Purkinje cells do not normally exhibit nNOS activity (Steinbusch *et al*., 2000), but reports have shown that chemical injuries can induce nNOS expression (Dawson *et al*., 1996; Wu, 2000). The determination of the dose at which nNOS expression will occur in cerebellum of juvenile and adult *Xenopus* frogs is therefore very important to understand the point of toxicity.

2.1.6 Cardiotoxicity of atrazine

The cardiovascular effects of atrazine include inhibition of frog atria contraction at high concentrations (Papaeftthimiou *et al*., 2003) and facilitating vaso-relaxation of the endothelium (Chan *et al.*, 2007). Other effects include enlargement and softening of heart and thickened valves in dogs (Pathak and Dikshit, 2011), as well as induction of oxidative stress in rat heart (Abarikwu, 2014). The *Xenopus* heart endothelial endocardium (EE) is uniquely the only barrier between the blood in the endocardium and the cardiac myocytes (Sys *et al.,* 1997) and is also in close proximity to Purkinje fibers. Thus, there is high possibility of atrazine adversely affecting

these fibres and perhaps the expression of homeostatic calcium gated receptors and enzymes on the Purkinje fibers and endothelial endocardium respectively.

2.1.6.1 Anatomy of *Xenopus* heart

The gross anatomy of the *Xenopus* heart, like most amphibians, is three chambered with upper left and right auricles (atrium) and one lower ventricle. Externally the *Xenopus* heart is avascular, with a double membrane pericardium followed by a fully muscular myocardium and most internally the endocardium.

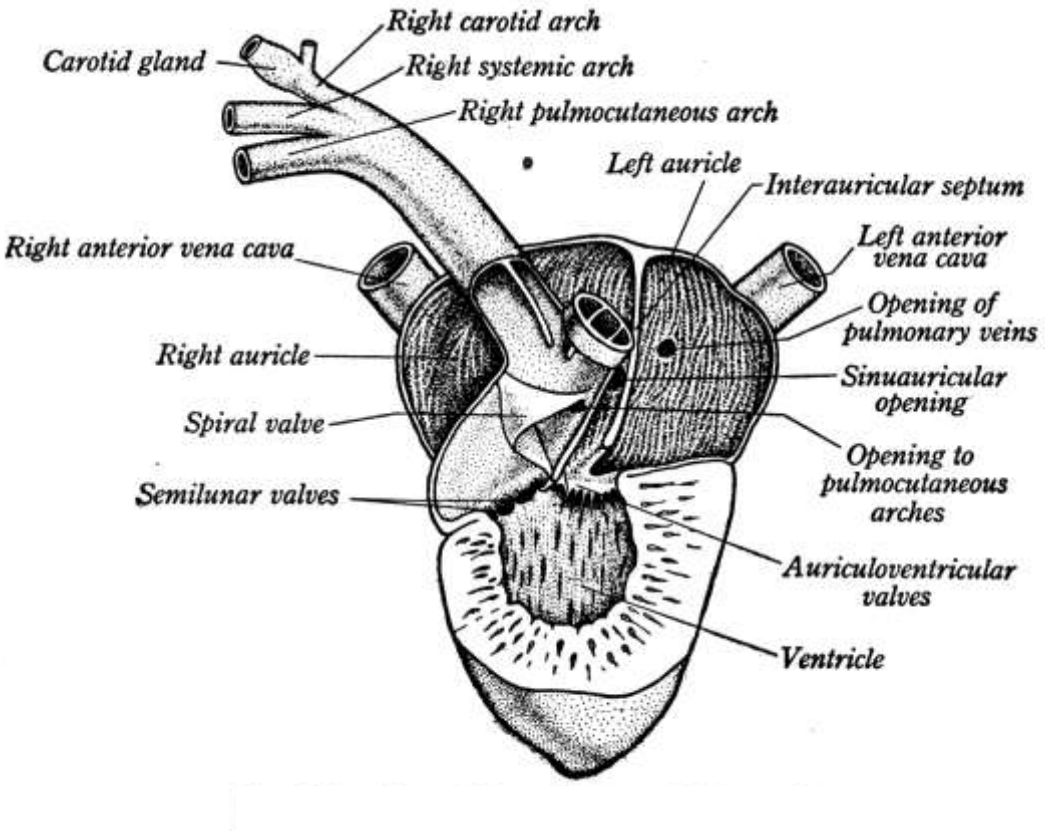


Figure 2.2 Illustrative diagram of the ventral dissection of frog heart. Adopted from Standardnote.blogspot.co.za (downloaded on 18/08/2017)

Histologically between the endocardium and myocardium are specialised modified cardiac cells; Purkinje fibres. The Purkinje fibres generate and rapidly transmit contractile impulse to various parts of the myocardium in specific sequence. Histologically these are large in size, with larger

nuclei and peripheral myofibrils and pale staining cytoplasm when compared to the myocytes (Fig 2.3).

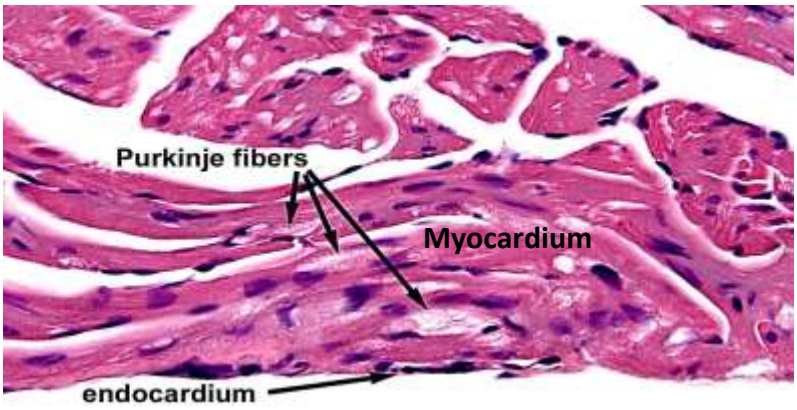


Figure 2.3 Photomicrograph of *Xenopus* frog cardiac tissue. Showing myocytes, Purkinje fibers and endocardium. Adopted from the book, Colour atlas of *Xenopus laevis* histology (Wiechmann and Wirsig-Wiechmann, 2003)

2.1.6.2 Role of calcium gated channels in cardiac tissue

An individual cardiac muscle cell is made up of myofibrils which require several active calcium transport for relaxation and contraction during systole and diastole (Fig 2.4A, B, C). One cycle is entirely intracellular and involves Ca^{2+} fluxes into and out of the sarcoplasmic reticulum (SR), as well as Ca^{2+} binding to and release from thin actin filament (Fig 2.4 B,C and D). The other extracellular Ca^{2+} cycle occurs when this Ca^{2+} ion moves into and out of the cell, from the extracellular fluid and is completed when Ca^{2+} is actively transported back to the extracellular fluid mediated by the sodium-calcium exchanger and the plasma membrane calcium pump (Fig 2.4B). Therefore, the homeostatic control of Ca^{2+} gated pumps is essential for the proper physiologic contractility of cardiac muscle fibres.

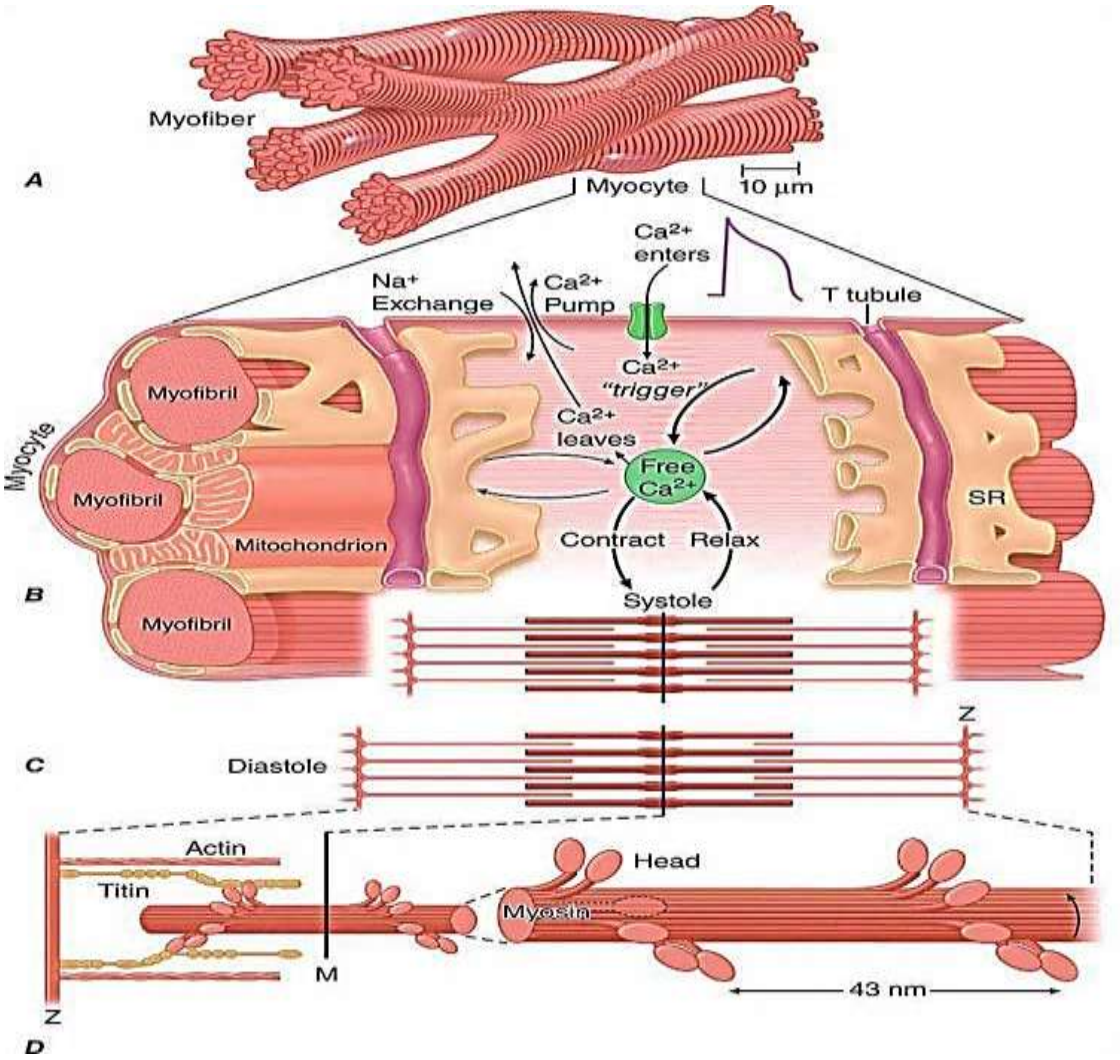


Figure 2.4 An illustrative figure on cardiac physiology. A: cardiac ultrastructure, B: calcium gated pumps in myofibril, C: contraction relaxation coupling in myofibrils, D: myosin filament. Adopted from Harrison's Principles of Internal Medicine (Braunwald *et al*., 2001)

2.1.6.3 Physiology and localization of 1, 4, 5-inisotol trisphosphate (IP_3) and IP_3 receptors (IP_3Rs)

1, 4, 5 inisotol- trisphosphate (IP_3) is the second messenger produced in the sarcolemma and travel through the cytoplasm to the endoplasmic reticulum (ER), where they bind with IP_3 receptors (IP_3Rs) on Ca^{2+} gated channels (Fig 2.3), and trigger the opening of the Ca^{2+} channel, and release of Ca^{2+} from the ER into the cytoplasm (Bers, 2001; Hund *et al.*, 2008).
Though cardiac excitation-contraction coupling depends majorly on ryanodine receptors (RyR2) (Fig 2.5, blue receptors), induced Ca^{2+} release more than IP_3Rs (in a ratio 100:1), specific interest in IP_3 and IP_3Rs mediated Ca^{2+} release has risen from researches that have shown the expression of IP_3Rs in and around the nucleus in cardiac myocytes (Bare *et al.*, 2005; Wu *et al.*, 2006; and Wu and Bers. 2006). This indicates a possible involvement in nuclear/ perinuclear Ca^{2+} signaling in atria and ventricular myocytes (Fig 2.5, red receptors dominate nuclear envelope) (Bare *et al.*, 2005; Mohler *et al.*, 2003, 2005). Furthermore, the expression of IP_3R is higher in Purkinje fibres than in other cardiac cells (Carmeliet and Vereecke, 2012), which could indicate vital implications if the function or regulation is impaired.

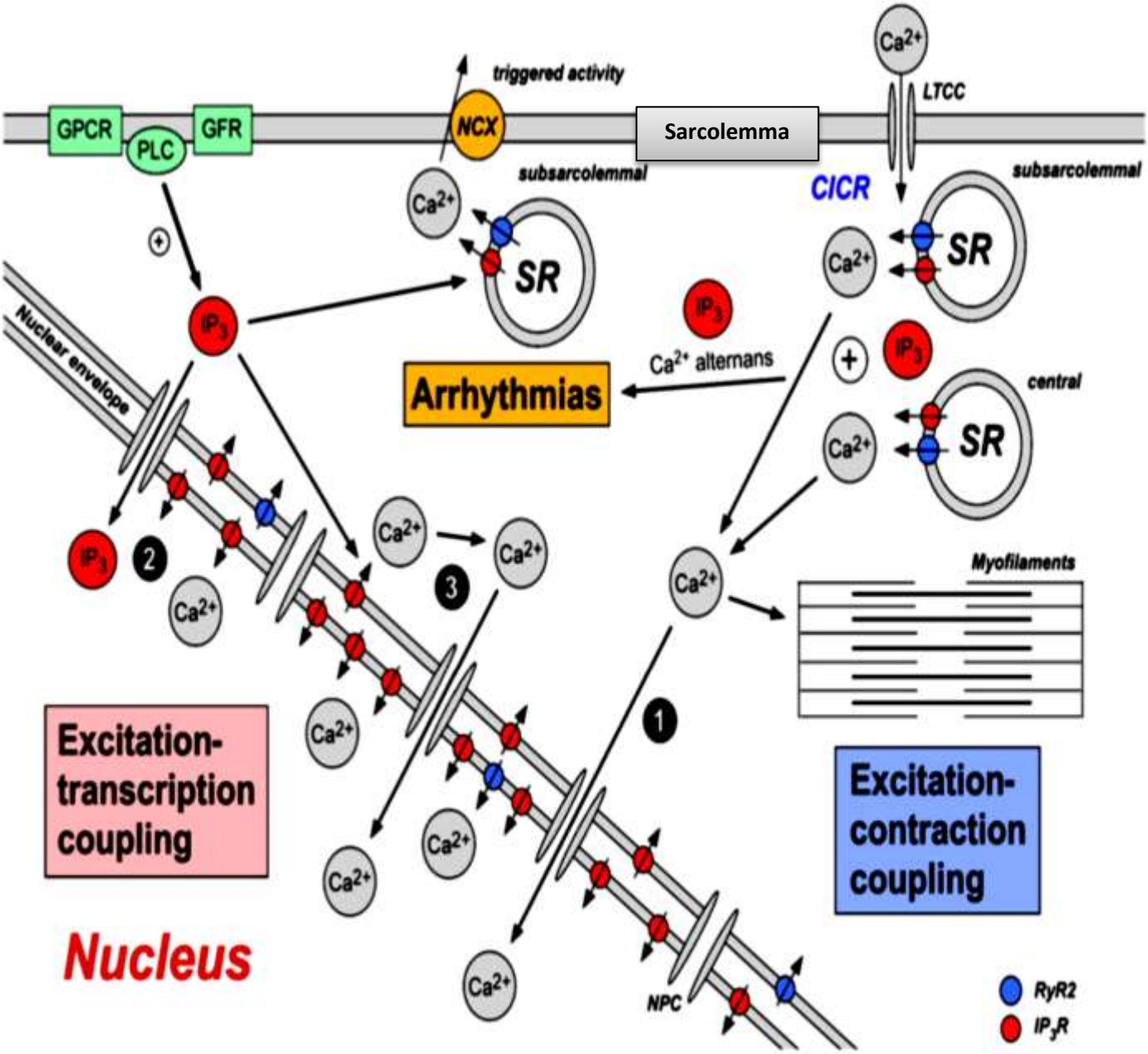


Figure 2.5 Illustration of Ca^{2+} gated channels and and IP_3 receptors between the sarcolemma, nucleus, and sarcoplasmic reticulum (SR). (Kockskamper., *et al* 2008)

2.1.6.4 Cardiac diseases associated with IP_3Rs

The unique location of IP_3Rs around the nucleus permits for automatic generation of Ca^{2+} signaling, and has been suggested to contribute to the regulation of cardiac gene transcription and controlling of cardiac hypertrophy (Kockskamper *et al*., 2008). In addition, the expression of cardiac IP_3Rs has been shown to be increased with age and in cases of atrial fibrillation (Kaplan *et al.,* 2007). Furthermore arrhythmias generally have been suggested to be triggered by both activation and loss of function of IP_3Rs (Kockskamper *et al*., 2008). Considering the sub-endocardial location, the histoarchitecture and the electrophysiology role of Purkinje fibres in excitation–contraction coupling, it is reasonable to attribute generation of arrhythmias to Purkinje fibres (Boyden *et al.,* 2009). It is therefore proposed that an environmental agent like atrazine may adversely affect the expression of IP_3Rs in the heart.

2.1.6.5 Endothelial nitric oxide synthase (eNOS): role in the cardiac tissue

All three isoforms of NO are expressed in the heart. But the isoform that has the greatest expression in the endothelium endocardium and cardiac myocytes is the eNOS (Brutsuert 2003). In addition the endocardial endothelium is the only place in the frog heart where endothelial nitric oxide synthase (eNOS) is found (Adler *et al.,* 2004).

eNOS plays a dominant role in the signalling and production of NO, which regulates myocardial contraction. Therefore eNOS functions as a homeostatic regulator of the contractility of the myocardium (Brutsuert, 2003; Mount *et al*., 2007). In addition, eNOS is a physiologic vasodilator and gives protection against thrombosis, can also inhibit proliferation of vascular smooth muscle; thereby making eNOS an anti-atherosclerotic agent (Li and Foretermann, 2000).

In the light of the physiological and pathological involvement of eNOS in both protective and excitotoxity process in cardiac endothelium and myocytes, it is of interest to this work to investigate eNOS expressions in cardiac tissue and connective tissue of both juvenile and adult *Xenopus* exposed to atrazine.

2.1.7 Pathophysiology of cardiomyopathy

Cardiomyopathy is a chronic disease of the heart muscle (myocardium), in which the muscle is abnormally enlarged, thickened, and /or stiffened. The weakened heart muscle loses the ability to pump blood effectively resulting in irregular heartbeats, arrhythmias and possibly even heart failure (Cardiomayopathy, 2012).

There are two types of cardiomyopathy of interest to this study: Dilated cardiomyopathy, in which the heart cavity is enlarged and stretched (cardiac dilation) (Fig 2.6a), resulting in weak and slow pumping of blood, formation of blood clots and abnormal heart rhythms and hypertrophic cardiomyopathy is characterised by the thickening of the heart muscle (Fig 2.6b) creating functional impairment and/or loss which could lead to sudden death.

Rajkovic *et al.*, (2014) was one of the first to research the effects of atrazine on the structure of rat myocardium, and reported that no significant changes occurred in the stereological parameters of cardiomyocytes such as volume, surface and connective tissue densities. However there is still hardly any information on the effects of atrazine on the histology and immunohistochemical expression of calcium gated channels/receptors in the heart.

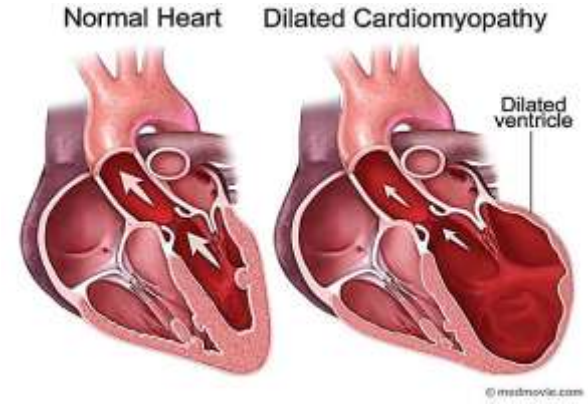


Figure 2.6a Animated image of dilated cardiomyopathy. Adopted from www.medmovie.com on March 20th 2017.

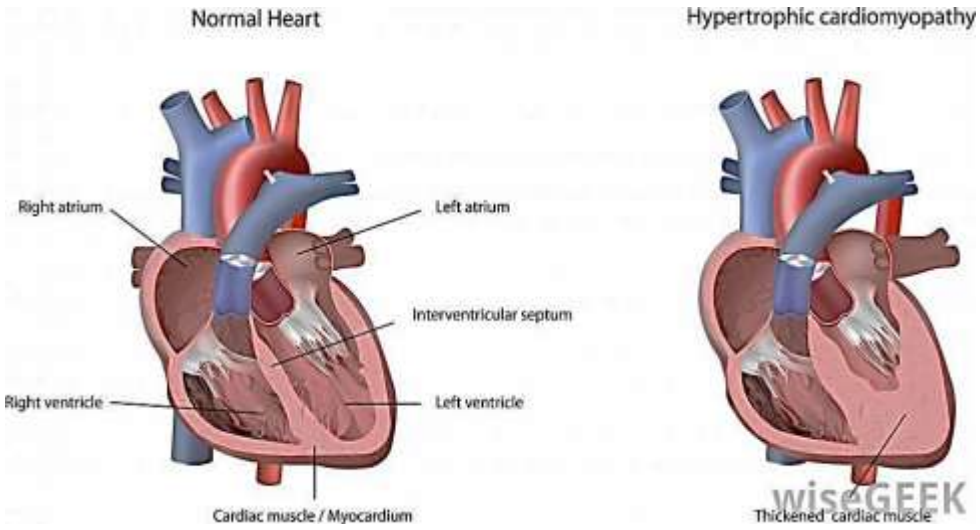


Figure 2.6b Animated images of hypertrophied cardiomyopathy. Adopted from www.wisegeek.com on March 20th 2017

Having given pertinent literature of the proposed study the next chapter describes the materials and methods used in carrying out the study.

CHAPTER THREE

MATERIALS AND METHOD

3.1.1 Animals

Both adult and juvenile frogs were used in the study which involved two sets of experiments for the adults and juvenile groups. A total of 360 frogs were approved (180 adults and 180 juvenile), taking into consideration any eventual mortality, but only 180 frogs were used in the study (60 adults and 120 juvenile frogs). The reduction in the number of frogs was because one aspect of the study that was to be carried out at the hartbeespoort dam was not carried out due to unavailability of research facilities and expiration of the permit to carry out the study at the dam.

Juvenile frogs

The juvenile frogs (tadpoles) were bred in the Central Animal Services unit of the University of Witwatersrand by the spawning of two adult *Xenopus* male and female frogs, the female was injected with gonadotropin hormone to stimulate egg production. Fertilized eggs were collected and allowed to develop into tadpoles for 10 days before the commencement of the experiment. The tadpoles were housed at a density of 15 tadpoles/ aquarium, in 60Litres glass tanks with a 12 hour light/dark cycle (on 06:00, off 18:00). Temperatures were maintained at 24± 2^{o}C using water heaters. Individual tank thermometers were verified using calibrated thermometers. Tadpole water was kept aerated by pumping air through clean sand and wool filter using a pump.

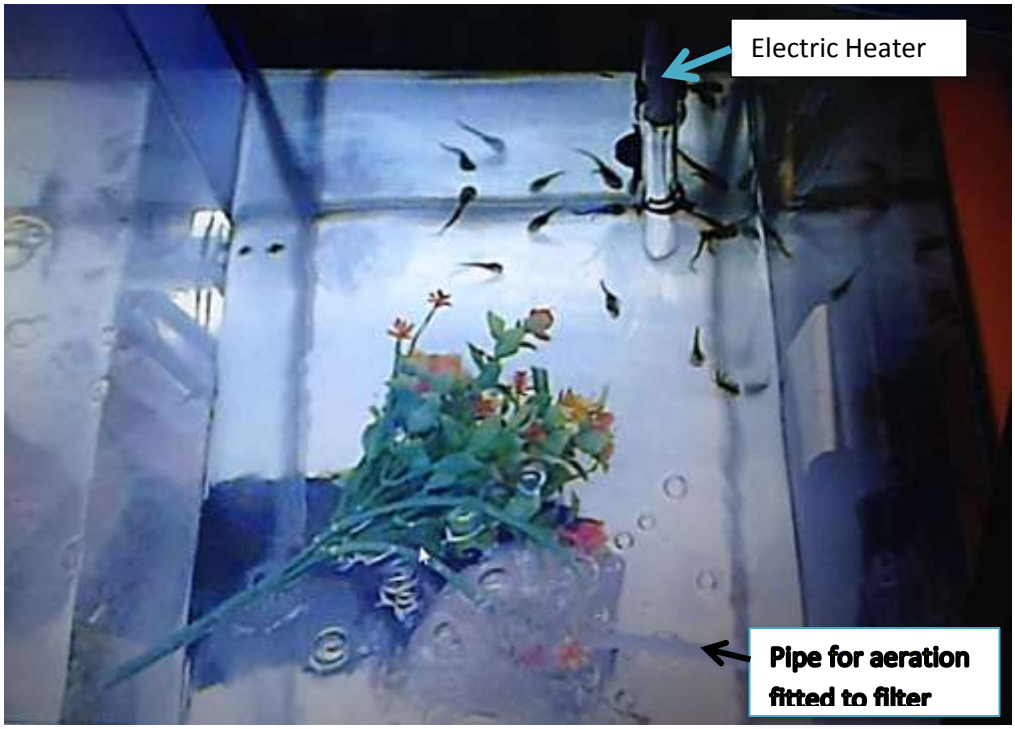


Figure 3.1 Photograph of experimental set up for juvenile group

Adult frogs

Sixty (60) apparently healthy adult male frogs (270 days old) obtained from breeders in a local African *Xenopus* facility (Knyser, South Africa) were used for this study. They were transported in water filled polystyrene plastic boxes under conditions suitable for the African clawed frog.

On arrival at the University of Witwatersrand, the frogs were received and housed in 225cm x 24cm x21cm steel tanks filled with 60 litres of water in a density of 15 frogs/ tank, and allowed to acclimatize to their environment for 2 weeks. Water temperatures were maintained at 22 ± 2^{o}C with a variable room heating system in a 12 hour light/dark cycle.

3.1.2 Feeding and treatment

Adult frog water was recycled (100%) 3 times a week after feeding to maintain a clean environment and to maintain atrazine levels. Water for tadpoles underwent 80% recycle for the first month to reduce stress to the tadpoles and 100% afterwards.

Tadpoles were fed with highly nutritious commercial ornamental fish micro flakes (TetraMini baby®; Johannesburg, Broadacres Shopping Centre, c/o Cedar and Valley Road, Fourways, Sandton; ©2017 Spectrum Brands - Pet, Home and Garden Division, Tetra-Fish) and received crushed fish pellets as from 75 days age. Adult frogs were fed with nutritious commercial fish pellets (Koi food; Daro Pet Products, Johannesburg, South Africa). The tadpoles and adult frogs were exposed to atrazine for 90 days in treatment tanks and thereafter euthanized using 0.2% benzocaine solution (a local anaesthetic).

3.1.3 Atrazine source and concentrations of exposure

Pure atrazine powder [CAS Number 1912-24-9, technical grade, 98.9% purity] was obtained from AccuStandard Inc. (New Haven, CT, USA) was used for this experiment. Three doses of atrazine (0.01 µg/L, 200 µg/L and 500 µg/L) were used in the present study. The low dose of 0.01µg/L was determined based on the concentrations found in the Hartbeespoort dam (located, south of Magaliesberg mountain range and north of the Witwatersberg mountain range, about 35 kilometers west of Pretoria, Gauteng Province); the second dose of 200 µg/L in par with that used by Jooste *et al*., (2005). The 500 µg/L was chosen accordingly with Hussain *et al*., 2011, 2012 who studied very high doses of 400 µg/L and 500 µg/L in Japanese Quail. The atrazine concentration was measured weekly (to ensure constant level of exposure) using solid phase micro extraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS). Atrazine concentrations were verified to be within ±5% for 0.01 µg L^{-1}, and 7% for both 200 and 500 µg L^{-1} of target concentrations.

A range of doses may normally be required for toxicological studies. The selection of 0.01 µg/L and 500 µg/L (the lowest and highest atrazine doses so far used in research studies) for the present study, aims to address the issue of previous inconclusive results by comparing the effects of the two doses. It is hoped that this high dose would produce a definite toxicological effects on the parameters being studied (heart and brain) and therefore create a reference point for extreme exposure to atrazine in high agricultural areas, and/or following accidental atrazine spillage.

3.2. Experimental design

The laboratory experiment was divided in to two phases, 1(juvenile) and (2) adult frogs. In both phases the animals (juveniles and adults) were divided into 4 experimental groups (A-D: juvenile; and E-H: adult frogs). Groups A (juvenile) and E (adult frogs) did not receive any atrazine treatment and served as controls, while groups B-D (juvenile) and F-H(adults) were each respectively treated with same concentrations of atrazine and served as the treatment groups.

Table 3.1 Table of animal groups and concentration of atrazine exposure

Juvenile groups	Exposure dose	Adult groups	Exposure dose
A	Control (No treatment)	E	Control (No treatment)
B	0.01 µg/L	F	0.01 µg/L
C	200 µg/L	G	200 µg/L
D	500 µg/L	H	500 µg/L

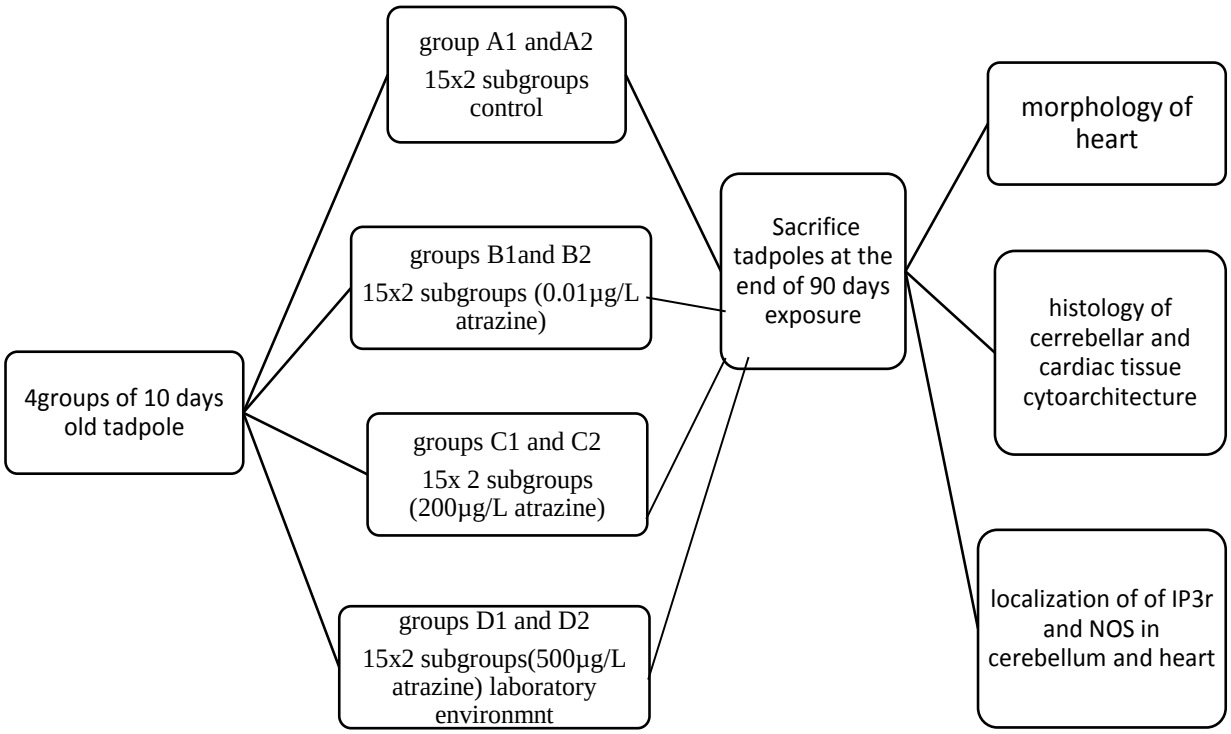
Juvenile phase

One hundred and twenty 10 days old tadpole were divided into 4 main groups A, B, C and D of 30 tadpoles each. Each group was further divided into two sub groups; A1, A2; B1, B2; C1, C2; D1 and D2 with 15 tadpoles each. Subgroups A1 and A2 did not receive any atrazine and served as control. Subgroups B1, B2, C1, C2, D1, and D2 were exposed to atrazine concentrations; B1 & B2 - 0.01 µg/L, C1 & C2 – 200 µg/L, D1 & D2- 500 µg/L respectively (Fig 3.2) for 90 days and thereafter euthanized with benzocaine anaesthetics and killed. The organs (brain, and heart) were harvested, weighed and recorded. The length (mm) and breath (mm) of the heart were measured and recorded. These organs were immediately fixed in buffered formaldehyde and 4% paraformaldehyde for brain tissue.

Adult phase

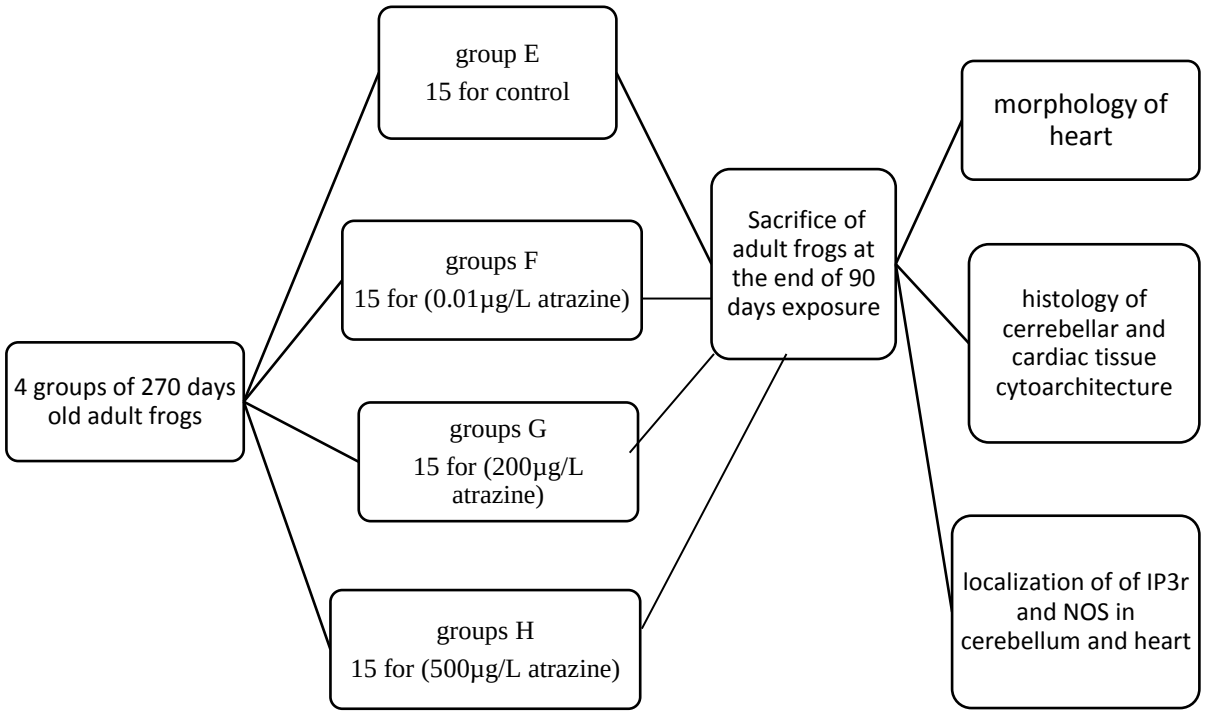
Similarly, in the adult group (Fig. 3.3), sixty adult frogs (270 days old) were divided into 4 groups E, F, G and H of 15 frogs each. Group E was not exposed to atrazine and serve as control. Groups F, G and H were similarly exposed to atrazine concentration of; F - 0.01 µg/L, G- 200 µg/L, H- 500 µg/L respectively for 90 days and thereafter killed as previously described in juvenile group. The heart and brain were harvested and immediately closely inspected visually

for any gross abnormality and then similarly weighed and all measurements recorded, before fixation in appropriate fixatives for subsequent analysis.



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Figure 3.2 Experimental design for juvenile frogs phase



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Figure 3.3 Experimental design for adult frogs phase

3.2.1 Sacrifice of animals

Juvenile and adult frogs were retrieved from their respective tanks individually, deeply anaesthetized by inhalation in glass bell jars containing 0.2 % benzocaine and then sacrificed.

3.3 Specimen collection and analysis

3.3.1 Organ and tissue parameters

At the end of 90 days of exposure, and following anaesthesia, a longitudinal abdominal incision was made to expose the abdominal cavity while the rib cartilages were carefully transected in order to expose the heart and the lungs. The heart was carefully harvested and weighed in a Mettler analytical balance and their wet weights recorded, the length and the width were also recorded for the determination of area. The rest of the internal organs (kidneys, liver, pancreas, spleen, adrenals, testes, epididymis, seminal vesicle, and prostate glands) were also harvested, weighed and their wet weight recorded for comparative analysis.

The heads of the frogs were incised at the level of the spinal cord that intersects between the top of both upper limbs. The soft skull bone was immediately cracked (opened up) using the skull forceps and the brains fixed. The fixed brain tissue was later carefully removed by neatly cracking and removing the soft skull bone piece by piece thereby exposing the complete brain. Thereafter the carcasses were disposed of by incineration.

3.3.2 Gross morphological observations and measurement of adult hearts

Weight of the heart of juvenile and adult frog hearts were recorded and the adult frogs hearts observed for any gross morphological abnormalities. The juvenile heart was not observed for any abnormalities due to their very small size. Furthermore, measurements of the length and breadth of the juvenile and adult frog hearts were done.

3.4 Histology of the heart

The heart and brain were trimmed of connective tissue, weighed, the length and breadth measured and all values recorded. The tissues were immediately placed in appropriately labelled specimen bottles, containing a large volume of 10% buffered formalin (heart) and 4% paraformaldehyde (brain) for fixation.

After fixation, the heart was wrapped in strips of filter paper in a tight parcel and placed in plastic cassettes and routinely dehydrated in a graded series percentages (%) of ethanol, and embedded in paraffin wax. Blocks of heart tissues were sectioned (longitudinal) at 5μm thickness using a rotary microtome. Tissue sections were picked up onto pre-cleaned glass microscopic slides, mounted, dried in an oven overnight and stained with haematoxylin and eosin (H&E) and cover-slipped with entellen. These sections were then viewed and analysed under the light microscope**.**

3.4.1 Connective tissue histology

Sections of the heart (cardiac tissue) were subjected to routine Mallory-Heidenhain rapid one-step staining procedure for cardiac connective tissue analysis. Mounted slides of cardiac tissue section were deparaffinised and rehydrated in water. Sections were stained for 5mins in staining solution, washed in running water for 3.5 minutes using a digital stopwatch. The tissues were dehydrated rapidly, cleared in xylene, mounted with entelen, and viewed with light microscope.

3.4.2 Histology of the cerebellum: Cresyl violet for cytoarchitecture of the cerebellar tissue

Whole brain tissue was processed in sucrose overnight, and then mounted on frozen microtome with 30% sucrose and coronal sections were made into 0.1MPB. Brain tissue sections were mounted on gelatine coated slides then placed in defating solution overnight, then subjected to cresyl violet staining process (Appendix V) for the analysis of the cytoarchitecture of the cerebellum.

3.5 Immunohistochemistry and immunofluorescence

3.5.1 Immunohistochemistry for eNOS expression in cardiac sections

Cardiac tissue sections were mounted on silane coated slides and processed using immunohistochemistry protocol for paraffin sections (Appendix 7.4.1). Normal goat serum was used, primary antibody was rabbit polyclonal antibody raised against rat eNOS diluted in the ratio 1:500, while secondary antibody was goat anti-rabbit antibody IgGs diluted in the ratio 1:1000. Stained sections where viewed with light microscope. Control sections were incubated with PBS.

3.5.2 Immunohistochemistry for IP_3R expression in cardiac sections

Cardiac tissue sections were mounted on silane coated slides and processed using immunohistochemistry protocol for paraffin section (Appendix 7.4.1). Normal goat serum was used, primary antibody was rabbit polyclonal antibody raised against rat IP_3R diluted in the ratio 1:1000, while secondary antibody was goat anti-rabbit antibody IgGs diluted in the ratio 1:1000. Stained sections were viewed with light microscope. In control sections primary antibody was substituted for PBS.

3.5.3 Immunofluorescence for eNOS expression in adult cardiac sections

Cardiac tissue sections of adult frogs only were processed by Immunofluorescence protocol (Appendix 7.4.2) was done in a dark room and tiny juvenile cardiac sections were impossible to manipulate in the dark and so were excluded. Normal goat serum was used, primary antibody was rabbit polyclonal antibody raised against rat eNOS diluted in the ratio 1:500. Secondary antibody used was Alexa 488 fluorescence conjugated anti rabbit antibody, diluted in the ration 1:500 and stained with DAPI 1:1000, washed and then cover slipped with fluoromount and viewed with Olympus Cens immunofluorescence microscope.

3.5.4 Immunohistochemistry for nNOS expression in the cerebellar cortex

Fixed brain tissue sections were collected in 48 well plates, and processed using immunohistochemistry protocol for free floating sections (Appendix 7.4.3). Several different normal serums were optimized (horse, goat, rabbit and swine) as donkey serum ordered but were not supplied, due to the agricultural licensing permit issues. Primary antibody was polyclonal antibody raised against sheep nNOS in 1:1000. While secondary antibody was donkey anti sheep IgGs, in control the primary antibody will be omitted. Slides were examined with a light microscope.

Several attempts were made to optimize nNOS expression in juvenile and adult frog brain tissue, by changing the dilution of the primary antibody, antigen retrieval in phosphate buffer and trying other several normal serums. But all were unsuccessful.

3.5.5 Immunohistochemistry for IP_3R expression in cerebellar cortex

Fixed brain tissue sections were collected in 48 wells plate. Tissue sections underwent immunohistochemistry protocol for free floating sections (Appendix 7.4.3). Normal goat serum was used, primary antibody was polyclonal antibody raised against rabbit IP_3R diluted in a ratio of 1:1000 while secondary antibody was goat anti rabbit antibody IgGs. Control sections were incubated 0.1PB, the primary antibody will be omitted. Slides will be examined with a light microscope.

3.6 Cell count

The point counting of IP_3R3 expressing cells in the cardiac (in adult frogs only) and cerebellar (in both juvenile and adult frogs) tissues was carried out using computer assisted images analysis software (Olympus Cell Sens 2011 software). Zeiss axioscope light microscope (Switzerland) fitted with a digital camera was used to capture the microscopic image of the sections on to the computer screen using x40 and x630 objective lens. Images were saved in tiff format on the computer and analysed quantitatively using Image-j image analysis software. However, eNOS expression in the juvenille and adult frog cardiac tissue was determined qualitatively. The count

of Purkinje cells that expressed IP_3Rs in photomicrographs of cardiac and cerebellar sections were carried out using image-j multi-point tool. On each tissue, all the cells expressing IP_3R per cross-section per field/frame sampled were carefully counted by the aid of the software pointing counting programme. Fields were randomly sampled with the aid of microscope stage and specimen controllers, ensuring that no single area of each tissue section was sampled more than once. Five randomly selected sections from each adult frog heart and brain (juvenile and adult frogs) were used. Twenty five fields were randomly selected from each treatment group and cell counted. The image-j software has an inbuilt cell differentiation capability (find edges) by varying the intensity of IP_3R3 expressed in each cell type. This makes for easy identification of expressed cells (Appendix VIII). In addition, the multi-point tool marks and counts the identified cells and totals the count per field and per section. These points were averaged to determine the mean number of cells per treatment. In the cardiac tissue, images were taken from the apex of the heart (ventricle) towards the atria (Appendix VI). And in the cerebellar cortex it was taken from left to right along the dorsal Purkinje cell layer (Appendix VII).

3.7 Statistical analysis

Statistical analysis was done using IBM SPSS 24. All data were expressed as mean ± Standard error (SEM). The tables and graphs were constructed using Microsoft Excel 2010. Data were tested for normality. Normal data were analysed by SPSS one way ANOVA and a post hoc Turkey test was conducted to determine differences between the groups. Non-parametric data were analysed by Kruskal Wallis test and a post hoc Mann Whitney test to determine differences between groups. All values of $P < 0.05$ were regarded as significant

3.8 Ethical and legal considerations

This study was approved by Gauteng Nature Conservation, permit numbers 0115 and 0120 (Appendix I). And all animal procedures were performed according to the standard protocol for research animal experimentation approved by the Animal Screening Ethics Committee (AESC) University of the Witwatersrand, Johannesburg, and clearance certificate number 2014/32/D (Appendix II).

CHAPTER FOUR

RESULTS

4.1 General

Mortality was observed in the juvenile during the experiment (Fig. 4.1). While there was high mortality (60%) in the treated juvenile group, only about 10% (3 frogs) mortality was recorded among the control juvenile group during the last 5 days to the end of the experiment. There was no autopsy done on demised frogs but bed letters monitoring welfare of frog showed normal readings. There was a gradual increase in mortality per dose of atrazine, but mortality rate per specific dose of atrazine at the duration of the experiment (90 days) was recorded as follows: 30%: 0.01 µg/L; 43.4%: 200 µg/L and 76.7%: 500 µg/L (Fig. 4.1). There was statistically significant mortality observed amongst treated groups relative to the control group of juvenile frogs ($p < 0.024$). In the adult frog groups, both controls and treated groups survived the 90 days atrazine exposure and no physical adverse features were observed. Throughout the 90 days experimental period, the feed intake was regular with minor fluctuations.

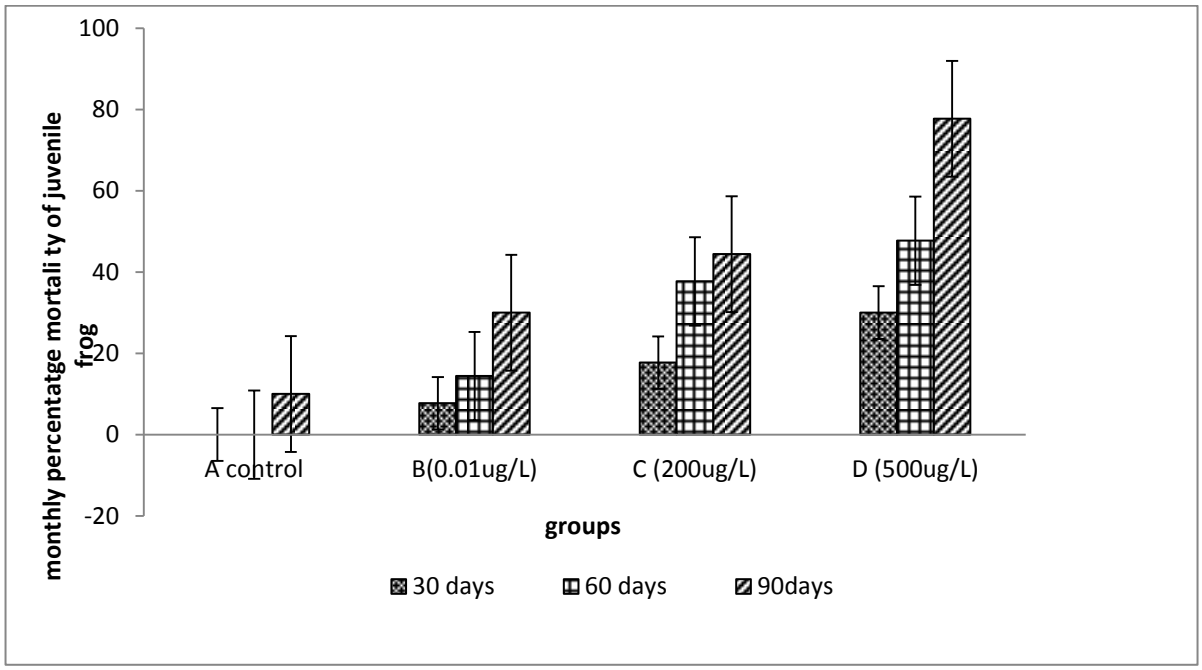


Figure 4.1 Time dependent and dose specific percentage mortality of juvenile frogs

4.2 Gross morphology observation and measurements of the hearts

4.2.1 Gross morphology of adult frog hearts

Morphological examinations of adult frog hearts showed normal heart shape, configuration, colour and size for the control group. The hearts in the 200 µg/L group were all enlarged (largest in all treated groups); followed in size by the 0.01 µg/L group and both latter groups appear to be a mixture of dark and light tissue colouration (Fig 4.2). The hearts in the 500 µg/L group appeared to be the same size as the control but had a deep dark-brown colouration. These observations were noted in all the treated adult groups.

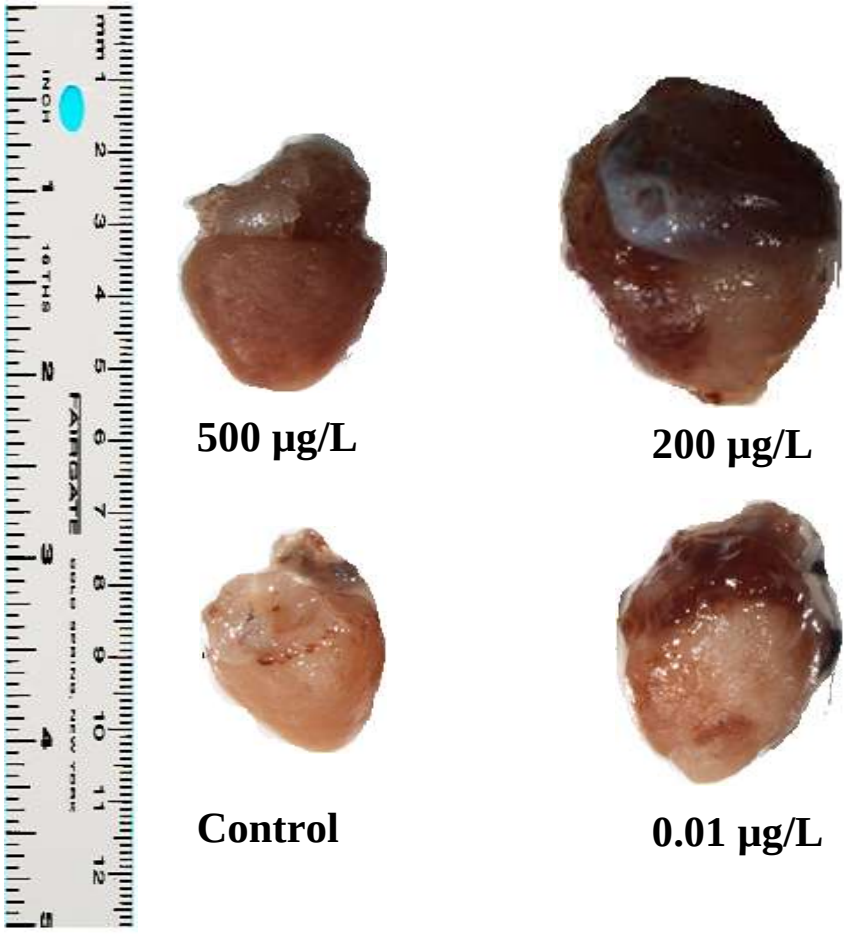


Figure 4.2 Photographs of dose dependent effect of atrazine on the gross morphology of adult frog hearts.

4.2.2 Gross measurement of juvenile hearts

Atrazine exposure affected the size and area measurements of juvenile heart of the treated groups. The exposure of juvenile frogs to 0.01µg/L and 200µg/L of atrazine showed no significant increase in mean weight of juvenile frog hearts, but a decrese in 500µg/L exposed frog heart compared with the control(Fig 4.4). The mean area of juvenile heart of the 500 µg/L treated group was significantly reduced in comparison to the control and 0.01µg/L treated frog groups (*P* < 0.045 and 0.031 respectively) (Fig. 4.3; Table 4.1).

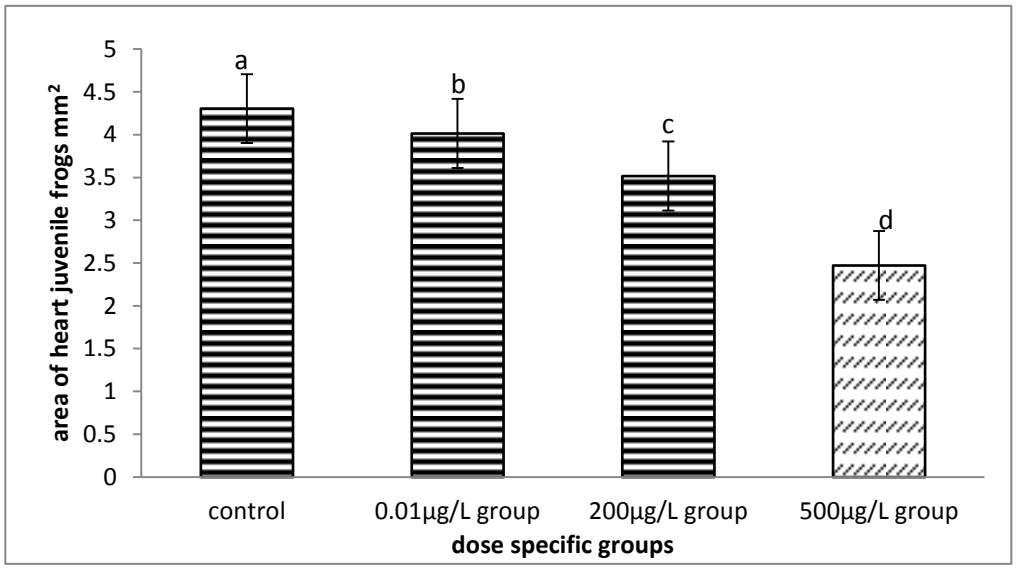


Figure 4.3 Dose dependent effects of atrazine on area of the juvenile heart. Mean area of juvenile heart; d (500 µg/L) is significantly reduced in comparison to 'a'(control) and 'b' (0.01 µg/L) at p < 0.045 and p < 0.031 respectively. No significant change between 'b' and 'c'.

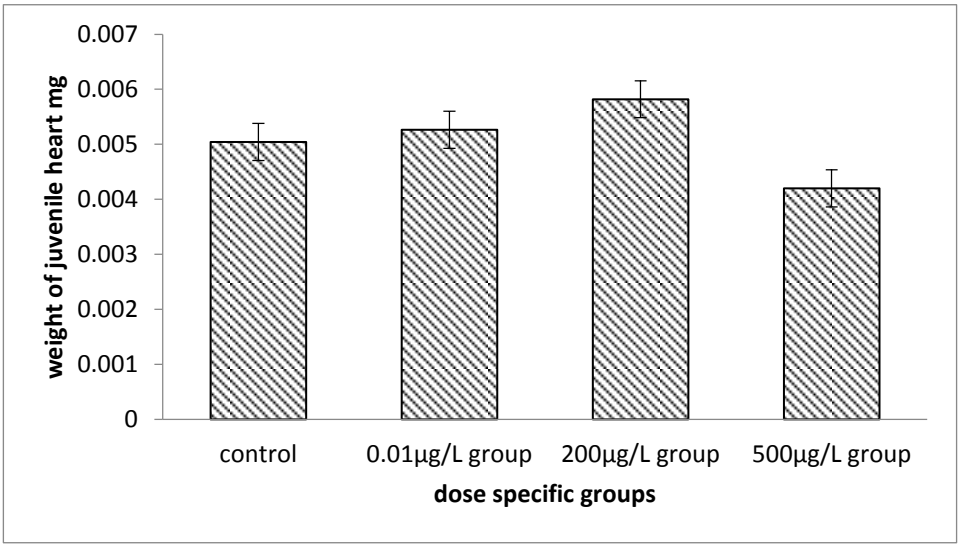


Figure 4.4 Dose dependent effect of atrazine on weight of juvenile heart. Mean weight of juvenile frogs exposure to atrazine for 90 days, $p < 0.434$, no significant difference between groups.

Table 4.1 Comparison of mean heart area and weight in adult and juvenile frogs between groups (Post hoc test p values)

	Control vs 0.01 µg/L	**Control vs 200 µg/L**	**Control vs 500 µg/L**	**0.0l µg/L vs 200 g/L**	**0.01 µg/L vs 500 µg/L**	**200 µg/L vs 500 µg/L**
Juvenile heart area	0.96	0.47	0.045*	0.525	0.031*	0.332
Adult frog heart area	9.29	0.001*	0.998	0.000*	0.964	0.00*
Adult frog heart weight	0.70	0.31	0.93	0.036*	0.96	0.10

*Significant difference

4.2.3 Gross measurement of adult frog hearts

There was a significant increase in the mean area of adult frog hearts between the 200µg/L group and the control, 0.01µg/L and 500µg/L atrazine exposed groups ($p < 0.001$ respectively; Table 4.1) The 0.01 µg/L treated group recorded the lowest value in heart area (Fig 4.5). Also significant increase in the mean weight of adult heart was recorded between the 200 µg/L group ($p < 0.036$) and the 0.01 µg/L group (Fig. 4.6, Table 4.1).

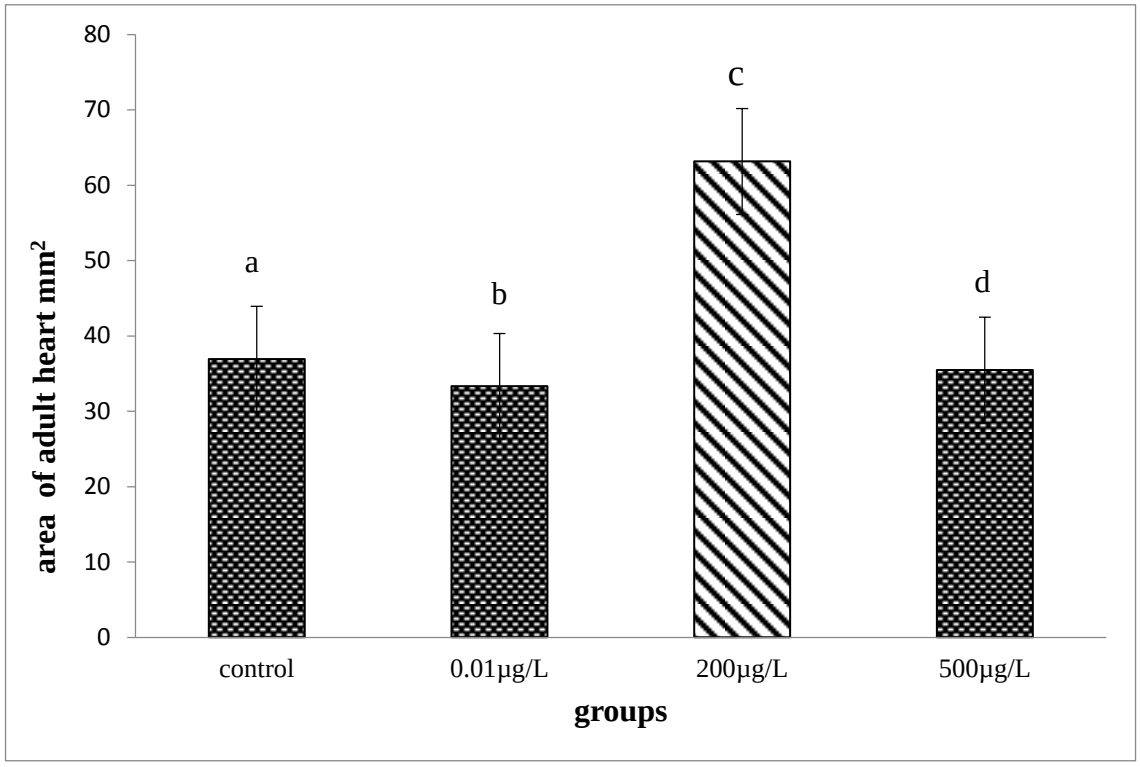


Figure 4.5 Dose dependent effect of atrazine on the area of adult frog heart.

'a' is similar to b and d; but 'c' is significantly increase relative to 'a, b and d' ($p < 0.001$)

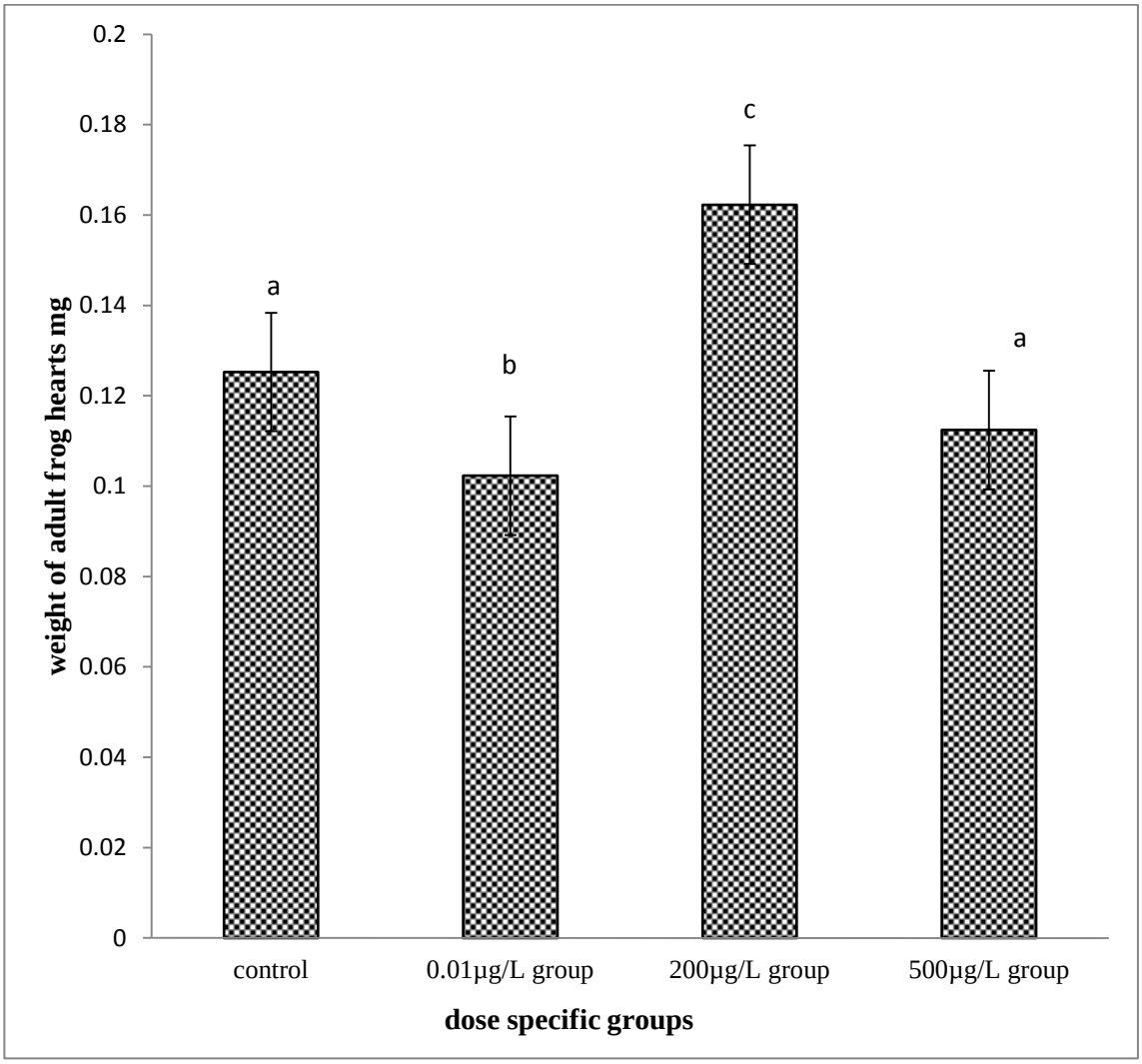


Figure 4.6 Dose dependent effect of atrazine on weight of adult frog heart.

Significant difference exists between control and atrazine treated groups ($p < 0.040$). 'b' is significantly decreased when compared with 'c' ($p < 0.037$) but not significantly reduced when compared with 'a'

4.3 Histology

4.3.1 Histology of cardiac tissue

Cardiac histology of the control animals (juvenile and adults) revealed normal appearance, showing regular branched cells having intercalated disks, centrally placed nucleus with intervening connective tissue/interstitial spaces evenly distributed among cardiac myocytes (Fig. 4.7 A and 4.8A respectively). Numerous endothelial cell nuclei were present, surrounding the cardiac fibres of the control group. In 0.01 µg/L group, mild hypertrophy/enlargement and disorientation of cardiac muscle fibres was observed, resulting in narrowing of the interstitial spaces. The endothelial cells nuclei were clearly seen but the intercalated discs were not visible (Fig. 4.7 B and 4.8B). In the 200 µg/L group the cardiac myocytes decreased in size and appeared very thin and wavy, and some diffused muscle fibres were observed in both the juvenile and adult frogs (Fig. 4.7 C and 4.8C). There was also breakdown of cardiac muscle and interstitial spaces which were more pronounced in the 200 µg/L group than in the control and 0.01 µg/L groups and the nuclei of cardiac myocytes were clearly seen. In the 500 µg/L group, diffused and disorganized branching of cardiac muscle fibres with reduction of striations were observed (Figs. 4.7D, 4.8D)

Figure 4.7 Photomicrograph of juvenile cardiac tissue, showing effects of atrazine in different groups.

A: control group, homogenous cardiac muscle fibers and interstitial spaces

B: 0.01 µg/L group fibers thickened uneven myofibrils

C: 200 µg/L group thin and wavy myocytes

D: 500 µg/L group show disorganized myofibrils.

AI (double arrow) width of control cardiac fiber, B1 (double arrow) width of 0.01 µg/L cardiac fiber, (short arrows) endothelial cells. H&E stain X630. Scale bar 100µm

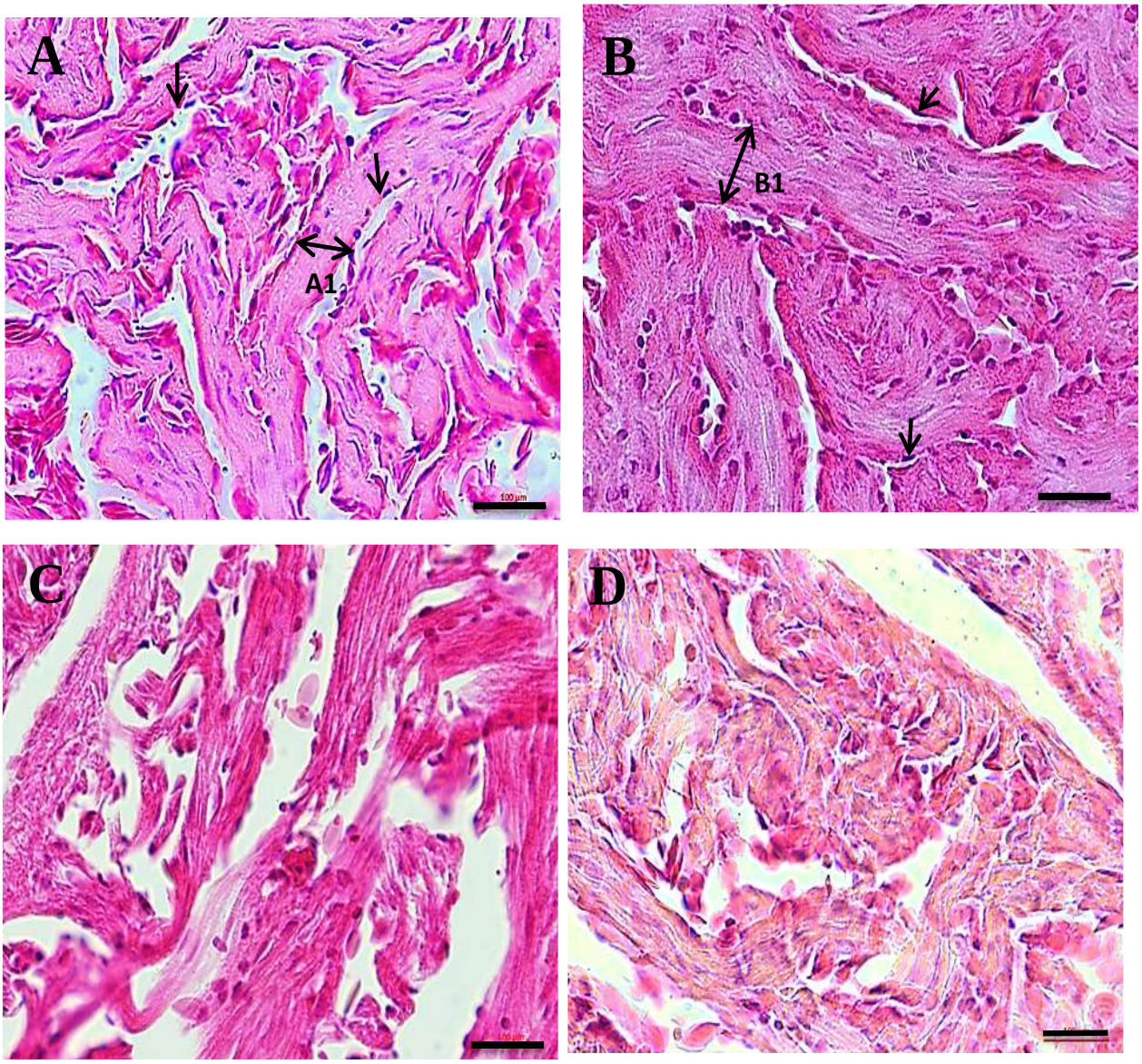

A
A1
B
B1
C
D

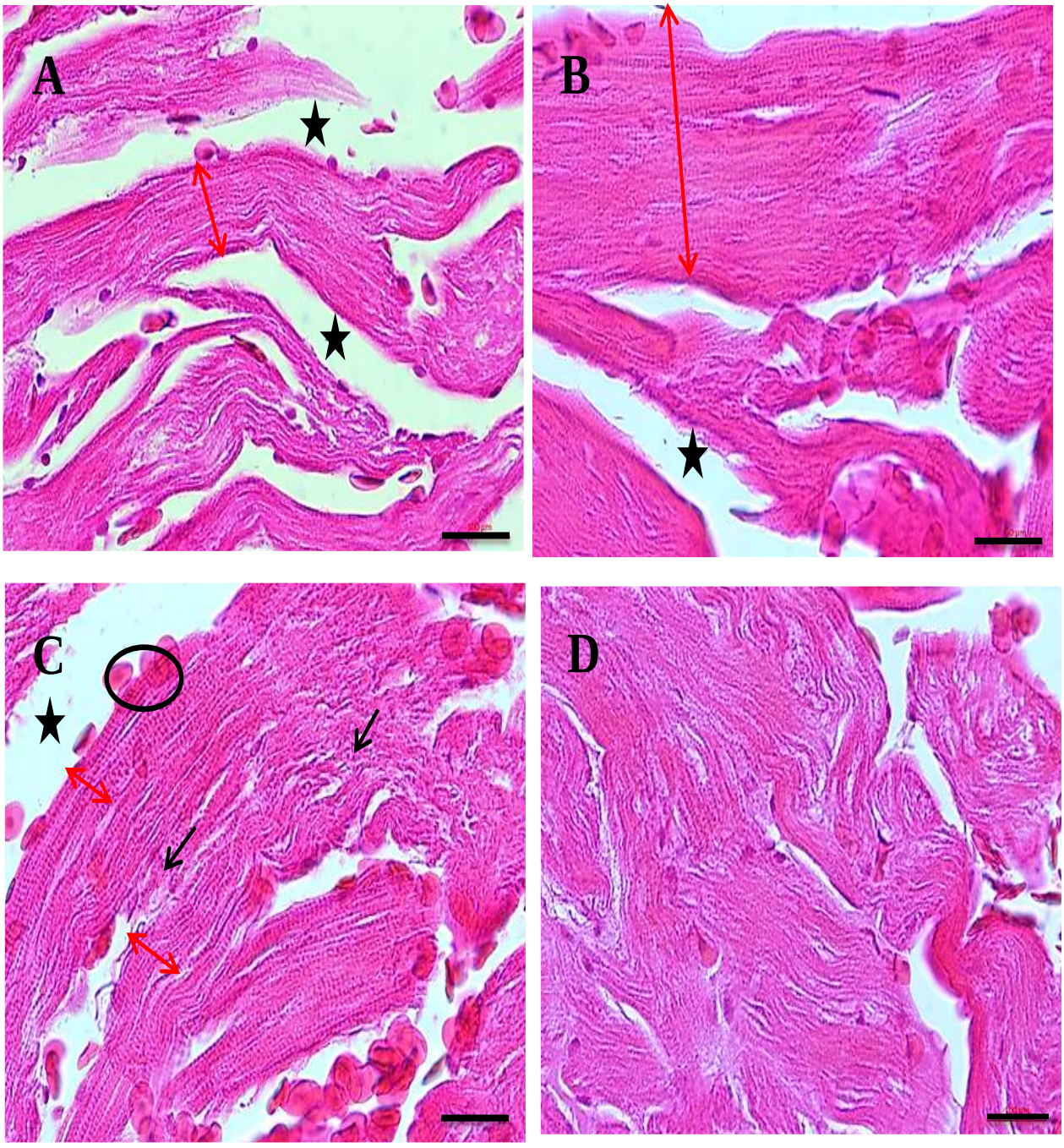
Figure 4.8 Photomicrograph of adult frog cardiac tissue showing effects of atrazine on the cardiac myocytes in different groups.

A: Control group, homogenous cardiac muscle fibres (double red arrows) and interstial spaces (black star) are observed.

B: 0.01 µg/L group, thicker cardiac fibres (double red arrows) and a slightly smaller interstitial space

C: 200 µg/L group, slight increase in cardiac fibres (double red arrows) stacked together with some diffused cardiac muscle cells observed (black arrows) in cardiac interstitial space. (Circle) red blood cells

D: 500 µg/L group, disarray of myofibrils whose width cannot be indicated by an arrow due to irregular branching of fibres H&E stain X630. Scale bar 100µm


A
B
C
D

.

4.3.2 Histology of cardiac connective tissue

The control groups (juvenile and adults) showed normal organisation of cardiac fibres with usual branching of fibres and well distributed interstitial spaces with bright pink and red Mallory-Heidenhain rapid one-step stain staining. The control group myocytes were observed with no blue connective tissue staining, except for a few minimal staining, but properly distributed to only the endomysium (Fig 4.10A). Mixture of cardiac muscle fibres and connective tissue fibre infiltration (Fig 4.9B, 4.10B; arrows) was observed amongst 0.01µg/L group, while predominantly blue connective tissue staining with some ruptured myocardial fibres (Fig 4.10C) was observed in the 200µg/L groups (Figs 4.9C, 4.10C). The 500µg/L exposed group showed intense red staining fibres indicating hypertrophied myocytes with gapping intercalated discs (black arrow) (Figs 4.9D).

Figure 4.9 Photomicrograph of juvenile cardiac tissue showing effects of atrazine on the connective tissue profile in different groups.

A: control group, no connective tissue observed with myofibrils

B: 0.01ug/L group, onset of infiltration of connective tissue into myofibrils (thick arrows)

C: 200ug/L group, general distribution of connective tissue replacing most of the myofibrils

D: 500ug/L group, disarray and dense myofibril and interstitial fibrosis, Intercalated disc (arrows), appear broken.

Circles- blood cells, stars- interstitial space, arrows- intercalated discs, block arrows- connective tissue infiltration. Mallory one step connective tissue stain X630. Scale bar 100µm

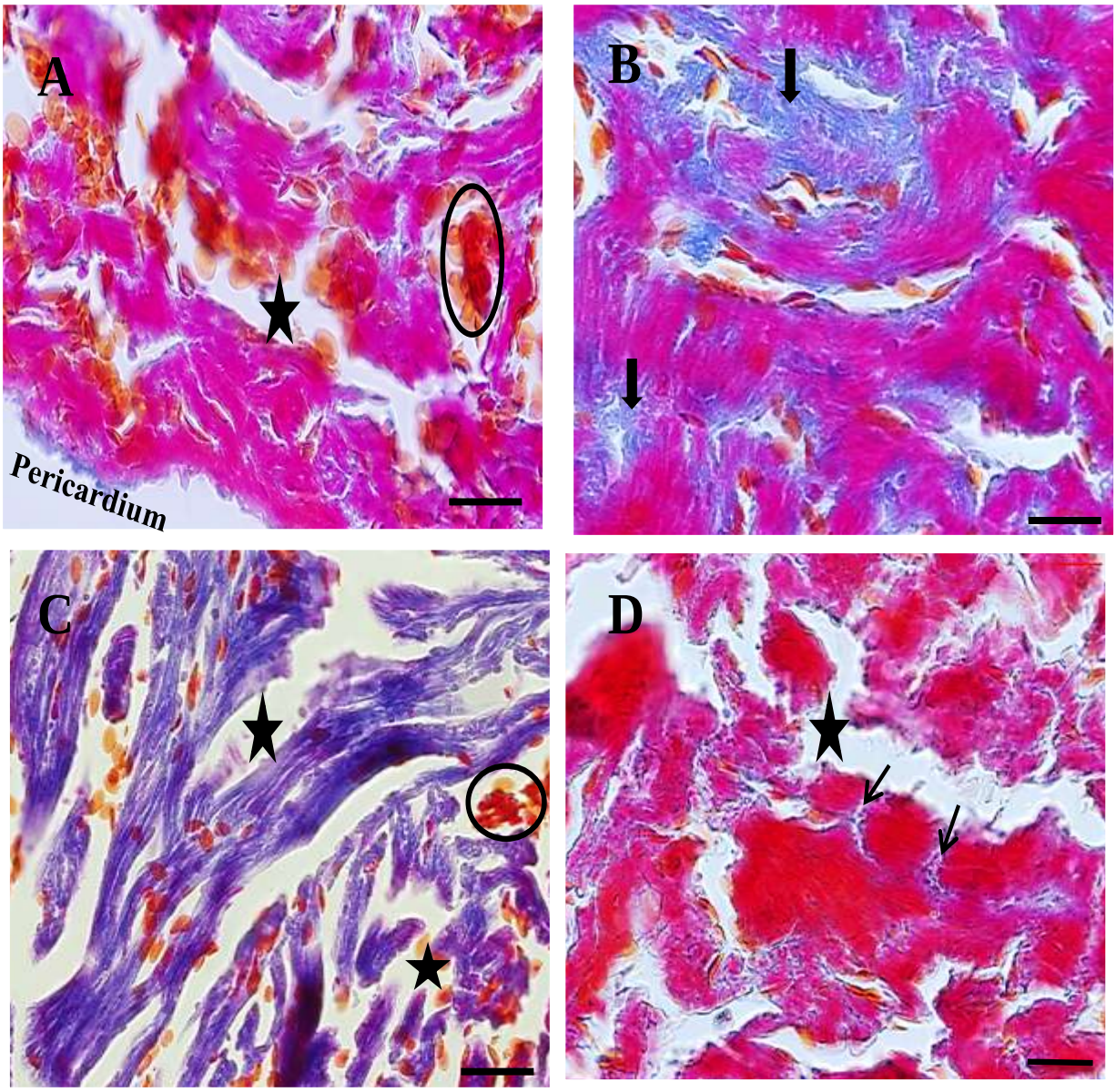

A
Pericardium
B
C
D

Figure 4.10 Photomicrograph of adult frog cardiac tissue showing effects of atrazine on the connective tissue profile in different groups.

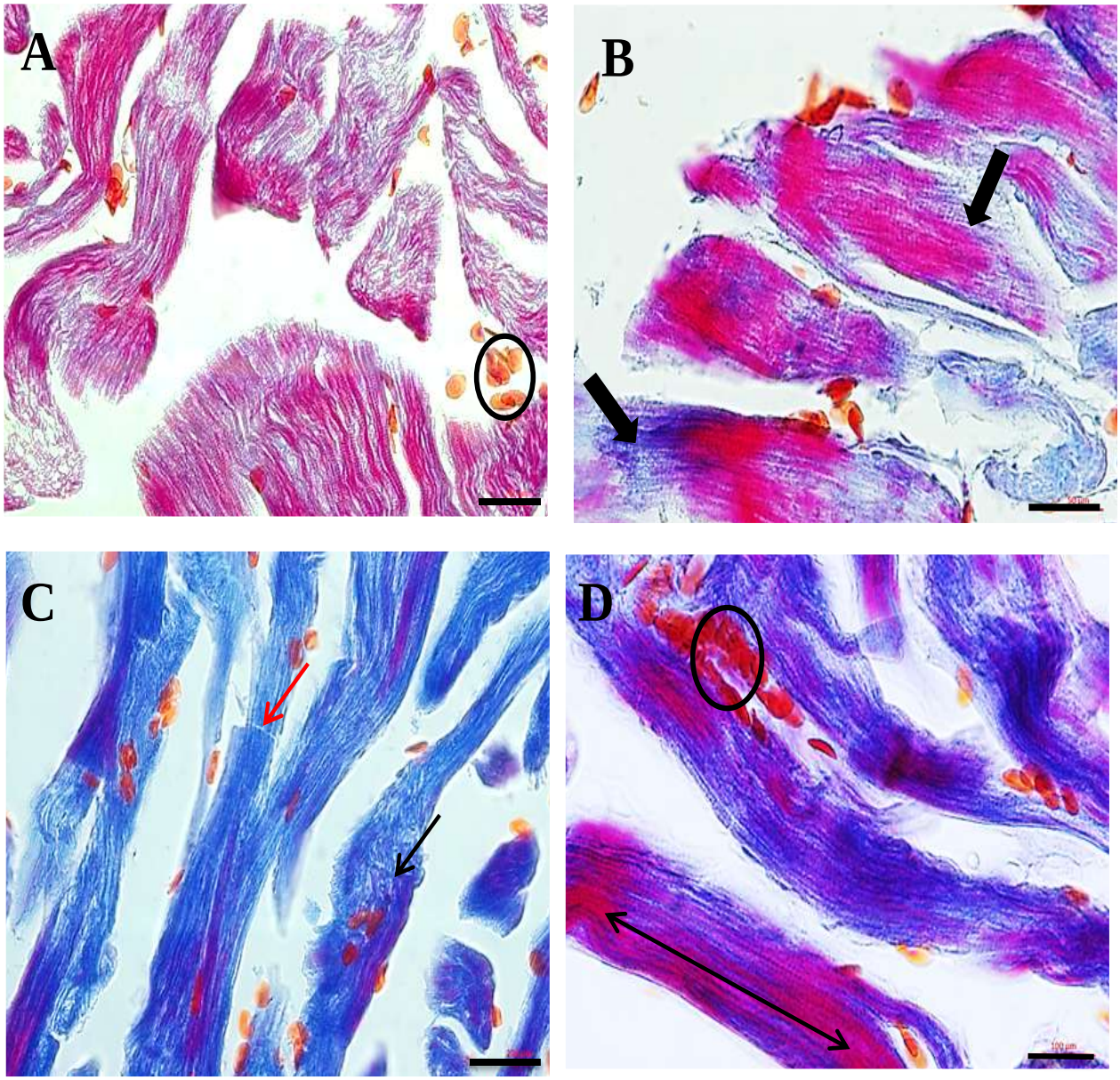
A: Control group, little to no connective tissue.

B: 0.01µg/L group, observation of the onset of infiltration of connective tissue to surround the myofibrils (thick arrows)

C: 200ug/L group show breakdown of normal orientation of cardiac fibres (black arrow), intercalated disk (red arrow) and general distribution of connective tissue replacing some of the myofibrils

D: 500ug/L group, onset of dark red staining myofibrils (double arrow)

Circles- red blood cells, Mallory one step connective tissue stain x630. Scale bar 100µm and interstial spaces (black star) are observed especially in 200ug/L and 500ug/L.


A
B
C
D

4.3.3 Histology of Cerebellar cortex

Figures 4.11 and 4.12 demonstrate the histology of juvenile and adult frog's cerebellar cortex using Cresyl violet staining technique. The three layers of the cerebellum from outside inwards (molecular, Purkinje and granular layers) are clearly visible in the control groups (Fig 4.11A, 4.12A) of juvenile and adult frogs. Pale staining pear shaped Purkinje neurons are observed with a vesicular nucleus, in the intermediate layer. In the inner granular layer are darkly stained round granular cells densely arranged, while the molecular layer has very scanty round basket cells. The granular layer cells were observed to increase in width more in treated groups than the control groups.

In the control groups of both the juvenile and adult frogs the Purkinje cell layers are densely packed with a thick cluster of numerous Purkinje neurons with vesicular nucleus (Fig 4.11A, 4.12A). The Purkinje layer gradually became more diffuse with fewer cells in the 0.01 µg/L and 200 µg/L groups (Fig 4.11B, 4.12B and 4.11C, 4.12C). The Purkinje cell layer was reduced to almost a single scanty row in 500 µg/L treated group (Fig 4.11D, 4.12D), and the nuclei were dark and enlarged in juvenile but small and round in adult 500 µg/L group.

Furthermore most of the Purkinje neurons in the juvenile and adult frog treated groups did not have vesicular nucleus but nuclei appear dark (pyknotic; triangle), fragmented (karyolitic; square) or smaller and shrunken (apoptotic). However the observed apoptotic nuclei were observed more in the juvenile treated groups compared to the control (Fig 4.11, 4.12), while apoptotic nuclei was only observed in the treated groups of the adult frogs and none in the control. Granular cell layer in juvenile treated groups appear wider than in the control.

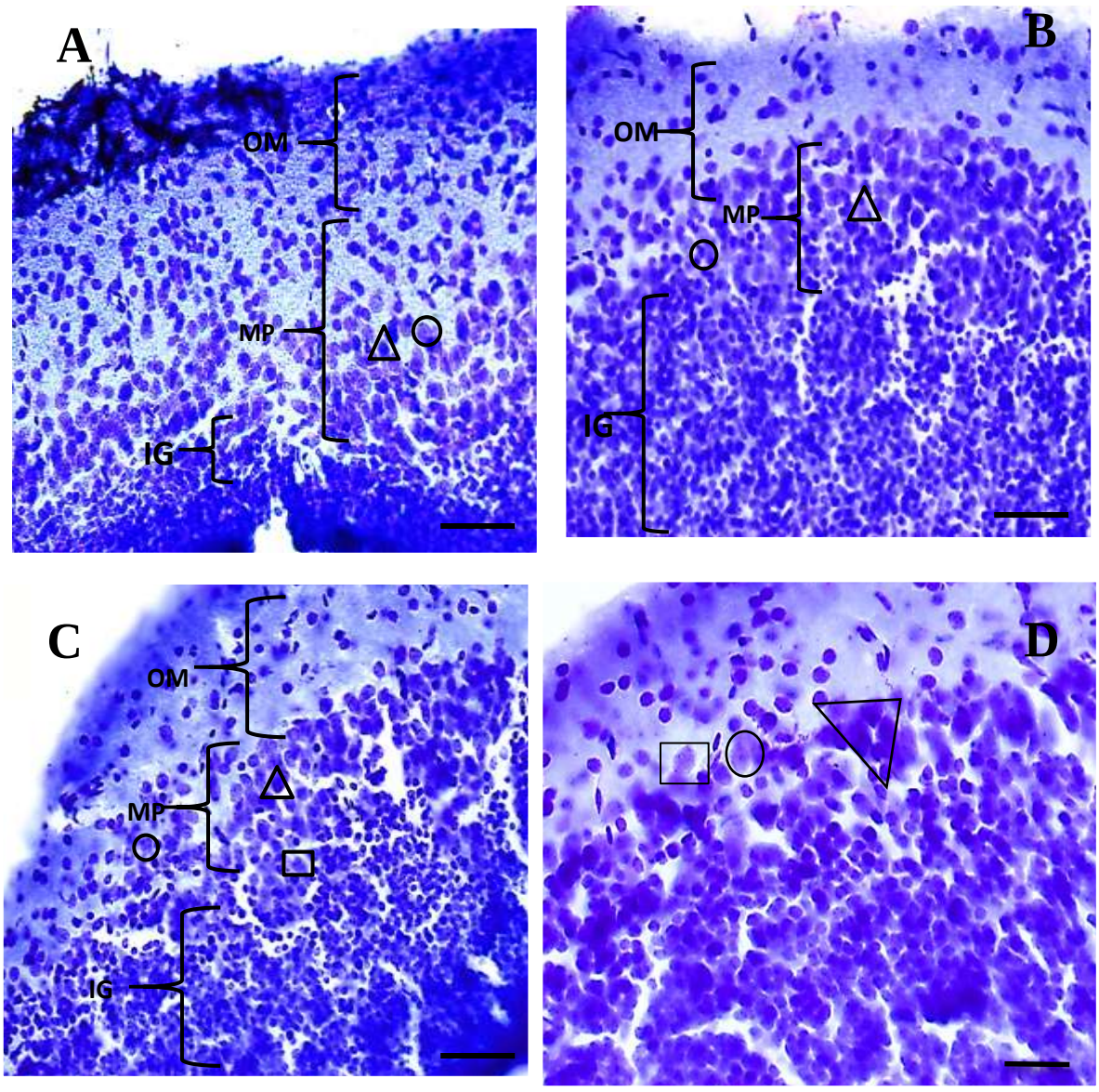
Figure 4.11 Photomicrograph of juvenile cerebellar cortex showing effects of atrazine in different groups.

A: Control group densely packed wider layer of numerous Purkinje neurons with pale vesicular nucleus (circle) and few apoptotic nuclei (triangle)

B: 0.01µg/L group, larger granular layer, thin layer of Purkinje cells with vesicular nucleus (circle) and few shrunken and dark stained apoptotic nuclei (triangle)

C: 200µg/L group few rows of diffuse Purkinje cells, some with vesicular nucleus (circle), dark pyknotic nucleus (triangle) and fragmented karyolytic nucleus (squares)

D: 500µg/L diffuse scanty Purkinje cells, few with vesicular nuclei (circles), darkly stained pyknotic nucleus (triangle) and karyolytic nucleus (squares)
Cresyl violet stain X630. The outer molecular layer OM, the middle Purkinje layer MP and the inner granular layer IG. Scale bar 100µm


A
OM
MP
IG
B
OM
MP
IG
C
OM
MP
IG
D

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Figure 4.12 Photomicrograph of adult frog cerebellar cortex, showing effect of atrazine in different groups.

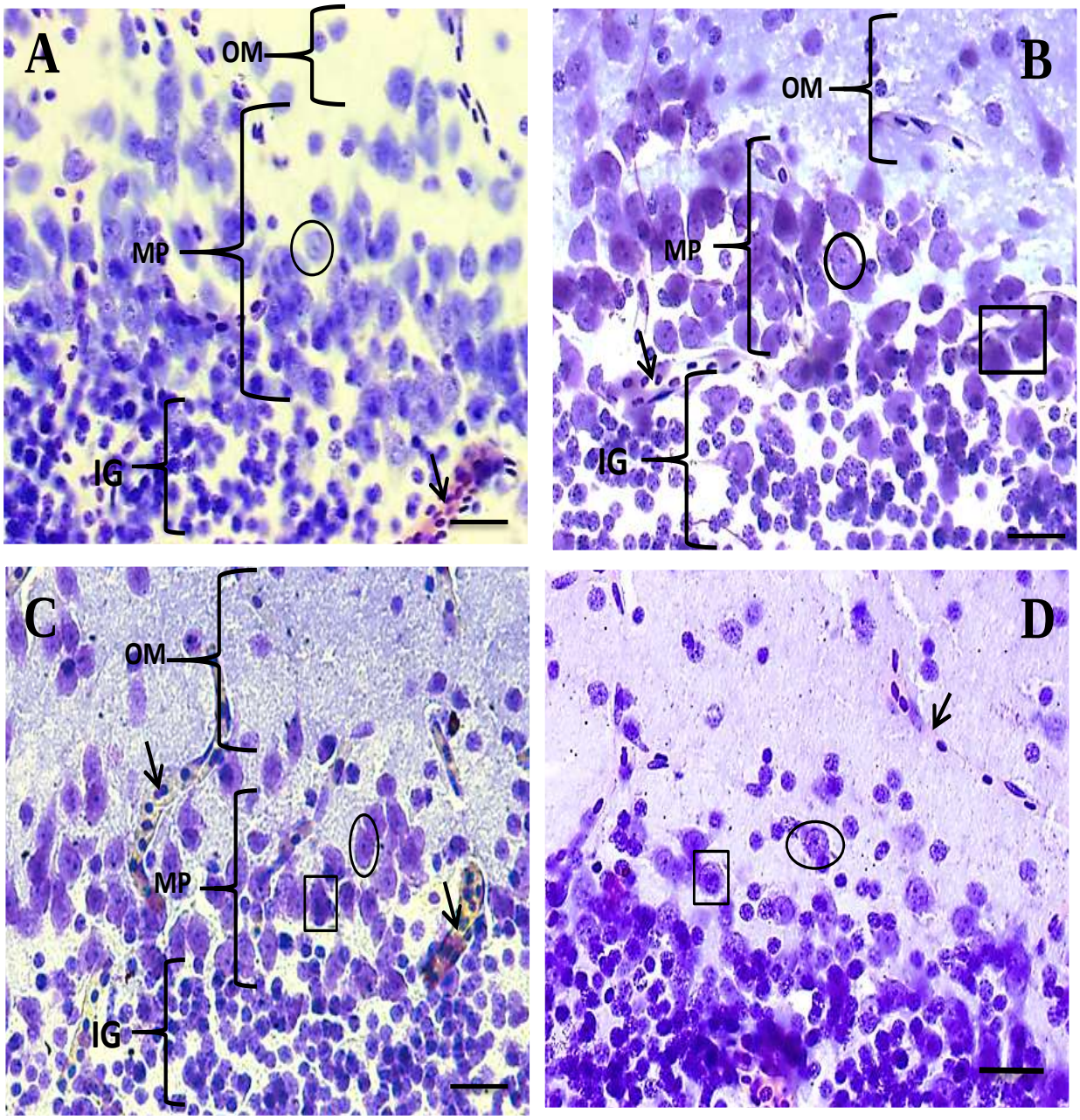
A-control group densely packed wider layer of Purkinje cells with vesicular nucleus (circle) that reach into the molecular layer

B-0.01ug/L group narrow layer of enlarged Purkinje cells, some with vesicular nucleus (circle) and few with dark apoptotic nucleus (squares)

C-200ug/L group slightly enlarged row of diffuse Purkinje cells, some with vesicular nucleus (circle) and few with dark apoptotic nucleus (squares)

D-500ug/L diffuse scanty and smaller round Purkinje cells with vesicular nucleus (circles), few dark apoptotic nucleus

Cresyl violet stain X630. Blood vessels (black arrow), the outer molecular layer OM, the middle Purkinje layer MP and the inner granular layer IG. Scale bar 100μm


A
OM
MP
IG
B
OM
MP
IG
C
OM
MP
IG
D

4.4 Immunohistochemistry and immunofluorescence

4.4.1 Expression of eNOS in cardiac tissue

In the juvenile and adult control groups, eNOS was expressed in distinct, intense and exclusive borders of the endothelium within the intact endocardium but the myocardium remained very pale and unstained (Fig 4.13A, 4.14A). In the 0.01 µg/L and 200 µg/L groups the penetration and staining of the myocardium by eNOS was observed, more in the 200 µg/L than in the 0.01 µg/L group in both juvenile and adults cardiac tissues (Figs.4.13B, 4.14B); additionally a mixture of stained and unstained endocardium endothelium (EE), ripped and un-ripped endothelium was also observed in the 200 µg/L (Fig 4.13C, 4.14C). However in the 500 µg/L group eNOS expression was not observed in the juvenile group (Fig 4.13D) but very weakly expressed in the adult groups (Fig 4.14D).

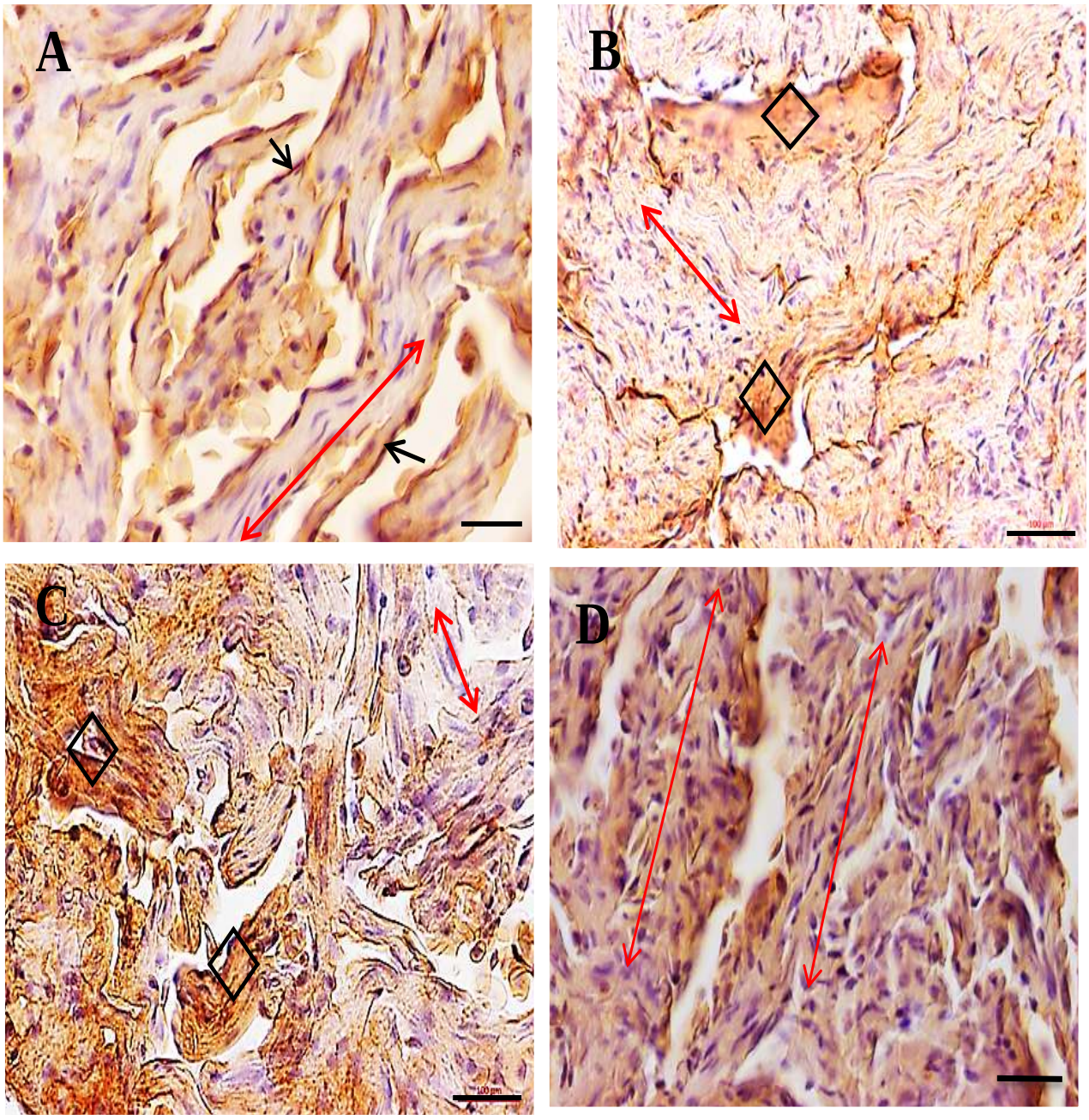
Figure 4.13 Photomicrograph of eNOS expression in juvenile cardiac tissue, showing effects of atrazine in different groups.

A: Control group, even and intense expression of eNOS in the endothelium of the endocardium (arrows) and very pale, unstained myocardium (double red arrow)

B: 0.01 µg/L group, expression of eNOS in the endothelium of the endocardium, few areas in the myocardium express eNOS(black diamonds).

C: 200 µg/L group, endocardium endothelium as well as the myocardium express eNOS (black diamonds), while the internal myocardial fibres receive very little to no expression of eNOS (red arrow)

D: 500µg/L group, myocardium remains completely unstained (double red arrows) and endothelium hardly expresses any eNOS. X630. Scale bar 100µm


A
B
C
D

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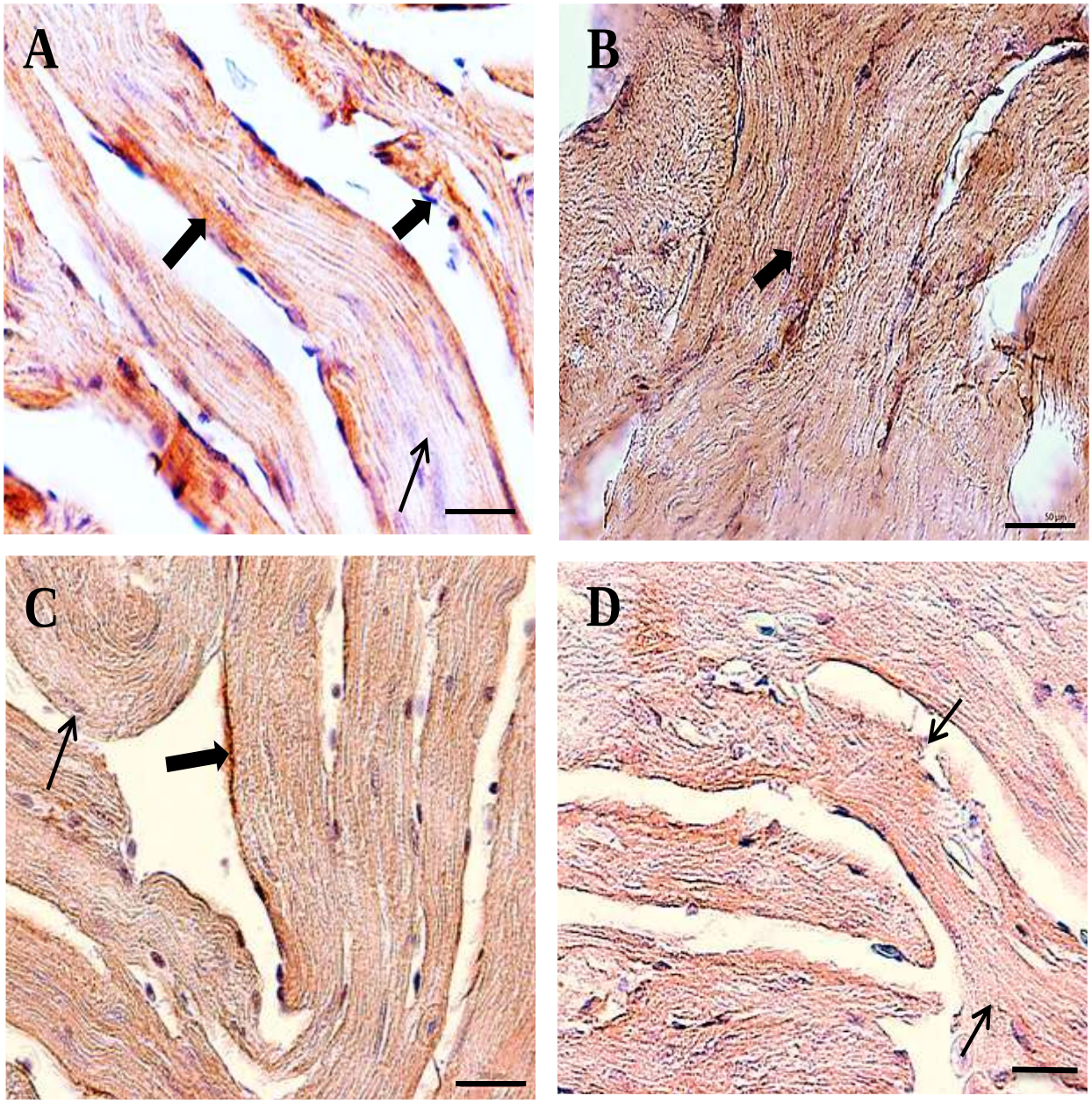
Figure 4.14 Photomicrograph of eNOS expression in adult frog cardiac tissue, showing atrazine effect in different groups.

A: Control group, even and intense expression of eNOS in the endothelium of intact endocardium (block arrows) and very pale, unstained myocardium (arrow)

B: 0.01ug/L group, intense expression of eNOS in the myocardium as well as endocardial endothelium (block arrow)

C: 200ug/L group, less expression of eNOS in intact EE (block arrow) but not expressed in ruptured areas (arrow)

D: 500 ug/L group, very pale to no staining of tissue, lots of ripped endocardial endothelium (arrows) X630. Scale bar 100μm


A
B
C
D

4.4.2 Immunofluorescence expression of cardiac eNOS (adult frogs)

Immunofluorescence labeling of eNOS in the adult *Xenopus* cardiac tissue eliminates the brown background staining in DAB immunohistochemistry and shows specific detail of eNOS expression exclusively in the endocardium endothelium (EE) of the control group (Fig 4.15aC). The NOS is diffusely expressed, spreading over from the EE to some part of the myocytes in the 0.01 µg/L group (Fig 4.15aD). In the 200 µg/L and 500 µg/L EE staining is very faint with fewer EE nuclei staining in the 500 µg/L exposed group (Fig4.15bC, D).

Figure 4.15a Photomicrograph of immunofluorescence expression of eNOS in adult cardiac tissue, showing atrazine effects in control and 0.01µg/L groups.

A: control group, DAPI nuclei stain are numerous

B: 0.01µg/L group, many DAPI nuclei

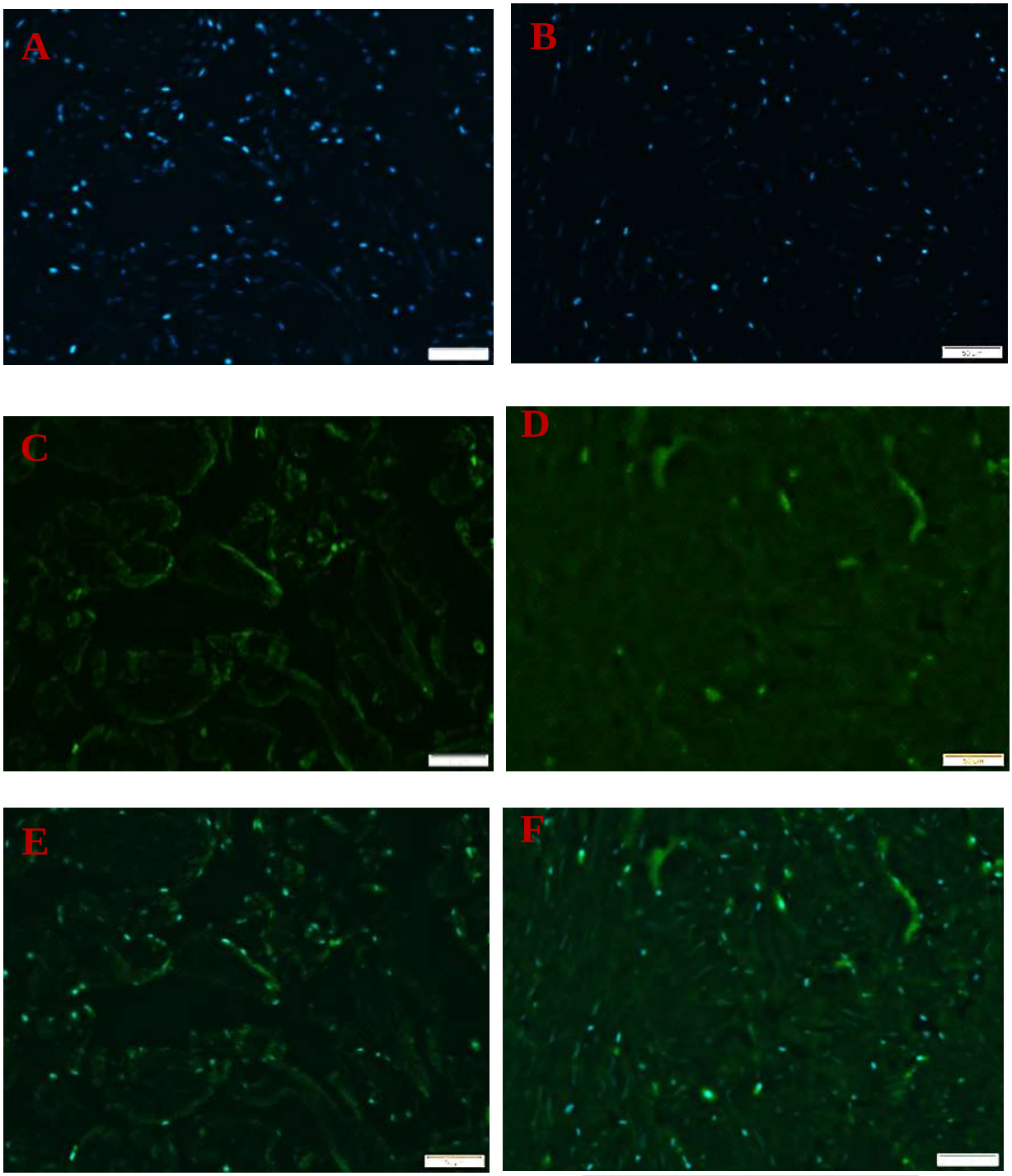
C: Control endocardium endothelium intensely expresses eNOS

D: 0.01µg/L group, uneven expression of eNOS in the endothelium and myocardium X400

E: Control group, overlay of eNOS expression and DAPI nuclei stain

F: 0.01µg/L group, overlay of eNOS expression and DAPI nuclei stain, scale bar 50µm

Figure 4.15a



Control group

0.01µg/L exposed group

Figure 4.15b Photomicrograph of immunofluorescence expression of eNOS in adult cardiac tissue, showing atrazine effects in 200µg/L and 500µg/L groups.

A: 200µg/L group, DAPI nuclei stain are few

B: 500µg/L group, DAPI nuclei are very scanty

C: 200µg/L group endothelium palely expresses eNOS

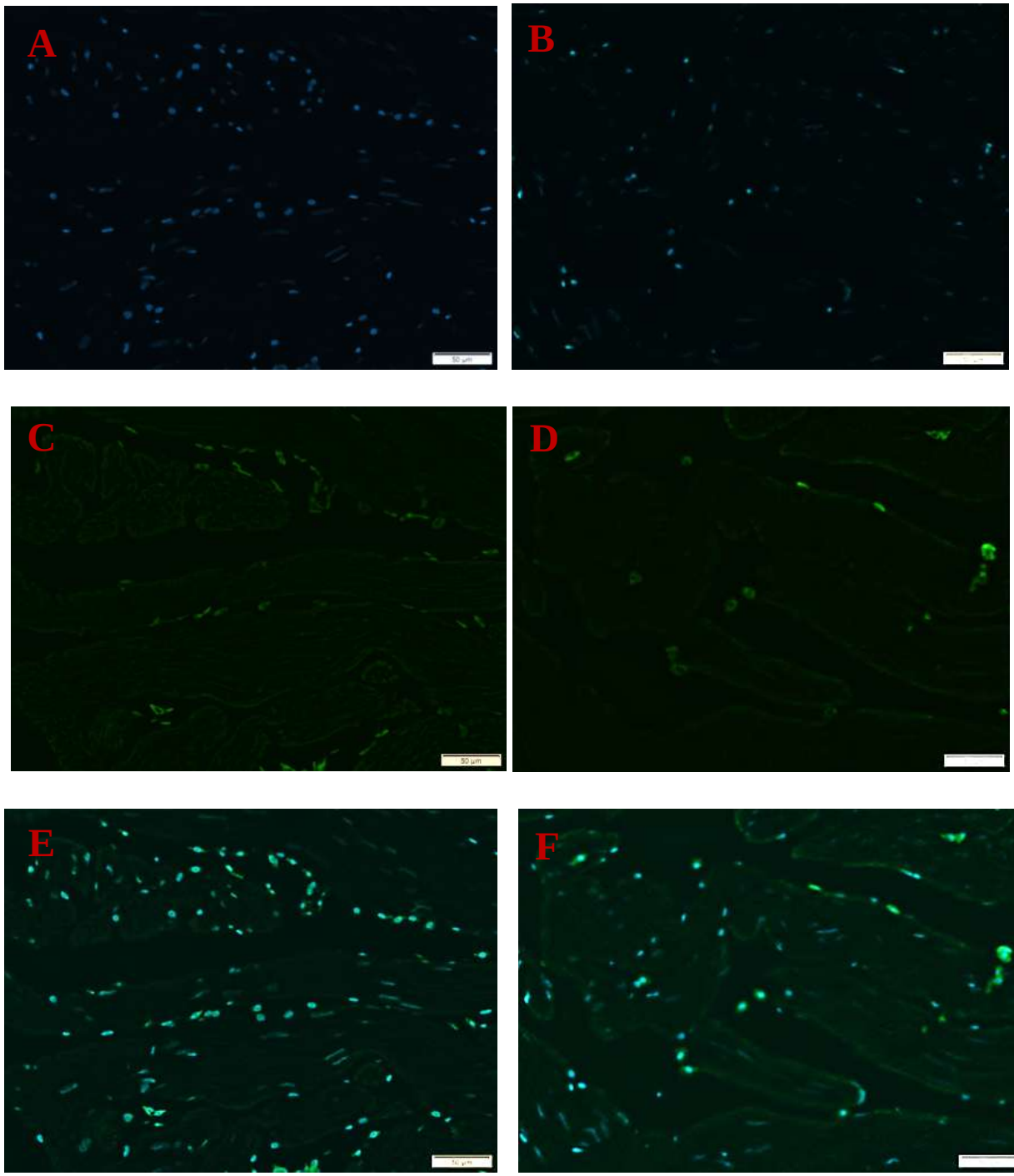
D: 500µg/L group, very pale expression of eNOS in the endothelium and myocardium X400

E: 200µg/L group, overlay of eNOS expression and DAPI nuclei stain

F: 500µg/L group, overlay of eNOS expression and DAPI nuclei stain X400, Scale bar 50µm

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Figure 4.15b



200µg/L group

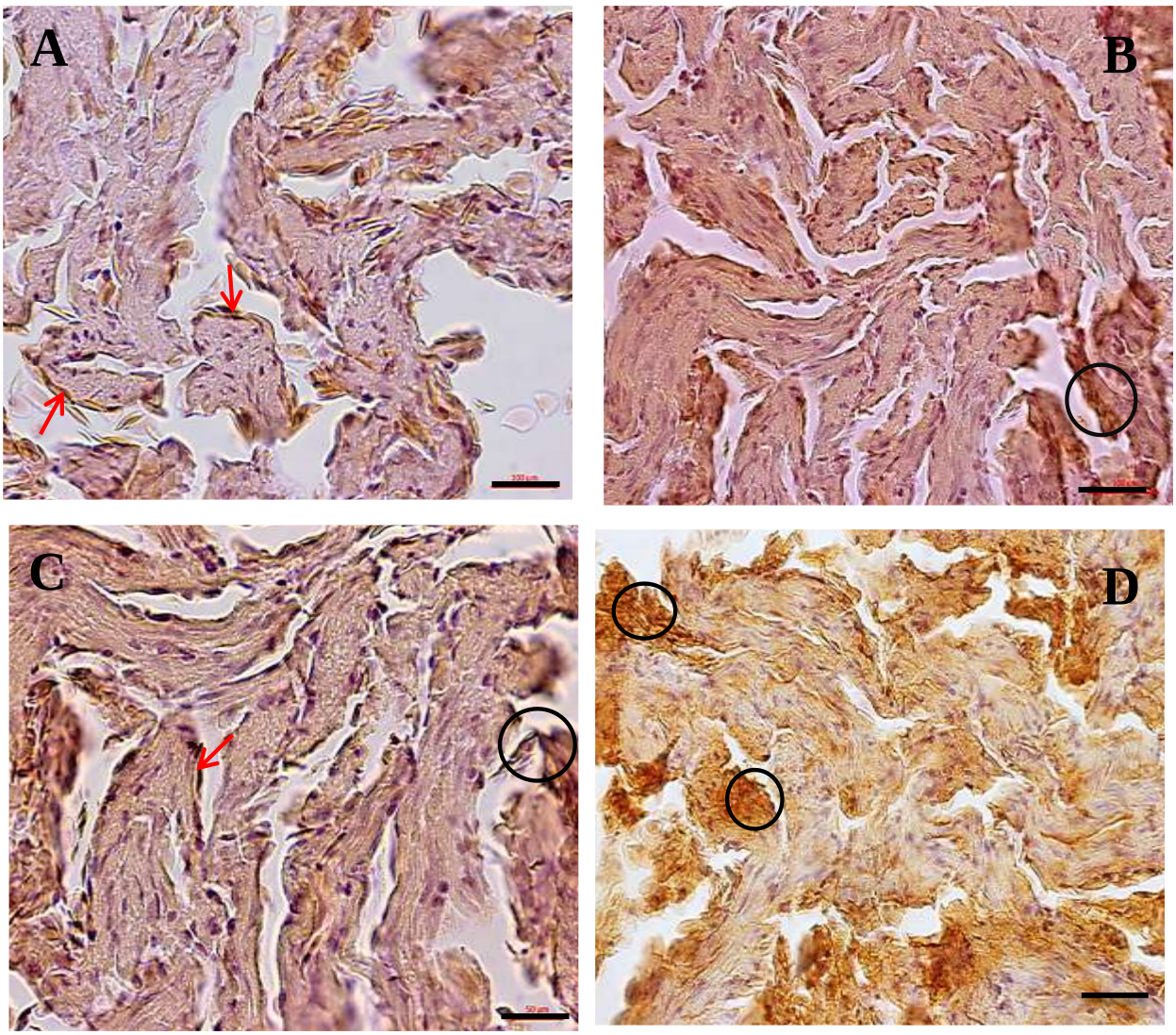
500µg/L group

4.4.3 Expression of IP_3Rs in cardiac tissue

Several dilutions of primary and secondary antibodies where carried out to optimize IP_3R expression; but the juvenile cardiac tissue failed to express any IP_3Rs (Fig 4.16). Both control and treated groups had similar background and non-specific brown staining, while cardiac myocytes remained unstained and did not express IP_3Rs (Fig 4.16).

In the adult cardiac tissue, IP_3Rs was clearly expressed in the perinuclear membrane of Purkinje fibers (control and 0.01 µg/L groups) and cardiac myocytes of all groups (Fig 4.17). Figure 4.18 shows the mean number of cells expressing IP_3R. An increase in the mean number of cells (cardiac or Purkinje cells) expressing IP_3R in 0.01 µg/L group and a decrease in the 200 µg/L and 500 µg/L groups were observed when compared with the control group.

Also the Kruskal Wallis test showed significant difference ($p < 0.006$) between groups (Table 7.9.3 Appendix). The post hoc test further showed the IP_3R expression significantly decreased ($p < 0.03$) in the 200 µg/L group relative to the control, and significantly decreased in the 200 µg/L and 500 µg/L groups compared to the 0.01 µg/L group ($p < 0.009$). The increase in the IP_3R expression in the 0.01 µg/L group was not significant compared to control.



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Figure 4.16 Photomicrograph of IP_3R immunohistochemistry in juvenile frog cardiac tissue of different groups.

A -control, B- 0.01µg/L, C- 200µg/L, D-500µg/L. Mostly blood cells (red arrows), and non-specific background staining (circles), no expression of IP_3Rs X630. Scale bar (black) 100µm

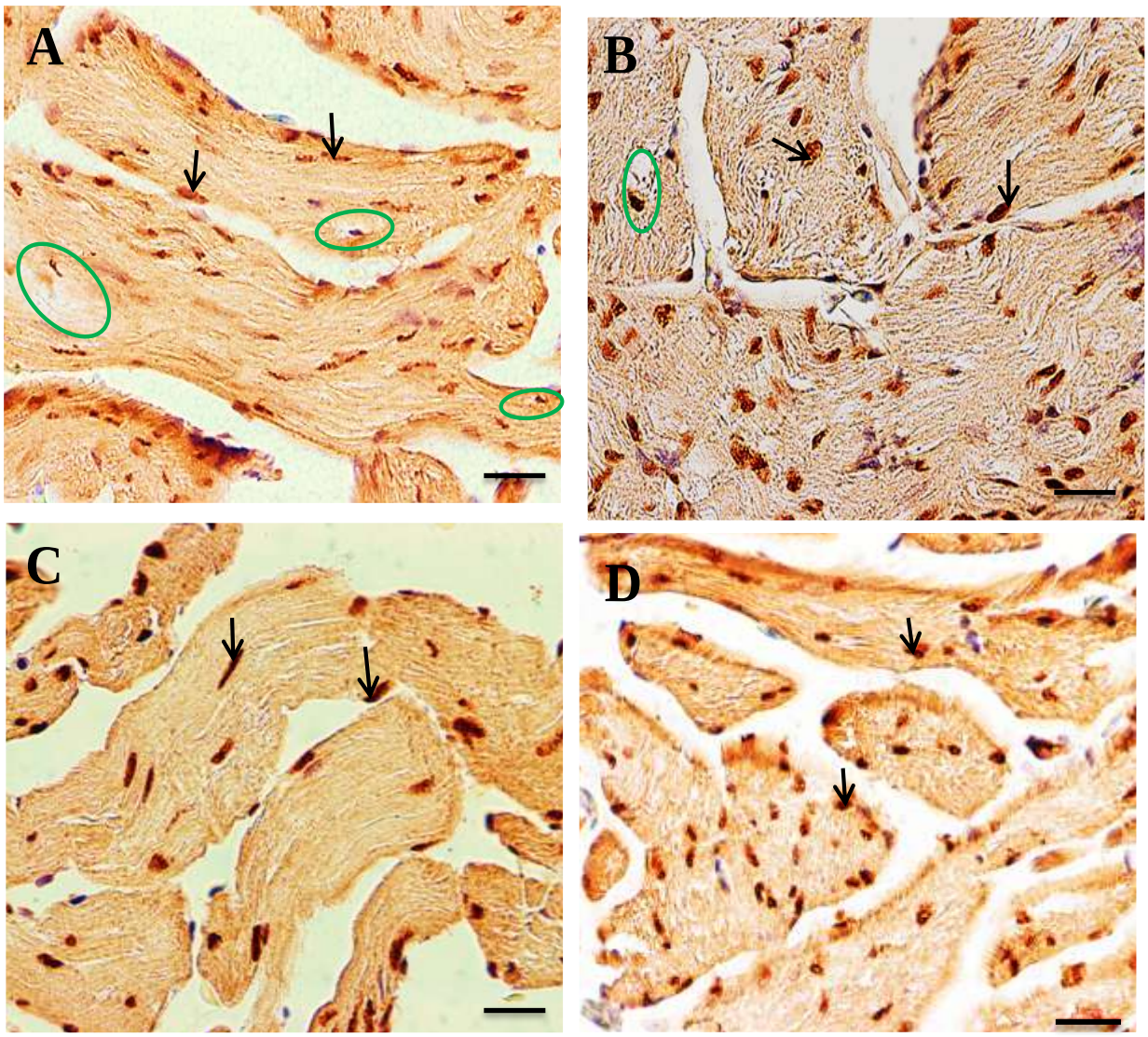


Figure 4.17 Photomicrograph of IP_3R expressions in adult frog cardiac tissue showing effects of atrazine in different groups.

A: Control IP_3R expression in perinuclear membrane of numerous Purkinje fibers (green circles) and cardiac myocytes (black arrows)

B: 0.01µg/L group, few Purkinje fibers (green circle) and highest amount of expressed IP_3Rs observed in cardiac myocytes (black arrows)

C: 200µg/L few expressions of IP_3Rs in cardiac myocytes

D: 500µg/L group, numerous expressions of IP_3Rs in cardiac myocytes (black arrows) X630. Scale bar 100µm

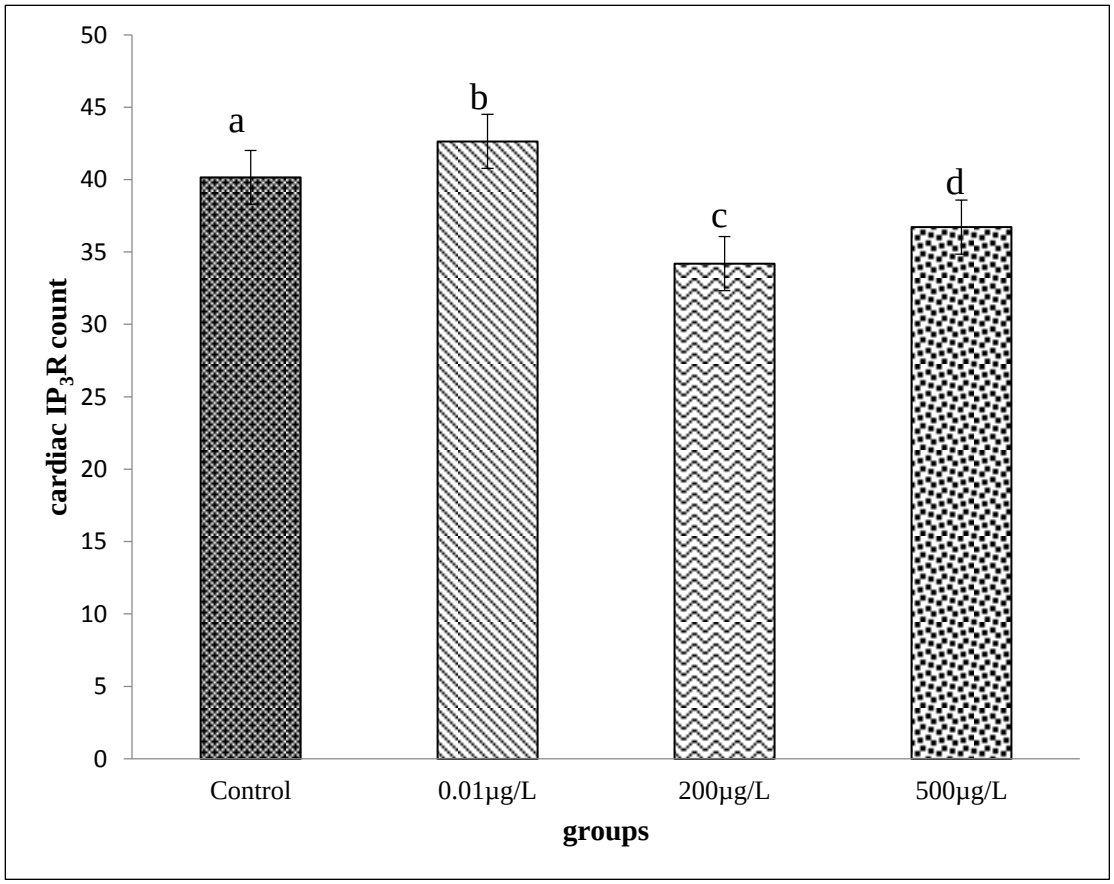


Figure 4.18 Dose dependent effect of atrazine on myocytes (count) expressing IP_3Rs in adult frog heart

'c' is significantly decreased relative to 'a' ($p<0.03$), 'b' significantly increased relative to 'c' and 'd' ($p< 0.05$; $p< 0.009$, respectively)

4.4.4 Expression of IP_3R in the cerebellar cortex

The cerebellar cortex in the juvenile frog

General observations across the groups indicate that the number of expressed IP_3Rs decreased as the concentration of atrazine exposure increased (Fig 4.19).

The count of cells expressing IP_3Rs in juvenile brains showed significant differences between the control and atrazine treated groups (p <0.000) (Table 7.9.5 Appendix). Further analysis (Mann Whitney post hoc test) showed significant decrease in the number of IP_3Rs expressed in the 200 µg/L ($p < 0.033$) and 500µg/L relative to the control; but the observed decrease in the 0.01 µg/L group was insignificant relative to the control ($p < 0.208$). In addition the 200 µg/L group was significantly decreased relative to the 0.01 µg/L group ($p < 0.029$), while the 500 µg/L was significantly decreased relative to 0.01 µg/L ($p < 0.01$) and 200 µg/L groups ($p < 0.01$) (Table 4.2).

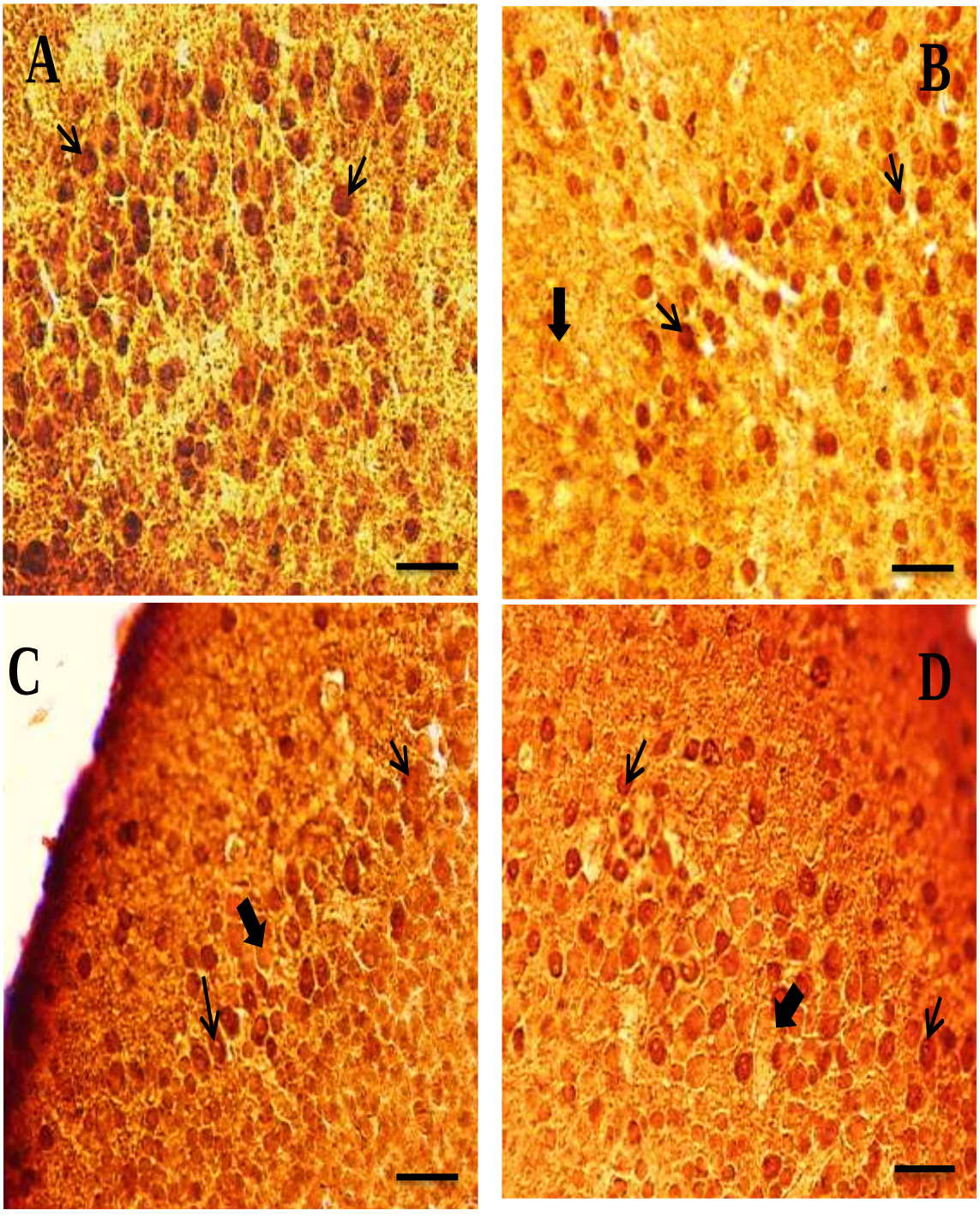
Figure 4.19 Photomicrograph of IP_3Rs expressions in juvenile cerebellar cortex, showing effects of atrazine in different groups.

A: Control numerous represent intensely expressed IP_3Rs (arrows)

B: 0.01µg/L group many expressed IP_3Rs (arrows) and few unexpressed IP3Rs (block arrow)

C: 200µg/L almost equal amount of expressed (arrows) and unexpressed IP_3Rs (block arrows)

D: 500µg/L numerous unexpressed IP3Rs (block arrow) an d fewer expressed IP_3Rs (arrows) X630. Scale bar 100µm


A
B
C
D

.

Table 4.2 Comparison of mean number of Purkinje cells expressing IP_3Rs in juvenile frog cerebellum of different atrazine treated groups
(Mann Whitney post-hoc test)

Groups	***p* values**
Control vs 0.01µg/L	0.269
Control vs 200µg/L	0.033*
Control vs 500µg/L	0.003*
0.0lµg/L vs 200µg/L	0.029*
0.01µg/L vs 500µg/L	0.00*
200µg/L vs 500µg/L	0.00*

*statistically significant difference between group means ($p<0.05$)

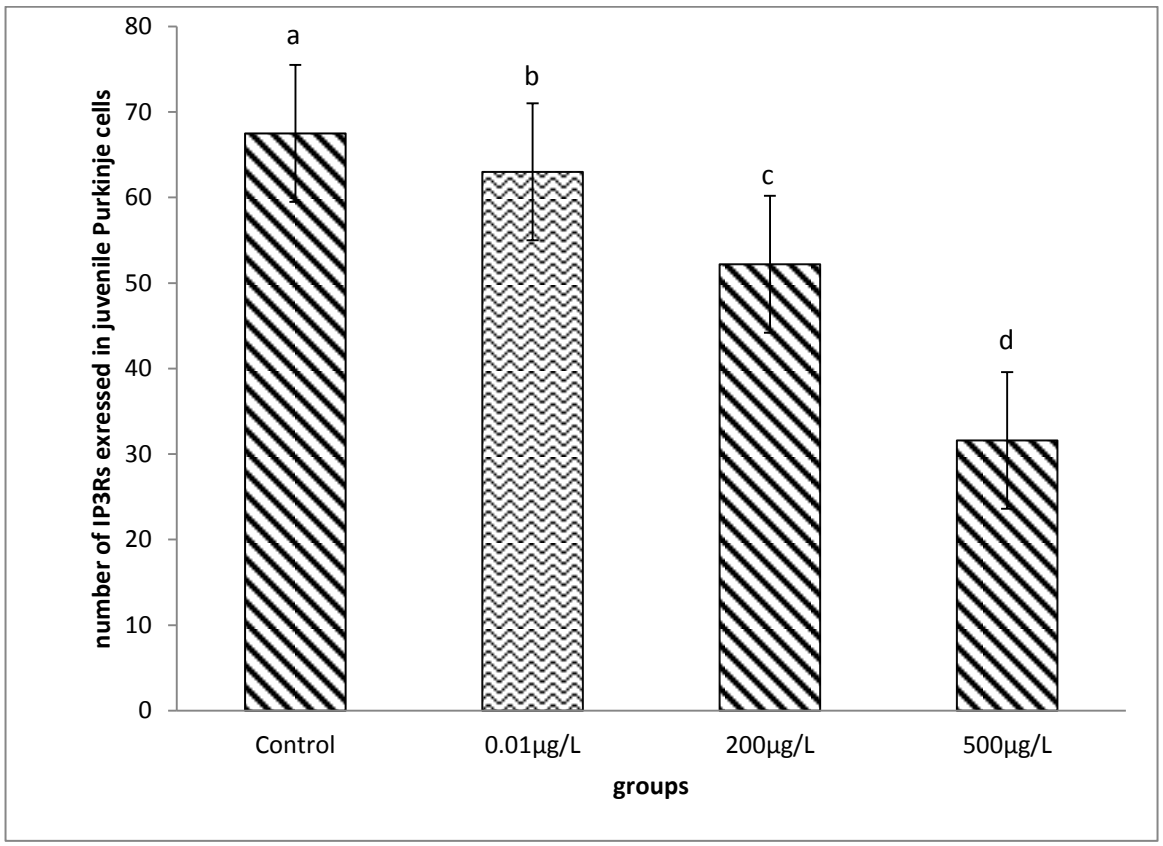


Figure 4.20 Dose dependent effect of atrazine on cell count of IP_3R expression in juvenile cerebellar Purkinje cells

'c' and 'd' (but not b) is significantly decreased relative to 'a'. d is significantly reduced relative to b and c.

The cerebellar cortex in the adult frog

In the adult cerebellum, a decrease in the number of expressed IP_3Rs was observed with increase in atrazine concentration (inverse relationship) (Fig 4.22).

The Kruskal Wallis test found significant difference in the cell count of expressed IP_3Rs between the control and treated groups $p < 0.00$ (Table 7.9.7 Appendix). Mann Whitney post hoc test reported significant decrease in the number of expressed IP_3Rs; in the 200 µg/L group ($p < 0.037$) and 500 µg/L ($p < 0.00$). However a non-significant decrease observed in the 0.01 µg/L relative to the control ($p < 0.208$) and in the 200 µg/L relative to 0.01 µg/L ($p < 0.262$).
Additionally the number of expressed IP_3Rs was reported to be significantly reduced in the 500 µg/L group when compared with the, 0.01 µg/L ($p< 0.00$) and the 200 µg/L groups $p<0.00$ (Table 4.3).

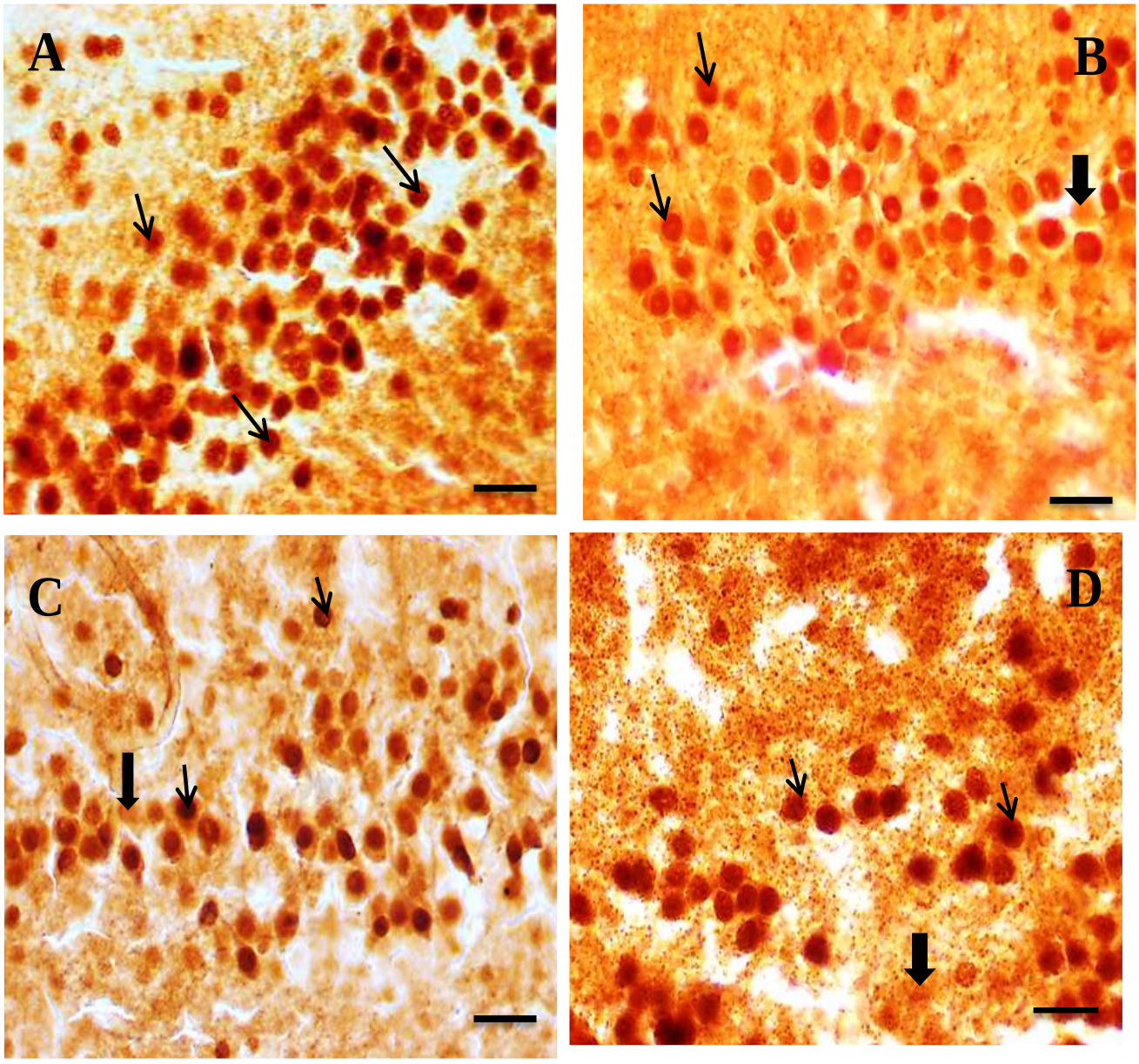
Figure 4.21 Photomicrograph of IP_3R expressions in adult cerebellar cortex showing effects of atrazine in different groups

A: Control, numerous Purkinje cells express IP3R (arrows)

B: 0.01ug/L group many cells observed intensely express IP3R (arrow) few unexpressed cells (block arrows)

C: 200ug/L and more non-expressed cells observed (block arrows) and few cells expressing IP_3R

D: 500ug/L fewer cells; many non-expressing IP_3R (arrows), many non-expressing cells (block arrows) X630. Scale bar 100µm


A
B
C
D

.

Table 4.3 Comparison of mean number of Purkinje cells expressing IP_3Rs in adult frog cerebellum of different atrazine treated groups
(Mann Whitney post-hoc test)

Groups	*p* values
Control vs 0.01µg/L	0..208
Control vs 200µg/L	0.037*
Control vs 500µg/L	0.003*
0.0lµg/L vs 200µg/L	0.262
0.01µg/L vs 500µg/L	0.00*
200µg/L vs 500µg/L	0.00*

*statistically significant difference between group means ($p < 0.05$)

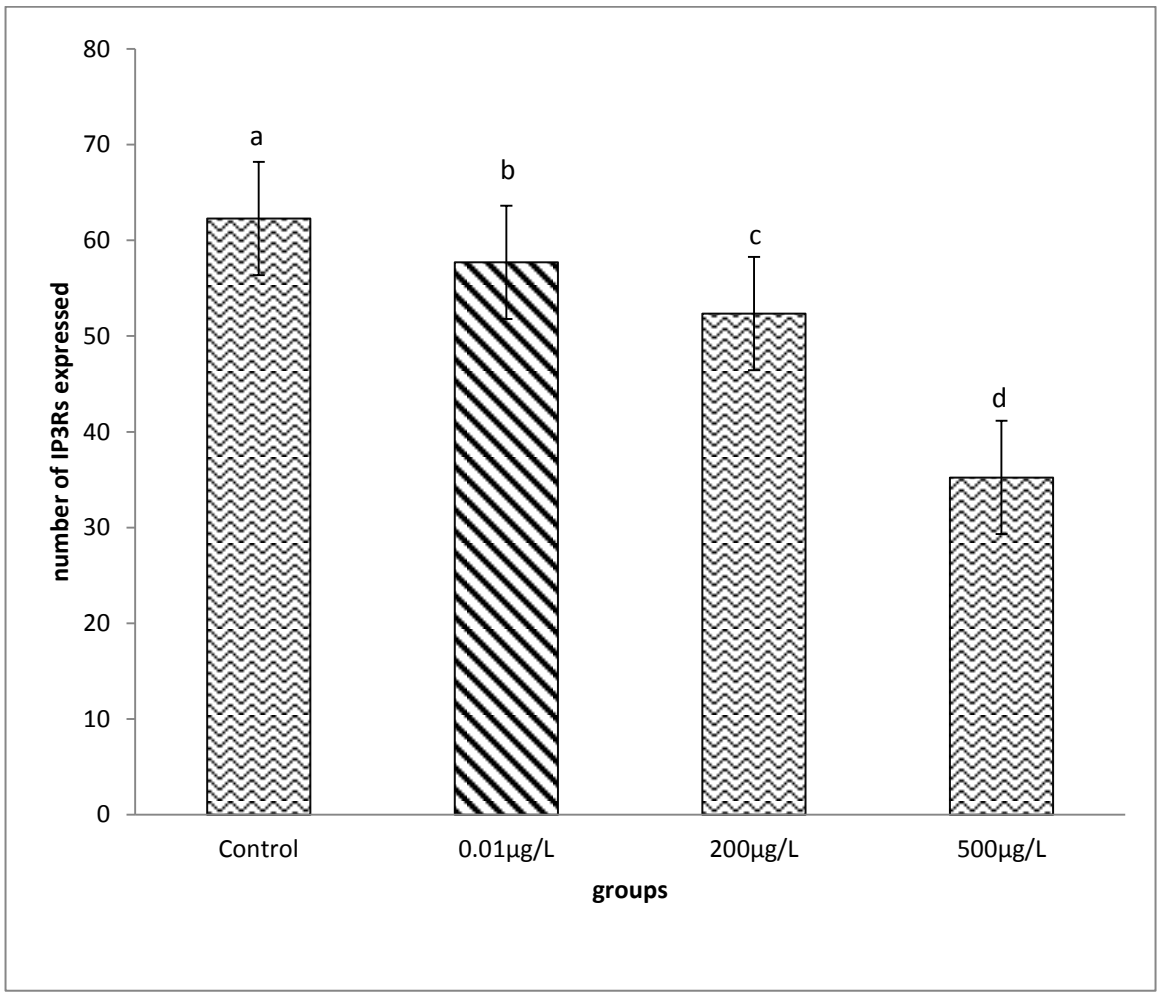


Figure 4.22 Dose dependent effects of atrazine on IP_3R expression in adult cerebellar Purkinje cells

Significant reduction in 'c' and'd' (but not 'b') relative to 'a' (p<0.037), 'd' significantly reduced relative to 'b' and 'c' (p< 0.00). Reduction in 'c' relative to 'b' is insignificant.

4.4.5 Expression of nNOS in the cerebellar cortex

The immunohistochemistry carried out for nNOS in juvenile and adult frog cerebellar tissue was all negative. Both juvenile and adult *Xenopus* cerebellar tissue, stained with Abcam brand of antibodies at different dilutions all failed to express nNOS (Fig 4.23)

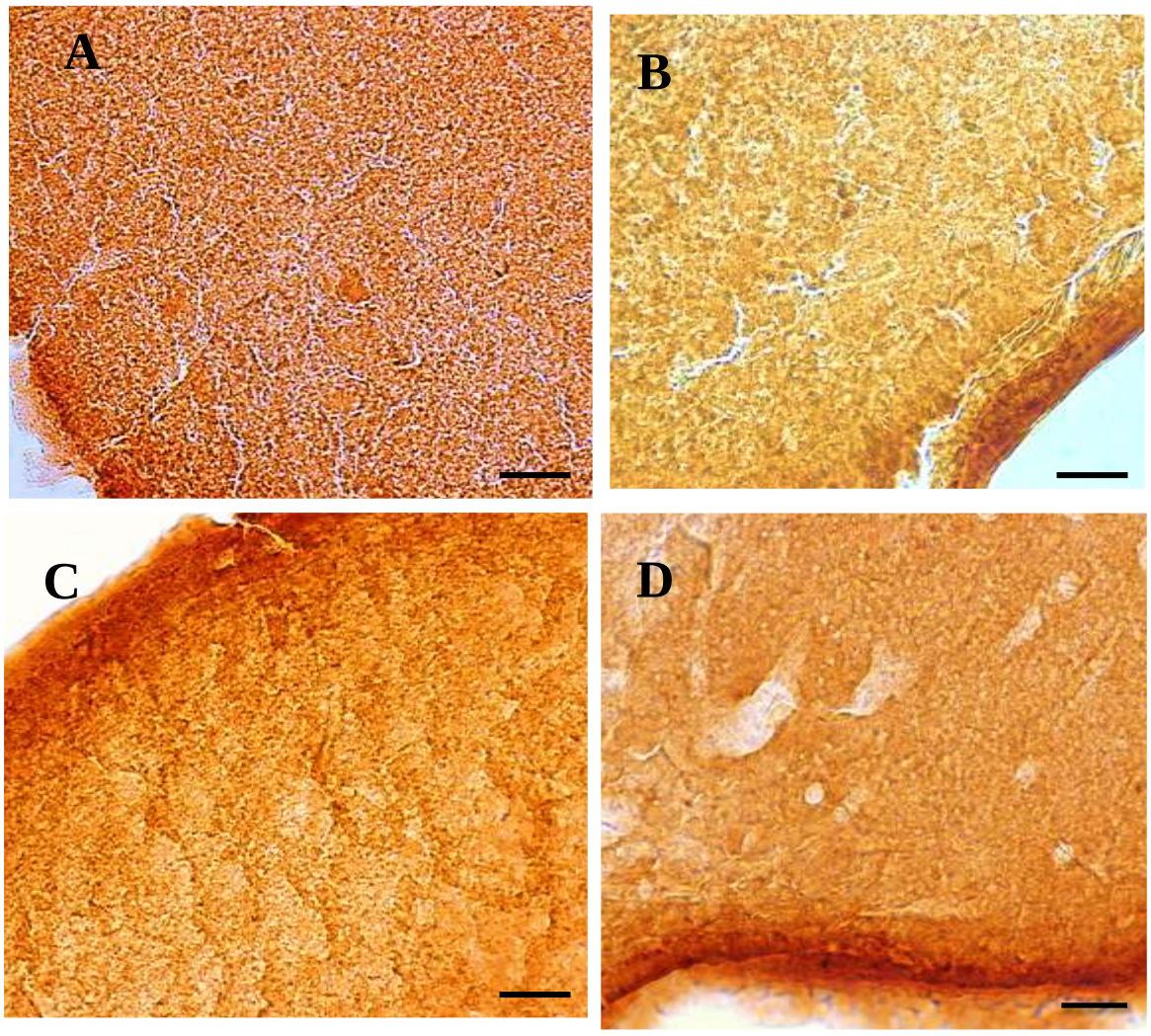


Figure 4.23 Photomicrograph of nNOS immunohistochemistry in juvenile and adult cerebellar cortex.

A and B- control groups in the juvenile and adult respectively, C- 200 µg/L juvenile group, D- 500 µg/L adult groups, No structure or cells expressed nNOS X630. Scale 100µm

CHAPTER FIVE

DISCUSSION

This present study was carried to determine the effects of atrazine on cardiac and cerebellar Purkinje cells. The laboratory experimental design was adopted to exclude the effects of other numerous contaminants in natural water sources that will obscure the effects of atrazine, and lead to inconclusive results.

Reports on the teratogenicity and embryotoxic effects of atrazine have mostly been conducted on amphibian and fish embryos between 48-96 hours post fertilization (Lenkowski *et al*., 2008, Ton *et al*., 2006, Allran and Karasov, 2000) which covers the egg and delicate embryonic stages. Thus, these studies excluded the tadpole stage of frog development. However, in the juvenile phase of this research, exposure to atrazine commenced with 10 day old tadpole through the delicate metamorphic development stages until they are matured into juvenile frogs. This enabled observation of atrazine toxic effects on the histology/morphology of such organs as the heart and brain

This study reports high mortality (60%) in the juvenile group and zero mortality amongst the adult group. The gradual increase in juvenile mortality over the duration of the experiment and the absence of mortality recorded in the adult group suggests that the juvenile frogs could be an endangered group to aquatic atrazine pollution (delicate developmental stage). The juvenile frogs are unable to withstand the toxic effects of atrazine unlike adult frogs which may be more resilient. It also suggests that increased use of atrazine in the agricultural sector and the subsequent presence of atrazine in water systems may threaten the existence of this frog species and generally unhealthy for consumption by anurans and aquatic animals (Duhigg, 2009) suggesting that the ecosystem equilibrium will be negatively disturb.

It has been reported that cardiac (left ventricular) mass peaks in adolescence and declines with age (Cain *et al.,* 2009). Also as younger elastic cardiac tissue atrophies with age, it stiffens (shrinks/ reduces area) and becomes weaker (Bhella, 2012). However in this study, morphological measurements revealed the area and volume of the heart were relatively highest in 200 µg/L atrazine and lowest in 500 µg/L atrazine exposed group of juvenile frogs and in 0.01

µg/L group of adult frogs. This suggests that exposure to atrazine can alter the size and mass of cardiac tissue in all age groups suggesting structural damage/alterations of the tissue which could interference with the normal function of the heart. Abnormalities in size of the heart may be associated with abnormalities of the cardiac muscle and connective tissue fibres which may result in malfunctioning of the heart. Additionally morphological examination of adult frog hearts reveals light cardiac tissue in the control group but a mixture of light and/or dark tissue in the treated groups suggesting adverse effects on some components (connective tissue) of the cardiac tissue in the treated frog hearts.

Histological examination of cardiac sections of frogs (juvenile and adult) exposed to atrazine also showed connective tissue infiltration suggestive of hyperactivity of cardiac fibers leading to hypertrophy and consequently cardiomegaly, a condition strongly associated with heart failure. Additionally, the hyperactivity of cardiac fibers may result to infiltration and compensatory replacement of cardiac fibers by connective (myocardial fibrosis). Fibrosis commonly results in heart attack (myocardial infarction) and eventual death (Sovari and Karagueuzian, 2011). This may explain the high mortality (60%) observed in the juvenile frog group. Radu *et al.*, 2012 previously described histopathological features of dilated cardiomyopathy such as interstial fibrosis and intense predominant collagen fibers which was observed in the connective tissue stain in the 200 µg/L groups of juvenile and adult groups. This corresponds with the very thin and wavy cardiac muscle fibers, identified mostly in the juvenile 200 µg/L group. Furthermore, the observed intertwined and disarray of myofibrils, reduced interstial spaces and darkened gross morphological observation in the 500 µg/L groups of the juvenile and adult frogs may suggests hypertrophied cardiomyopathy; a condition characterized by enlarged, thickened cardiac muscle which may be replaced by scar tissue and consequently cardiac malfunction and death as observed in the juvenile group. Therefore exposure to atrazine at different concentrations may induce different kinds of cardiomyopathy effects on the cardiac tissue of developing and adult species.

Observations of eNOS immunohistochemistry in the cardiac muscle sections of juvenile and adult frogs showed expressions in the both myocardium and endothelial endocardium of the treated groups (0.01 µg/L and 200 µg/L) when compared to the control, and very little to no expressions in the 500 µg/L group. This finding implies that exposure to atrazine reduces or

inhibits the expression of eNOS in the endocardium endothelium; or eNOS is expressed but are inactive as cell membranes are ruptured and eNOS attaches to the underlying myocardium (Adler *et al.,* 2004). The observation, further suggests that atrazine disrupts the endocardium endothelium's unique expression of eNOS, and possibly interfere with NO production, and hence the anti-atherosclerotic function and regulation of homeostatic activities of the vascular system associated with NO. The fluorescence immunohistochemistry results agreed with the normal eNOS immunohistochemistry and showed the presence of both properly functional and inactive eNOS in the treated adult frog endothelium. Since atrazine is shown to disrupt eNOS expression in the endothelium; which functions as a homeostatic regulator of myocardial contractility (Mount *et al.,* 2007). Atrazine may adversely affect the rhythmic contractility of the heart and consequently lead to dire heart conditions. As has been reported by Papaefthimiou *et al*. (2003), in vitro atrazine exposure resulted in the decreased atria contraction and vasorelaxation in rat aorta (Chan *et al*., 2007). Thus, atrazine can adversely affect the homeostatic activity of the vascular system through endothelial toxicity in *Xenopus* frogs.

Arrhythmias have been suggested to be triggered by both activation and loss of function of IP_3Rs (Kockskamper *et al*., 2008). In this study atrazine significantly reduced IP_3Rs expression in adult frog Purkinje fibers and cardiac myocytes in the 200 µg/L group but none significantly decreased in 500µg/L and increased in 0.01 µg/L groups relative to the control. Such changes (both increase and decrease) in cardiac IP_3Rs have been implicated with aging and in atrial fibrillation a common form of arrhythmia (Kaplan *et al.,* 2007). Therefore atrazine exposure may be implicated in inducing arrhythmic conditions in frogs. The lack of IP_3Rs expression in the juvenile frog cardiac tissue sections may be that the juvenile's heart development had not progressed to a stage where they homeostatic calcium gated channel receptors such as IP_3Rs are functional. This may be a novel finding associated with atrazine effects. Certain genes are known to be activated by age.

Purkinje cells have been reported to be found stacked on top of each other and grouped into clusters, and not regularly spaced in a single row as described in mammalian cerebellum and uniquely identified by their pear/ oval shape and light staining vesicular nucleus (Sotelo; 1976).

In this study, we observed few apoptotic Purkinje nuclei in the control juvenile group but none in the adult control group. Additionally we observed pyknosis and karyolysis in the Purkinje nuclei of juvenile and adult treated groups. This combination of findings support the report of Dusart and Flamant (2012) who reported the differences between naturally occurring developmental cell death (in control of juvenile) and neuronal degeneration (treated groups of juvenile and adult). Liu *et al.,* (2016) also reported decreased number of Purkinje cells commencing in post-natal development and degeneration decreasing with age, which explains why adult control groups where unaffected. This observed apoptosis and possible autophagy in the treated groups is indicative of neuronal degeneration (Garman, 2011) and can lead to decrease in the Purkinje neuronal population.

A decrease in the density and increase size of Purkinje neurons have been implicated in bipolar disorders, schizophrenia, and autism spectrum disorders in humans (Maloku *et al*., 2010; Sudarov, 2013). This study observed similar results in the Purkinje neuron of frogs treated with atrazine. These complex neurological disorders associated with Purkinje cells in humans and observed in frogs exposed to atrazine suggests possible extrapolation of these atrazine effects to human population exposure to this chemical and therefore need further studies. This is very imperative as atrazine use in agriculture continue on the increase.

A study by Podda and colleagues (1997) reported a reduction in the spontaneous firing rate of Purkinje cells and reduction in evoked activity of the cerebellar cortex in rats exposed to atrazine. These reported pathophysiological effects of atrazine on Purkinje neurons could be correlated with the morphological alterations of Purkinje neurons observed in this study. Motor control is essential to the survival of frog and anuran species and as such, the morphological alteration of the Purkinje neuron (a major motor neuron) by atrazine may negatively affect the survival of this frog species and probably its extinction.

In cerebellar Purkinje cells, IP_3Rs mediated Ca^{2+} release plays a major role in generation of long-term depression (LTD) of synaptic strength (Ito, 2001; Higley and Sabatini, 2008). This cerebellar plasticity and has been considered as a critical cellular mechanism for motor learning (Yang and Lisenberger, 2014), motor performance and stimulation on eye movement performance during training (Nguyen-Vu *et al.,* 2013).

The stereological analysis of IP_3R expression in cerebellar Purkinje cells found decreasing number of Purkinje cells expressing IP_3R with increasing atrazine concentration. However the observed decrease in cell number in the 0.01µg/L group compared to the control was

insignificant in both the juvenile and adult groups, but at 200 µg/L and 500 µg/L exposure, the Purkinje cells number expressing IP_3R was generally significantly ($p < 0.05$) reduced in juvenile adult frogs compared to the control group. This is in line with the observed slightly larger but fewer Purkinje cells with vesicular nuclei in the histological images of the 0.01 µg/L treated groups. These significant reductions in IP_3Rs are indicative of the adverse effects that atrazine may have on the cerebellar plasticity which will deter the agility of *Xenopus* frogs and compromise their ability to maneuver and thrive in their habitat.

Additionally this study showed significant reduction IP_3R expressions of the 200 µg/L group relative to 0.01 µg/L in the juvenile frogs but an insignificant reduction was found in the adult frog. This implies the presence of possible subtle compensatory mechanism in adult frogs to atrazine effects between the concentrations of 0.01 µg/L and 200 µg/L.

This study also presents findings that suggest some resilience of adult species to adverse effects of atrazine on IP_3R expression between the concentrations of 0.01 µg/L and 200 µg/L.

The nNOS levels in juvenile and adult frog cerebellar tissue were all negative (not expressed) in this study. nNOS has been implicated in pesticide mediated toxicity (Oritz-Oritz *et al.*, 2009), however cerebellar Purkinje cells do not normally exhibit nNOS activity (Steinbusch *et al*., 2000) but reports have shown that chemical injuries can induce nNOS expression (Wu, 2000; Dawson *et al.*, 1996). It is not known if this particular brand of nNOS antibodies does not stain nNOS in *Xenopus* species, or if the atrazine exposure does not solicit sufficient chemical injury to induce the expression of nNOS in the cerebellum.

CHAPTER SIX

CONCLUSION

In summary atrazine exposure at different concentration induced changes in the morphology, histology and expressions of IP_3R and eNOS in cardiac tissue and expression of IP_3R in cerebellar Purkinje cells. These changes indicate probable cardiomyopathy, arrhythmia, motor and visual detriments that animals exposed to atrazine may encounter. Evidence of cardiac fibrosis in all the treated groups and loss of Purkinje cell in the high concentration treated groups (200 µg/L and 500 µg/L) could possibly be due to cardiac and cerebellar toxicity in juvenile frogs. Furthermore most effects were more severe in juvenile than adult, but all concentrations of atrazine from low to very high have some observable histological and pathological implications for this frog species.

This study reveals that atrazine exposure disrupts morphology, connective tissue profile and impairs IP_3R and eNOS expression in the cardiac tissue of both juvenile and adult frogs. These observed effects in different atrazine treated groups (increased weight and area of the heart, connective tissue infiltration, decreased expression of eNOS and disrupted IP_3R expression) suggest different forms of cardiomyopathy and arrhythmias conditions in the cardiac tissue.

Furthermore the IP_3Rs immunohistochemistry and histology of Purkinje cells suggest atrazine may impair motor learning and coordination, by reducing the amount of Purkinje cells in both juvenile and adult frog cerebellum as well as reducing the expression of IP_3Rs especially in the juvenile frogs.

Atrazine pollutes water and could lead to neurotoxicity and cardiotoxicity. These results call for clear and detailed regulation in the use of atrazine in agricultural systems and around our environments to monitor the presence of this chemical in drinking water and water resources. Restrictions on atrazine use should be enforced to avoid or reduce to the barest minimum its associated health hazards among *Xenopus lavies* population and potentially other aquatic animals and biodiversity in general.

5.1 RESEARCH LIMITATION

Inability to determine the effect of atrazine on the thyroid hormones, to correlate endocrine disruption effects of atrazine.

Cardiac IP_3Rs unexpressed in all juvenile groups and thus inability to compare with adult groups.

5.2 RECOMMENDATIONS FOR FURTHER STUDIES

Stereological quantitative studies from serial sections throughout the entire organ (brain or heart) to evaluate to total Purkinje neurons/ fibers and analyse the percentage of eNOS and IP3Rs expressed.

Detailed studies on other neurological centers in the *Xenopus* brain are required, in order to understand possible hindrances to their motor control and metabolic well-being.

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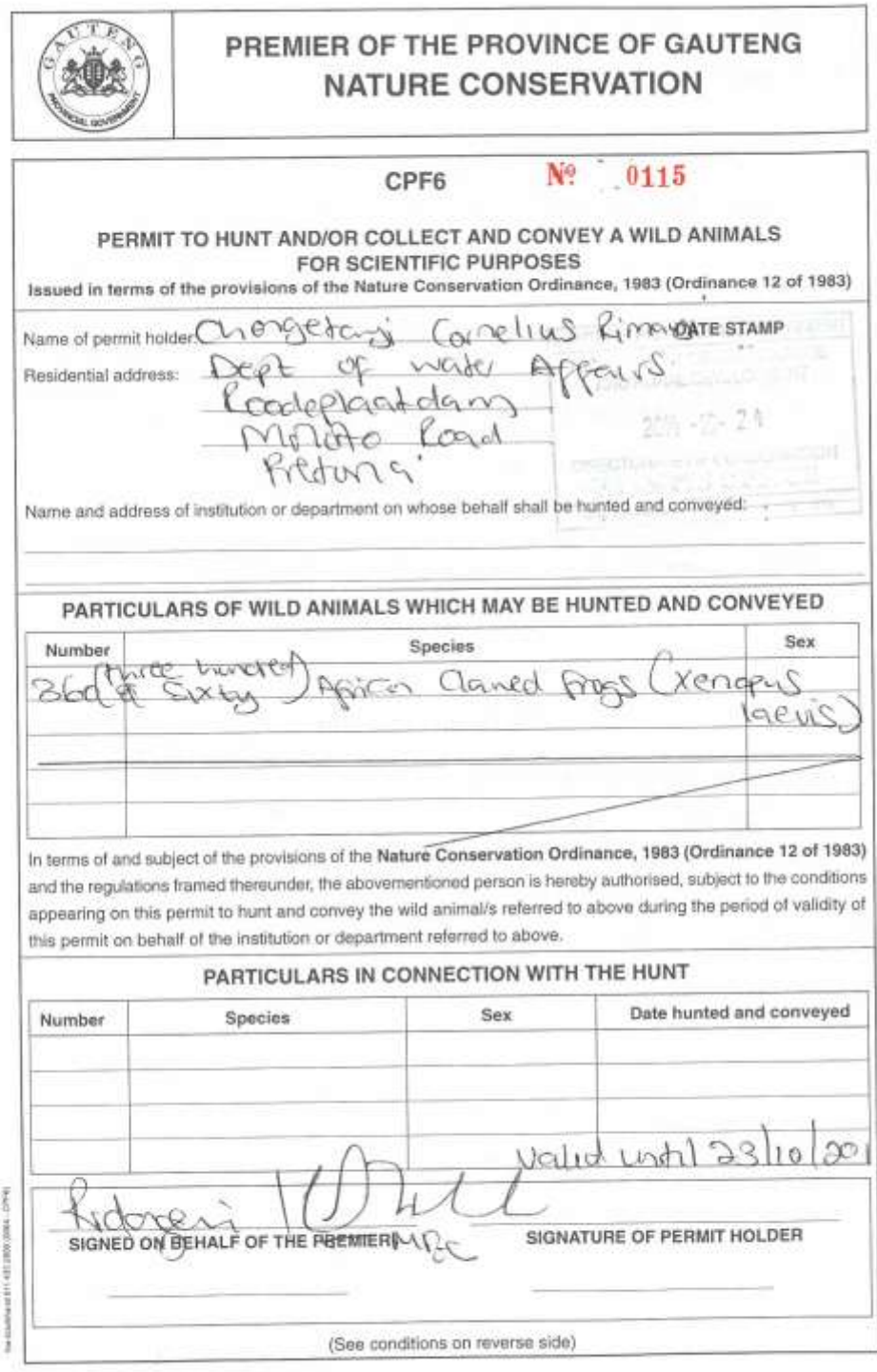
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APPENDIX I: NATURE CONSERVATION PERMIT



PREMIER OF THE PROVINCE OF GAUTENG
NATURE CONSERVATION

CPF6 № 0115

PERMIT TO HUNT AND/OR COLLECT AND CONVEY A WILD ANIMALS FOR SCIENTIFIC PURPOSES

Issued in terms of the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)

Name of permit holder: Chogeta-ji Cornelius Rimayi

DATE STAMP

Residential address: Dept of water Affairs
Roodeplaatdam
Moloto Road
Pretoria

Name and address of institution or department on whose behalf shall be hunted and conveyed:

PARTICULARS OF WILD ANIMALS WHICH MAY BE HUNTED AND CONVEYED

Number	Species	Sex
360 (three hundred and Sixty)	African Clawed Frogs (Xenopus laevis)	

In terms of and subject of the provisions of the **Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983)** and the regulations framed thereunder, the abovementioned person is hereby authorised, subject to the conditions appearing on this permit to hunt and convey the wild animal/s referred to above during the period of validity of this permit on behalf of the institution or department referred to above.

PARTICULARS IN CONNECTION WITH THE HUNT

Number	Species	Sex	Date hunted and conveyed

Valid until 23/10/2015

SIGNED ON BEHALF OF THE PREMIER

SIGNATURE OF PERMIT HOLDER

(See conditions on reverse side)

7.2 APPENDIX II: ETHICS CLEARANCE

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Chengetayi Cornelius Rimayi.....

b. Department: Chemistry.....

c. Experiment to be modified / extended **AESC NO:2014/32/D**

Original AESC number	2014	32	D
Other M&E's : n/a			

d. Project Title: Frogs (Xenopus laevis) as an indicator for environmental organic pollution

e.	Number and species of animals **originally approved**:	360	
f.	Number of additional animals **previously allocated on M&Es**:	n/a	
g.	Total number of animals **allocated** to the experiment to date:	360	
	h. Number of animals **used** to date:	360	

i. Specific modification / extension requested:

a) To include the use of the scheduled hormone, Gonadotropin for inducing spawning in female frogs for production of the required tadpoles. N.B an authorised registered worker will purchase and use the gonadotropin on Mr Rimayi's behalf.

b) Inclusion of co-workers Jaclyn Asouzu and Lynette Rufaro Sena who will also care for the frogs and also conduct histological analyses on the frog organs I will not be conducting histological analysis on and which I would otherwise be discarding.

j. Motivation for modification / extension:

a) It would be less expensive to produce the tadpoles using the Gonadotropin hormone at the Wits Central Animal Service than to buy them from an external organizations due to limited funds

available.

b) There are organs which I will not do histological analysis on such as the heart, brain, spinal cord and bones which the co-workers can use after I sacrifice the frogs. Instead of discarding and wasting the organs, the co-workers can use them for their own histological studies.

Date: 05/12/2014..... Signature: 

RECOMMENDATIONS: **APPROVED**

i. Use of gonadotropin to induce spawning in frogs.

ii. Inclusion of Jaclyn Asouzu and Lynette Rufaro Sena as co-workers that will collect organs and tissues post mortem from the frogs for histological studies.



Date: 11 December 2014 Signature:

Chairman, AESC

2/2

7.3 APPENDIX III: IMMUNOHISTOCHEMISTRY SOLUTIONS

7.3.1 Citrate Buffer pH6 with 0,05% Tween 20

Tri-Sodium Citrate (Di-hydrate)	2.9g
Distilled water	1000ml

Mix to dissolve and adjust pH to 6 with 1M HCl

7.3.2 1M Phosphate Buffer pH7.4

NaCl	16g
Na_2HPO_4	3.5g
KCl	0.4g
KH_2PO_4	0.4g
Distilled water	2000ml

7.3.3 Antibody Diluent

PBS	100ml
Triton-X	0.2ml (200µl)

7.3.4 Normal Goat serum (make up only the amount required for the no of slides)

Normal goat serum	10µl
PBS	100µl

7.3.5 DAB working solution

(a)In a clean bijou bottle measure out

1mg DAB (0.001g) .To this, add 2ml Tris HCl and mix well.

(b) In a separate tube add:

29ul cold distilled water

1µl hydrogen peroxide (100%)

Add 20µl of sol (b) to DAB sol (a)

7.3.6 ABC:

25ul A + 25ul B + 1.25ml PBS **make up 30min before use**

7.4 APPENDIX IV: IMMUNOHISTOCHEMISTRY PROTOCOLS

7.4.1 Immunohistochemistry protocol for paraffin sections

Day1

1. Deparaffinize sections in Xylene for	2x5mins
2. Rehydrate sections through graded alcohols	
3. Wash slides in running water	5mins
4. Antigen retrieval of sections in Citrate Buffer pH6	
5. Microwave @med high 720	10mins
6. Allow to cool at room temperature for	20mins
7. Rinse slides in PBS	5mins
8. Block endogenous peroxidase with 1% H_2O_2 in methanol	15mins
9. Wash in PBS pH7.4	3x 5mins
10. Incubate with normal serum 5%	45mins
11. Tap serum off slides. DO NOT WASH	
12. Incubate with primary antibody IP 3 overnight @4°C	

Day 2

13. Wash in PBS pH7.4	3x5mins
14. Incubate with biotinylated secondary antibody (vector labs BA- 1000)	30mins
15. Wash in PBSpH7.4	3x5mins
16. Incubate with ABC (make up ABC 30mins before use)	30mins
17. Wash in PBS pH7.4	3x5mins

18. Incubate with DAB working solution 5mins

19. Rinse in running tap water 5mins

20. Counter stain in Haematoxylin 1min

21. Dehydrate through graded alcohols, clear in xylene and mount with Entellen.

7.4.2 Immunofluorescence on paraffin sections

Dewax slides:

Xylene	10min
Xylene	10min
Abs. alcohol – 70% alcohol	10 dips each
Wash in water	5min
Draw circles around each section with PAP pen	
Microwave sections in Citrate Buffer pH6	10min
Allow sections to cool to room temperature	20min
Wash in PBS/0,2% Triton –X	3 x5min
Block sections with 10% Normal serum	1hr@Room Temperature
Incubate with Primary antibody	Over Night @4°C in a humid chamber

<u>Day 2</u>

Wash in PBS	3 x5min

Incubate **in the DARK** in secondary antibody – goat anti rabbit Alexa fluor 488 1:500

1hr @ Room Temperature or 30min @ 37°C

Wash in PBS	3 x5min
Incubate with DAPI in PBS 1:10 000	5min
Wash in PBS	3 x5min

Mount with anti-fade and store in the dark at 4°C until viewing

7.4.3 Immunohistochemistry protocol for free floating sections

Allow the tissue to defrost at room temperature for 30mins. Thereafter wash the sucrose off with 3 x 10min washes in 0.1M PB under gentle shaking. For all subsequent washes and incubations use 1ml per well/48well plates

1. Quench tissue for endogenous peroxidase by placing in:

Methanol	50ml
PB (0.1M)	50ml
H_2O_2	1.66ml of 30% H_2O_2

For 30min @ Room Temperature under gentle shaking (1ml per well)

2. Wash in PB (0.1M) 3 x10min (@RT under gentle shaking)

3. Preincubate with blocking buffer for 2hrs @RT under gentle shaking

Blocking buffer:	0.25% Triton –X 100 in PB (0.1M)	100ml
	Normal serum 3%	3ml
	BSA 2%	2ml

Triton –X is a detergent and creates pores in the cell membrane allowing the antibody to penetrate the tissue. Normal serum and BSA are both used to prevent non-specific staining.

NB: Do not wash between the primary antibody and the blocking step. Allow solution to drip off.

4. Primary antibody incubation 48hrs (@4°C under gentle shaking)

Primary antibody dilution Buffer

0.25% Triton –X100 in PB (0.1M)

3% Normal serum

2% BSA

Primary antibody at the recommended dilution

Control sections: Omit the primary antibody

Omit the secondary antibody

After sections have been in the fridge allow them to reach room temperature by placing them on the bench for 15min.

5. Wash 3 X10min in 0.1M PB @Room Temperature under gentle shaking.

6. Secondary antibody incubation 1-2hrs @ Room Temperature under gentle shaking.

3% Normal serum

2% BSA

PB (0.1M)

Biotinylated secondary antibody at the recommended dilution

7. Wash 3 X 10min in PB (0.1M) @Room Temperature under gentle shaking

8. Incubate sections in ABC-Avidin Biotin Complex -1hr @Room Temperature under gentle shaking

ABC complex has to be made 30min before required

40ul A +40ul B in 5ml of PB (0.1M) with 3% Normal serum,2%BSA

9. Wash 3 X10min in PB (0.1M) @RT under gentle shaking.

10. Incubate sections in the DAB chromogen for 5min @RT under gentle shaking

0.05% DAB in 0.1M PB.

Make total volume of DAB required

Place 0.7ml per well and allow to stand for 5min

Thereafter add 3ul of 30% H_2O_2 per 1ml of DAB solution. Add this to the remaining

DAB solution. Add this to the wells and allow color to develop. TIP when placing DAB with H_2O_2 into wells allow for 30secs between each well to make sure that each well is incubated with DAB for the same length of time. Check microscopically for development and then wash to stop the DAB reaction.

11. Wash 3 X10min in PB0.1M @RT under gentle shaking

12. Sections can be left in PB overnight before being mounted.

13. Mount sections on 0.5% gelatin coated slides and leave to dry for 2-3days.

14. Dehydrate in a series of alcohols:

70%	2hrs
95%	5min
100%	5min
100%	5min
Xylene	5min
Xylene	5min

15. Coverslip sections with DPX or Entellen mounting medium

7.5 APPENDIX V: CRESYL VIOLET HISTOLOGY METHOD

Fixative: Paraformaldehyde

Staining solution: Add 1g Cresyl Fast Violet Acetate to 100ml of dist. water

Allow to mix on stirrer for 1 week, cover with foil while mixing.

NB: The solution should not be exposed to light.

Method

For Neuro sections 25-50um thick.

1. Once the Nissl sections have been mounted and are dry, place sections in a solution of 50% alcohol and 50% chloroform overnight. (1part 100%alcohol: 1 part chloroform)
2. Rehydrate in alcohol:

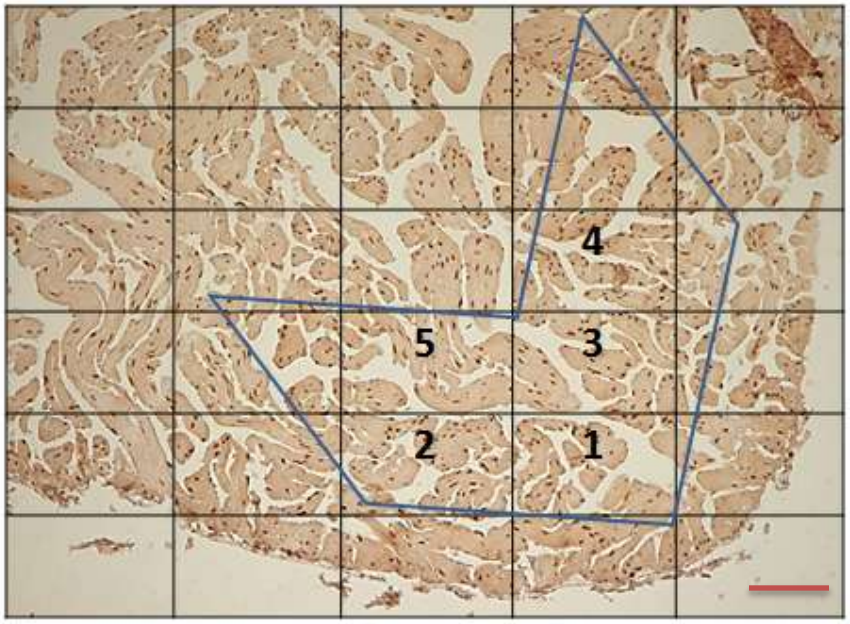
100%	5min
100%	5min
95%	2min
70%	2min
50%	2min

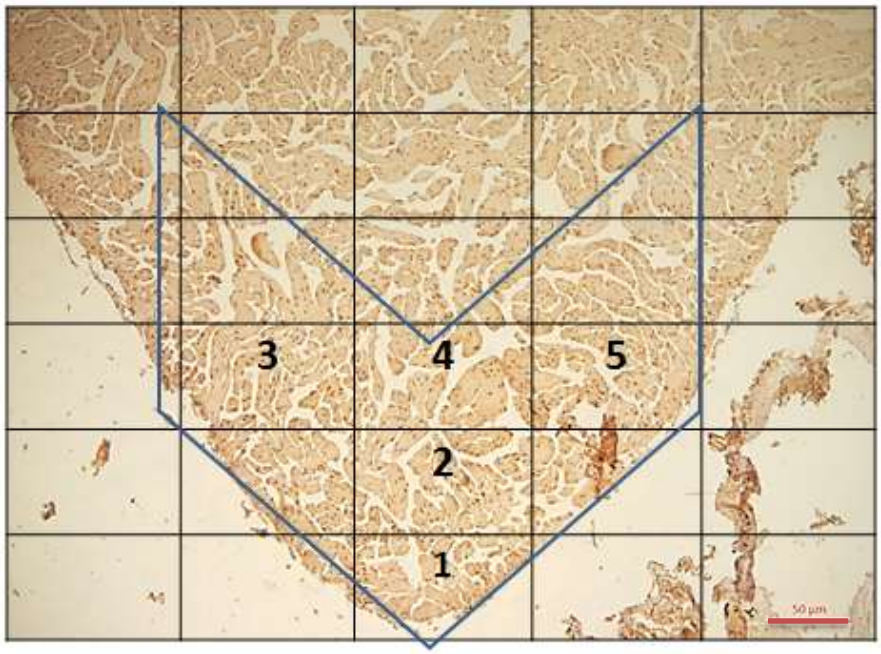
3. Cresyl violet 1min
4. Distilled water 1min
5. Dehydrate in alcohol:

50%	2min
70%	variable time check microscopically
95%	2min
100%	5min
Xylene	5min
Xylene	5min

Coverslip and mount with mounting medium

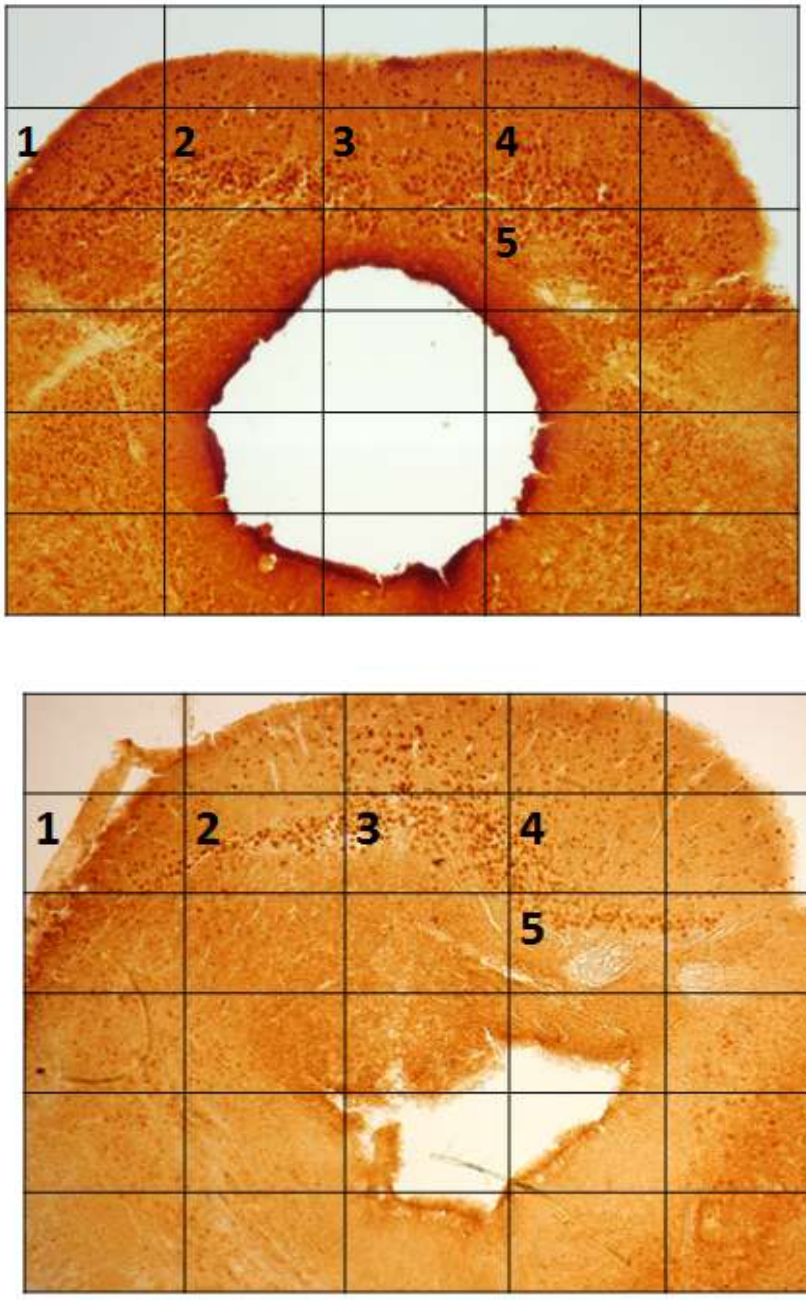
7.6 APPENDIX VI: IMAGE SELECTION FOR CELL COUNT IN CARDIAC SECTIONS





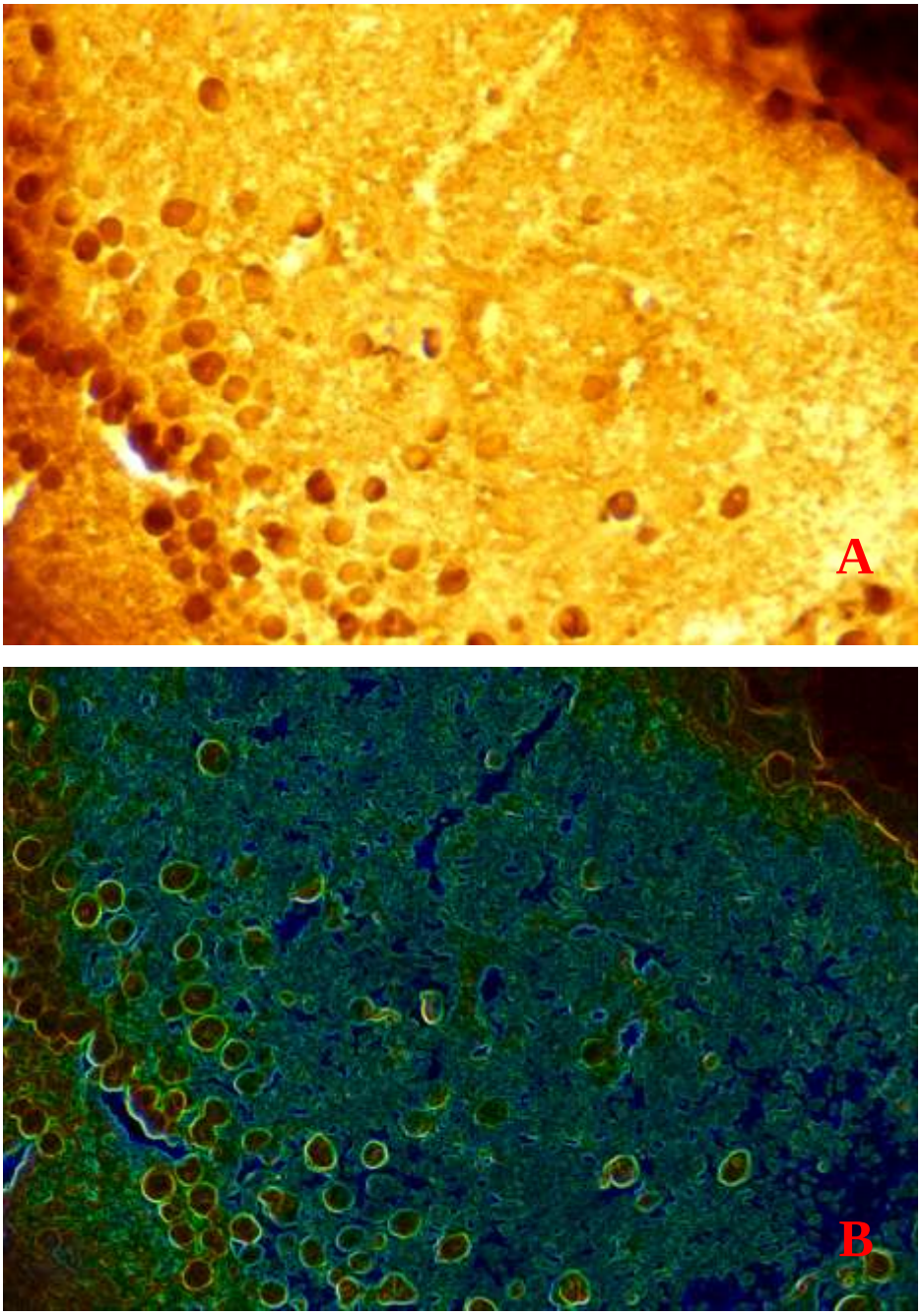
Photomicrograph of cardiac sections showing specifically selected regions of the five image taken, from the apex of the heart towards the atria on both sides. Images were taken in the first five grids within the area of interest, which fill up to 75% of the grid.

7.7 APPENDIX VII: IMAGE SELECTION FOR CELL COUNT IN BRAIN (CEREBELLAR) SECTIONS



Photomicrograph of brain sections showing specifically selected regions of the five images taken, from the left to the right along the Purkinje layer of the cerebellum. Images were taken of the first five grids within the area of interest, which fill up to 75% of the grid.

7.8 APPENDIX VIII: IMAGE ANALYSIS WITH IMAGE –J FOR CELL COUNTING



A- Immunohistochemistry for expression of IP_3R in cerebellar cortex

B- Image analysis of image A, using image-j software/ find edges for easy identification and counting of expressed cells.

7.9 APPENDIX IX: DATA ANALYSIS TABLES

Table 7.9.1 One way ANOVA for juvenile cardiac area and weight

		Sum of Squares	Df	Mean Square	F	Sig.
Juvenile area	Between Groups	18.251	3	6.084	3.480	0.023*
	Within Groups	82.158	47	1.748		
	Total	100.409	50			
Juvenile weight	Between Groups	.000	3	.000	.929	0.434
	Within Groups	.000	47	.000		
	Total	.000	50			

* significant difference

Table 7.9.2 One way ANOVA of adult cardiac weight and area

		Sum of Squares	df	Mean Square	F	Sig.
Adult weight	Between Groups	.021	3	.007	3.069	0.040*
	Within Groups	.081	36	.002		
	Total	.101	39			
Adult area	Between Groups	2888.916	3	962.972	14.215	0.000*
	Within Groups	1083.865	16	67.742		
	Total	3972.781	19			

*significant difference

Table 7.9.3 Kruskal Wallis test for adult frog cardiac IP_3Rs count between groups

	IP_3R cardiac count
Chi-Square	12.507
Df	3
Asymp. Sig.	0.006*

* Significant difference

Table 7.9.4 Normality test for IP_3R count in juvenile cerebellar Purkinje cells

Tests of Normality							
	doses of atrazine	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	p value Sig.
number of IP3Rs expressed	control	.228	8	.200	.881	8	0.194
	0.01µg/L	.172	8	.200	.930	8	0.512
	200µg/L	.171	10	.200	.969	10	0.881
	500µg/L	.177	10	.200	.932	10	0.463

Table 7.9.5 Kruskal Wallis test for IP_3R count in juvenile cerebellar Purkinje cells

	Number of IP3Rs expressed
Chi-Square	21.594
Df	3
Asymp. Sig.	0.00*

*p value

Table 7.9.6 Normality test of IP_3Rs count in adult frog cerebellar Purkinje cells

Tests of Normality							
	doses of atrazine	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	p value Sig.
number of IP_3Rs expressed	control	0.178	21	0.079	0.919	21	0.082
	0.01µg/L	0.083	21	0.200	0.960	21	0.525
	200µg/L	0.153	20	0.200	0.952	20	0.404
	500µg/L	0.152	18	0.200	0.933	18	0.221

Table 7.9.7 Kruskal Wallis test of IP_3Rs count in adult frog cerebellar Purkinje cells between groups

	number of IP_3Rs expressed
Chi-Square	28.311
Df	3
Asymp. Sig.	0.00*

p value*