Development of *'In vitro'* intestinal models to study the pharmacology of drugs affecting the gastrointestinal tract in normal and diseased conditions

Development of a cell culture model for intestinal pharmacology

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BSc (Hons) Biomedical Sciences

Thesis submitted for the degree of Doctor of Philosophy

School of Pharmacy
University of Bradford

2009

Development of *'In vitro'* intestinal models to study the pharmacology of drugs affecting the gastrointestinal tract in normal and diseased conditions

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Key words: cell culture, neonatal rat intestine, 5-HT, inflammation, and cryopreservation.

ABSTRACT: Studies investigating the effect of 5-HT receptors mediating a response in the neonatal intestine have been limited. There are evidences that the development of new neurones continues past postnatal term and this suggests that receptors expression may differ during maturation. Thus, *'in vitro'* experiments were carried out to investigate the effects of ACh, atropine, 5-HT and its related drugs on intact intestinal segments taken from the ileum of adult and neonate rats. The application of ACh (3nM-1mM) and 5-HT (3nM-1mM) induced contractions in a concentration dependent manner in all tissues examined. The 5-HT induced contractions were only sensitive to antagonism by atropine (1μM) in segments taken from the neonates but not adults. The pre-treatment with methysergide ($5\text{-HT}_{1/2/5\text{-}7}$ receptor antagonist), ritanserin (5-HT_2 receptor antagonist), granisetron (5-HT_3 receptor antagonist) and RS 23597 (5-HT_4 receptor antagonist) at 1μM or a combination of ritanserin, granisetron, plus RS 23597 at 1μM significantly reduced or abolished contractile responses induced by 5-HT. SB 269970A (5-HT_7 receptor antagonist) and WAY 100635 (5-HT_{1A} receptor antagonist) at 1μM failed to influence contractile responses induced by 5-HT or the challenges to 5-HT receptor agonists, 5-CT ($5\text{-HT}_{1A/7}$ receptor agonist) and 8-OH-DPAT (5-HT_{1A} receptor agonist) at a concentration range of 10nM-0.1mM, indicating the unlikely involvement of 5-HT_{1A} and 5-HT_7 receptors in the mediation of contractile responses in the neonatal rat ileum. Results indicate differences in cholinergic receptor involvement during postnatal maturation and suggest the involvement of 5-HT_2, 5-HT_3 and 5-HT_4 receptors in the mediation of contractile responses to 5-HT in the neonatal rat ileum.

There is a growing need to decrease animal usage in pharmacological experiments. This may be achieved by the development of *'in vitro'* cell culture models. Thus attempts were also made to develop a cell culture model of neonatal intestine to further investigate the action of pharmacologically active agents. The isolation of individual cell populations from segments taken from the intestine of rat neonates were achieved by ligation of both ends of the intestine prior to incubation in trypsin so that a gradual dissociation could be monitored. This was supported by histological procedures, determining the time required to extract large numbers of cells from different intestinal layers. Differential adhesion and selective cytotoxicity techniques were used for further purification of intestinal smooth muscle cells (ISMC), neuronal cells, and a coculture of ISMC and neuronal cells, and these were characterised through immunostaining with antibodies to α-smooth muscle actin, α-actinin and the 5-HT_3 receptor. A protocol for cryopreservation of ISMC was designed in order to protect cells against genetic instability, enhance cell availability and reduce animal usage. Results showed that cells extracted from the intestine are viable for up to 4-months. ISMC functionality was analysed via the application of known pharmacologically active drugs on ISMC, which were plated onto glass and silicone elastomer substrate. The cultured ISMC responded to the application of drugs such as potassium chloride (KCl), carbachol, 5-HT and noradrenaline (NA). Large population of cocultures seeded onto silicone elastomers or cholesteric liquid crystal substrates (LC) were assessed for their ability to produce a collective response to KCl application. Attempts were made to detect any deformations of the substrate surface due to the exposure to KCl and NA. Cholesteric LC substrates seemed to be the most suitable material for investigating the cellular tensions.

The availability of cell cultures allowed the development of an intestinal model of inflammation. This was achieved through the use of lipopolysaccharide (LPS)-induced inflammation and was confirmed by assessing the levels pro-inflammatory mediators interleukin (IL-8) and nitric oxide (NO), which were significantly elevated. Reduction of IL-8 ad NO was also examined using granisetron and L-NAME and Chaga mushroom extract. Granisetron and L-NAME reduced the NO production during short incubation times. However, an elevated level of NO was observed when longer treatment times were examined. The Chaga mushroom extract caused a significant reduction in NO production in the model of inflammation. This indicates that this model may be a valuable tool for the investigation of other pro-inflammatory mediators and may contribute for the investigation of more selective drugs in the management of intestinal inflammation in neonates.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis.

I am particularly grateful to my supervisors **Dr. Farideh A. Javid, Dr. Morgan Denyer**, and **Dr. Stephen Britland**, whose knowledge, encouragement and thoughtful guidance have helped me tremendously in the course of my research. I was very fortunate to have had them as my supervisors.

Special gratitude is extended to all the technical staff for their support, especially Richard Bottomly, Mark Fibly, Zoë Smith and Chris Buckskill.

I would like to thank the Federation for Women Graduate for the award of the Foundation Grant, which was of great help during the final year of my research.

Finally, I thank God for everything and I dedicate thesis to my parents Marcelino Batista Lobo and Maria Rodrigues Batista Lobo for their endless support.

STATEMENT OF ORIGINALITY

To the best of my knowledge, material or the contents presented in this thesis are original except where otherwise noted within the text. None of this research has been submitted in whole or in part for a degree at this or any other university.

Samira Batista Lobo

STATEMENT OF ETHICAL INTENT

All procedures involving laboratory animals reported in this Thesis were carried out in accordance with the Home Office Legislation for Scientific Procedures on Living Animals as outlined in the Animal (Scientific Procedures) Act 1986 (ISBN 010 218290-6-HMSO).

Samira Batista Lobo

PUBLICATIONS ASSOCIATED WITH THIS THESIS

Papers

1. **S. Batista Lobo, M. Denyer, S. T. Britland, F. A. Javid (2009)** Investigation of the 5-hydroxytryptamine receptors mediating a response in the intestine of rat neonates. (To be submitted to British Journal of Pharmacology).

2. **S. Batista Lobo, M. Denyer, S. T. Britland, F. A. Javid (2009)** Development of an intestinal model of inflammation for the investigation of anti-inflammatory drugs (To be submitted to British Journal of Pharmacology).

3. **S. Batista Lobo, M. Denyer, R. Gopalan, F. A. Javid (2008)** Technique for cryopreservation of intestinal smooth muscle cells. Cryobiology. Vol 57, pp186-189.

4. **Jamil M. M. Abdul, F. Sefat, S. A. Khaghani, S. B. Lobo, F. A. Javid, M. Youseffi, S. Britland, S. G. Liu, C. W See, M. G. Somekh, M. Denyer (2008)** Cell imaging with the widefield surface plasmon microscope. IFMBE Proceedings, Vol 21, issue 1-2, pp528-531.

5. **S. Batista Lobo, M. Denyer, S. Britland, F. A. Javid (2007)** Development of an intestinal cell culture model to obtain smooth muscle cells and myenteric neurones. Journal of Anatomy. Vol 211, pp 819-829.

Abstracts

1. **S. Batista Lobo, M. Denyer, F. A. Javid (2009)** Characterisation of the 5-hydroxytryptamine receptors mediating contraction in the intestine of rat neonates. (To be presented at the British Pharmacological Society Meeting).

2. **S. Batista Lobo, M. Denyer, F. A. Javid (2009)** Comparison of the effects of 5-HT on the intestinal segments taken from the adult and neonate rats. (To be presented at the British Pharmacological Society Meeting).

3. **S. Batista Lobo, M. Denyer, F. A. Javid (2009)** 5-hydroxytryptamine and cholinergic receptors involvement in contraction of the rat small intestine during maturation. (To be presented at the British Pharmacological Society Meeting).

4. **S. Batista Lobo, M. Denyer, F. A. Javid (2009)** *"In vitro'* model of intestinal inflammation (To be presented at the British Pharmacological Society Meeting).

5. **C. F. Soon, M. Denyer, P. Twigg, M. Youseffi, N. Blagden, F. A. Javid, S. B. Lobo (2009)** Development of a novel cell forces transducing assay based on shear sensitive cholesteric liquid crystals. School of Life Science Research Open Day, University of Bradford.

6. **S. Batista Lobo, M. Denyer, F. A. Javid (2007)** Development of a simplified method for isolation of highly purified enteric ganglia neurones. Br. Pharm. Society. Vol 5, Issue 2, abs 131P, pA_2 online.

7. **S. Batista Lobo, M. Denyer, F. A. Javid (2007)** Development of an intestinal smooth muscle cell culture model using scorpion venom. 6^{th} Ambercrombie Meeting. Oxford.

8. **S. Batista Lobo, M. Denyer, F. A. Javid (2006)** Technique for the cryopreservation of intestinal smooth muscle cells from rat neonates. IRCE: Pharmacy and Pharmacological, Institute of Pharmaceutical Innovation, University of Bradford. Available on line: http://www.irce.org/va_html.

9. **S. Batista Lobo, M. Denyer, F. A. Javid (2006)** Development of a new intestinal smooth muscle cells culture model from rat neonates. Neurogastroenterology and Motility Joint International Meeting 2006. Boston, USA.

ABBREVIATIONS

ACh	acetylcholine
ATP	adenosine triphosphate
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
Ca^{2+}	calcium
5-CT	5-carboxyamidotryptamine
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco modified eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetracetic acid
ENS	enteric nervous system
FCS	fetal calf serum
$FeCl_3$	ferric chloride
FITC	fluorescein isothiocyanate
GI	gastrointestinal
HBSS	Hank's balanced salt solution
HCL	hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h	hour
5-HT	5-hydroxytrytamine
ICCs	interstitial cells of Cajal
IBD	inflammatory bowel disease
IL	interleukin
ISMC	intestinal smooth muscle cells
LC	liquid crystal
$LiCO_3$	lithium carbonate
L-NAME	L- N (G)-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
KCl	potassium chloride
$KH2PO_4$	potassium dihydrogen phosphate
$MgSO_4$	magnesium Sulphate
min	minutes
NaCl	sodium chloride

NA	noradrenaline
$NaHCO_3$	sodium hydrogen carbonate
NaOH	sodium hydroxide
NO	nitric oxide
8-OH-DPAT	8-hydroxyl-di-n-propylamino tetralin
PBS	phosphate balanced salt
PDMS	polidimethylsiloxane
RS	rat serum
sec	seconds
SOM	somatostatin
Th	T helper cells
TK	tachykinin
TNF	tumour necrosis factor
Tris	(hydroxymethyl) aminomethane
TRITC	tetramethylrhodamine isothiocyanate
VIP	vasoactive intestinal peptide

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CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Globally it is estimated that 6-60 billion cases of gastrointestinal (GI) illness occur annually (Guandalini, 2004). Advances in pharmacological approaches have improved therapeutic options for the treatment of GI diseases. Such approaches focus on well-identified cellular components of the GI tract including receptors and neuroendocrine substances. However, peadiatric pharmacology remains an area that does not provide adequate information about many drugs. The scientific interpretation of data for therapeutics prescribed for infants continues to be a challenge due to the difficulties in establishing a relationship between pharmacokinetics (what the body does with the drug) and pharmacodynamics (what the drug does to the body). Thus, it is necessary to test the efficacy and toxicity of these potential drug candidates in relation to the potential end user. Due to differences across species, animal data obtained from experimental pharmacology must be modeled appropriately and ma

de relevant to specific mechanisms to improve drug dosing in infants.

The complexity of the cell signal within the GI makes the interpretation of the cellular response to chemical agents very difficult. Thus, therapies based on the direct delivery of these chemical agents into single cells have become an area of focus. This is based on isolation of specific cell populations from a primary tissue source with subsequent remodeling into suitable substrates to create functional templates that mimic the physiological aspects of the GI tract. Development of such '*in vitro*' models can be potentially used to improve our present understanding of the basic functions of the GI tract in health and disease.

1.2 THE ANATOMY OF THE GASTROINTESTINAL TRACT

The GI tract system consists of the digestive tract (Figure 1.2.1), whose functions are the ingestion and transport, and the digestion and absorption of nutrients along the GI tract. Food contents following ingestion are passed from the oral cavity to the esophagus, which connects to the stomach. The luminal contents are propelled along the length of the tract at a rate appropriate to the digestive and absorptive processes occurring at each part of the tract. This is achievable through the transit component of the GI tract, which is controlled and coordinated by the nerves and hormones originating the motility pattern of the GI tract (Kirszenbaum, 2002).

The wall of the GI tract is composed of four main components (Figure 1.2.2). The mucosa is highly specialised in each of the regions of the GI tract, where it plays a role in absorbing a multitude of substances in the small intestine and absorbing specific quantities of water in the large intestine. In the small intestine the structure consists of a membrane of columnar epithelium folded into villi. The submucosa consists of irregular connective tissue with large blood vessels, lymphatic vessels and nerves branching into the mucosa and muscularis. The muscularis externae consists of circular inner muscular layer and longitudinal outer muscular layer and between the two muscle layers are the myenteric plexus, collectively they are responsible for the gross movement of gut contents towards the distal end of the tract. Lastly, is the serosa, a thin membrane of connective tissue that encapsulates the gut (Kirszenbaum, 2002).

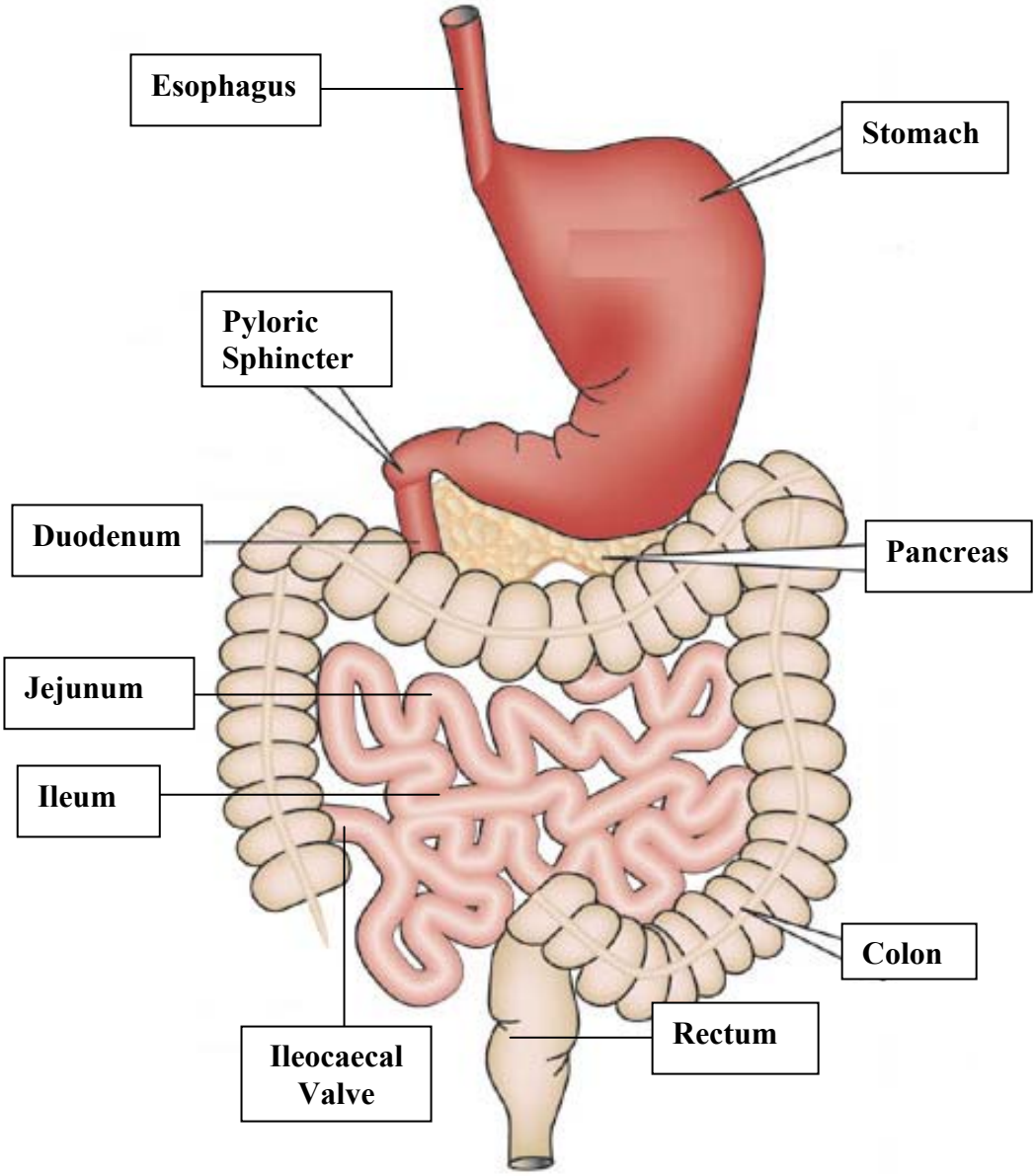


Figure 1.2.1: Diagrammatic illustration of the gastrointestinal tract (Murphy & Bloom, 2006).

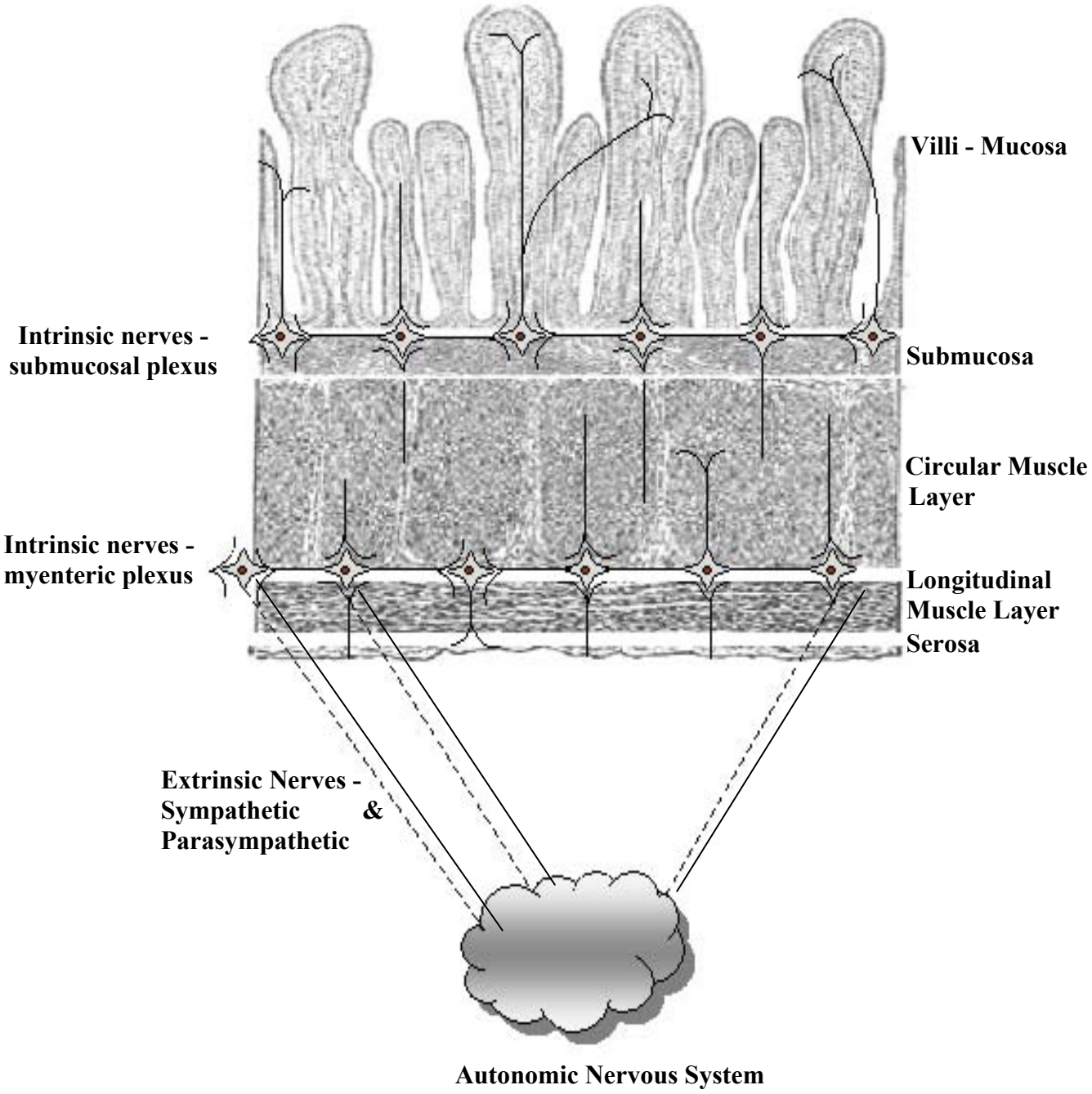


Figure 1.2.2: Diagrammatic representation of the GI tract layer's distribution (Gray, 1918). Additional illustration represents the extrinsic and intrinsic innervations controlling the digestive tract motility (Roman & Gonella, 1987).

1.3 GASTROINTESTINAL MOTILITY

A complex network of nerves is embedded in the walls of the digestive tract. This network extends from the pharynx to the anal sphincter and is composed of a large collection of intrinsic neurones (enteric neurones) and processes of both vagal afferents (sensory neurones) and efferents sympathetic and parasympathetic extrinsic neurones. From these two ganglionated plexus, smaller bundles of nerves emerge from non-ganglionated plexus in the longitudinal muscle, the circular muscle, around the blood vessels, the submucosa, around the bases of the mucosal glands, and within the cores of the villi (Weisbrodt, 1987) (Figure 1.2.2).

The intrinsic cycle, which derives from two patterns of electrical activity across the membrane referred as spikes, coordinates a rhythmic spontaneous depolarisation, which subsequently reaches threshold and generates an action potential (Axelsson, 1970, Somlyo & Somlyo, 1968, Yamazawa & Iono, 2002). In the smooth muscle this excitation-contraction coupling (electrochemical) results in calcium permeating the membrane through the cell membrane from the extracellular space into the cytosol of the smooth muscle cells (Axelsson, 1970).

In the small intestine, early dissection studies led to the conclusion that the spontaneous electrical activity was originated in the longitudinal muscle (Bortoff, 1965). Following studies suggested that the interstitial cells of Cajal (ICCs) generated electrical waves that paced rhythmic contractions through nerve-to-cell communication. These were reported to have a synaptic relationship to the nerve processes and form gap junctions with smooth muscle cells, thus giving rise to a pathway from the nerve to the muscle that paralleled to the pathway of direct innervations of the smooth muscle (Thuneberg, 1982, Thuneberg et al., 1995). More recent studies demonstrated that spontaneous electrical activity, intrinsic to smooth muscle tissue, is generated by the ICCs that are electrically coupled to the smooth muscle cells. In addition to this level of control, inputs from the enteric nervous

system and hormonal influences were shown to regulate normal activity during normal physiological responses (Sanders et al., 2006).

The extrinsic innervations, on the other hand, are divided into sympathetic and parasympathetic branches. The parasympathetic branches innervates the GI tract via preganglionic nerves and the sympathetic branches innervates the gut via postganglionic nerves, both of which synapse mainly with cells of the enteric nervous system. The enteric nerve stimulation originated from the extrinsic innervations, forms networks dispersed through the smooth muscle cells to influence GI motility. Here pharmacochemical coupling takes place, in which receptors are activated by drugs or hormones leading to modulation of intrinsic patterns of GI smooth muscle contraction, through calcium release from an internal source – the endoplasmic reticulum (Somlyo & Somlyo, 1968, Chen & Breemen, 1992, Somlyo & Somlyo, 1994, Somlyo et al., 1999).

Both extrinsic and intrinsic nerve pathways induces depolarisation and contribute to the increase in intracellular calcium, resulting in calcium associating with the calmodulin complex (CaM), which activates smooth muscle myosin light chain kinase (MLCK). This in turn creates a complex with actin filaments and the muscle contracts (Stull et al., 1996).

The coordination of both intrinsic and extrinsic neuronal reflexes results in the appropriate GI motility through coordinated contractions of smooth muscle cells from the muscularis region of the GI tract (Figure 1.3.1).

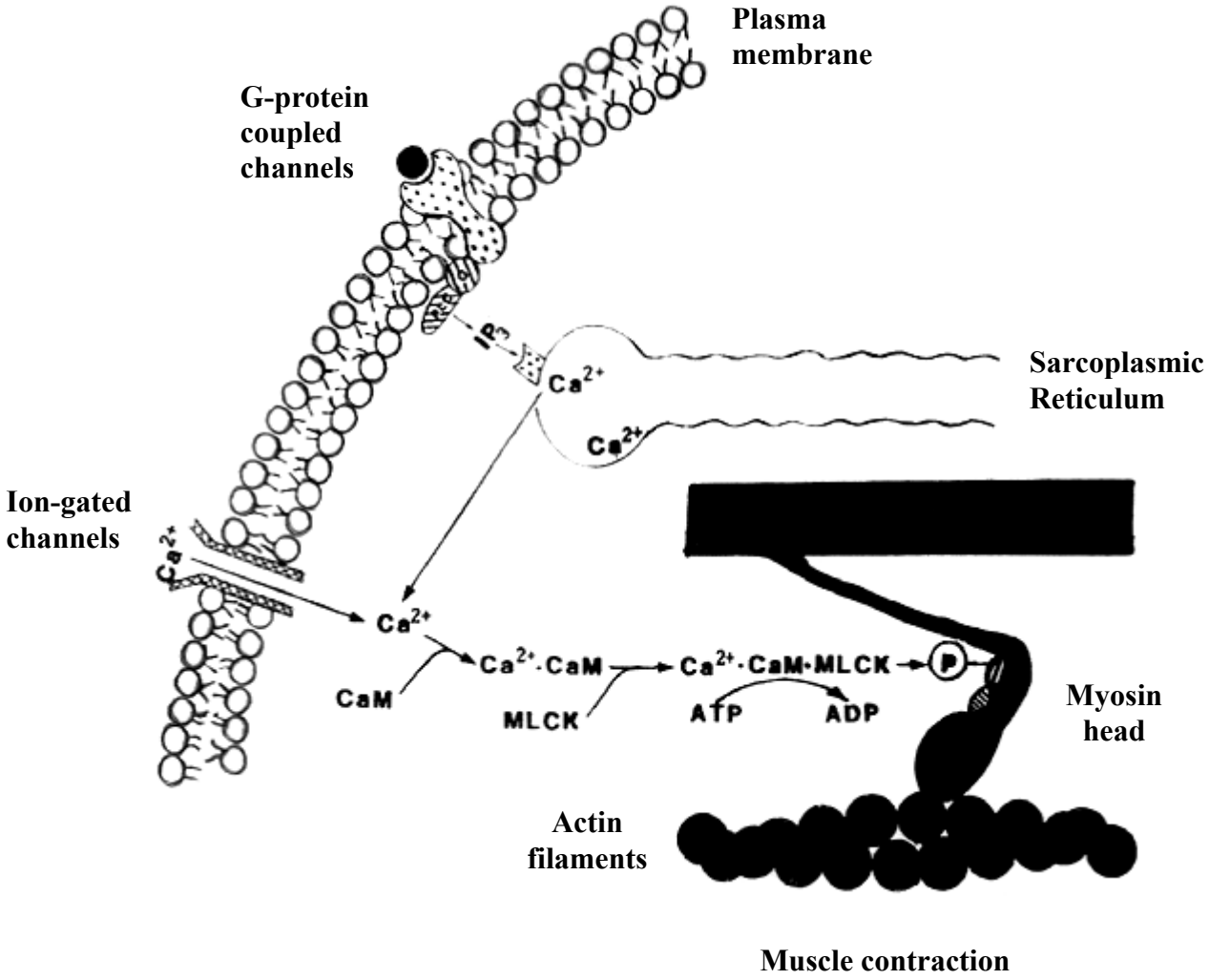


Figure1.3.1: Diagrammatic representation of the signaling transduction pathways involved in smooth muscle contraction (Stull et al., 1996).

1.4 THE SMOOTH MUSCLE CELL

Smooth muscle cell cytoskeleton is arranged into three classes of myofilaments: the thin filaments correspond to the actin (Hanson & Lowy, 1963, Shoenberg, 1965), the thick filaments are identified as myosin (Cooke & Fay, 1972, Devine & Somlyo, 1971, Lowy & Small, 1970), and the intermediate filaments (Lazarides, 1982) (Figure 1.4.1).

With advent of high resolution electron microscopy, myofilaments were first found to constitute a single class of contractile proteins within the smooth muscle cell with a diameter of 60-80nm (Hanson & Lowy, 1963, Shoenberg, 1965). Further work, indicated that when smooth muscle cells were fixed under "physiological" conditions, they contained numerous thick myofilaments (170nm) and x-ray reflection characteristic of myosin filaments were found (Cooke & Fay, 1972, Devine & Somlyo, 1971, Lowy & Small, 1970). More recent ultrastructural studies revealed the presence of three distinct classes of myofilaments in smooth muscle cells (Cooke & Fay, 1972, Fay & Cooke, 1973, Small & Squire, 1972). The third of which were identified as intermediate filaments with a circular cross-sectional profile around 100nm in diameter (Lazarides, 1982).

Actin filaments are the most abundant, they are packed into bundles of filaments showing considerably regular center-to-center interfilaments spacing. They also appeared homogeneously distributed throughout the sarcoplasm (Cooke, 1976). There are at least six different actin isoforms, however, those that have been studied in greatest deal are the muscle α and y actin associated with contractile filaments and the cytoplasmic β and y actin associated with non-contractile filaments (North et al., 1994).

Myosin filaments are consistently found in the smooth muscle, but their absolute abundance and distribution are highly variable (Cooke, 1976). Moreover, with the introduction of electron microscopy it became clear that both contractile proteins (actin and myosin) existed as organised filaments, which were obliquely orientated to the longitudinal axis of the cell (Bagby et al., 1971). Following numerous studies smooth muscle-myosin is

now a well-characterised marker of smooth muscle phenotype, which is exclusively expressed in smooth muscle cells (Miano et al., 1994, Owens, 1995). The known smooth muscle-myosin heavy chain isoforms are products of an alternative splicing of a single gene. This splicing has enabled the production of at least four heavy chain isoforms including smooth muscle myosin 1 (SM-1); smooth muscle-myosin 2 (SM-2); smooth muscle-myosin A (SMA) and smooth muscle-myosin B (SMB) (White et al., 1993).

The molecular structure and organisation of the contractile apparatus in the smooth muscle cells enables force generation (contraction) through sliding of the adjacent α-actin and heavy chain myosin filaments.

The intermediate filaments were first demonstrated through use of electron microscope and revealed that numerous connections were made between the dense bodies and bundles of intermediate filaments (Cooke, 1976). The direct structural connections between the intermediate filaments and the dense bodies suggested that these structures constituted a three-dimensional filamentous network within each smooth muscle cell (Cooke, 1976). Following various studies it is now accepted that intermediate filaments constitutes the major component of the smooth muscle cytoskeleton, serving a structural role in the cell body and supporting the contractile apparatus (Malmqvist et al., 1991).

In smooth muscle, the intermediate filaments are mainly composed by two major proteins: desmin and vimentin (Berner et al., 1981, Osborn et al., 1981). Desmin intermediate filaments bind to the dense bodies (Small & Sobieszek, 1977, Small, 1995) and might provide an anchoring of the contractile units (Wede et al., 2002).

As a result, smooth muscle cells possess a contractile apparatus of actin and myosin filaments and the cytoskeleton structurally supported by the intermediate filaments, which provides the interface between the contractile machinery on the inside of the cell and the extracellular matrix on the outside, to which force must be transmitted. Force transmission between smooth muscle cells is achieved by gap junctions, which provide low-resistance

channels between cells allowing easy and rapid spread of electrical currents (Peracchia, 1980, Spray & Bennett, 1985).

Gap junctions of small intestine are found between the circular muscle cells (Henderson et al., 1971, Thuneberg, 1982), between the ICCs of the deep muscular plexus and adjacent outer circular muscle (Thuneberg, 1982, Horiguchi & Komuro, 1998). Gap junctions between longitudinal muscle layers or between cells of the inner circular muscle have not been reported (Daniel et al., 1998, Thuneberg et al., 1995). It is agreed that gap junctions provides transit between cells. This was demonstrated through techniques using dye injection into one and its spread into adjacent cells (Farraway et al., 1995). Similarly, techniques using electrode systems provided evidence that gap junctions couple cells. In such experiments adjacent cells were impaled with potassium chloride filled glassed microelectrodes so that one cell could be depolarised by the injection of current and the corresponding depolarisation of the second cell could be monitored (Verheule et al., 1997).

In addition to this level of control, studies have been reported on independent intestinal smooth muscle contraction in response to stretch. This stretch activation mechanisms, suggests that mechanosensitivity and mechanotransduction can occur at the level of muscle tissue. These studies went on to conclude that stretch activation pathway displayed an increase intracellular pressure leading to calcium release. Thus, stretch-activated calcium channels in the GI smooth muscle may also regulate smooth muscle tone and intestinal motility (Farrugia et al., 1999).

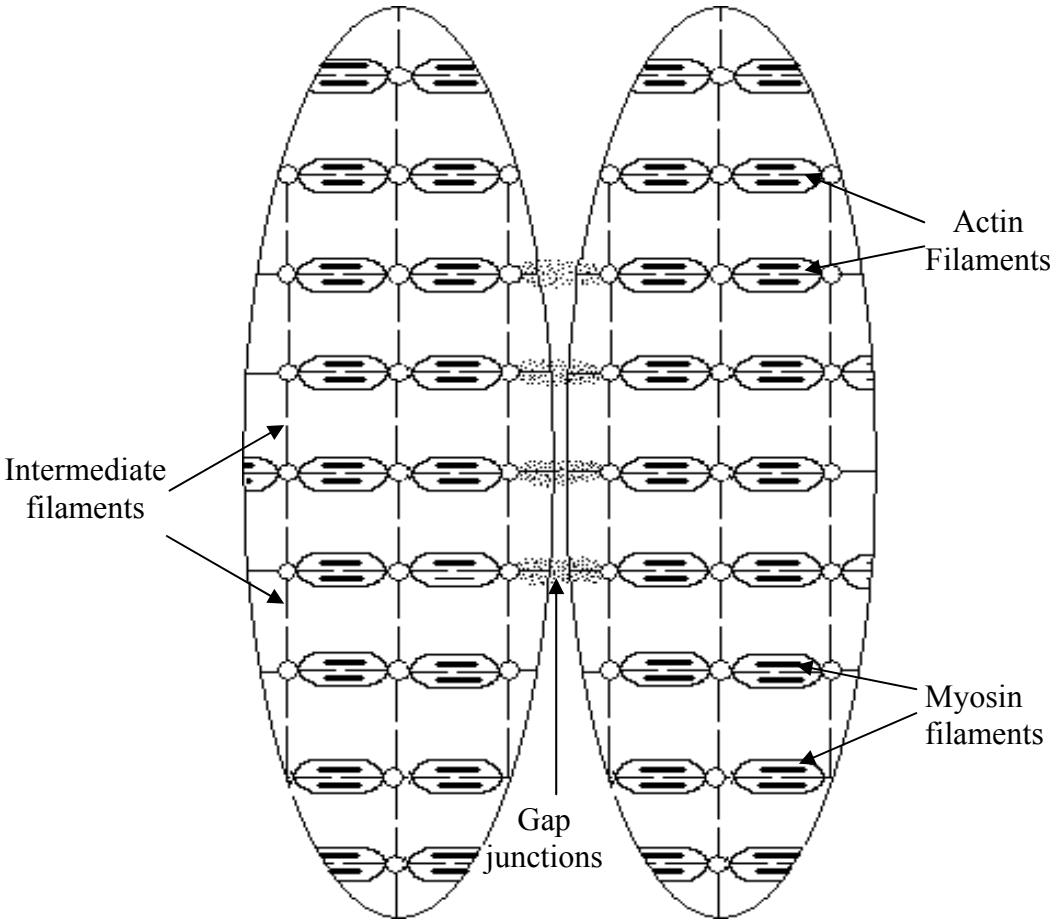


Figure 1.4.1: Diagrammatic representation of spindle-like smooth muscle cells composed of thick myosin and thin actin filaments, which constitutes the contractile proteins. The thin filaments anchored either from the plasma membrane or the structures known as dense bodies, which are attached to a network of intermediate filaments. The intermediate filaments form an internal cytoskeleton in which contractile filaments are anchored. Contraction of smooth muscle occurs due to the sliding filament mechanism with cross-bridge formation occurring between the overlapping thin and thick filaments. The cells function as an effector unit because cells are functionally coupled to one another. Opposed cell membranes are fused to form gap junctions that allow the spread of excitation from one cell to another.

1.5 THE ENTERIC NEURONES

For several decades it was accepted that the autonomic nervous system consisted of the parasympathetic and sympathetic nervous system. In 1921 pioneering work carried out by Langley established the enteric nervous system as the third division of the autonomic nervous system containing two efferent pathways: the cholinergic and adrenergic neurones. The cholinergic pathway was assumed to act at the nicotinic/acetylcholine (ACh) receptors to cause excitation of the enteric nerves and muscarinic receptors to cause contraction of the GI function. Inhibition of the GI function was believed to take place through the action of norepinephrine released from the sympathetic nerves. At the time Langley also postulated the hypothesis that many of the neurones present in the enteric nervous system were not connected to the central nervous system (Langley, 1921). Succeeding studies demonstrated that the enteric nerves could release non-cholinergic excitatory and non-adrenergic inhibitory neurotransmitters (Burnstock et al., 1966, Ambache & Freeman, 1968).

Intensive studies over the last 20 years have led to identification of the major neuronal types in the enteric nervous system and it is now appreciated that the enteric nervous system possesses several neuronal mechanisms similar to the central nervous system, but has autonomic and physiological independence from the central nervous system (Bornstein et al., 1994). Further work continued into investigation of their mechanisms of action revealing that the muscle layers of the intestine are dually innervated by excitatory and inhibitory motor neurones located in the gut. With the introduction of selective antagonists, ACh was soon recognised as the primary transmitter of the excitatory motor neurones. However, it was also observed that cholinergic blockers did not completely abolish the excitatory transmission. This transmission was later found to be blocked by antagonism of tachykinin (TK) receptors (Burnstock et al., 1966, Ambache & Freeman, 1968).

Classification of the mechanisms of transmission from excitatory motor neurones was concluded through immunohistochemical studies, which demonstrated that smooth muscle is innervated by one class of excitatory neurones that is immunoreactive for both choline acetyl-transferase (through which ACh is synthesised) and tachykinin (Galligan et al., 1986), thus confirming that both these components of transmission are derived from single neurons (Galligan et al., 1986). The main inhibitory neurotransmitters have been suggested to include nitric oxide (NO), adenosine triphosphate (ATP) and vasoactive intestinal peptide (VIP) (Mashimo & Goyal, 1999), however, the roles of these transmitters are not yet fully resolved. Additionally, inhibition of the GI function also takes place through the release of noradrenaline at most post-ganglionic sympathetic nerve endings causing muscle relaxation (Lungren, 2000).

Evidence for the existence of interneurones in the enteric nerve network arose from structural studies, which revealed the existence of neurones with cell bodies in the myenteric ganglia and in the terminal ganglia. There are at least one class of ascending interneurones and three classes of descending interneurones. It is challenging to study interneurones physiologically, however, direct recording have shown that interneurones respond to reflex stimuli with fast excitatory postsynaptic potentials. Data obtained from organ bath pharmacology and the microelectrode recording studies indicated that in ascending pathways, transmission between interneurones and excitatory muscle motor neurones is cholinergic via the nicotinic receptors. Therefore, ascending interneurones contain choline acetyl-transferase and tachykinins, and they transmit to each other via ACh at the nicotinic receptors, but to excitatory motor neurones via both ACh and tachykinins (Bornstein et al., 2004).

To date three types of descending interneurones have been recognised and one transmits to the inhibitory motor neurones via adenosine triphosphate. The second one is thought to occur via NO synthase-containing interneurones and the third transmission to and from

descending interneurones pathway is thought to occur either via choline acetyl-transferase/serotonin and/or choline acetyl-transferase/somatostatin, but this is yet to be characterised (Bornstein et al., 2004) (Table 1.1). The neurones in the intrinsic nerve circuits are the intrinsic primary afferent neurones also known as sensory neurones. These nerve fibers were recognised through studies using intracellular electrodes, in which it was found that some neurones were excited by sensory stimuli. Extensive work in this area demonstrated that the sensory nerve fibers in the wall of the GI tract detect mucosal pH and osmolarity and can respond to temperature, tension and touch (Berthoud et al., 2004).

On the basis of this information, when changes in the environment of the intestine are detected by a receptor cell, which synapses with a sensory neurone it carries the impulse from the site of stimulus to the central nervous system. Here it synapses with an interneurone, which in turn synapses with a motor neurone carrying the impulse out to an effector cell, thus restoring normal homeostasis (Berthoud et al., 2004) (Figure 1.5.1).

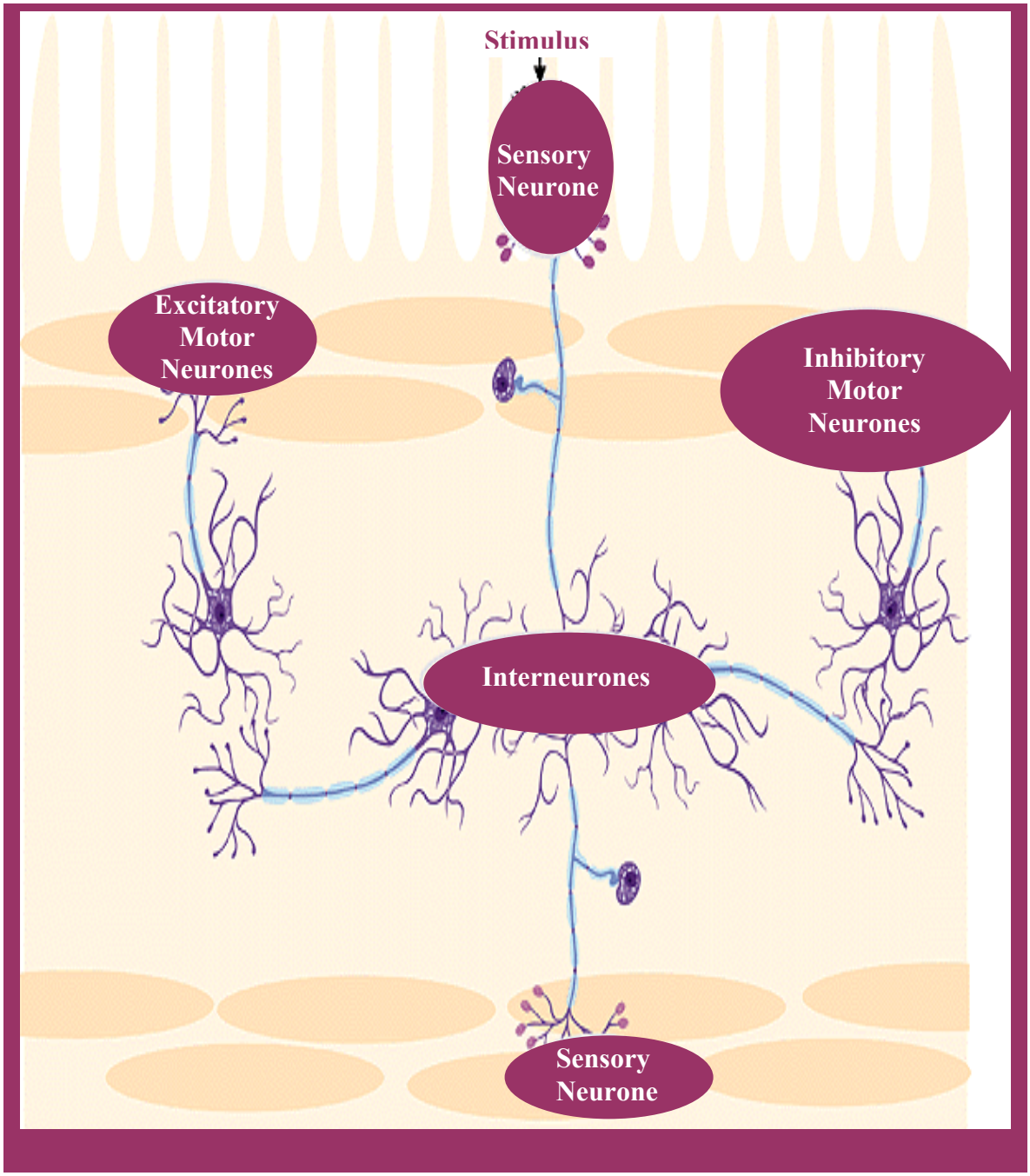


Figure 1.5.1: The organisation of the three types of enteric ganglia neurones (sensory, interneurone and motor neurones) and the circuit network from the triggered stimuli to the response elicited in the GI musculature.

Table 1.1: Summary of the types of neurones in the enteric nervous system and some of their defining characteristics in the gastrointestinal tract (Bornstein et al, 2004).

Type of neurone	Function
Excitatory motor neurones	**Primary transmitter ACh, co-transmitter TK**
Inhibitory motor neurones	**Several co-transmitters with varying prominence: ATP, NO and/or VIP**
Ascending interneurones	**Primary transmitter ACh**
Descending interneurones (excitatory secrotomotor effect)	**Primary transmitter ACh, co-transmitter 5-HT or SOM**
Descending interneurones (inhibitory secrotomotor effect)	**Primary transmitter ATP and NO**

ACh, acetylcholine; ATP, adenosine triphosphate; NO, nitric oxide; 5-HT, 5-Hydroxytrytamine; SOM, somatostatin; TK, tachykinin; VIP, vasoactive intestinal peptide.

1.6 HORMONAL REGULATION

In the past it was widely accepted that the concept of GI endocrinology was that a few peptides were released to the circulation from the endocrine cells interspersed among other mucosal cells in upper GI tract (Starling, 1905, Edkins, 1905, Edkins, 1906). However, with the advances in cell and molecular biology it is now more complex to describe the effect of substances in response to bodily stimulus arising from within or outside the body (Rehfeld, 1998). This is because bodily stimulus may act directly on the responding cell or on a sensory cell that in turn sends a chemical messenger to the responding cell. These chemicals messenger are transmitted by one of the three different mechanisms: neurocrine, endocrine, or paracrine (Figure 1.6.1).

In neurocrine transmission the signalling molecules of the nervous system of the species are chemicals called neurotransmitters. These are released at the axon end of one nerve cell referred as the synaptic cleft to target cells: receptor cells, nerves cells, and neuromuscular junctions. The paracrine transmission takes place as the signalling cells released by one cell travels through the extracellular environment and acts on the receptor molecule of the adjacent cells in close proximity. These are normally growth regulators that react with the receptor cell and are removed from the environment. Lastly, the endocrine transmission signalling molecules act on target cells distant from their site of synthesis. These are often specialised cells that release the hormones into vessels of the circulatory system, so that they can travel to the target cells in other parts of the body. However, endocrine transmission is more complex as there is evidence of scattered cells rather than discrete glands that act as organs and endocrine cells that affect themselves. This autocrine transmission suggests that endocrine cell activity may be modulated directly within that endocrine gland itself, without the need of hormones entering the blood stream (Sporn & Roberts, 1992).

Communication between cells via neurotransmitters or hormones can elicit a response within a cell if receptor is present. This cascade is then amplified by interaction with a number of proteins embedded in the cell membrane referred as second messengers by which a ligand generate a specific intracellular event. However, in studies of release mechanisms it is difficult to distinguish material derived from the neurocrine or endocrine or paracrine cells, since the unifying links between these systems is that all three utilise amino acids, peptides, and amines as second messengers, just as some of the same peptides are found in both nerves and endocrine cells. Conversely, the responses evoked by exogenous applications of substances may reflect actions usually exerted in the paracrine, endocrine or neuroregulatory systems (Arimura et al., 1975).

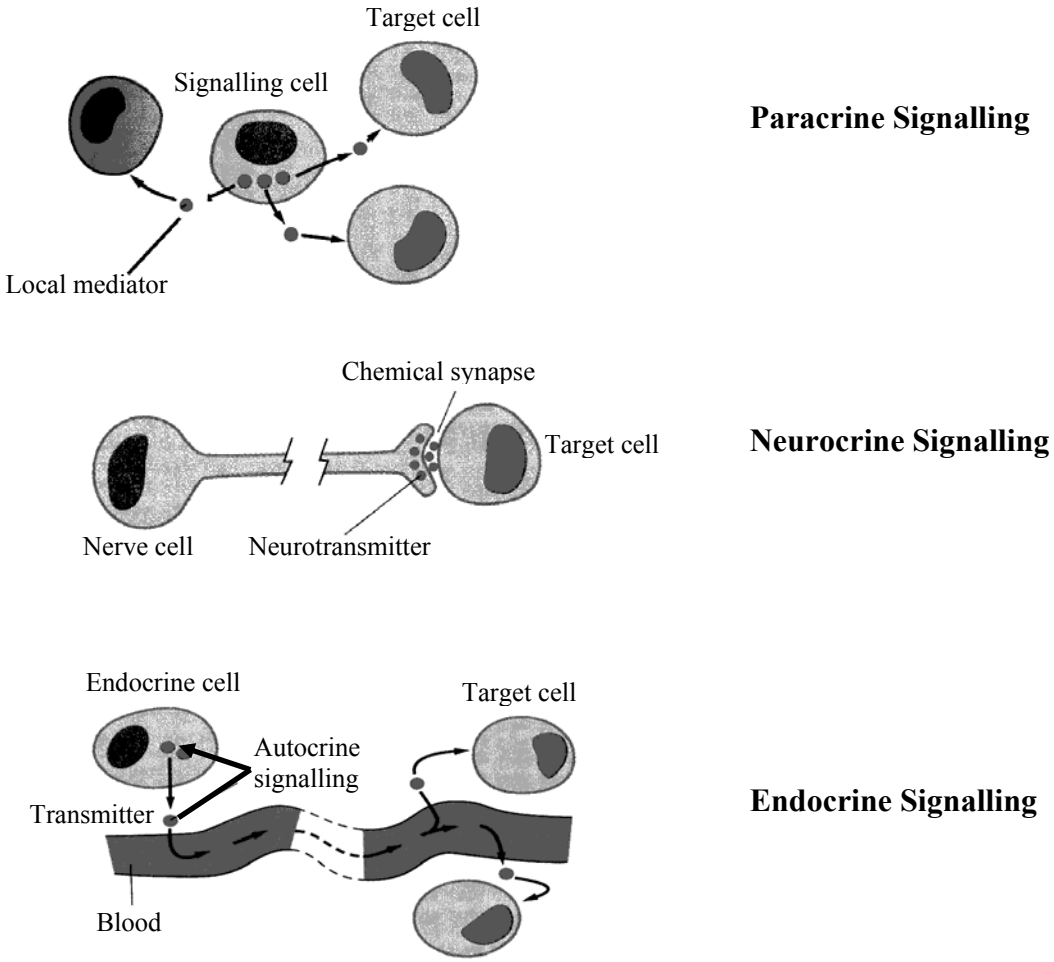


Figure 1.6.1: Images adapted from Alberts et al, to illustrate the different signalling mechanisms (paracrine, endocrine, and neurocrine) used by chemical messengers to travel to target cell (Alberts et al., 1994).

1.7 THE GASTROINTESTINAL TRACT IN HEALTH AND DISEASE STATE

Functional GI disorders comprise alterations in motor and secretory activity. The causes and mechanisms underlying most of functional GI disorders are still largely unknown, however, alterations seem to arise primarily in inflammation with consequent changes in localised neurotransmitters released locally, that induce pathological reflex mechanisms occurring in response to specific stimuli. Because the GI tract is the natural habitat to a large and dynamic population of bacteria, the continuous exposure to even normal physiological conditions such as food ingestion, displays an immunological state in response to the pathogens that is manifested by the abundance of pro-inflammatory agents. These agents constitute the GI immune system and their main function is to restore homeostasis by maintaining a delicate balance between the immune response to the microbial pathogens and tolerance to the dietary pathogens and the local microflora (Brandtzaeg et al., 1989). The initiation of the immune response against antigens within the intestine involves primarily the selective migration of T lymphocytes into the regions. Of these, the lymphocytes that play a significant role are T helper cells (Th), which are later divided into subsets of Th1, Th2, Th3, Th0, Thp, and Tr1 cells (Seder & Mosmann, 1999). These cells, when activated during the immune response, synthesise and release pro-inflammatory cytokines such as tumour necrosis factor (TNF) α; interleukin (IL) 1β, IL-6, IL8, IL-12, IL18; metalloproteinases (collagenase, gelatinase), arachidonate metabolites (platelet-activating factor) reactive oxygen species and nitrogen metabolites (superoxide, hydrogen peroxide, nitric oxide) (Abbas et al., 2000).

Conversely, gut flora might also be an essential factor in certain pathological conditions leading to chronic intestinal inflammation. The reasons for the breakdown in the regulation of the intestinal immune system are yet to be identified. However, there is evidence

suggesting the possible involvement of Th cells in attacking bacteria in the gut, hence, leading to intestinal inflammation. This has been shown in studies generated in genetically engineered rodent models of inflammatory bowel disease (IBD). This works by using vector systems that can efficiently transduce epithelial and sub-epithelial areas of the intestine to overproduce a pro-inflammatory mediator of interest inducing the disease state (Wirtz & Neurath, 2000). It has been found that a failure to regulate protective immune regulators resulted in uncontrolled overproduction of pro-inflammatory cytokines and mediators. This can cause severe and extended inflammation of the inner epithelial lining of the gut leading to the onset of fibrosis. It can subsequently lead to scarring, which reduces the width of the lumen known as strictures, giving rise to functional GI disorders. Although, intensive research has been carried out towards understanding the aetiology and specific pathogenic mechanisms, to date advances have been minimal.

Despite the substantial new data that has been obtained in recent studies, it has been increasingly difficult to accommodate this into a unifying disease model. However, when looking at the exact mechanisms that trigger immune response breakdown in the intestine, it continues to be apparent that the immune system plays a crucial role in promoting chronic gut inflammation and that the antigens that initiates and cause the onset of the disease are part of the normal gut flora (Laroux et al., 2001).

1.8 THE ROLE OF 5-HT IN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL PROCESSES IN THE GI TRACT

The pharmacological understanding of the nervous and endocrine controls of the GI tract and its importance for gut function in several states of disease has advanced greatly over recent years. Thus, opening doors into new therapeutic concepts based on the roles of key neurotransmitters and neuroendocrine substances.

One clear example is 5-hydroxytryptamine (5-HT), one of the neurotransmitters in the GI tract, possessing complex actions within the gut. 5-HT is synthesised, stored and released by enterochromaffin cells and enteric neurones, and participates in sensory, motor, and mucosal sensory stimulation. The serotonergic myenteric interneurones project into the gut but not into the luminal area. The sensory stimulus by the enteric nervous system and the central nervous system is achieved by paracrine transmission, which activates specialised epithelial detectors, such as enterochromaffin cells underlying the intrinsic and extrinsic afferent nerves (Gershon, 2002). Thus, the main source of 5-HT in the gut is the enterochromaffin cells from which 5-HT is released in response to increased luminal contents and stimulus such as food; pathophysiological conditions such as bacteria or toxins; or pharmacologically in response to chemical agents. The released 5-HT exerts its effects through receptors that are located on intrinsic primary afferents and extrinsic afferents, ganglion cells, axonal membranes, nerve terminals, enterochromaffin cells, enterocytes, and immuno-related cells (Gershon, 1999).

The effects of 5-HT and its pharmacological interaction with the serotonergic pathways on gut functions have proven over the years to be complex and unpredictable. This seems to be caused by the action of 5-HT and its multiple receptors.

1.9 5-HT RECEPTOR CLASSIFICATION

The classification of 5-HT receptors began in 1957, when 5-HT was first found to affect the guinea pig ileum and that this action could be blocked by morphine (M) and partially by dibenzyline (D) (Gaddum & Picarelli, 1957). However, neither morphine nor dibenzyline were selective to the 5-HT receptors. In 1976 while performing radio-ligand binding studies, the presence of 5-HT was reported in the brain. A few years later it was reported that two distinct 5-HT binding sites existed (Peroutka & Snyder, 1979). The D receptor was found to correspond pharmacologically to the two receptors reported by Peroutoka and Snyder, and the M receptor was distinct. As a result, Bradley and colleagues proposed the existence of three groups of 5-HT receptors, named 5-HT_1-like, 5-HT_2, 5-HT_3, the latter corresponding to the M receptor type (Bradley et al., 1986). In the mid 1980s due to the increased use of radioligands, followed by an increased understanding about the second messenger systems and the advances in genetic recombination techniques in the mid 90's, a vast number of 5-HT receptor subtypes were identified. Currently seven 5-HT receptor families have been recognised. The 5-HT_1 receptor class comprises five receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}). These operate via ligand-gated ion channels and are negatively coupled by adenyl cyclase to inhibit cyclic AMP formation. In the GI tract, $5HT_{1A}$ has been reported to act as inhibitory modulators of fast excitatory postsynaptic neurones (Hannon & Hoyer, 2002).

The 5-HT_2 receptor class is divided into 5-HT_{2A}. 5-HT_{2B}, 5-HT_{2C} and operate via ligand-gated ion channels and are thought to increase in inositol phosphates and cytosolic calcium concentration by positive coupling to phospholipase C. 5-HT_{2A} is widely distributed in the periphery including ileal and urinary smooth muscle. 5-HT_{2B} receptors contract longitudinal muscle in human small intestine (Hannon & Hoyer, 2002).

5-HT_3 receptors (M receptors of Gaddun and Picarelli 1957) are unique in that they couple to ions channels, which gate sodium, potassium, and calcium (Hannon & Hoyer, 2002). In

the GI tract the 5-HT_3 receptors are believed to be involved in motility and intestinal secretion.

The 5-HT_4, 5-HT_6, 5-HT_7 receptors are positively coupled to phospholipase C and thus generate inositol phosphates and mobilise intracellular calcium when activated to mediate effects of 5-HT. The 5-HT_4 receptor activation has been reported to trigger ACh release in the guinea-pig ileum, it also stimulates secretory responses to 5-HT in the intestinal mucosa (Bockaert et al., 1992). The 5-HT_5 receptors are believed to stimulate cyclic adenosine monophosphate (AMP) production, however, a physiological functional response or specific 5-HT_5 binding are still missing (Bockaert et al., 1992). The 5-HT_6 receptors are found in the striatum, amygdala, nucleus accumbens, hippocampus, cortex and olfactory tubercle, but have not been found in the peripheral organs (Hannon & Hoyer, 2002).

The 5-HT_7 receptor is the previously proposed '5-HT_{1A}-like' receptor and it has been shown to mediate relaxation in the guinea-pig ileum (Hannon & Hoyer, 2002) (Figure 1.9.1).

5-HT acts on cell membranes, which have specific 5-HT receptors, furthermore, the sub-types of these receptors are present in different cells associated with different actions of 5-HT. This is further compounded with the ability of 5-HT to act indirectly either by stimulating or inhibiting the release other neurotransmitters. For instance increased 5-HT release causes stimulation of the ACh release from the myenteric neurones increasing intestinal contractions and secretions, however, 5-HT also directly activates smooth muscle cells causing contraction (Costall & Naylor, 1990, Gershon et al., 1990) . Therefore, it is not surprising that when there is dysfunction of the enteric nervous system several of the recognised functional GI disorders are related to alterations in 5-HT levels (Thompson, 2002). 5-HT has been suggested to play an important role in controlling both inhibition and facilitation of motor functions of the oesophagus, stomach, small intestine, ileocaecal

sphincter and colon, as well as modulating small intestine and colon secretion (Jule, 1980, Goldberg et al., 1986, Costa & furness, 1976, Bulbring & Gershon, 1967, Beubler & Horina, 1990).

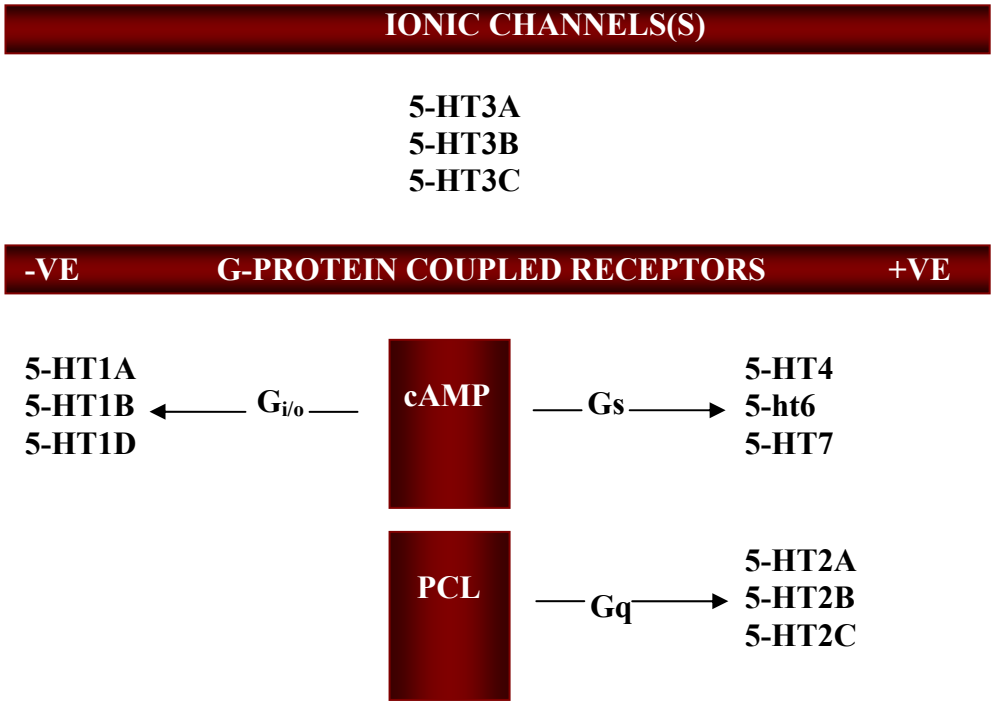


Figure 1.9.1: Graphical representation of the current classification of 5-HT receptors. Receptor subtypes represented by lower cases designates receptors that have not been demonstrated to completely function in the native systems. Abbreviations: 3'-5' cyclic adenosine monophosphate (cAMP); phospholipase C (PLC); negative (-ve); positive (+ve). Adapted from (Hannon & Hoyer, 2002).

On the basis of this information, the 5-HT receptor family has become a target of intense research with the identification of more potent and selective ligands for the different receptor subtypes as a major goal. Such selective markers should assist in better defining the functions of these receptors, furthermore, leading to potential drug treatments.

However, when seeking to determine the 'identity of action', it is essential to know not only the mechanisms of action interconnecting the muscular layers to the neuronal plexuses but all the co-existing effects exerted by neurotransmitters, hormones, and other peptides in the gut. Thereby the urge to create models representative of individual

intestinal layers in order to investigate the mechanisms of action of selective agonists and antagonists and attempt to relate these to the unifying links in communication among the different intestinal layers and the hormones and neurotransmitters produced.

Studies of stimulus-contraction coupling of smooth muscle cells have been largely performed on intact tissues, considerable information about the function of these tissues have been acquired. However, results obtained from the intact tissue normally emerge from the complex network of organised smooth muscle cells into a multicellular unit rather than from the individual properties of cells themselves.

More recently, attention has been directed to the use of cells isolated from the intact tissue as a source for studies of some basic physiology and biochemical properties of muscle cells (Lieberman et al., 1987).

1.10 PRIMARY CELL CULTURE

Cell culture was first devised at the beginning of the century (Harrison, 1907, Carrel, 1912) as a method for studying the behaviour of animal cells free from systemic variations that might arise both during normal homeostasis and under stress caused by external stimulus during an experiment. In cell culture, when specific cells are extracted from primary tissues and cultured, they usually propagate, expand and divide into identical replicates assuming a uniform constitution (Freshney, 2005). Cell culture refers to culture derived from dispersed cells taken from the original tissue, by enzymatic, mechanical or chemical disaggregation. Since this provides the accessibility to study particular cell populations within the tissue or organ, this has created a new wave of interest based in the cultivation of tissue *'in vitro'* (Freshney, 1994) and subsequently became a valuable technique for studies of physiological properties of cells (Lieberman et al., 1987). Moreover, due to the requirement of animals for continuation of preclinical trials of new pharmaceuticals, there is a widespread concern about the extensive use of animals in drug development. Hence, there is an ever-increasing need for more *'in vitro'* assays, which can subsequently at later stages of evaluation be validated with data from animal experiments (Freshney, 1994).

1.11 INTESTINAL CELL CULTURE

The culture of different cell populations extracted from the small intestine has proven challenging. For intestinal smooth muscle cells (ISMC) this is primarily related to the loss in phenotypic characteristics (Thyberg et al., 1983). Muscle cells *'in vitro'* are highly differentiated which is evident from the large proportion of contractile filaments within the cell and thus must undergo a process referred as "phenotypic modulation" before mitosis so an increase in the cell number may occur (Chamley-Campbell et al., 1979). There is

evidence that such proliferation processes lead to a decrease in the number of contractile proteins, hence changes in the phenotypic properties of cells, resulting in a loss of contractility (Chamley-Campbell et al., 1979). Furthermore, the complex layered nature of the GI tract makes it difficult to acquire pure preparations of individual cell types. These difficulties are further compounded by the limited availability of adult animal species from which successful ISMC cultures can be established (Bitar & Makahlouf, 1986, Benham et al., 1986, Oishi et al., 2000).

1.12 COMPARISON OF NEONATAL AND ADULT INTESTINAL CELL CULTURE MODELS

In establishing primary culture models, the major concerns are obtaining sufficient number of cells, obtaining a population with a high degree of purity, and, most importantly, having a cell population that will survive the culture. The increasing difficulty in obtaining viable proliferating cells with increasing age is mainly due to the onset of differentiation, the increase fibrous connective tissue and the decrease in undifferentiated proliferating cells (Freshney, 2005). Conversely, embryonic or neonatal tissue disperses more readily and gives higher number of proliferating cells compared to the adult tissue (Freshney, 2005). It has been reported that the phenotype of cultured neonatal smooth muscle cells are very stable in terms of their contractile profile (Yamashita et al., 1994) and that they are less susceptible to damaged by the dissociation process than ISMC obtained from adult tissue (Chlocikova et al., 2001).

However, when dealing with neonatal tissue, techniques for cellular dissociation requires the support of advanced optical equipment due to the small size of the tissues (Smirnov et al., 1992, Frings et al., 2000). Although, enzymatic and mechanical techniques of disaggregation usually gives a high yield in the neonatal tissue due to its softness compared

to the adult tissue (Schaffer et al., 1997), it can be disadvantageous since cell cultures yielded via this technique are more representative of the whole tissue (Freshney, 2005). Nevertheless, such limitations in the disaggregation of cells from neonatal tissue can be minimised if methods are employed to monitor the gradual dissociation from a specific region of the intestine. This technique, combined with cell sorting techniques and morphological monitoring may provide the parameters necessary for acquisition of highly pure cell preparations from neonatal tissue.

1.13 PURIFICATION TECHNIQUES FOR INTESTINAL CELLS

Preparations of homogenous samples of cells from tissues are considered to be an obligatory step, not only in the study of physiological processes, they are also required for modern methods in molecular biology, drug screening, diagnosis and cell replacement therapy. It has been estimated that 90% of the cost and 95% of the time needed to obtain molecular diagnostic data today are associated with sample collection and preparation. The inadequacy of effective sample preparations may be one of the major shortcomings in past cell based and molecular analysis systems (Gascoyne & Vykoukal, 2004).

Although, there have been improvements in the instrumentation and techniques used for separating specific target cells from a mixed preparation, challenges in this area are still encountered due to the relatively high cost in the separation of large cell populations.

1.13.1 SUBSTRATUM ADHESIVENESS

Some methods exploited the differences in substratum adhesiveness between the cells to be separated (Yaffe, 1968). This method has been successful in the past enabling separation of myogenic cells from fibroblasts (Richler & Yafee, 1970, Gareth et al., 1990, Rando & Blau, 1994, Qu et al., 1998). This is because the method implies that when cells of differing affinities are mixed, differences in cell adhesion can drive cell sorting. If two different cell populations are mixed they will re-arrange on basis of strength of cell-cell interaction. If cells have equivalent adhesions, they will mix randomly, however, if their adhesion molecules are more expressed in one cell population that the other, they will sort to the surface separately (McNeill, 2000) (Figure 1.13.1). Thus, this is performed by continuous monitoring of the rate of cell attachment for different populations, which subsequently provides estimation of the time required for a specific cell population to attach to the surface.

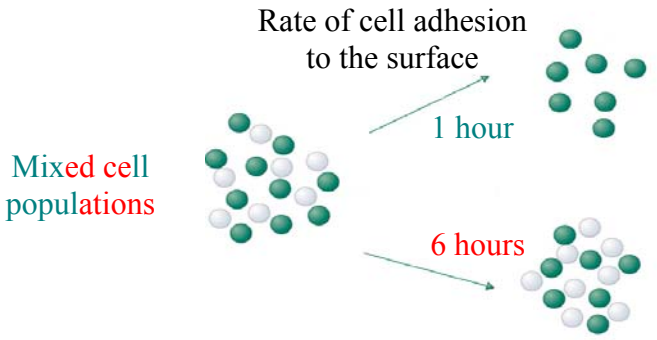


Figure 1.13.1: Schematic diagram of the cell separation system: substratum adhesiveness.

1.13.2 PERCOLL GRADIENT

Other methods exploited the differences in cell density when separated in a Percoll gradient (Price et al., 1978). This conventional method comprises a cell suspension that is layered over a polymer gradient column in a centrifuge tube. The cell populations are then separated by centrifugation based on the cell density and shape (Figure 1.13.2). Although clean cell population can be isolated using this method, depending on conditions, isolation times can be lengthy causing cell viability to decrease. Density gradient fractionation can also pose risk to the cells in two distinct ways: primarily, if solution of gradient material is viscous at high density, and secondly if it exerts high osmotic effects at low concentration. As a result it may cause cells to band at a different density from their physiological density. In separate occasions it can also lead to the solution of the gradient material with low molecular weight penetrating the cells (Pertoft, 2000).

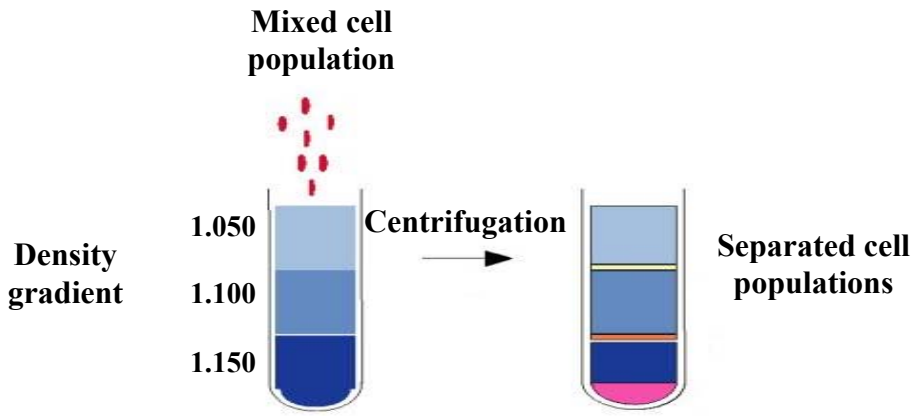


Figure 1.13.2: Schematic diagram of the cell separation system: density gradient cell separation.

1.13.3 PANNING METHOD

Those early methods, however, failed to produce an entirely pure population of cells.

More selective techniques involve the panning method, which was developed for the separation cells in mixed populations. This technique requires the use of a specific antibody, which binds to the target cells' specific isotope. The unbound fraction is then removed simply by pouring the supernatant from the dish (Figure 1.13.3). Although this technique results in the production of an enriched homogenous fraction of cells with a high recovery of approximately 90%-95% of the original population (Jones et al., 1990) its limitations is that it cannot be applied universally because of the lack of antibodies and known surface markers for some cell types.

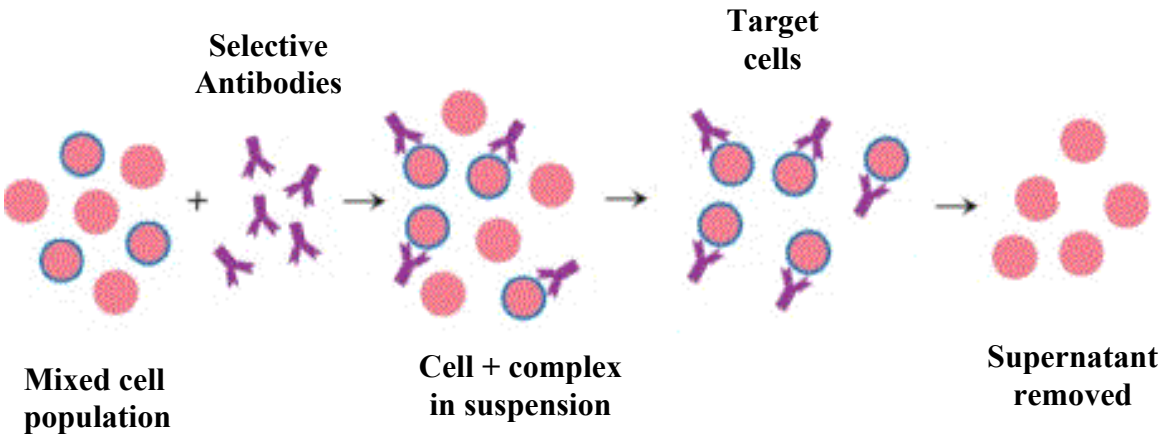


Figure 1.13.3: Schematic diagram of the cell separation system: panning method.

1.13.4 MAGNETIC AND FLUORESCENCE ACTIVATED CELL SORTING METHODS

The most recently devised method for separating cells centers on the use of cell sorting machinery. Fluorescence-activated cell sorter and magnetic-activated cell sorter are both typically used for separating specific target cells and like the panning method they require immunolabelling to separate target cells. Additionally, magnetic-activated method combines the antibodies used to tag cells of interest with small magnetic microbeads. Magnetic-activated method is favoured over fluorescence-activated method, primarily because a larger cell population yield was attained with magnetic cell sorting (Blau et al., 1983) (Figure 1.13.4).

Both these cell separation systems provide convenient pre-enriched cell suspensions before they run downstream sorting through flow cytometry. The use of flow cytometry has been developed to improve the specificity of the cell separation. As samples are processed through a column and placed within the separator instrument, the cells retained are subsequently analysed and separated, one cell at a time, thus determining the purity of the cells sorted. The enrichment of samples through magnetic or fluorescence separation provides an essential step that isolate cells of interest in a specific manner, which can subsequently ensure an accurate separation through flow cytometry. The data is then analysed by computer and can be plotted in several ways. Cell separation through these methods can lead to extremely pure preparations and can subsequently ensure an accurate cell separation. Nonetheless, this technique requires specialised and expensive equipment and is not employed within standard cell culture facilities. Although sophisticated, this technique presents drawbacks associated with sterility and cost.

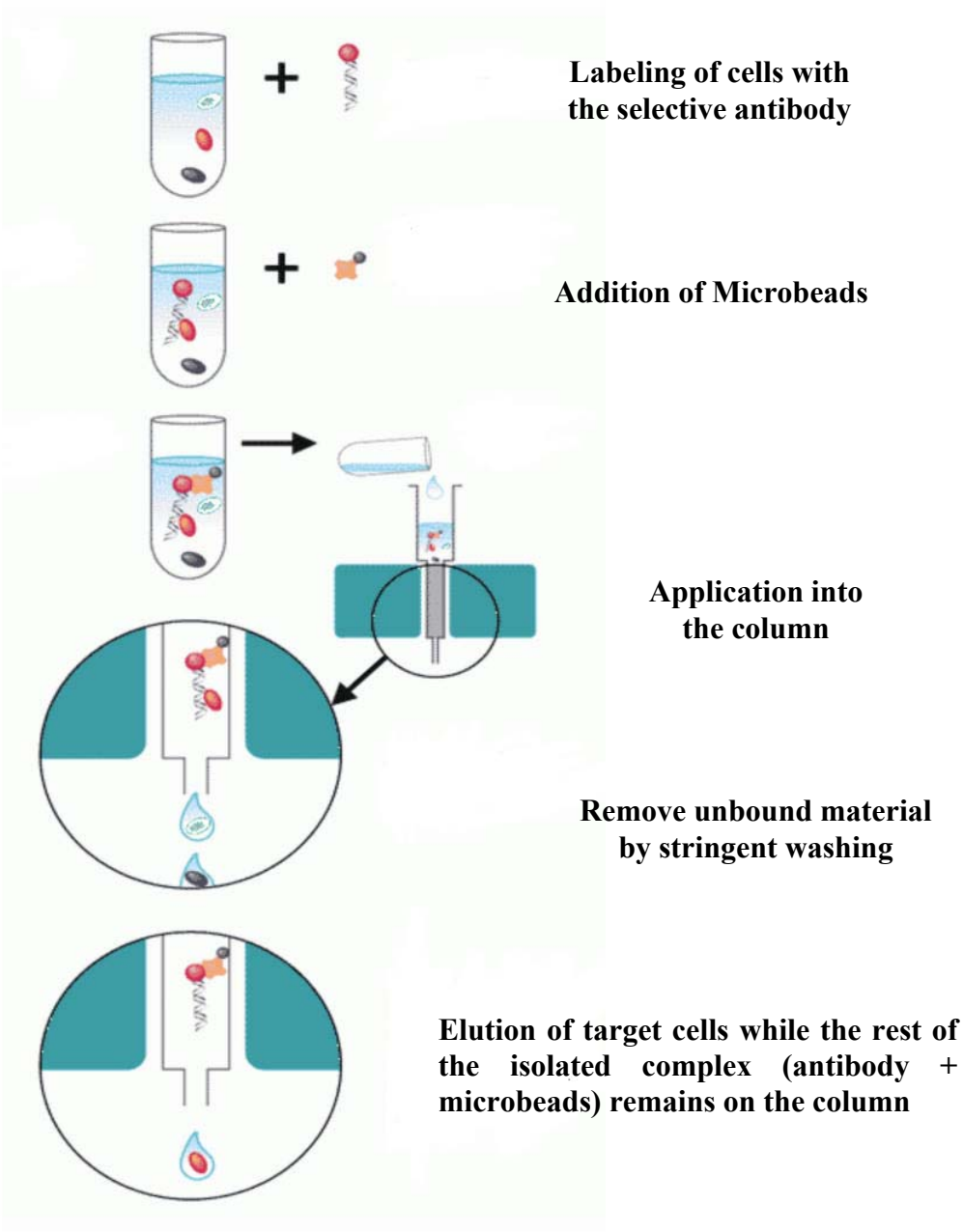


Figure 1.13.4: Schematic diagram of cell separation technique adapted from Biomagnetic research and technology Reviews (Zaiyed et al., 2003): Magnetic activated cell sorting.

1.13.5 SELECTIVE CYTOTOXICITY

In the foregoing procedures, cells are isolated and purified before culture. In some instances this is not feasible. Instead, cultures can be enriched in specific cell types by culture conditions. Selective cytotoxicity is a method that allows selection by favouring the proliferation of one of the cell types in the mixture (Ham, 1984). The technique has been adopted from developmental neurotoxicity. Studies of the central and peripheral nervous systems have benefited greatly from the systematic application of the systems toxicology approach. The ability of certain compounds to have an adverse effect on structure or function, or physical agent that diminishes the ability of an organism to survive, reproduce or adapt to its environment creates a useful tool for studies of structure and function of the specific organ systems. Thus, as adapted for toxicology and referred to as systems toxicology, it involves the perturbation by chemicals and stressors by monitoring alterations in the culture environment (Figure 1.13.5).

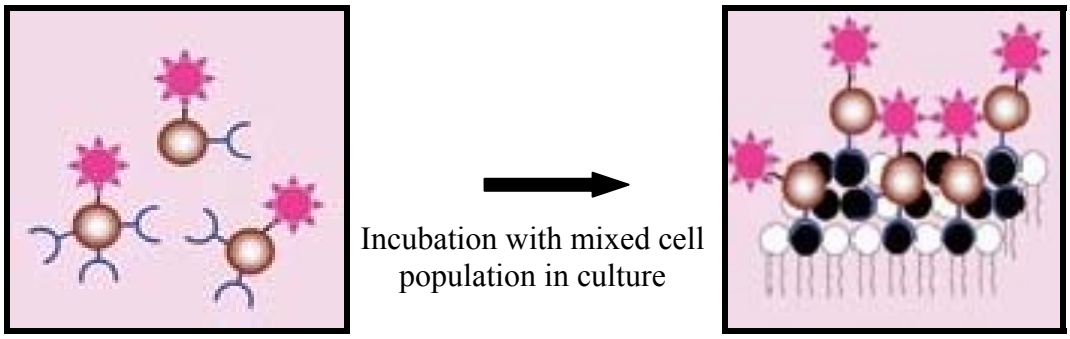


Figure 1.13.5: Schematic diagram of cell separation technique adapted from Nano Letter articles (Quinti et al., 2006): selective cytotoxicity.

Hence, this approach requires the understanding of functional interactions between key components of cells and how they change with toxicant exposure (Waters et al., 2003).

In tissue culture it has been demonstrated that specific use of toxicology systems resulted in apoptotic cell death of targeted cell populations. Some of the studies include those by Blennerhassett and Lourenssen, where scorpion venom was used to target neuronal cells in a mixed cell culture. This led to targeted neurotoxicity without disturbing neighboring cells (Blennerhassett & Lourenssen, 2000). Additionally, some toxicants are yet more specific to molecular components of the cell. One example is the antimitotic cytosine arabinoside that targets non-neuronal cells. Treatment with this toxicant has been shown to incorporate efficiently into the RNA-primed DNA fragments to discontinue DNA synthesis in cells (Harrington & Perrino, 1995). Limitations for this technique are that drug specifications must be accurate so that it specifically targets the proposed cell population. Furthermore, it only allows separation of one cell population at a time and like immunolabeling due to the drug specification required it is not available to all cell types.

1.14 TECHNIQUES FOR INVESTIGATING THE SIGNALLING TRANSDUCTION PATHWAYS IN THE GASTROINTESTINAL TRACT

1.14.1 BATH PERFUSED PREPARATIONS

A variety of '*in vitro'* methods are available for studying the signalling pathways mechanisms of intestinal motility. The most commonly used method for qualitative pharmacological studies '*in vitro'* are the conventional bath-perfused preparations to study contractility of smooth muscle through identification of specific nerve firing during

contraction, their projections, their mediators and the transduction mechanisms used by their mediators (Furness, 2000). The preparations are normally strips containing the whole musculature that are set up in an organ bath and connected to a strain gauge. The contraction is then recorded and contractile responses measured as agonists are added in increasing concentrations to obtain a complete concentration-effect curve. This can subsequently be used to normalise the responses recorded as a percentage of the maximal response to a full agonists, thus, giving information about the ability of various agonists to attain maximal responses. It also provides quantitative information about the affinity of the antagonist and the nature of its interaction with the receptor (Schild, 1969, Schild, 1975, Schild, 1997, Arunlakshana & Schild, 1997). The GI tissue strips, however, have a large population of intrinsic nerves, some of which may contribute in hidden modulation of responses, thus agonists may act in part by a releasing mediator either increasing or decreasing the potency of the response (Daniel et al., 2001). On the basis of this information, the relation of structure to function of smooth muscle cells within multicellular preparations has proven difficult to analyse owing to the variable interactions, electrical and mechanical between neighbouring cells and the intracellular matrix.

As a result, new approaches investigating the force generated by single cells have been introduced (Bitar et al., 1984).

1.14.2 COMPUTERISED CELL COUNTER

The first methods reported for measurement of contraction and relaxation of isolated muscle cells were achieved through an estimate in the average of cell dimension of large population of cells. These included methods reported by Singer and Fay in which a computerised cell counter technique was used to obtain an estimate of change in cell width. The technique required the measurement of large numbers of cells in order to generate histograms on cell dimensions (Singer & Fay, 1977).

1.14.3 IMAGE-SPLITTING MICROMETRY

Methods that followed computerised cell counter involved the use of image splitting micrometry to obtain estimates of change in length of cells in successive microscope fields in response to different drug. The cell suspension incubated with the test agent is measured for cell length by looking at initial and successive microscopic images and relative percentage decrease in cell length (Bitar & Makhlouf, 1982a). Both these methods were supported by micrographs and were proven to be accurate. However, it is important to note that validation of such methods implied measurement of large population of fixed cells. Furthermore, it limits the characterisation of specific kinetics and reversibility of response since only one concentration can be tested at a time.

1.14.4 ELECTROPHYSIOLOGICAL STUDIES

With the introduction of single cell electrophysiological studies of smooth muscle, this permitted the measurement of the kinetics and study of single and multiple ion channels in cells.

These include the patch clamping technique that is used to measure ion current across the cell membrane. Current patch-clamp recording allows the detection and measurement of action potential in excitable cells. For these events to be captured the technique requires the use of an extremely fine hollow glass pipette held against the cell membrane by suction to achieve a seal between the glass pipette and the cell membrane. Recording of cell electrophysiology are then acquired by filling the interior of the pipette with solution to match the ionic salts in the bath solution. In this system pipette electrode measures changes in current in relation to a chloride coated earthing/reference wire (Benham et al., 1982, Benham et al., 1983) (Figure 1.14.1). Although this technique is very powerful and allows measurement of the current flowing through a single ion channel, if cells are perforated the

cell contents may be diluted, hence, producing recordings that do not reflect the entire cell activity (Hamil et al., 1981).

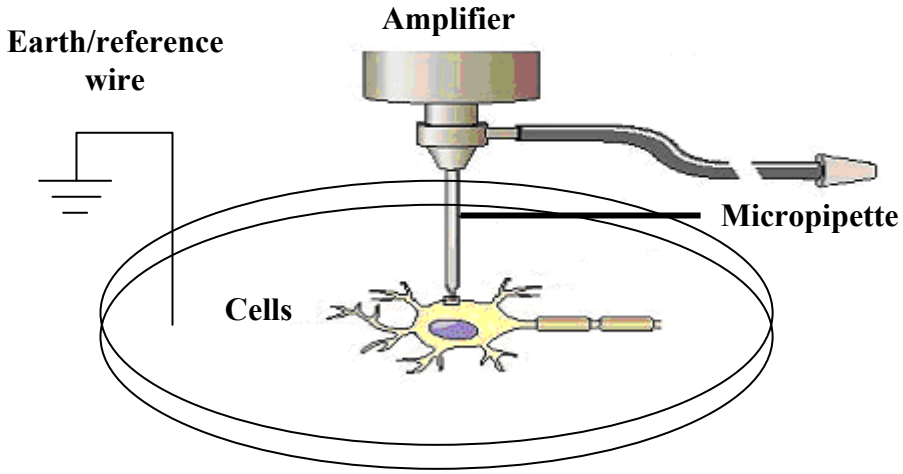


Figure 1.14.1: Diagram illustrating the single cell patch-clamping procedure. Adapted from Addison Wesley Longman Inc.

1.14.5 INTRACELLULAR CALCIUM MEASUREMENTS

The introduction of calcium sensitive dyes was a result of intensive research into membrane depolarisation and its correlation with calcium release. Calcium was taken as an indicator to cells in response to a trigger, after it was established that calcium is released as long as the membrane depolarisation is maintained during contraction. In essence, cells are loaded with a stipulated dye concentration for a pre-determined time period. The cell preparation is subsequently loaded onto the coverslip on a set microscope stage and allowed to attach to the surface. Commercial instrumentation such as dynamic fluorescence digital imaging and laser confocal fluorescent microscope are then used for spatial mapping of calcium mobilisation in the different regions of single smooth muscle cells (Drummond et al., 2000). The calibration of the fluorescence signals can be transformed into pseudocolour for easy mapping and visualisation of the concentration gradient across the entire area of the cell or in a focal area of interest. When a stimulus is induced the

contractile events can be monitored simultaneously in real time with a change in fluorescence ratio such that the changes of cytosolic calcium concentration can be related to the contractile events (Figure 1.14.2).

Limitations to the technique may arise in data interpretation, as baseline of fluorescence signal tends to decrease with time due to photo-bleaching and other conditions (Daniel et al., 2001).

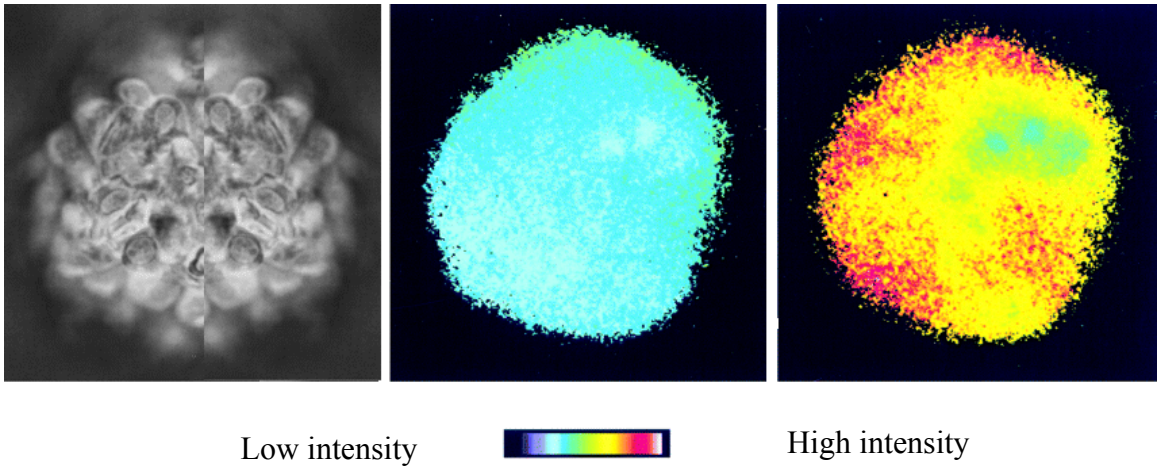


Figure 1.14.2: Diagrammatic illustration of intracellular calcium response of isolated rat glomerulus to selective agonist using fluorescence video microscopy and calcium indicator dye (Nitschke et al., 2000)

1.15 INTESTINAL RESTITUTION: PROGRESSION IN THE FABRICATION OF RESPONSIVE CELL CULTURE SURFACES

Methods for studying the intrinsic properties of muscle in the GI tract has led to the dissociation and purification of smooth muscle from the intact intestine so that they could be easily manipulated *'in vitro'*. However, disengagement of cells from the extracellular architecture leads to phenotypic changes in culture and therefore impairs the ability of muscle cells to contract.

As a result, it was observed that when attempting to create a representative model to investigate mechanical and biochemical controls of smooth muscle physiology as well as pathophysiology it is necessary to recreate the appropriate conditions. Since most common attachment sites for mammalian cell is another similar cell or extracellular matrix, this indicates that normal tissue cells probe elasticity as they anchor and pull on their surroundings. These are materials that have elastic moduli unlike the commonly used glass or plastic surfaces. Thus, there is a need to develop cell culture substrates with mechanical properties mimicking those of the '*in vivo'* environment. However, there have been enormous challenges over the selection of an appropriate substrate to embody the cell surroundings, mainly due to the dynamic relationship that exists between cells and the extracellular matrix. While many tools exist to manipulate the biochemical adhesiveness of experimental substrates, for example, the culturing of cells using laminin and collagen allow them to express muscle-specific contractile proteins (Thyberg & Hultgardh-Nilsson, 1994). Fewer approaches have been successful towards the development of suitable substrates to study the mechanical forces of contraction. More recently with the introduction of soft materials such as polymerised hydrogels or silicone elastomers, this has allowed the study of force exerted by cells. Since these materials allow the control of mechanical compliance (Harris et al., 1980) when cells are seeded and attach appropriately to a compliant substrate, this creates local deformations to the substrate, which can

subsequently be used to calculate the force created by cells (Dembo & Wang, 1999). Thus, it is apparent that to develop functional models of muscle cells, substrates should be sufficiently strong to resist damage from the contracting cell population. Conversely, cell adhesion should be sufficient to permit attachment and generate tractional force for movement, yet not so adhesive as to inhibit capture of the contractile signals induced in response to selective drugs.

Accomplishing both these criteria has proven challenging over the years because cells on soft substrates are not able to generate sufficient tractional force, conversely stiff substrates are less compliant to cytoskeleton-generated contractile forces (Peyton & Putnam, 2005).

However, with the recent advances in understanding how the extracellular matrix affects cell function, it is clear that matrix compliance is an important controllable variable (Georges & Janmey, 2005).

Thus, optimising the polymer substrate to mimic the extracellular matrix flexibility of the tissue appears to be the primary goal in establishing a responsive model of contractile cells.

1.16 THE AIM OF THE PRESENT STUDY

The first aim of the present study was to pharmacologically investigate the role of 5-HT in the regulation of motility in the neonatal rat small intestine. The study focused on the "*in vitro*" measurement of changes in the tension following the exogenous addition of drugs using the traditional organ bath set ups.

Secondly, the study aimed at developing cell culture models representative of the intact intestine. Such studies were designed to culture different layers of the intestine separately and/or in combination. This was sought to have cells disengaged from extracellular architecture of the intact intestine and into an environment that enabled an easy assess, control, and manipulation of the cells. Additionally, the study intended to assess the functionality of isolated single cells and compare the patterns of contraction to those observed in the intact intestinal preparations using the organ bath set up. Furthermore, the study also focused on the culturing of cells on different substrates to create a reproducible responsive model of contractile cells.

Having established a model of primary cell culture, the final aim was to exploit the model for investigation of the inflammatory response. The study focused on establishing the inflammatory response and then exploring the characteristics associated with smooth muscle phenotype, cell adhesion, proliferation, and immunological regulators. Furthermore, the effects of granisetron, a 5-HT_3 receptor antagonist, L-NAME, a NO synthase inhibitor, and Chaga mushroom extract on inflammatory response were investigated.

CHAPTER TWO

PHARMACOLOGICAL CHARACTERISATION OF 5-HT RECEPTORS IN THE RAT NEONATAL ILEUM

2.1 INTRODUCTION

Since the discovery of 5-hydroxytryptamine (5-HT) in the 1930s, and subsequent extraction of 5-HT from the intestine in 1937 (Erspamer & Vialli, 1937), it has became clear that 5-HT is implicated in various GI physiological functions via interactions with multiple 5-HT receptors (Barnes & Sharp, 1999, Hoyer, 2002). Presently, fourteen 5-HT receptors including 5-HT_{1A-AF}, 5-HT_{2A-2C}, 5-HT_{3A-3C}, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 have been identified (Hoyer et al., 1994, Hoyer et al., 2002). Extensive research into the 5-HT receptor families and their subtypes has led to the development of selective agonists and antagonist that are now used as valuable tools to study the role of each 5-HT receptor in GI function. Most importantly, 5-HT has also been associated with more complex pathophysiological activities in the GI tract such as emesis and diarrhoea. Preclinical studies also indicate therapeutic potentials for drugs acting on 5-HT receptors in the treatment of emesis (Sanger & Andrews, 2006), diarrhoea and visceral pain (Meerveld, 2007). For instance, the application of 5-HT_3 receptor antagonists such as ondansetron, granisetron, tropisetron, ramosetron, and alosetron have been shown to increase whole gut, small intestine, or colonic transit time in mice, rats, and guinea pigs, presumably by attenuating endogenous 5-HT through 5-HT_3 receptors-mediated prokinetic activity (Clayton et al., 1999, Brown et al., 1993). Thus, 5-HT_3 receptor antagonists have considerable value, not only as pharmacological tools but also as clinically potential therapeutics for nausea and vomiting (Sanger & Andrews, 2006), and diarrhoea predominant irritable bowel disease (IBS) sufferers (Camilleri et al., 2000). However, it remains to determine which of the many actions of 5-HT might be of physiological significance to the neonates. One could argue that results obtained in the physiological studies of adults might be used to interpret those in the neonates. However, it has been well documented in the past that there may be great variations in the way the gut muscles from

different species respond to drugs (Gaddum & Picarelli, 1957). Variations were also reported in different GI regions from the same animal (Bennett & Fleshler, 1969). These variations are further compounded by the possibilities that responses to GI muscle might be influenced by the age of the animal.

In the adults, GI motility is regulated by the enteric nervous system, which controls muscle contraction. Neurones in the gut can be directly coupled to smooth muscle or may affect the muscle cells via ICCs, which generate synchronised electrical slow waves that are thought to be the driving force of spontaneous motor activity in the smooth muscle (Thuneberg et al., 1982). In the embryos and neonates, the enteric neurones migrate principally from the vagal neural crest and colonise the gut (Epperlein et al., 1996). Smooth muscle maturation follows with the formation of circular and longitudinal muscles. Finally the ICCs network develop (Wallace & Burns, 2005). GI motility in the human neonate develops from a weak and disorganised motor activity into an organised cyclic pattern. Thus, there is a clear pattern of maturation with the gestational age. The pattern is characterised by an increase in the contractility and frequency of contraction of intestinal smooth muscle cells associated with an increase in the degree of propagative activity. This eventual development of a cyclic pattern characterises intestinal motility in older infants and adults (Bisset et al., 1988, Vantrappen et al., 1977).

In terms of enteric nervous system activity, it is apparent that although the gut and the enteric nerves must be functioning at the time of birth to cope with feeding, both the gut and the enteric nervous system enlarge as a function of postnatal maturation (Phillips & Powley, 2001). In mice it has been demonstrated that the development of new neurones continues at least through to postnatal day 21 (Pharm et al, 1991). The corresponding age has not been fully documented in humans, although, it has been recently demonstrated that the postnatal gut contains stem cells (Kruger et al., 2002). This evidence suggests that there

is a possibility of new neurones being added to the enteric nervous system during postnatal life.

Although, many studies have been reported on the role of 5-HT receptor family in the adult intestine of various species, little is known about 5-HT receptor distribution and activity in neonatal models. Moreover, due to the relative immaturity of the motor function in neonates and the scant understanding about the relationship between gestational age and motor development, one should investigate the participation of 5-HT in the modulation of GI motility in the neonates. A better understanding of the role of 5-HT and its associated receptors in the physiology of the GI tract in neonates will certainly help to identify the 5-HT selective compounds that target specific 5-HT receptors in the neonatal preparations. The correct analysis of drug actions provides insight into normal and pathological systems that control motility in the GI tract and may provide therapeutic candidates that in the future may prove useful in the treatment of functional GI diseases in neonates.

2.2 AIM

The present study aimed at the pharmacological characterisation of the 5-HT receptors that may be involved in the regulation of motility in the neonatal rat small intestine. The study focused on the '*in vitro*' measurement of changes in the tension of the intact intestinal segments taken from rats to exogenously added acetylcholine (ACh), 5-HT, and 5-HT receptor agonists in the absence and presence of different antagonists such as atropine or 5-HT related antagonists.

2.3 METHODS

2.3.1 ANIMAL AND HOUSING CONDITIONS

The experiments were carried out on adult (4 months old) and neonatal (5-8 days old) Sprague-Dawley rats (Bradford University strain). Adult rats were housed in cages (385Wx590Lx200HMM) supplied with food and water *ad libitum* and maintained at humidity between 45-50% at 24°C and illuminated between 21.00 and 07.00. The cage floors were covered with sawdust and cleaned twice a week and food and water checked every day. Female rats were mated and delivered pups. After delivery the necessary number of pups was separated and experiments were carried out within 30-60 minutes following separation from the mother.

2.3.2 PREPARATION OF ISOLATED TISSUES

Rats were killed by dislocation of the neck. The whole intestine was removed and immediately placed in freshly prepared Krebs solution (composition mM: NaCl 118, KCl 4.7, KH2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 10) and gassed with 95% O2 and 5% CO2 at room temperature. The mesentery fatty tissue was removed and intestine emptied of its contents by flushing Krebs solution gently through the intestine using a 10ml syringe. The length of the intestine was approximately 15cm in the neonatal rats and 70cm in the adult rat in its contracted form. A segment of approximately 6cm was taken 1-7cm proximal to the Ileocaecal junction. The segment was then cut into four pieces of approximately 1.5cm each segment. The preparations were alternated between their use as a control or treatment in paired experiments. Each segment was connected in a 20ml water-jacketed organ bath (Figure 2.1). The Krebs solution was maintained at a temperature of 37°C ± 0.5°C and gassed continuously with a mixture of 95% O2 and 5% CO2. The free end of the isolated tissue was connected to an isometric transducer

(Dynamometer UFI) and the tension adjusted to 0.40g. Each tissue was left to equilibrate in the presence or absence of antagonist for 1h, and washed every 20 min. The resting tension was readjusted to 0.40g when required. Changes in the tension were recorded by using Power Lab Chart V4.0.4.

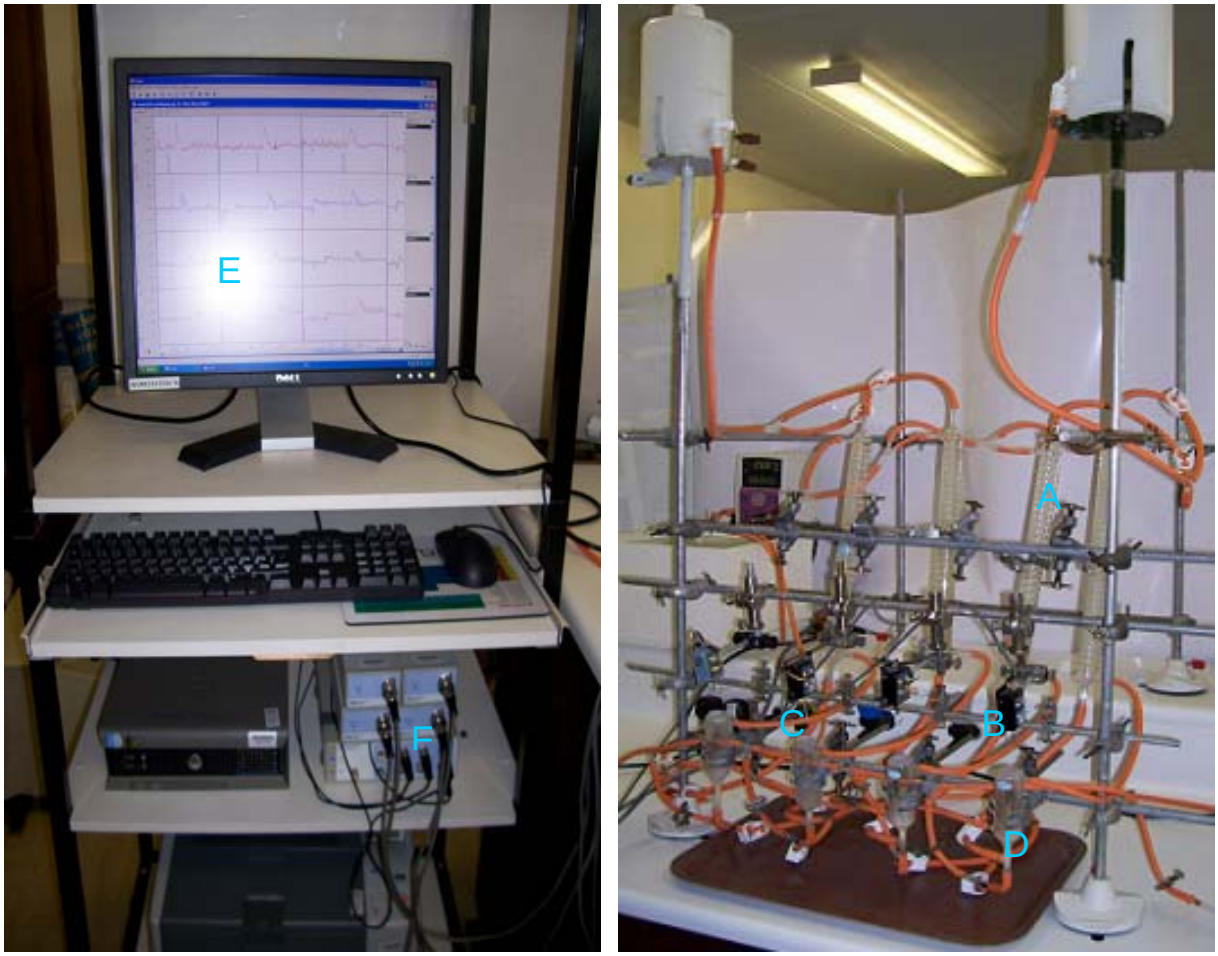


Figure 2.3.1: Photograph of the equipment used to measure the tension changes in the tissues obtained from the intestine of rat neonates.

A. Heating coil
B. Grass force displacement transducer
C. Tissue holder
D. Water-jacket bath
E. Computer connected to Power Lab recording system
F. Power Lab recording system

2.3.3 APPLICATION OF DRUGS

Drugs were added to the organ bath in single doses rather than cumulatively (Figure 2.3.2). The volumes of the drugs did not exceed 1.5% of the bath volume. The contact time between tissue and the agonist was 1 min, which was followed by two washings with 1 min in between. Agonists were injected every 15 min and when the influence of antagonists on the responses to an agonist was studied, tissues were bathed in the constant presence of the antagonist.

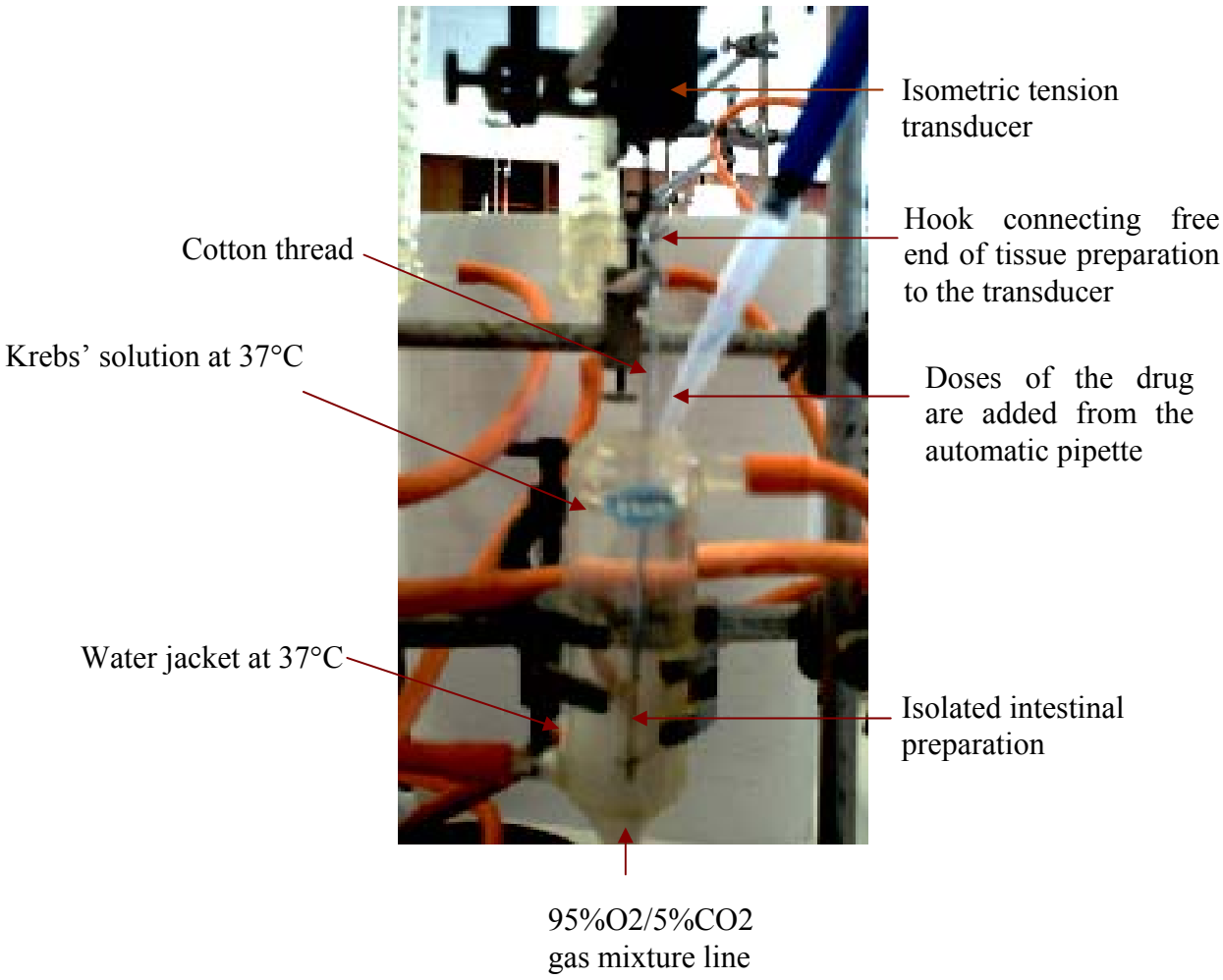


Figure 2.3.2: Image illustration of a single tissue bath apparatus, showing on attachment of an intestinal preparation isolated from the rat neonate.

Drugs were used freshly or prepared from their frozen stock solution every day. Throughout the present work concentrations of the drug refers to the final bath concentration (FBC). The effects of the agonists in the presence or absence of antagonists was expressed as the absolute value in grams tension, which were obtained after each dose application. In each comparison 'n' represents the number of animals used for the particular experiment. Only one concentration of the antagonist was used in any one tissue unless otherwise stated.

2.3.4 EXPERIMENTAL DESIGN 'PAIRED DESIGNED'

Segments were taken from the ileum of rat neonates and were set up as described in section 2.3.2. After 1h equilibrium time, non-cumulative dose response curves to ACh at concentration range of 3nM to 1mM and 5-HT at the concentration range of 3nM to 0.1mM were constructed. In such experiments, two adjacent segments from each region of the intestine were taken 1-7cm proximal to the ileocaecal junction, one served as the control and the other as the test tissue. Results in paired tissues were comparable. Therefore, a paired experimental design was used for subsequent experiments (Figure 2.4.1a-b).

2.3.5 ESTABLISHMENT OF CONCENTRATION – RESPONSE CURVES TO ACh AND 5-HT IN TISSUES TAKEN FROM THE ILEUM OF RAT NEONATES

Tissues were set up and separate non-cumulative dose response curves to ACh (3nM - 1mM) and 5-HT (3nM – 0.1mM) were constructed.

2.3.6 THE AFFECTS OF 5-HT, 5-CT AND 8-OH-DPAT IN TISSUES TAKEN FROM THE ILEUM OF RAT NEONATES

Experiments were carried out to compare the responses induced by exogenous applications of 5-HT and 5-HT receptor agonists. In such experiments, concentration response curves to 5-HT (10nM – 0.1mM), 5-HT_{1A} receptor agonist, 8-OH-DPAT (10nM – 0.1mM), and 5-$HT_{1A/7}$ receptors agonist, 5-CT (10nM – 0.1mM) were established in segments taken from the neonatal rat ileum.

2.3.7 THE EFFECT OF ATROPINE ON 5-HT INDUCED CONTRACTILE RESPONSES IN THE ADULT AND NEONATAL RAT SMALL INTESTINE

Experiments were carried out to investigate the effect of 5-HT (3nM – 1mM) in the absence and presence of atropine (0.1μM) in the neonatal rat ileum.

In separate experiments, using the adult rat ileum, segments were exposed to 5-HT (3nM – 1mM) in the absence and presence of atropine (0.1μM).

2.3.8 THE EFFECTS OF 5-HT RECEPTOR ANTAGONISTS ON 5-HT-INDUCED CONTRACTILE RESPONSES IN THE ISOLATED NEONATAL RAT ILEUM

Using the paired experimental design, tissues were set up and the concentration-response curves to 5-HT (30nM – 1mM) in the absence and presence of a 5-HT_2 receptor antagonist, ritanserin (0.1μM), a 5-HT_3 receptor antagonist, granisetron (0.1μM), a 5-$HT_{1/2/5-7}$ receptors antagonist, methysergide (0.1μM), a 5-HT_4 receptor antagonist, RS 23597-190 Hydrochloride (0.1μM), a 5-HT_7 receptor antagonist, SB269970A (0.1μM), and a

combination of ritanserin (0.1μM), granisetron (0.1μM), RS 23597-190 hydrochloride (0.1μM), plus SB269970A (0.1μM) were investigated.

2.3.9 THE AFFECTS OF WAY 100635 AND SB269970A ON RESPONSES TO 5-HT, 8-OH-DPAT, 5-CT IN TISSUES TAKEN FROM ILEUM OF RAT NEONATES

Segments were set up and the effects of 5-HT (30nM – 1mM) in the absence and presence of a selective 5-HT_{1A} receptor antagonists, WAY 100635 (0.1μM) was investigated in the segments taken from the neonatal rat ileum.

In separate experiments, segments were set up and the effects of 8-OH-DPAT, a 5-HT_{1A} receptor agonist (10nM – 0.1mM) in the absence and presence of WAY 100635 (0.1μM) were investigated.

In another series of experiments the effects of 5-CT, a 5-HT receptor agonist (10nM – 0.1mM) were investigated in the absence and presence of WAY 100635 (0.1μM) and SB269970A, a selective 5-HT_7 receptor antagonists (0.1μM).

2.3.10 ANALYSIS OF RESULTS

Changes in g tension were expressed as a mean of the absolute values and their standard error of the mean (mean ± s.e.mean). The potency of the agonists were expressed as the absolute value (g) of the tension originated.

The significance in differences between control and the test responses were determined using the Student's paired t-test, where *$p<0.05$, **$p<0.01$, ***$p<0.001$ were taken as significant.

Table 2.1 Drugs used in the present study, their solvent and suppliers.

Drugs	**Solvents**	**Suppliers**
Acetylcholine	Distilled water	Sigma
Atropine sulphate	Distilled water	Sigma
Granisetron HCL $2H_20$	Distilled water	Glaxo smithkline
Methysergide hydrogen maleate	Distilled water	Sigma
Ritanserin	Methanol	Sigma
RS 23597-190 Hydrochloride	Distilled water	Tocris
SB 269970 (R)-3-[2-(2-(4-methyl-1-piperidinyl) ethyl] pyrolidine-1-sulphonyl) phenol)	Distilled water	Glaxo Smithkline
WAY-100365 maleate (N-[2-[4-(2-methoxyphenyl)-1-piperaziny] ethyl]-N- (2-pyridinyl) cyclohexanecarboxamiidetrihydrochloride)	Distilled water	Sigma
5-CT (5-Carboxamidotryptamine)	Distilled water	Tocris
5-Hydroxytryptamine	Distilled water	Sigma
8-HO-DPAT	Distilled water	Tocris

2.4 RESULTS

2.4.1 GENERAL OBSERVATIONS

The isolated intestine of the adult rat showed spontaneous activity under a basal 1g tension and for the neonatal rat this was achieved under a basal 0.40g tension. The magnitude of contraction oscillated between the 0.1-0.8g in the adult rat and 0.01-0.05g in the neonatal rat. The rate of spontaneous activity was approximately 24-38 cycles per min in the adult intestine and 27-31 cycles in the neonatal intestine.

2.4.2 ESTABLISHING THE EXPERIMENTAL DESIGN

Four segments were taken 1-7cm proximal to the ileocaecal junction. The concentration-response curves to 5-HT and ACh separately overlapped in all four segments (Figure 2.4.1a-b). It was decided to use the paired experimental design for the subsequent experiments. In such experiments two segments were taken as control and the other two as test tissues.

2.4.3 THE EFFECTS OF ACh AND 5-HT INDUCED CONTRACTION IN THE NEONATAL RAT SMALL INTESTINE

The non-cumulative addition of ACh at concentration range of 3nM-1mM and 5-HT at concentration range of 3nM-0.1mM produced concentration dependent contractions in all segments taken from the ileum of the neonatal rat intestine. The administration of ACh induced a rapid sustained contraction. A representative tracing is shown in Figure 2.4.2. The maximum tension of 0.26g±0.004 induced by ACh was observed at 10 μM. The EC50 values for ACh was 1.3μM ±0.21.

The contraction induced by 5-HT developed slowly and was followed by a contraction maintained at a slightly lower level. A representative tracing is shown in Figure 2.4.3. The maximum tension of 0.185g±0.004 induced by 5-HT was observed at 10μM. The EC50 values for 5-HT was 1.8μM ±0.4.

No relaxation response occurred following the administration of ACh or 5-HT, in all tissues examined.

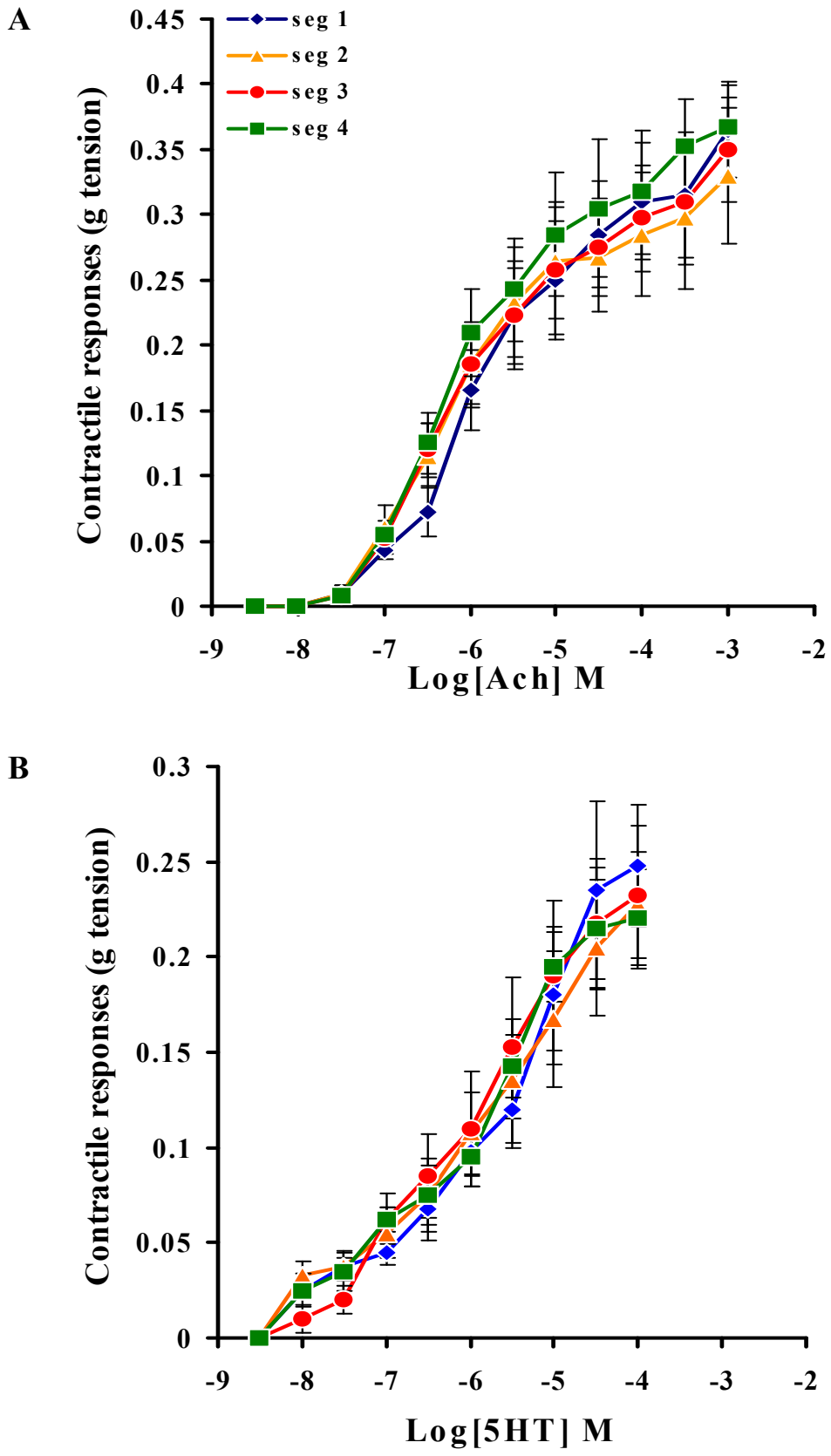


Figure 2.4.1: The contractile responses induced by ACh (A) and 5-HT (B) in segments taken from the neonatal rat ileum. Segments 1, 2, 3, 4 were taken from 6-4.5cm; 4.5-3.0cm; 3.0-1.5cm; and 1.5-0cm proximal to the ileocaeal junction, respectively. Each point represents the mean±s.e.mean; n=4.

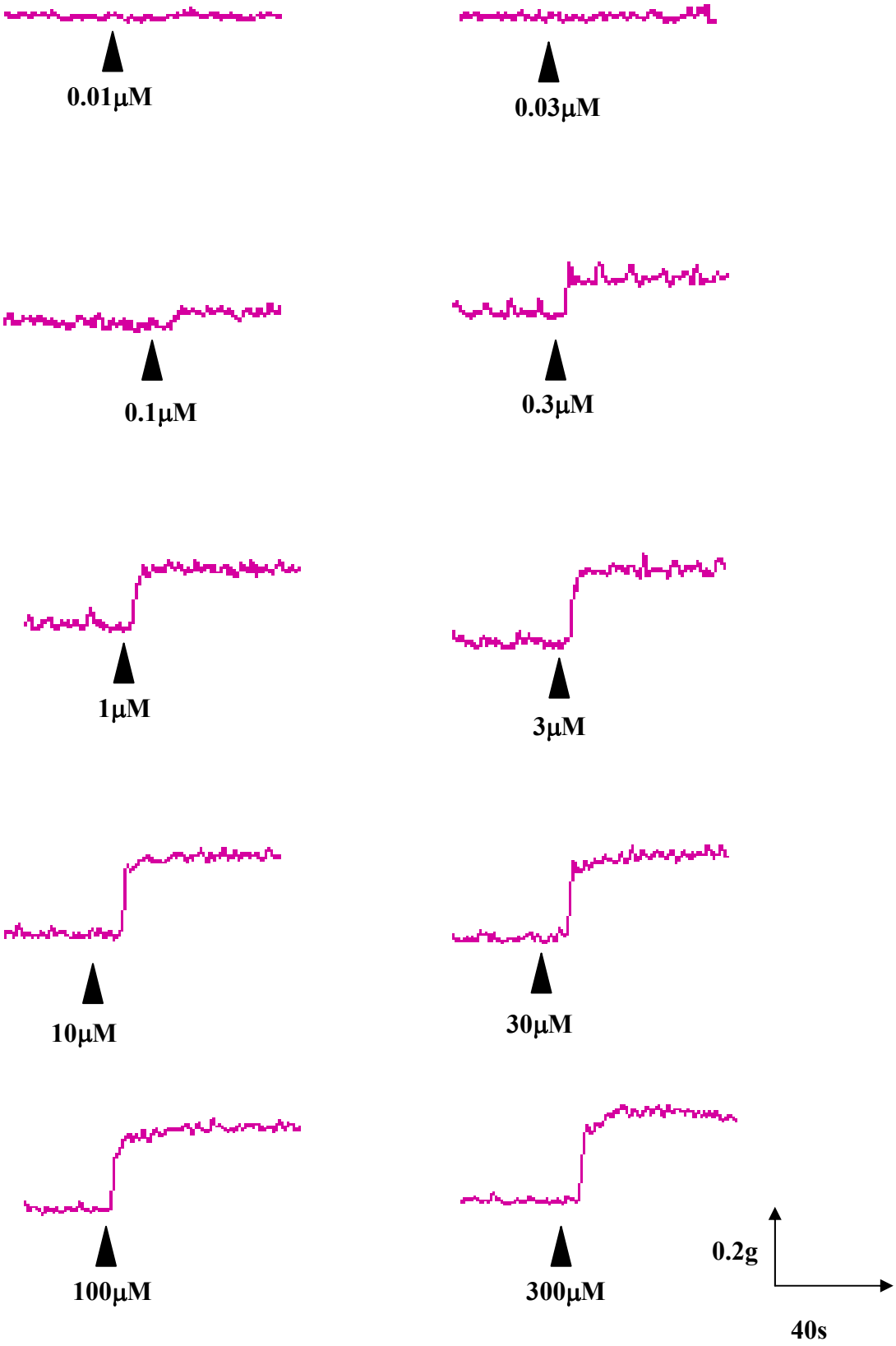


Figure 2.4.2: Representative tracings showing the contractile responses induced by increasing concentrations of ACh in the isolated ileum of the rat neonates.

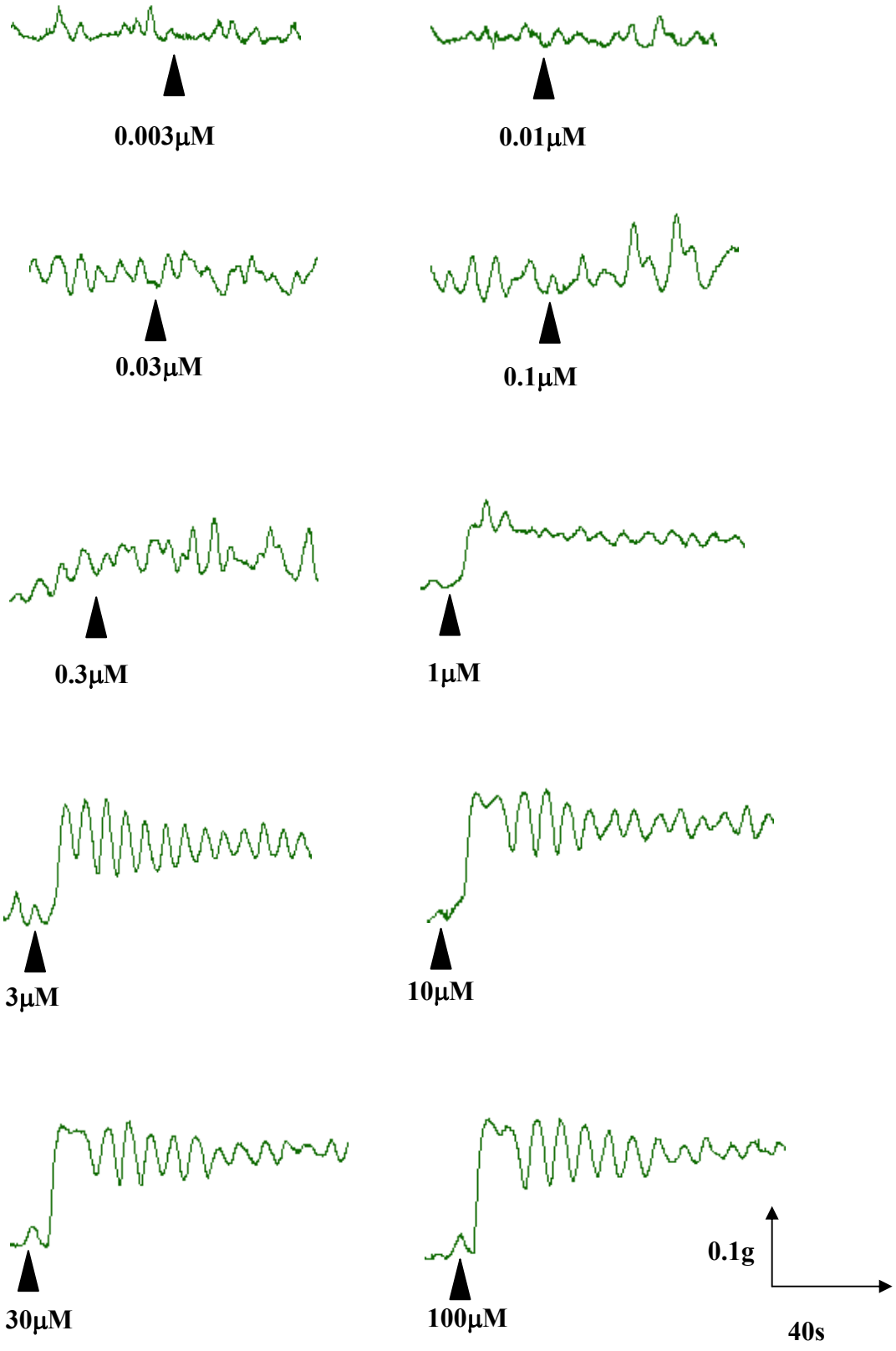


Figure 2.4.3: Representative tracings showing the contractile responses induced by increasing concentrations of 5-HT in the isolated ileum of the rat neonates.

2.4.4 COMPARISON OF THE EFFECT OF 5-HT ON THE ILEAL TISSUES TAKEN FROM ADULT AND NEONATAL RAT SMALL INTESTINE

The non-cumulative addition of 5-HT (3nM –1mM) in segments taken from the rat neonatal intestine and (3nM-1mM) in the rat adult intestine induced a concentration-dependent contraction response in all segments examined. The maximum tension produced by 5-HT in the adult intestine was in the range of 1.2-4.4g. However, in segments taken from the neonatal intestine, application of 5-HT induced contractile responses ranged from 0.11-0.23g. The EC50 values for 5-HT in the adult intestine was 7.9μM±0.96 and 1.8μM ±0.4 in the neonatal intestine (Figure 2.4.4a-b).

2.4.5 THE EFFECTS OF 5-HT, 5-CT, AND 8-OH-DPAT ON SEGMENTS TAKEN FROM THE NEONATAL RAT INTESTINE

Non-cumulative application of 5-HT and 5-CT at concentration ranges of 10nM to 0.1mM produced concentration dependent contractions in segments taken from the ileum of the neonatal rat intestine. The contractile responses induced by 5-CT were significantly ($p<0.05$-0.01) smaller compared to contractile responses induced by the exogenous application of 5-HT (Figure 2.4.5a). The non-cumulative application of 8-OH-DPAT at concentrations range of 10nM to 0.1mM produced hardly any contractile responses in the segments taken from the ileum of the neonatal rat intestine (Figure 2.4.5b).

The maximum tension of 0.074g±0.010 induced by 5-CT was observed at 30μM compared to 0.19g±0.024 induced by 5-HT at 10μM.

No relaxation response occurred following the administration of 5-CT.

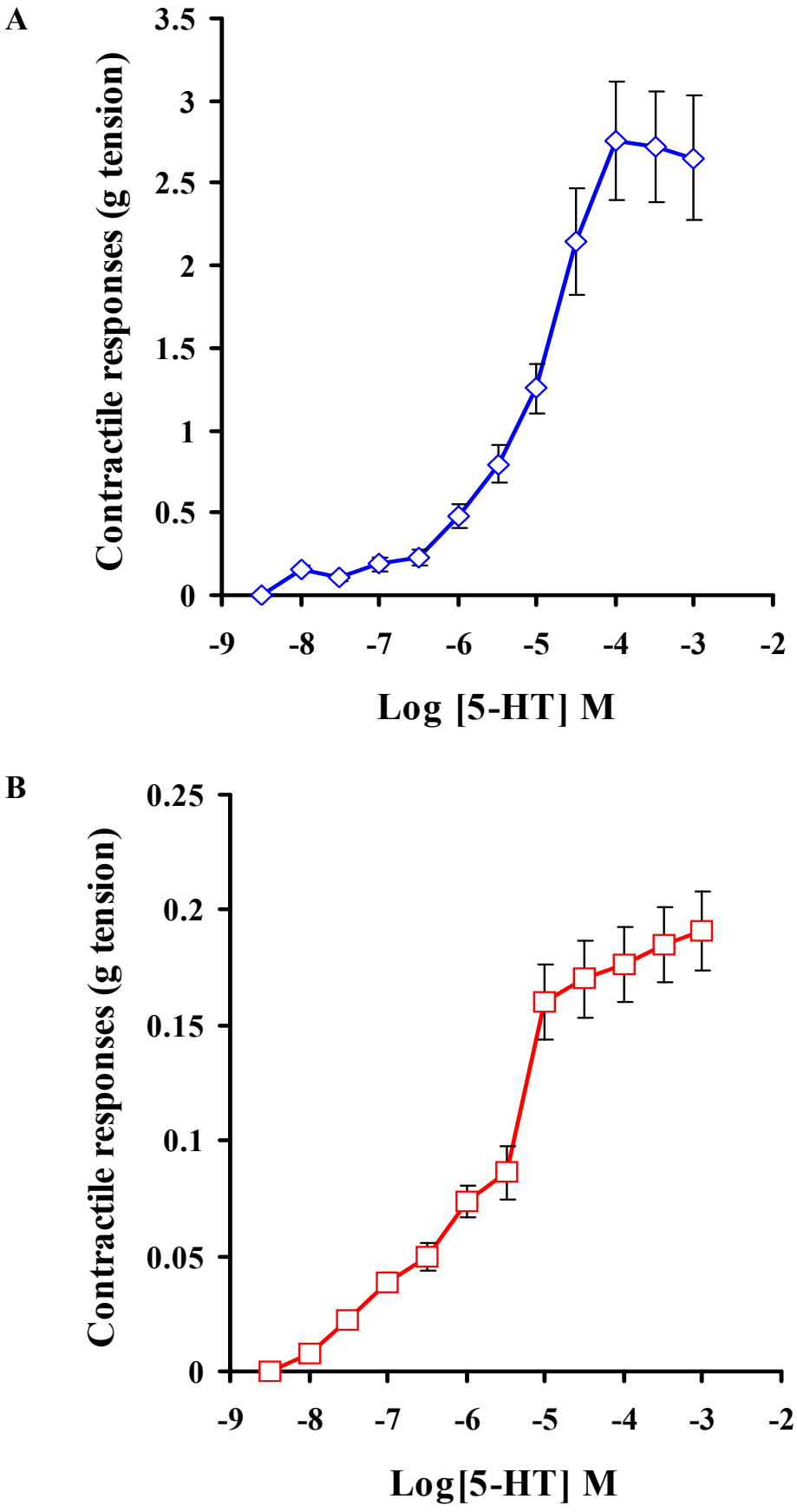


Figure 2.2.4: The contractile responses induced by 5-HT (3nM-1mM) in segments taken from the adult (A) and neonatal (B) rat ileum. Each point represents the mean±s.e.mean; n=4.

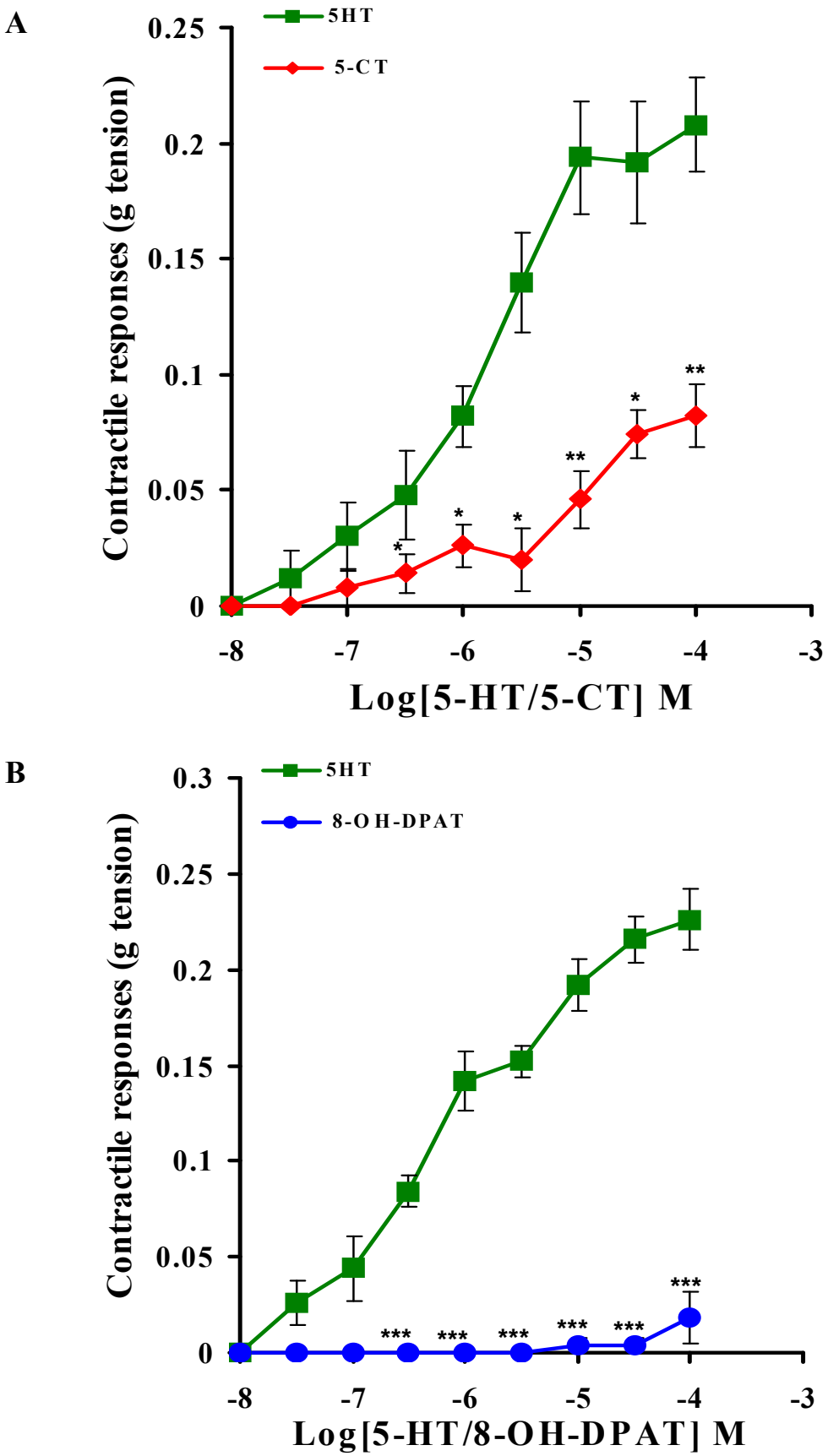


*Figure 2.4.5: Comparison between the contractile responses induced by 5-HT (10nM-0.1mM) and to 5-HT$_{1A/7}$ receptor agonists 5-CT (10nM - 0.1mM) (A) and 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (10nM - 0.1Mm) (B) in segments taken from the ileum of rat neonates. Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

2.4.6 THE ABILITY OF ATROPINE TO MODIFY 5-HT INDUCED CONTRACTION IN THE ADULT AND NEONATAL RAT SMALL INTESTINE

Tissues were set up as previously described in section 2.1.2 and contraction response curves to the non-cumulative addition of 5-HT (3nM-1Mm) were constructed in the absence and presence of atropine (0.1μM). In all experiments tissues were exposed to a single concentration of the antagonist. Previous experiments revealed that the concentrations chosen for the antagonists were effective in blocking related receptors (Obreshkove, 1941). Results presented in Figure 2.4.6 show that, in tissues taken from ileum of adult rats, atropine at 0.1μM reduced the contraction induced by 5-HT at concentrations higher than 10μM, however, these observations were not significantly different as compared to the control tissues. However, in tissues taken from the ileum of rat neonates, atropine (0.1μM) significantly ($p<0.05$-$p<0.001$) reduced the contraction induced by 5-HT at ≥ 10nM concentrations. Representative tracings are shown in Figures 2.4.7 and 2.4.8.

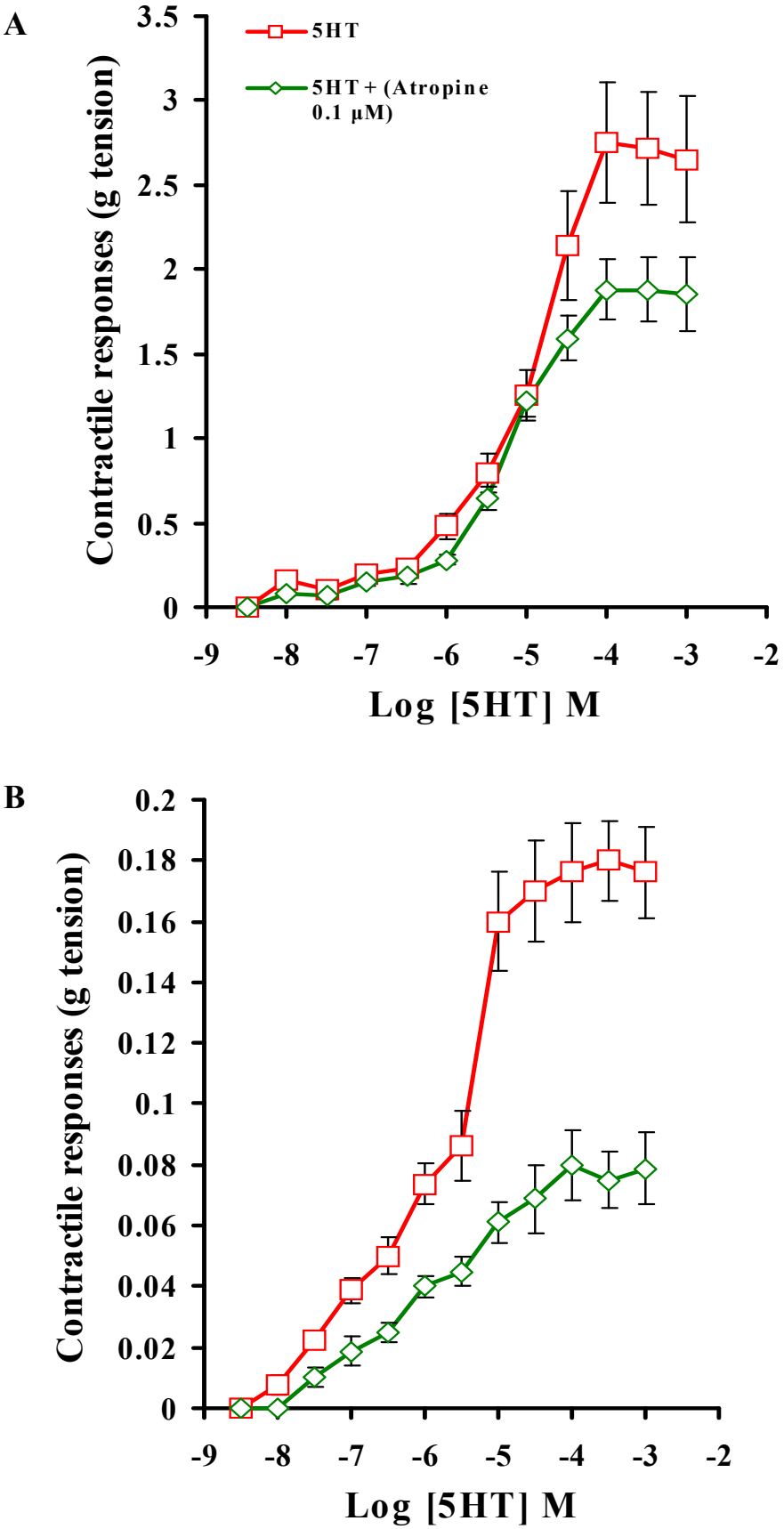


*Figure 2.4.6: The contractile responses induced by 5-HT (3nM-1mM) in segments taken from the adult (A) and neonate (B) rat ileum in the absence and presence of a selective muscarinic receptor antagonist, atropine at 0.1µM. Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

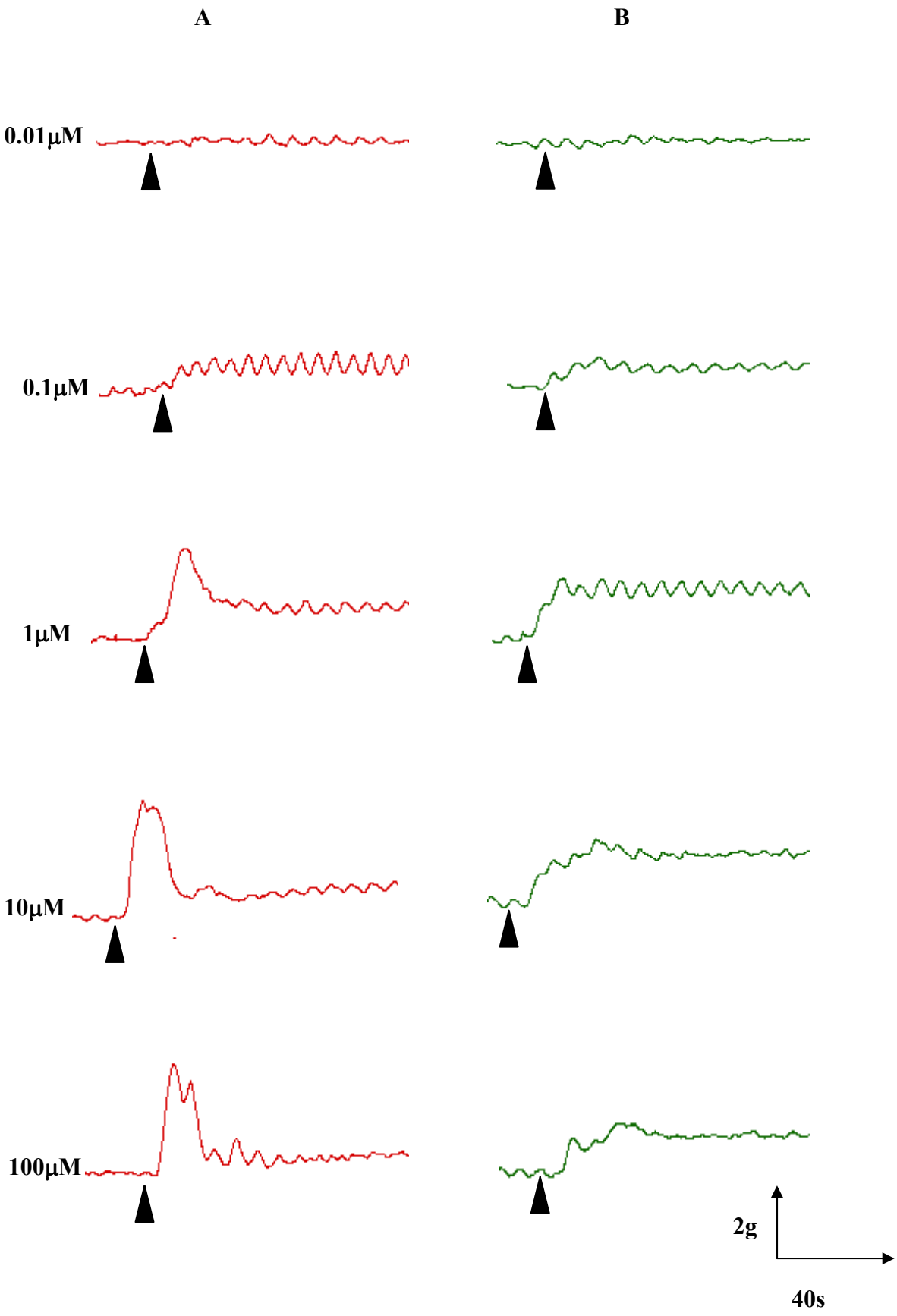


Figure 2.4.7: Representative tracings showing the contractile responses induced by increasing concentrations of 5-HT in the adult ileum in the absence (A) and in presence of 0.1µM atropine (B).

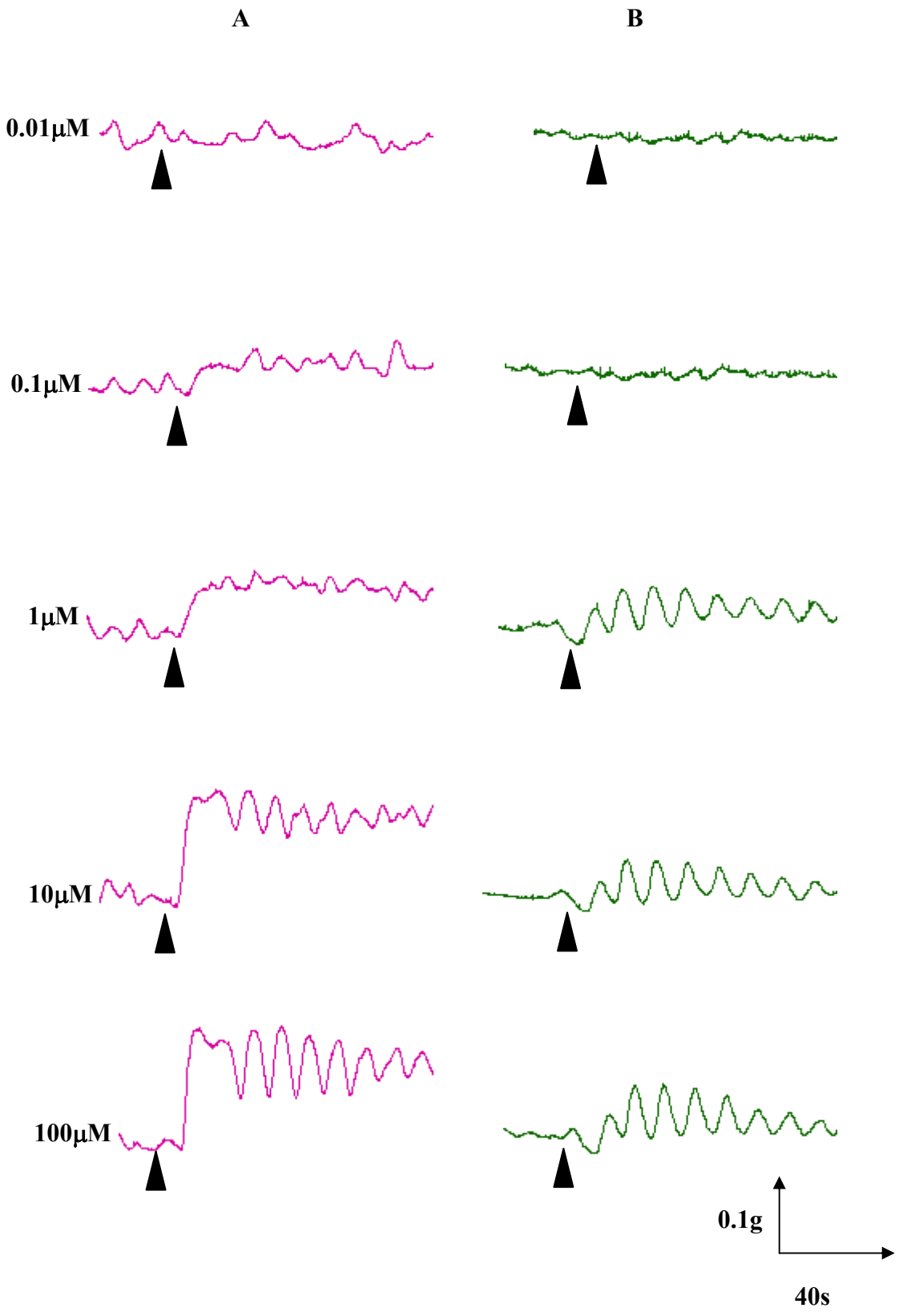


Figure 2.4.8: Representative tracings showing the contractile responses induced by increasing concentrations of 5-HT in segments taken from the ileum of rat neonates in the absence (A) and in presence of 0.1µM atropine (B).

2.2.7 THE ABILITY OF METHYSERGIDE, RITANSERIN, GRANISETRON AND RS 23597 190 TO MODIFY 5HT-INDUCED CONTRACTION IN NEONATAL RAT SMALL INTESTINE

In segments taken from the ileum of rat neonates, separate application of methysergide at 0.1μM and ritanserin at 0.1μM significantly ($p<0.05$-$p<0.01$) reduced the contraction induced by 5-HT at concentrations lower than 3μM (Figure 2.4.9a-b). The application of the selective 5-HT_4 receptor antagonist, RS 23597 190 at 0.1μM significantly ($p<0.05$-$p<0.01$) reduced the contraction induced by 5-HT at concentrations lower than 1μM, and also at a concentration of 100μM 5-HT ($p<0.05$) (Figure 2.4.9d). In the presence of methysergide (0.1μM), ritanserin (0.1μM), or RS 23597 190 hydrochloride (0.1μM), higher doses of 5-HT induced contractions, where the maximum responses were comparable to those obtained in the absence of the antagonists. The administration of granisetron at 0.1μM significantly ($p<0.01$-$p<0.001$) reduced the contraction induced by 5-HT at concentrations higher than 10μM (Figure 2.4.9c).

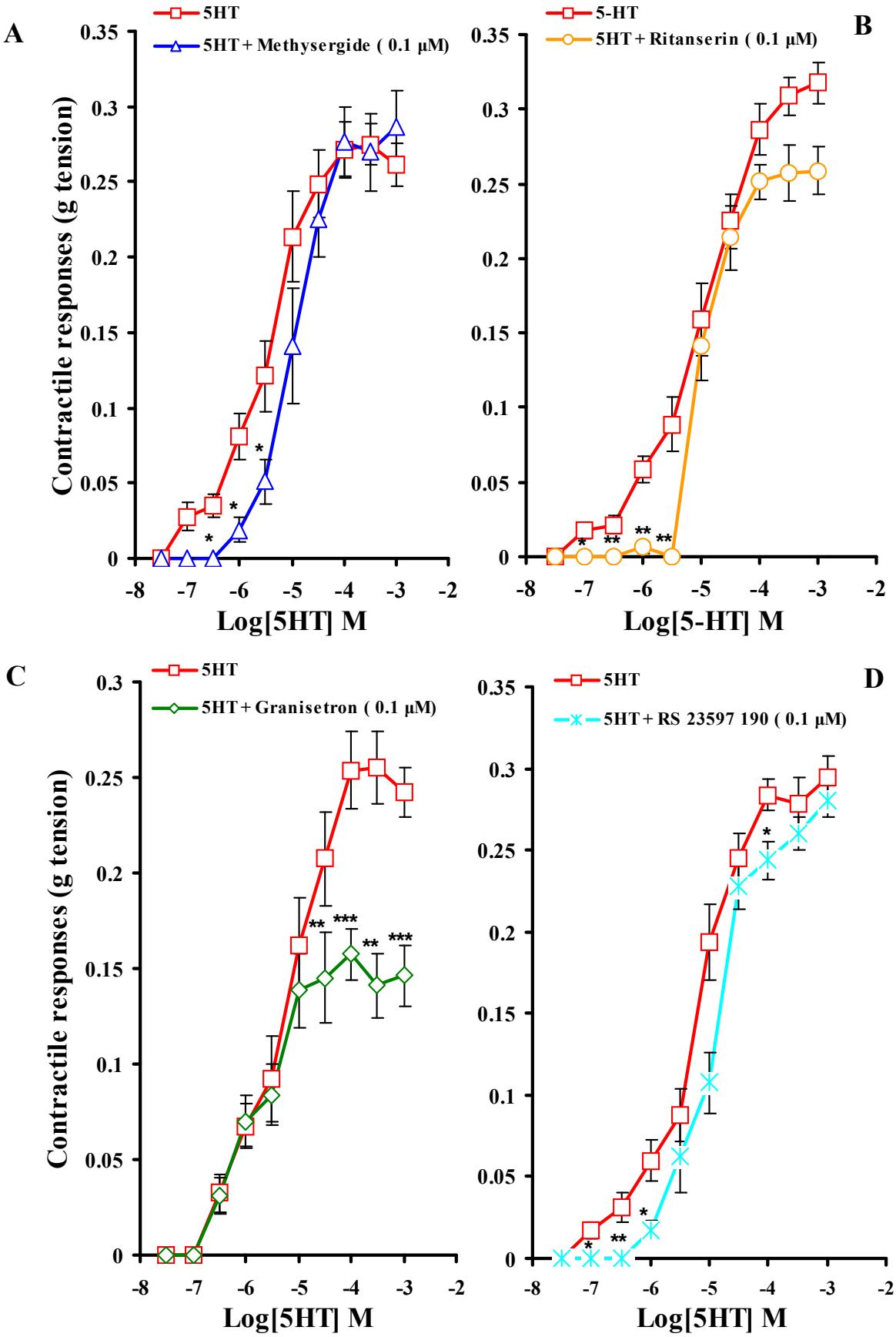


*Figure 2.4.9: The contractile responses induced by 5-HT (30nM-1mM) in the neonatal rat small intestine in absence (control) and presence of selective 5-HT receptor antagonists 0.1µM methysergide (A), 0.1µM ritanserin (B), 0.1µM granisetron (C), 0.1µM RS 23597 190 hydrochloride (D). Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

2.2.8 THE ABILITY OF SB269970A ALONE AND IN THE PRESENCE OF A COMBINATION OF RITANSERIN, AND GRANISETRON, PLUS RS 23597 190 TO MODIFY 5HT-INDUCED CONTRACTIONS IN THE NEONATAL RAT SMALL INTESTINE

The administration of a combination of ritanserin, and granisetron, plus RS 23597 190 at 0.1μM significantly ($p<0.01$-$p<0.001$) reduced the contractions induced by 5-HT at concentrations higher than 1μM. The contractile responses were nearly abolished, with some possible spontaneous activity remaining.

The administration of a 5-HT_7 receptor antagonist, SB267790A alone at 0.1μM failed to significantly modify contractions induced by 5-HT in all segments taken from the terminal regions of the ileum. The contractions to 5-HT were comparable to those in control tissues (Figure 2.4.10). Furthermore, addition of SB267790A at 0.1μM to a combination of ritanserin, and granisetron, plus RS 23597 190 at 0.1μM did not further antagonise 5-HT induced responses in the segments taken from the neonatal rat ileum (Figure 2.4.10).

2.2.9 THE ABILITY OF WAY 100635 AND SB269970A TO MODIFY 5-HT, 8-OH-DPAT, 5-CT-INDUCED RESPONSES IN TISSUES TAKEN FROM ILEUM OF RAT NEONATES

In segments taken from the ileum of rat neonates, separate application of WAY 100635 at 0.1μM failed to reduce the contractile responses induced by 5-HT (Figure 2.4.11a) Similarly, the separate application of WAY 100635 and SB267790A at 0.1μM failed to influence the contractile responses induced by 5-CT (Figure 2.4.11b). Applications of 8-OH-DPAT in the presence of WAY 100635 (1μM) induced responses comparable to those of 8-OH-DPAT when applied alone (Figure 2.4.11c).

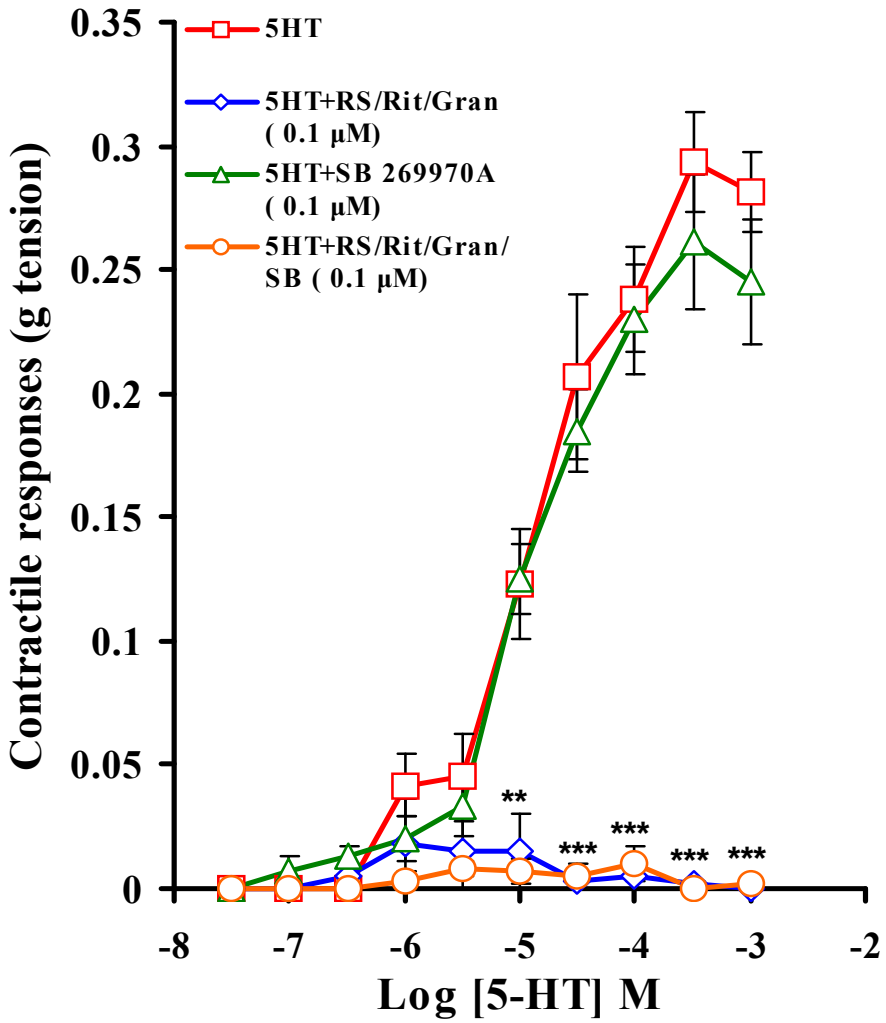


*Figure 2.4.10: The contractile responses induced by 5-HT (30nM-1mM) alone and in presence of 0.1µM SB269970A, a combination of 0.1µM ritanserin/granisetron/RS23597 190, and a combination of 0.1µM ritanserin/granisetron/RS23597 plus SB267790A in the segments taken from the ileum of rat neonates. Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

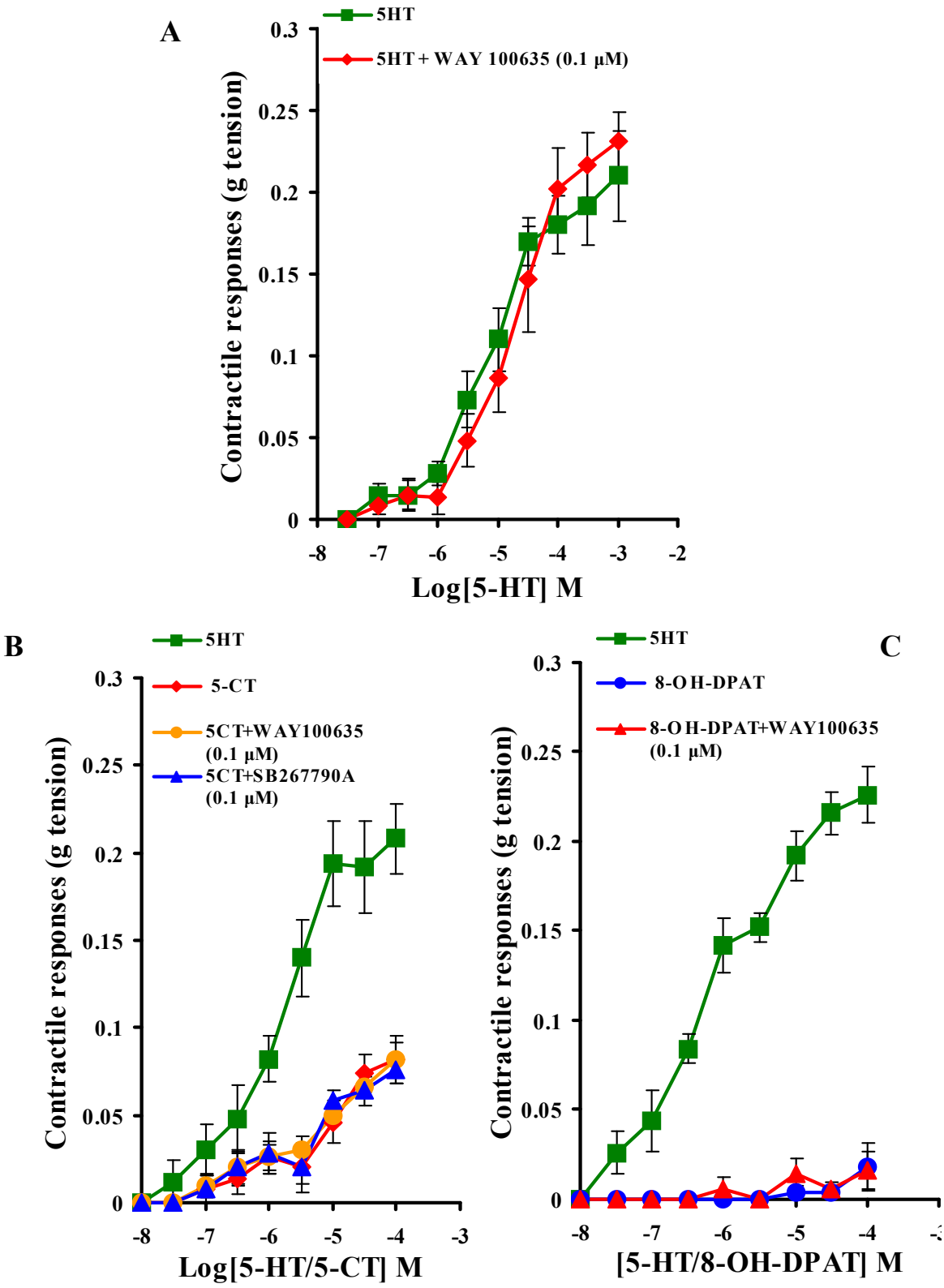


*Figure 2.4.11: Contractile responses induced by 5-HT (30nM-1Mm) in the absence and presence of WAY 100635 0.1µM (A). Contractile responses induced by 5-HT (10nM-0.1Mm) and the comparison between contractile responses induced by 5-CT (10nM - 0.1mM), and 8-OH-DPAT (10nM - 0.1mM) in the absence and presence of WAY 100635 and SB 267790A 0.1µM (B, C) in segments taken from the ileum of rat neonates. Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

2.5 DISCUSSION

In the present studies attempts were made to investigate the involvement of 5-HT receptor subtypes mediating contractile responses in the neonatal rat small intestine.

The GI tract is a complex and highly organised system that regulates and coordinates the absorption, secretion, sensation, and motility of gut contents. This is achievable through a network of nerves embedded into the walls of the digestive tube (Gershon & Tack, 2007). The ENS consists of the myenteric and submucosal plexus and a complex network of intrinsic and extrinsic afferent neurones, ascending and descending interneurones, excitatory and inhibitory motor neurones, which interact with the longitudinal and circular smooth muscle and mucosal endocrine cells. Additionally, the myenteric motor neurones innervate smooth muscle via the ICCs and regulate mechanical activity (Gershon, 2004, Gershon & Tack, 2007).

It has been well reported that the main source of 5-HT is in the gut, more precisely in the enterochromaffin cells, where it is synthesised and stored. 5-HT can be released into the gut lumen as a reaction to pressure and nervous or alimentary stimuli. As a result it acts on 5-HT receptors located on smooth muscle, enterocytes, nerves, ICCs through their distinct receptor subtypes to induce muscle contraction or relaxation (Hannon & Hoyer, 2002, Gershon & Tack, 2007). Enterocytes, in turn express the serotonin reuptake transporter (SERT), which plays a critical role in terminating 5-HT (Ramamoorthy et al., 1993) and ensuring that 5-HT mediated toxicity and receptor desensitisation that are usually observed as consequences of maintained agonist exposure, are avoided (Chen et al., 1998, Camilleri et al., 2007).

Therefore, the coordinated movement of food along the GI tract is dependent on 5-HT-mediated regulation of smooth muscle tone, peristalsis, mucosal secretion, and visceral perception (Baker, 2005, Hansen, 1997, Jin et al., 1999) via regulated signalling with the

intrinsic enteric and the extrinsic afferent neurones, the ICCs, smooth muscle cells, and enterocytes (Gershon & Tack, 2007, Read & Gwee, 1994, Wouters et al., 2007).

The involvement of 5-HT-receptor subtypes in 5-HT-induced intestinal contraction has been well examined in the adult guinea pigs (Costa & Furness, 1979, Woollard et al., 1994). This involvement has been less extensively investigated in the adult rat isolated intestine. There is evidence, however, that species difference exists in the involvement of 5-HT-receptor subtypes in intestinal motility. Experimental evidence demonstrated that the 5-HT induced contraction in the ferret, piglet and rat ileum were mediated by the activation of different 5-HT receptors. 5-HT-induced contraction in the ferret ileum was mediated by the activation of 5-HT_1 on smooth muscle cells and ACh release through the activation of 5-HT_3 receptors. Previous studies have also shown that, in the piglet ileum, the 5-HT-induced contractions were mediated by the release of neurotransmitters other than ACh via 5-HT_1 receptors. The 5-HT induced contraction in the rat ileum was evoked by the activation of 5-HT_1 and 5-HT_2 receptors located on the muscle cells (Yamano et al., 1997). Recent studies have indicated significant differences in the 5-HT-induced contraction amongst adult and neonates (Bian et al., 2007). Evidence supporting these arose in studies comparing 5-HT disposition in the intestinal mucosa of neonatal and adult guinea pigs. The results revealed that although there were no differences in 5-HT content between neonates and adults, the enterochromaffin cells where 5-HT is stored, were found in the crypts in neonatal and along the villi in adults intestinal segments. As a result 5-HT expression is low in neonates, and this is associated with high levels of free mucosal 5-HT and reduced metabolism (Bian et al., 2007). Therefore, due to clear physiological differences related to 5-HT distribution during GI tract postnatal maturation, the importance of experimental pharmacology is fundamental to identify the site and mode of action of 5-HT signalling in the control of intestinal motor reflexes in neonates.

Initial experiments focused on the comparative investigation of the involvement of cholinergic pathways in the 5-HT-induced contraction in the adult and neonatal rat intestine. In the present study the administration of 5-HT induced contractile responses, which were sensitive to antagonism by atropine in segments taken from the neonatal rat intestine. However, the application of atropine failed to significantly modify the contractile response to 5-HT in the adult rat small intestine. This finding correlates with previous studies that demonstrated that contractile response to 5-HT is insensitive to antagonism by atropine in the isolated rat ileum and colon (Sakai, 1979, Lu et al., 1995). However, pre-treatment with atropine of the neonatal rat ileum preparations significantly reduced the contractile response induced by 5-HT. The antagonistic action of atropine was insurmountable, depressing the maximum response to 5-HT. Therefore, the effect of atropine on neonatal preparations implies that intestinal contraction induced by exogenously applied 5-HT is partially cholinergically mediated since atropine is a highly selective cholinergic receptor antagonist (Ambache, 1955). The present data is in line with human studies by Prins et al., where it was demonstrated that 5-HT_4 receptors on cholinergic neurones facilitate contractility, partially through the release of ACh (Prins et al., 2000). Moreover, studies examining 5-HT receptors mediating contractile responses and ACh release in the guinea-pig ileum, have clearly demonstrated that evoked contraction through ACh release are mediated via 5-HT_3 receptor activation (Fox & Morton, 1990).

In more recent studies investigating the physiological role of 5-HT_3 receptors, it was confirmed that 5-HT_3 receptors activation followed by rapid depolarisation causes a rapid rise in cytosolic calcium concentration, as well as, modulation of various neurotransmitters including ACh leading to muscle contraction (Greenshow & Silverstone, 1997). Thus, there is evidence that 5-HT receptor activation produce an ACh-mediated contraction. In the present study, atropine caused significant reduction of the 5-HT-induced contractions

in segments taken from the ileum of rat neonates, however, in adult preparations atropine only caused a slight reduction to 5-HT-induced contractions, which did not achieve significance. This was an interesting observation and raised questions as to why the ileum loses its sensitivity to atropine as the animal ages. It is possible that other systems dominate and may mask the cholinergic involvement in mediating a response to 5-HT. It is also possible that the involvements of some systems or receptors are diminished as the animal ages. Indeed recent studies have shown the involvement of 5-HT_4 in the enteric neuronal survival to be age-dependent (Gershon & Liu, 2007). Such studies showed that the number of enteric neurones in the gut increase in the postnatal life, reaching a peak and then stabilising. Aging subsequently is associated with a postpeak decline, hence, fewer neurones than in the young mature GI tract. These findings revealed that 5-HT_4 receptor partial agonists such as tagaserod increase the number of neurones in cultured populations, therefore enhancing cell survival or increasing differentiation of neurones from precursors, or both. The ability of 5-HT_4 agonists to promote enteric neurogenesis from precursors 'in vitro' supports that 5-HT_4 receptors when activated may also promote the development of new enteric neurones from the stem cells that have been reported to be present in the young gut (Gershon & Liu, 2007). This may suggest the nomination of other mechanisms in mediating the responses to 5-HT as animals mature.

Following the above observations, experiments were designed to characterise the 5-HT receptors present in the 5-HT-induced contraction in the intestine of rat neonates. In the presence of methysergide the contractile responses to 5-HT were significantly reduced. The antagonistic action of methysergide (pA_2 8.12±0.14) in the neonatal isolated intestine was surmountable, with no alteration of the maximum response induced by exogenously applied 5-HT. Methysergide has been shown to be an antagonist of 5-HT_{2A} (pA_2 7.1), 5-HT_{2B} (7.1-8.2) and 5-HT_{2C} receptors (pA_2 8.9), but also believed to act as a weak agonist at 5-HT_{1D} receptors (pEC50 7.0). It also has a moderate affinity at 5-HT_{1F} (pA_2 7.5), and 5-

HT_{5-7} receptors (pA_2~7.0) (Briejer et al., 1997, Routsalainen, 2003). In previous experiments performed in the isolated adult rat ileum, the contractile responses to 5-HT were sensitive to methysergide (Yamano et al., 1997, Javid & Naylor, 1998) and the surmountable antagonistic action correlated to those observed in the neonatal intestine in the present studies.

In separate experiments the effect of ritanserin, a potent 5-HT_2 receptors antagonist were performed. Ritanserin (pA_2 7.99±0.32) demonstrated similar patterns of the antagonistic action compared to those of methysergide (pA_2 8.12.±0.14). Ritanserin displayed surmountable antagonistic activity, with no significant difference of the maximum response induced by 5-HT as compared to control values. In line with these observations, studies investigating the effect of ketanserin (5-HT_{2A} receptor antagonist) in the adult rat distal ileum (Yamano et al., 1997) equally showed inhibitory effects of ketanserin on the 5-HT-induced contractions. The 5-HT_2 receptor family comprises of the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor subtypes. There is no evidence for a functional role of 5-HT_{2C} in the GI tract physiology, whereas 5-HT_{2A} (Leysen, 2004) and 5-HT_{2B} (Borman et al., 2002) receptor subtypes have been localised in longitudinal smooth muscle cells and to a lesser extent in the myenteric neurones and circular smooth muscle cells. Moreover, 5-HT_{2B} is also expressed on ICCs in the human and mouse intestine (Wouters et al., 2007).

In the present study, the antagonism afforded by methysergide and ritanserin to the contractile responses, suggests the involvement of 5-HT_2 receptors in mediating 5-HT responses in the segments taken from the neonatal rat ileum.

The involvement of 5-HT_3 receptors in the 5-HT induced contraction was investigated in presence of the selective 5-HT_3 receptors antagonist granisetron (Tamura et al., 1996). The antagonist action of granisetron on the neonatal isolated intestine was insurmountable, with a significant depression of the maximum response to 5-HT. Previous studies investigating

the effect of $5HT_3$ receptors in the 5-HT-induced contraction in the adult rat ileum reported no influence of 5-HT_3 receptors (Yamano et al., 1997). 5-HT_3 receptors are believed to be located in the enteric neurones of the GI tract and are implicated in the control of motility and secretion throughout the entire GI tract (Hoyer et al., 1994, DePonti & Tonini, 2001). The pharmacology data acquired from the present study on the isolated neonatal intestine suggests the involvement of 5-HT_3 receptors in the contractile response elicited by higher concentrations of 5-HT.

Further experiments using RS 23597 190 hydrochloride, a selective 5-HT_4 receptor antagonist (Bonhaus et al., 1994) revealed a surmountable antagonistic action (pA_2 7.9±0.35) in the neonatal isolated intestine, with no alteration of the maximum response induced by exogenously applied 5-HT. However, previous studies revealed that the pre-treatment with 5-HT_4 receptor antagonists (GR113808) in the adult rat ileum failed to influence the 5-HT-induded contractions. In fact, GR113808 enhanced contractile response elicited by 5-HT (Yamano et al., 1997). In the GI tract, 5-HT receptors are located on the myenteric neurones and also on smooth muscle cells. In the enteric nervous system, the presence of 5-HT_4 receptors has been demonstrated on several functionally distinct types of neurones, including intrinsic primary afferent neurones, ascending cholinergic/tachykinergic excitatory pathways and descending non-adrenergic and, non-cholinergic inhibitory pathways (Foxx-Orenstein et al., 1996). Activation of the 5-HT_4 receptors is associated with the release of transmitters, where the excitatory responses appear to be mediated indirectly via facilitatory cholinergic or non-cholinergic transmission, whereas the inhibitory responses are caused by direct smooth muscle relaxation (Wouters et al., 2007). Thus, it is apparent that 5-HT_4 receptors mediate both inhibition and activation of smooth muscle involve myogenic as well as neuronal mechanisms. In the present studies, pre-treatment with RS 23597 190, significantly reduced the contraction induced by 5-HT. The present results suggest the likely

involvement of 5-HT_4-receptors in mediating contraction induced by 5-HT in the ileum taken from neonatal GI tract.

In summary, there is apparent involvement of 5-HT_2 and 5-HT_4 receptors being activated at lower concentrations and 5-HT_3 being activated at higher concentrations of 5-HT-induced contraction in ileal segments taken from rat neonates. Therefore, subsequent experiments were designed to investigate whether the combined antagonism of all three receptors on the isolated neonatal intestine would result in complete abolition of the contraction elicited by exogenously added 5-HT. The results revealed that the combination of methysergide and granisetron plus RS 23597 190 greatly diminished the contractile responses elicited 5-HT administration on the neonatal isolated intestine, with hardly any responses remaining. These observations provide strong evidence that the 5-HT induced contractions in the ileum of rat neonates are mediated through 5-HT_2, 5-HT_3, and 5-HT_4 receptor subtypes.

Nonetheless, additional experiments were designed to investigate the possible involvement of additional 5-HT receptor subtypes known to be present in the GI tract. These include 5-HT_{1A} and 5-HT_7.

5-HT_{1A} has been localised in the smooth muscle of the GI tract where it is expected to induce an excitatory contractile response, when coupled with adenylate cyclase, which is the primary coupling mechanism of this receptor (Raymond et al., 1999). Analysis of 5-HT_{1A} receptors involvement was performed in the presence of a selective 5-HT_{1A} receptor antagonist, WAY 100635 (Corradetti et al., 1998), which failed to influence 5-HT induced contractions in the neonatal rat ileum. Preclinical studies using isolated GI smooth muscle preparations have demonstrated 5-HT_{1A} receptor-mediated activities. 5-HT_{1A} receptors activation is associated with inhibition of electrically evoked contractions in the guinea pig stomach (Buchheit & Buhl, 1994) and relaxation of the dog proximal stomach (Janssen et al., 2003).

The involvement of 5-HT_7 receptors in the 5-HT induced contraction was primarily investigated in the presence of the selective 5-HT_7 receptors antagonist, SB 269970A and separately in combination with previously described methysergide, granisetron and of RS 23597 190 hydrochloride. In both instances, SB 269970A failed to influence the 5-HT induced contraction in the rat neonatal intestine. 5-HT_7 receptors involvement has been demonstrated in the ENS where it is postulated to play a role in GI physiology (Hemedah et al., 1999). Stimulation of 5-HT_7 receptors in the smooth muscle of the ileum and colon produces relaxation (Carter et al., 1995) and in the rat jejunum it induces contractile responses (McLean & Coupar, 1996).

Further experiments of 5-HT_{1A} and 5-HT_7 receptors involvement in mediating a response in the neonatal intestine were performed by primarily establishing concentration response curves to 5-$HT_{1A/7}$ receptor agonist, 5-CT (Christ & Surprenant, 1987, Lucchelli et al., 2000) and 5-HT_{1A} receptor agonist, 8-OH-DPAT (Christ & Surprenant, 1987). This was followed by the investigation of the effect of SB 267790A and WAY 100635 antagonists on the 5-CT and 8-OH-DPAT induced responses. Although the dose response curve to 5-CT induced measureable contractions in a concentration-dependent manner, there were of less potency and efficacy compared to those elicited by 5-HT. The application of 8-OH-DPAT did not induce contractile responses in the neonatal rat ileum. Moreover, WAY 100635 and SB 267790A failed to influence 5-CT induced contractions. Since the application of 8-OH-DPAT did not induce a measurable response, further experiments were carried out to investigate if in the presence a highly selective 5-HT_{1A} receptor antagonist, WAY 100635, 8-OH-DPAT would induce a measurable response. However, the application of 8-OH-DPAT in the presence of WAY 100635 induced responses comparable to those of 8-OH-DPAT when applied alone.

5-CT has been extensively used in the central nervous system and enteric nervous system to localise 5-HT_7 receptors (Lucchelli et al., 2000). 8-OH-DPAT has been shown to

preferentially bind to 5-HT_{1A} receptors than other 5-HT receptor subtypes (Galligan et al., 1988). WAY 100635, is a highly selective 5-HT_{1A} receptor antagonist and a valuable tool to characterise these receptors (Hannon & Hoyer, 2002). SB267790A, is a highly selective 5-HT_7 receptor (Tonini et al., 2005). The failure of WAY100635 and SB267790A to modify the responses to 5-HT and 5-CT indicates the unlikely involvement of 5-HT_{1A} and 5-HT_7 in mediating contractile responses in the neonatal rat ileum.

Further experiments are required to investigate a possible relaxation response induced by 8-OH-DPAT on pre-contracted tissues. Such approach would also be useful for the characterisation of 5-HT receptors involvement in the relaxation responses to 5-HT in the intestine from rat neonates.

Additionally, it would also be very interesting to establish a methodology to investigate the reduction of 5-HT_4 receptors in adults as compared to those in neonates.

In summary, data obtained from the present experiments suggest that there are significant differences in the receptor responsiveness between the adult and neonate intestinal tissues. This strongly supports the view that the generation of pharmacological data is necessary for the development of treatments in infants.

Nonetheless, alternative methods that reduced, refine, or replace the traditional animal methods for generation of pharmacological data has indeed been an area of significant interest. Such methods involve '*in vitro'* systems, which aim at the development and validation of individual cell culture models for use in early stages of pharmacological, biochemical and immunological analyses. Data obtained from *'in vitro'* methods can then be validated at a later stage using data from animal experiments.

CHAPTER THREE

DEVELOPMENT OF CELL CULTURE MODEL TO OBTAIN INTESTINAL SMOOTH MUSCLE CELLS AND ENTERIC NEURONES

3.1 INTRODUCTION

Cell isolation techniques have been employed to obtain a variety of different cell types from different organs such as the heart, stomach, gallbladder, and intestinal tissues (Bitar & Makhlouf, 1982a, Collins & Gardner, 1982, Seidel & Johnson, 1983). Cells in suspension have proven useful in characterising the mechanical, biochemical and physiological properties of cells.

In the intestine the availability of individual cell types in culture has led to characterisation of receptors for neuropeptides using immunochemical techniques such as radioligand binding assay (Seidel & Johnson, 1983) and pharmacological techniques such as the measurement of single cell contraction and relaxation responses using specific agonists and antagonist for a particular receptor (Bitar & Makhlouf, 1982a, Souquet et al., 1984) . Such information is important since the functional data obtained from the intact intestine normally emerges from a complex network of organised smooth muscle cells in a multicellular unit rather that from individual properties of contractile cells themselves. When cell culture techniques became available, specific cell types were extracted from the primary source and kept in culture under controlled environment. This allowed them to propagate, expand, and divide into identical replicates, assuming a uniform constitution.

Experiments in the cultures of different cell population from the small intestine, however, have proven difficult. This is primarily because of the complex layered nature of the GI tract making it difficult to acquire pure preparations of individual cell types.

Nevertheless, such limitations in the disaggregation of cells from the intestine can be minimised when methods are employed to monitor the gradual dissociation from specific regions of the intestine. The cell extracts can be further monitored for purity through observations of morphological features or the use of cell sorting techniques including differential adhesion and selective cytotoxicity.

Although, the combination of cell sorting techniques may create the required parameters for the acquisition of highly purified cell populations from the intestine, further challenges remain on the continuity of primary cultures. This is due to the limited growth potential of the cells and the limited access to healthy cell lines. As a result there is increasing interest in the development of cryopreservation techniques of organs and tissues enabling constant availability. A well-established cryopreservation technique for use in the storage of intestinal cell population would allow storage, whilst enhancing availability and decreasing animal usage.

The functional assessment of smooth muscle cell contractility is an essential part of determining the phenotypic status of smooth muscle cells in culture and it is an important aspect of hypothesis testing the validity of '*in vitro'* cell culture models. This type of functional measurement complements the ability to assess cell responses to different conditions, particularly when this measurement is limited in the intact intestine.

3.2 AIM

The aims of this study were firstly to develop a cell culture model to establish and characterise the following cell populations from the intestine: intestinal smooth muscle cells (ISMC), myenteric neurones, and a coculture of ISMC and myenteric neurones. This was achieved through cell viability tests, cell confluence tests, histological techniques, and cell purification techniques to determine the optimum parameters for cell dissociation of different cell types originated from the primary tissues. Secondly, the study focused on the development of selective purification techniques to obtain specific cell populations from different intestinal layers and to characterise fully the individual cell types via immunocytochemistry methods. Thirdly, attempts were made to develop a suitable cryopreservation technique for long-term storage of ISMC. Lastly, the functionality of single cells was assessed using different agonists capable of activating specific receptors or

systems to induce a contraction or relaxation responses. Further experiments were carried out to assess the cell functionality in larger population of cells. This was achieved by seeding the cells onto substrates with different stiffness prior to monitoring their response to specific agonists.

3.3 METHODS

3.3.1 INTESTINAL DISSECTION

Animal housing and conditions were kept as described in section 2.3.1. Rat neonates were killed by dislocation of the neck. Bodies were subsequently transferred to a flow hood under sterile conditions and the whole intestine was removed and immediately placed in Hanks balanced salt solution (HBSS). The mesentery fatty tissue was removed and the intestine emptied of its content by flushing HBSS gently through the intestine using a 1ml syringe. The length of the intestine was approximately 15cm in its contracted form. In order to create a reproducible dissection of the different parts of the small intestine, one segment (1.5cm length) was taken from each region of the intestine (2-4cm, 7-9cm, 12-14cm distal to the pyloric region) and were considered as the duodenum, jejunum and ileum, respectively.

3.3.2 CELL CULTURE TECHNIQUE

Each respective segment was ligated in both ends using sterile cotton thread. Subsequently, each segment was transferred to a 15ml centrifuge tube containing 1ml of 0.25% porcine trypsin and 0.1% EDTA (Ethylene diamine tetraacetic acid) and incubated at 37°C at varying dissociation times of 30, 45, 60, 75, 90 min.

Preparations were removed respectively from the incubation and triturated by gently pipetting the preparation up and down for approximately 5 min. The supernatant was carefully transferred to a clean centrifuge tube and 1ml of media added: Dulbecco modified eagle's medium (DMEM) with HEPES modification (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and supplemented with 10% fetal calf serum (FCS), 100U

ml^{-1} penicillin and 100μg mL^{-1} streptomycin solution, 2mM L-glutamine, 2.5% rat serum (RS), and 0.2% amphotericin B.

The preparations were centrifuged for 5 min at 450xg. Supernatant was discarded and 5ml media was added. The cell suspensions were pipetted up and down so as to obtain a uniform suspension of single cells and then transferred to $25cm^2$ cell culture flasks and incubated at 37°C (Figure 3.3.1). Cells were routinely monitored and medium was changed at 48h intervals.

Once cultures reached approximately 70-80% confluence (Log-phase), cells were further dissociated in 0.05% trypsin/EDTA for approximately 2 min at 37°C and cell detachment monitored under the microscope. When cells had detached, 5ml of media was added in order to stop trypsin action. Medium was flushed through the cell culture flask to wash remaining cells that were still attached to the surface. Cell suspensions were subsequently transferred to a 15ml centrifuge tube and centrifuged for 5 min at 450xg. Supernatant was discarded and pellets were re-suspended in 5ml of medium. Cell suspensions were gently triturated as to obtain a suspension of single cells, and extra media was added accordingly to make up desirable dilution for proceeding passages. In most occasions 2ml of cell suspension was added to 4ml of media, hence, resulting in a 1:3 dilution (approximately $5x10^4$ cells/ml).

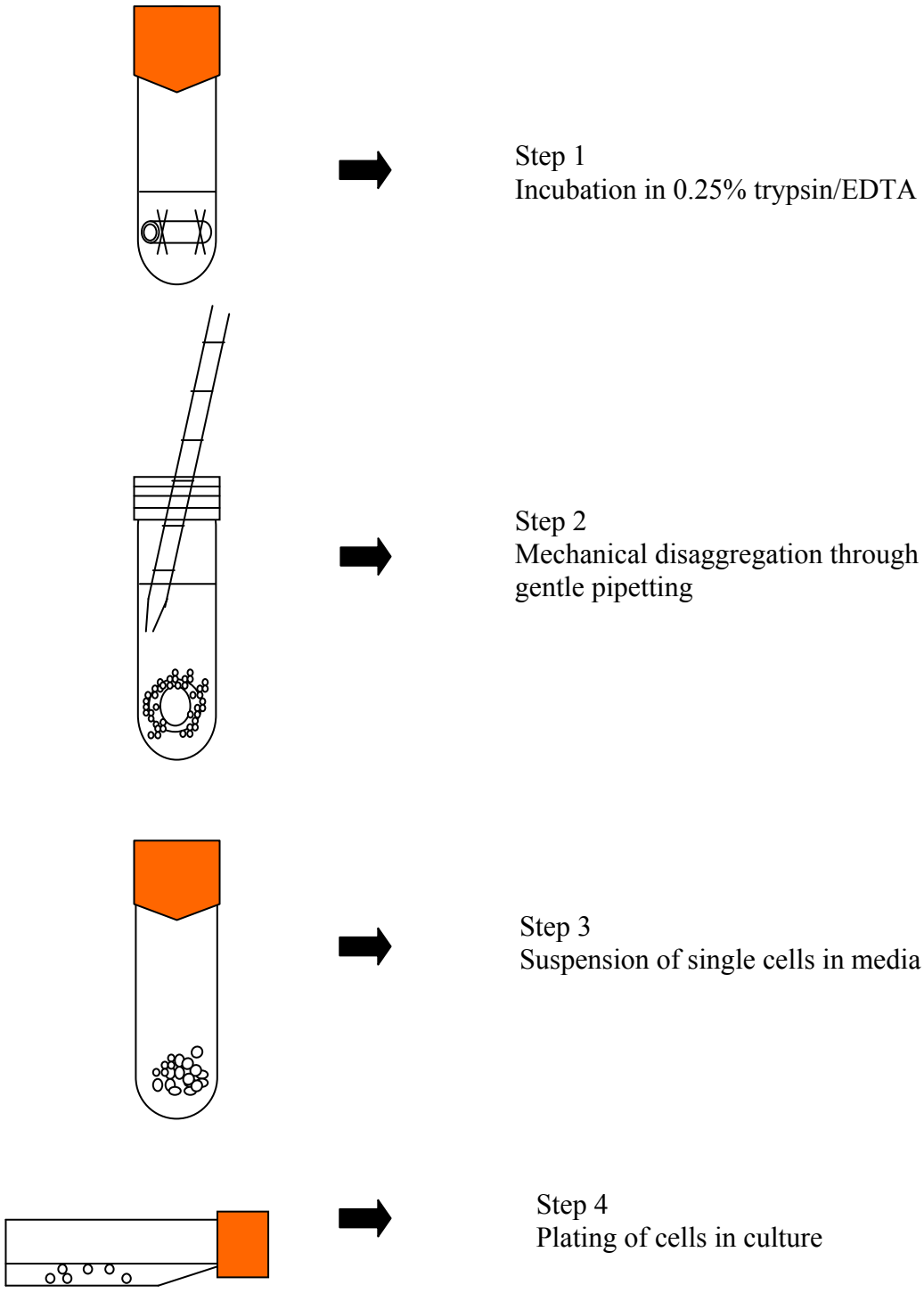


Figure 3.3.1: Schematic illustration of the cell culture technique.

3.3.3 OPTIMISATION OF CELL CULTURE PARAMETERS

Preliminary experiments were designed in order to investigate and optimise the main parameters for a successful cell culture. These included dissociation time, source of tissue and age of the animal on primary acquired intestinal cells. Cells were isolated from the different intestinal regions (duodenum, jejunum, and ileum, n=16), and the optimal dissociation time per intestinal region was determined by varying the trypsinisation times (30, 45, 60, 75, 90 min, n=4). The experiments were repeated for tissues derived from animals that were 1, 2, 3, 4 days old (n=4). For each preparation, isolated cells were monitored for cell growth, and cultures were assessed for their ability to reach confluence within a 7-day period. In this study a cell culture was considered confluent when the entire cell culture surface was completely covered by well spread cells.

3.3.4 CELL VIABILITY EXPERIMENTS

Cells were extracted from segments taken from the different intestinal regions of the rat neonates (1-4 days old) and exposed to the varying dissociation times. Subsequently, cells were centrifuged for 5 min at 450xg, the pellet was re-suspended in 5ml of medium and cell suspension was gently triturated to obtain a uniform suspension. Once re-suspended an accurate cell count was performed by diluting 50µl of 0.4% trypan blue into 100µl of the cell suspension making a dilution of 1:2. The preparation was mixed several times and allowed to stabilise at room temperature for 2 min. A cover slip was placed over the cell counting chamber (haemocytometer) and 20µl of the mix (0.4% trypan blue + cell suspension) was loaded into the chamber by allowing fluid to run into the chamber to the edges of the grooves only. The counting chamber was then placed under the microscope using x10 objective and the grid lines on the chamber were brought to focus. Only the cells laying within the areas bound by parallel lines were counted (4 x 1mm^2 per preparation).

Total number of stained and unstained cells were counted (dead cells take up the dye and appear blue under the microscope and living cells exclude the trypan blue and appear white). The haemocytometer was then washed with water first, followed by 70% alcohol, and allowed to air dry.

3.3.5 HISTOLOGY

Intestinal segments taken from the duodenum, jejunum and ileum of rat neonates (1-4 days old) were incubated in 0.25% trypsin for 15, 30, 45, 60, 75, 90 min. Segments were then rinsed in HBSS and fixed in 10% buferred formaldehyde and left overnight. Preparations were processed by dehydration in serial alcohols (70%, 80%, 90% each for 3 h and in 100% three times each for 2h). This was followed by impregnation in Histoclear I (1h) Histoclear II and Histoclear III (for 2h each). Segments were then infiltrated by pure wax (2 changes of wax, each 2h) and embedded in fresh wax with melting point of 61°C when transferred to the mold cassestes. Blocks were then labelled and stored at 4°C. For sectioning, wax blocks were removed individually from the refrigerator and immediately attached to a pre-cooled microtome. Sections were cut at thickness of 5-7μm, these were floated in a preheated water-bath (30°C) and picked up singly on slides pre-coated with poly-L-lysine. The slides were left to air dry at room temperature until stained.

After deparaffination (2 changes of Histoclear each for 5 min) and hydration in descending grades of alcohols (2 changes of 100%, 70%, and tap water each for 5 min), slides were stained with Herovici's picropolychrome (Herovici, 1963). Tissue sections were stained progressively from the nucleus (black) by incubation for 5 min each in two solutions, A and B. Solution A consisted of 0.25g celestine blue, 2.5g iron alum, dissolved and boiled for 3 min in 50ml of distilled water. Subsequent to this solution A was supplemented with 10ml of cold glycerol. Solution B consisted of 100ml 5% aqueous solution of aluminum sulphate heated to boiling point to which a further 50ml of 1% of alcoholic solution of

haematoxylin was added over 5 min and the resultant solution was boiled for a further 3 min. After cooling, 100ml of distilled water, 10ml 4% aqueous $FeCl_3$ and 1ml HCL was added. Subsequently, the cytoplasmic stain (yellow-green) was performed by incubation of the sections for 2 min in solution C, where solution C consisted of a mixture of 0.25g metanil yellow, 60ml distilled water, and 5 drops acetic acid. This was followed by incubation for 2 min in solution D (mixture of 60ml distilled water and 5 drops of acetic acid) and a further 2 min incubation in solution E (a mixture of 60ml distilled water and 2 drops of saturated aqueous $LiCO_3$). The sections were finally stained for connective tissue stain (red and blue) by incubation for 2 min in solution F (a mixture of a solution 1: 0.05g methyl blue in 50ml distilled water and solution 2: 0.1g of acid fuchsin in 50ml of saturated aqueous picric acid). After mixing these two solutions, the resultant solution was supplemented with 10ml of glycerol and 0.5ml of saturated aqueous $LiCO_3$. Between applications of solutions A-F the sections were washed for 2 min in running water. After the final incubation in solution F the sections were incubated for a further 2 min in 1% acetic acid. Lastly, tissue sections were dehydrated in serial alcohol solutions of 100%, 90%, and 70% (1 min each), incubated in Histoclear for 2 min each, mounted on glass slides using Histomount and examined using a Nikon Eclipse 80i bright field microscope.

3.3.6 ISOLATION OF INTESTINAL SMOOTH MUSCLE CELLS AND MYENTERIC NEURONES

Following cell confluence, cell viability and histological procedures, results revealed that intestinal segments taken from the ileum and a trypsinisation time of 30 min were the most suitable parameters for all the subsequent experiments. These parameters were selected based on the segments that gave the best cell yield and the shortest trypsin contact time necessary for the dissociation of a considerable number of smooth muscle cells and

myenteric neurones. Thus, the cells extracted from the ileum and trypsinised for 30 min produced viable cells, which were plated onto cell culture flasks and medium changed after 48h. The obtained cultures consisted predominantly of ISMC, neuronal cells and fibroblasts.

In subsequent experiments these cells were purified through differential adhesion technique to reduce the number of fibroblasts and produce a coculture consisting mainly of ISMC and neurones. The differential adhesion is a commonly utilised procedure to minimise the number of fibroblasts in culture since they characteristically adhere to the surface faster in a cell culture monolayer than other cell types (Yaffe, 1968). The differential adhesion technique was carried out by plating suspensions of mixed cell population (ISMC, neuronal cells and fibroblasts) into plastic cell culture flasks and incubating at 37ºC. Following 10 min incubation, flasks were monitored for cell adhesion under the microscope. This procedure was repeated for every 10 min until not only fibroblasts but also characteristic spindle-like shape ISMC were observed adhering to the cell culture flask. This was observed after 30 min of continuous monitoring. At this point the remaining non-adherent presumably consisting of ISMC and neurones were removed from the cell culture flask and transferred to a centrifuge tube and centrifuged for 5 min at 450xg. Cells were then re-suspended in 5ml of media by gentle trituration, plated onto a second cell culture flask and incubated at 37ºC until reaching confluence. By this stage the cell population consisted of a coculture of mostly ISMC and myenteric neurones with a significantly reduced number of fibroblasts.

In separate series of experiments attempts were made to purify the myenteric neurones through selective cytotoxicity technique. This procedure enables the cell purification by favouring the proliferation of one of the cell types in the mixture (Ham, 1984). For instance, for removal of non-neuronal cells, preparations were pre-treated with cytosine arabinoside, an anti-mitotic drug that inhibits non-neuronal DNA synthesis. After seeding

of the isolated cell mixture containing fibroblasts, ISMC and neurones in cell culture flasks for 24h, the cell culture media was replaced with media containing 6μM of cytosine arabinoside and incubated for 72h at 37ºC. Treatment with cytosine arabinoside containing media was replaced with normal medium and cells were allowed to proliferate for another 96h by incubating at 37ºC. Single dose treatment with cytosine arabinoside caused a significant reduction of non-neuronal cells including ISMC and fibroblasts with cultures displaying an approximate 80-90% neuronal cell population.

In another series of experiments attempts were made to purify ISMC. This was performed primarily through differential adhesion technique for 30 min to minimise the number of fibroblasts in culture. The remaining non-adherent cells consisting of ISMC and neurones were removed with the medium into a second flask. The cells were allowed to adhere to the cell culture flask for 5h. This procedure was carried out as an attempt to reduce the number of neurones in culture. By monitoring cell adhesion, it was observed that neurones took the longest to adhere to the surface compared to other cell types present in the mixture. It was also found that neurones displayed a distinct morphology as they formed clusters during cell adhesion to the surface. Hence, cell morphology monitoring revealed that neurones start to adhere to the surface after 5h elapsed. At this point, the media with the non-adherent cells were aspirated from the cell culture flask, replaced by fresh medium and cells were incubated at 37ºC and allowed to proliferate for 24h. A substantial reduction in neurones was observed following this procedure, however, a small number of neurones were still present in the culture. Subsequently, selective cytotoxicity technique was again applied for the removal of the remaining neurones. This was enabled by using scorpion venom *maurus palmatus*, an effective neurotoxin that disrupts neuronal activity without disturbing ISMC (Blennerhassett & Lourenssen, 2000). After cells were purified via the two-step differential adhesion procedure explained above and allowed to proliferate for 24h, cell culture media was replaced with media containing 30μg/ml *maurus palmatus* and

incubated at 37°C for 24h before being replaced with normal media. At confluence, the cell population in culture consisted mainly of ISMC, however, some neurones were still visible. Hence, cells were passaged as described in section 3.3.2, plated at a 1:2 dilution and allowed to proliferate for 24h before they were again exposed to a second treatment with scorpion venom *maurus palmatus* (30μg/ml) for 24h at 37°C. Following this treatment, cell cultures displayed a uniform distribution of ISMC with purity levels of approximately 80- 90%.

3.3.7 IMMUNOCYTOCHEMISTRY

Intestinal segments obtained from the ileum of rat neonates (1-4 days old) were exposed to 30 min trypsinisation as described in section 3.3.2. Three different cell cultures were prepared: the first culture consisted of a combination of ISMC and neurones, the second culture consisted of purified neuronal cells, and the third culture consisted of purified ISMC cells as explained in section 3.3.6.

Immunocytochemistry procedures were subsequently used to confirm the nature of the cells in each preparation following the purification protocols. At confluence cocultures and purified cell populations of neurones and ISMC were seeded on glass cover slides overnight using a 1:5 dilution and then they were fixed in 2% formaldehyde for 5 min and kept in PBS until used for immunostaining experiments. This dilution was selected, as lower cell numbers promote better cell dispersion in the cover slides and subsequently better images of cell morphology and immunoreactivity.

Dual-label immunocytochemistry with mouse anti-α-smooth muscle actin and anti-rabbit $5HT_3$ receptors antibodies were carried out to verify the nature of cells growing in the cocultures and moreover to confirm the purity of newly obtained ISMC and myenteric neurones in culture. Briefly, cell were exposed to PBS containing 0.1% Triton X-100 for 15 min. Slides were then bathed in PBS containing 2% goat serum for 30 min before

incubation for 60 min with mouse anti-α-smooth muscle actin (1:100 in PBS-1%BSA) at room temperature. This was followed by incubation with mouse TRITC-labeled secondary antibody (1:30 in PBS-1%BSA) for 60 min at room temperature. Slides were subsequently incubated with the neuronal marker rabbit anti-$5HT_3$ receptors antibody (5μg/ml) for 120 min at room temperature followed by incubation with rabbit FITC-labeled secondary antibody (1:80 in PBS-1%BSA) for 60 min room temperature. Incubations were preceded by 3 washes with PBS.

Antibodies to α-actinin were also used to confirm the nature of the purified ISMC in culture. Slides were incubated with mouse anti-α-actinin antibodies (1:400 PBS-1%BSA) for 60 min at room temperature followed by incubation in mouse secondary antibody labeled with Alexo fluor (1:400 PBS-1%BSA) for 60 min at room temperature. Both incubations were preceded through by 3 washes with PBS.

Negative controls were carried out, in which primary antibodies were replaced with PBS. Preparations were mounted using DAPI, this forms fluorescent complexes with natural double stranded DNA, hence is useful for marking the cell nucleus (Sulston et al., 1983). Stained samples were observed with Nikon Eclipse 80i Fluorescence, and images were digitally captured with SOP (ACT-2U) software. Digital recombination of images using Adobe Photoshop was applied for the dual labeling of neurones and ISMC.

3.3.8 CRYOPRESERVATION

In an attempt to develop a method for long-term ISMC storage, cryopreservation techniques were examined. Cells were prepared from ileum segments taken from rat neonates (1-4 days old) by exposing to 30 min trypsinisation as explained in section 3.3.2 and ISMC were purified as explained in section 3.3.6. Cell suspensions were plated onto cell culture flasks and medium changed after 48h until cells reached confluence. Cells were

subsequently passaged once as described in section 3.3.2. The cell suspensions were centrifuged for 5 min at 450xg and the pellet was re-suspended in 4 ml of media (dimethyl sulfoxide + 10% FCS) that was previously warmed up to 37°C, making up a suspension of 1-2x10^6cells/ml. Dimethyl sulfoxide is a commonly used cryoprotectant agent, which protects cells during cryopreservation. By warming the freezing media this promotes a balance between the cells and the surrounding environment and reduces the changes of disturbing the cell membrane.

Subsequently, different protocols for cell freezing were applied: In protocol 1 after the pellet containing the dispersed cells from the ileum were re-suspended in 4ml of freezing media, 1ml at a time were transferred into 1ml cryovials. The 4 cryovials were directly plunged into a –80°C freezer followed by liquid nitrogen (-196°C). In protocol 2, the cell pellet was again re-suspended in 4ml of freezing media and transferred into 1ml cryovials. However, this time the freezing protocol involved several steps, commencing with incubation of cryovials for 15 min at room temperature to allow cooling of samples (37°C to 21°C) and promote the diffusion of the freezing media across the cell membrane. These vials were then placed on ice for 20 min to help bring the samples to subzero conditions (21°C to -1°C). Subsequently, cryovials were rapidly transferred to a polystyrene container wrapped with thermo isolators and stored at -80°C freezer for 12h. This enables a slow cooling of samples from –1°C to –80°C before storage in liquid nitrogen (-196°C). This gradual decrease of the temperature was employed in order to try and reproduce a simple technique, which would allow controlled-rate freezing without the use of expensive equipment. The samples were stored for periods of 1-week, 1-month and 4-months. Thawing was achieved by immersing the vials into a water bath (39°C) until the contents thawed completely. The cells were then suspended in 10ml of the culture medium and centrifuged for 5 min at 450x*g*. Pellet was re-suspended in 5ml of media, plated into cell culture flasks and monitored daily to assess morphological changes.

3.3.9 COMET ASSAY

To qualitatively assess the viability of the cells in response to freezing, the percentage of viable cells for each of these preparations were measured by the trypan blue exclusion assay as explained in section 3.3.4. In Separate experiments, 100µl of the cell suspension were collected and used to quantitatively assess DNA damage using comet assay.

The comet assay is a sensitive method for analysing and quantifying DNA damage in individual cells. Briefly, a mixture of cell suspension (50µl) and a 1% low melting-point agarose (50µl) were dipped onto regular microscope slides coated with 1% normal melting-point agarose. A coverslip was then placed on the mixture and the slides were then placed on ice for 5 min. Next, the coverslips were removed and a final layer of 0.5% of low melting point agarose (100µl) was placed on the slide. The coverslip was replaced and the slide kept on ice for 5 min. Coverslips were then removed and slides were lysed in detergent overnight [178ml lysing solution (2.5 M NaCl + 100 mM EDTA + 10 mM Tris making up the volume to 890ml, PH adjusted to 10 with NaOH) + 2ml TritonX-100 + 20 ml of Mg_2SO]. Slides were transferred to an electrophoresis unit and incubated for 15 min with electrophoresis buffer (60ml of 60M NaOH + 10ml of 200 mM EDTA + 1930ml distilled water). The final step promotes the disruption of cell membrane such that the chromatin can be assessed and fragmentated DNA can migrate from the nucleus towards the anode during electrophoresis at 25V and 300mA (electrical field strength corresponding to 0.7 V/cm) for 30 min. After electrophoresis, the slides were rinsed 3 times with 400mM Tris, pH 7.5, stained with ethidium bromide (20µg/ml) and coverslip placed (Figure 3.3.2).

From each slide, 50 cells were randomly selected, nuclei were analysed using a fluorescence microscope. Comet length measurements were taken starting from the left

margin of the fluorescence constituting the head of the comet, to the furthest visible pixel in the tail via a computerised image analysis system (Komet version 3.1).

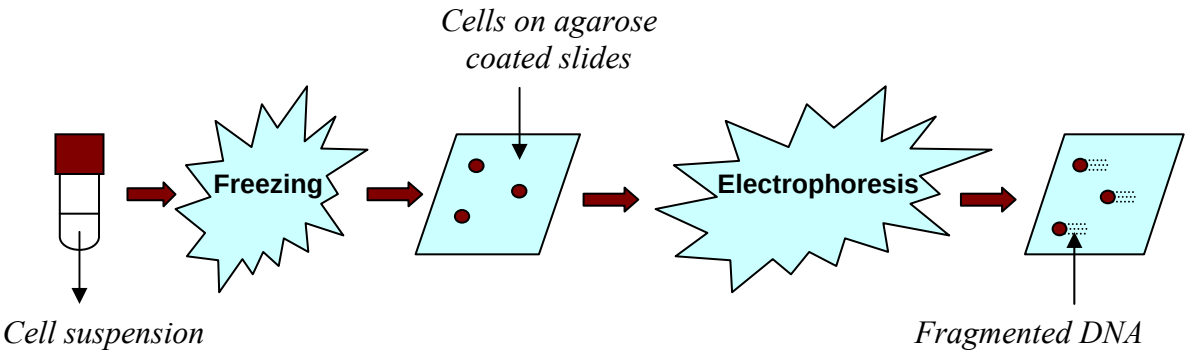


Figure 3.3.2: Schematic illustration of comet assay analysis. Cells in suspension are cryopreserved and subsequently thawed. Each sample preparations is smeared onto a agar treated-coverslip and lysed to release cell chromatin. Samples are finally ran in an electrophoresis system allowing the identification and quatification of the degree of DNA damage elicited by different treatments.

3.3.10 FUNCTIONALITY STUDIES (PLASTIC SURFACE)

In subsequent experiments attempts were made to investigate the functionality of single ISMC isolated from segments taken from the ileum of rat neonates. This was performed to verify if single ISMC responses to known agonists were comparable to those in the intact tissue. In such experiments cells were taken from the ileum and trypsinised for 30 min, centrifuged, re-suspended, and plated as explained in section 3.3.2. Once cultures reached confluence, they were passaged as explained in section 3.3.2. Cell suspensions were centrifuged for 5 min at 450xg and pellet re-suspended with 5 ml of media. Cells suspensions were subsequently plated into cell culture flasks using a 1:6 dilution and incubated overnight at 37°C. This dilution was chosen to promote low cell proliferation and enable clear morphological selection of ISMC for the functionality studies.

An inverted microscope with fitted Camera and heating stage kept at 37°C was used collectively with image processing software NIH Scion Image software for live capture of

cell responses to varying drug concentrations such as potassium chloride (KCl) (0.03, 0.1, 1, 6, 10, and 30mM), carbachol (0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3μM), and 5-HT (0.03, 0.1, 0.3, 1, 3, 10, 30, and 100nM). Drugs were applied and images were captured for 5 min in each administration. Preparations were washed twice with fresh medium kept at 37°C and allowed to settle for 10 min before the subsequent dose was added.

For every different concentration of the drugs, 10-point pixels were carefully selected around the inner border of the cell and the responses were assessed as a measure of variation in gray-scale intensities as cell dimensions changed in response to each drug applications. The 10-pixel points were selected before drug application (0 sec) and were kept fixed throughout the 5 min of drug exposure. The measurements of the contrast differences were subsequently taken for 0, 15, 60, 120, 180, 240, and 300 sec. Therefore, as cell displacement occurred in response to drug application, gray-scale measurements were accurately captured over a 5 min period. This reflected the ISMC contractile responses to any drug application (Figure 3.3.3a).

The cells displayed a spread appearance after plating on the plastic surface. The application of any drug caused the cells to slightly narrow in width without any observable shortening of the length. In response to these observations, pixel points were placed on one side of the cell to maximise the capture of cell movement in response to drug application. At each given concentration of a drug seven contact times of 0, 15, 60, 120, 180, 240, and 300 sec were measured per pixel point. This was repeated for all 10-pixel points (n=10). A complete concentration response curve to each drug was repeated 4 times (n=4).

3.3.11 FUNCTIONALITY STUDIES USING SILICONE ELASTOMER SUBSTRATES (SYLGARD SURFACE)

As ISMC demonstrated the ability to respond to drugs on the plastic surfaces, attempts were made to amplify these responses by plating ISMC into a softer surface.

Cells obtained from segments taken from the ileum of rat neonates were trypsinised for 30 min, centrifuged, re-suspended, and plated as explained in section 3.3.2. Once cultures reached confluence, they were passaged as explained in section 3.3.2. Cell suspensions were centrifuged for 5 min at 450xg and pellet re-suspended with 5 ml of media. Cell suspensions were subsequently plated onto cover slides previously coated with silicon elastomer using a 1:6 dilution and incubated overnight at 37°C. The silicone elastomer mixture (9 base: 1 curing agent) was coated onto the cover slides using a spin coater. A drop of the mixture was applied to the centre of the cover slide and gradually the rotation was increased up to 12V until the mixture had spread evenly across the entire surface. Subsequently, the coated cover slides were allowed to set for 12h before transferring into petri-dishes, where cells were seeded onto the coated substrates and incubated overnight at 37°C. Preparations were used to establish concentration response curves to non-cumulative addition of KCl (1, 10, 100μM, 1 and 3 mM), 5-HT (0.03, 0.1, 0.3, 1, 3, 10 and 30 nM), carbachol (0.1, 0.3, 1, 3, 10, 30, and 100 nM) in the absence and presence of atropine (0.1μM), and cumulative administration of carbachol (0.3, 1, 3, 10, 30, and 100 nM). In separate experiments, noradrenaline (1, 3, 10, 100nM, 1, 3, 10μM) was applied to investigate relaxation properties of single ISMC on the silicone elastomer substrates.

Parameters of measurements were kept the same as in section 3.3.10 with two exceptions. ISMC when plated on silicone elastomer surfaces displayed a different morphology compared to ISMC plated on plastic surfaces. The flexibility of the material enabled ISMC to attach to the silicone substrates and display a shorten appearance. As a result, responses to drug application caused not only changes in cell width but also in cell length. Therefore,

the 10-pixel points were placed all around the cell inner border to maximise the capture of changes in cell dimension following drug application. The other exception was applied on the measurement of ISMC responses to noradrenaline, in which the 10-pixel points were selected on the outer border of the cell. The reason for this change in the protocol was because noradrenaline is a known relaxant at the ISMC. Thus, if methods used to measure contraction were applied to measure cell relaxation, the variation in gray scale intensities would not be captured since relaxation induced by noradrenaline would be expected to produce flattened and expanded appearance of the cells and these changes would not be captured by pixel points placement at the inner border of the cells (Figure 3.3.3b).

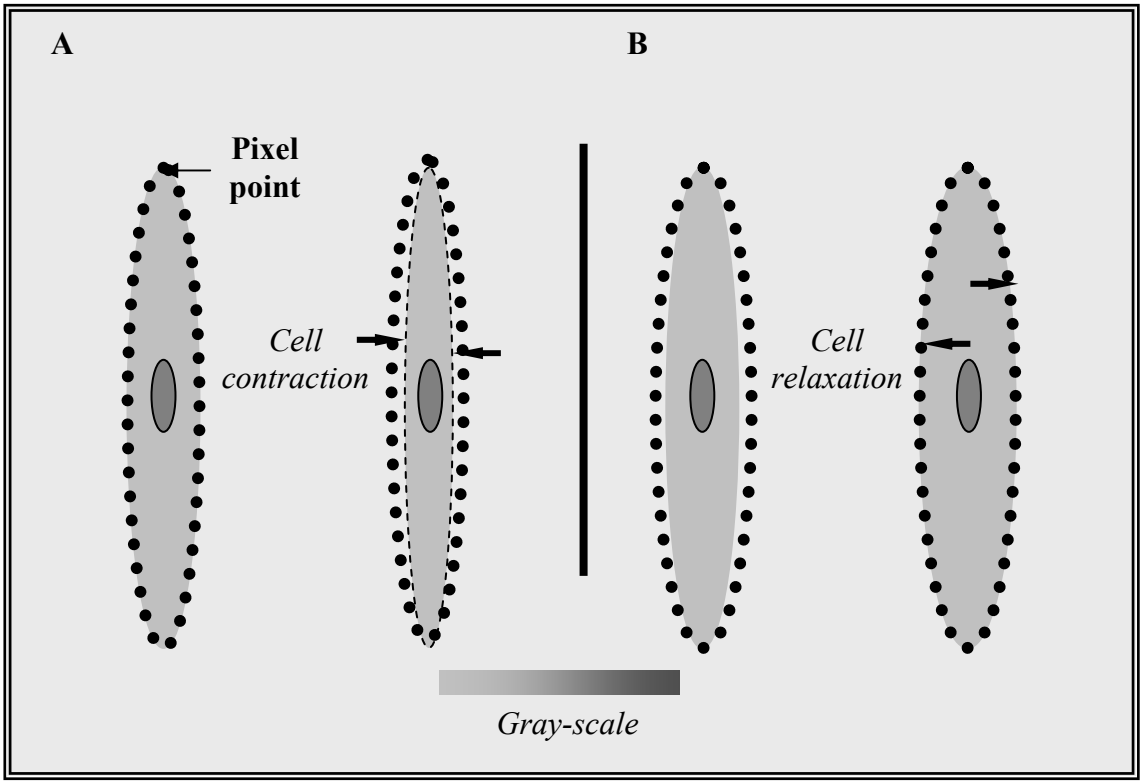


Figure 3.3.3: Image illustrating method of measurement of cell contraction (A) and relaxation (B). When ISMC were exposed to contractile drugs, pixel points were placed at the inner border of the cell where the higher intensities of gray are present. As cell dimension changed in response to drug application, the selected pixel points captured the variations in gray intensities. When ISMC were exposed to relaxant drugs pixel points were placed at the outer border of the cell where the lower intensities of gray are present. Thus, as cells flatten in response to drug application, the selected pixel points captured the variations in gray scale intensities. The differences in gray–scale values before and after drug application were proportional to cell responses to drug application.

3.3.12 MICROPATTERNING - CELL ALIGNMENT STUDIES

Experiments were carried out to investigate the ability of isolated intestinal cells taken from the neonatal rat ileum to respond to guidance cues. Such a technique is important controlling the organisation of cells in culture and in re-instituting the architecture attributed to cells in the intact intestine. The most commonly used method for promoting cell organisation and alignment is by patterning surfaces with extracellular matrix proteins such as fibronectin. This was achieved by casting silicone elastomer (polydimethylsiloxane, PDMS) in the desired patterns and subsequently coating the PDMS with fibronectin to be transferred to the substrate surface. The masterstamps of 3.8, 5, 12.5 and 25 micron were pre-fabricated at the University of Glasgow. The silicone elastomer was thoroughly mixed with the curing agent in a 9:1 ratio. The masterstamps were placed into a petri dish with the patterned grooves facing up. Subsequently, enough silicone elastomer mixture was poured into the petri dish covering the master stamps. The petri dish was placed for 30 min under a partial vacuum to remove bubbles from the elastomer and cured for 72h at room temperature. The stamps were carefully removed from the master stamps, washed in ethanol and distilled water to remove residuals from the surface and allowed to air dry.

Initially, cover slides were washed in distilled water followed by ethanol and allowed to dry in sterile conditions. Consequently, a mixture of 20µl fibronectin plus 80µl sterile water (1µg/ml) was prepared to use as ink in the stamping of the different pattern sizes.

The different stamp sizes were dipped in the ink for 1 min and subsequently the excess was wiped off from the edges and allowed to air dry prior to stamping. Stamping was then performed by firmly pressing the inked stamp onto the cover slide for approximately 1min. The stamped cover slides were transferred to small petri dishes and a coculture of purified ISMC and neurones taken from segments of the ileum as described in section 3.3.2 and

purified as described in section 3.3.6 were plated at cell densities of 1:7 onto the cover slides patterned with 3.8, 5.0, 12.5, and 25 micron wide fibronectin repeat grating. A low cell density of 1:7 was selected to ease cell movement and promote their ability to follow the stamped patterns. The petri dishes were then sealed with parafilm and incubated at 37°C overnight.

Following incubation the cell preparations were fixed in 2% formaldehyde for 5 min and washed three times with HBSS. Cell alignment to the different sized patterns was then determined by measuring the angle between the long axis of the cells and the stamp-patterned proteins repeat grating using image processing software NIH scion image. In these measurements a mean angle of 0˚ represents 100% alignment and a mean angle of approximately 45˚ represents random alignment.

3.3.13 SYLGARD PATTERNED THIN SUBSTRATES

Following determination of the optimal fibronectin grating for the alignment of ISMC and neurones, attempts were made to create thin sylgard patterned substrates casted from the 12.5 micron masterstamp and coated with fibronectin. The use of topographical cues derived from the 12.5 micron grating enabled the surface area micropatterned with fibronectin at dimensions optimal for cell alignment to be doubled. Such substrates were designed to grow large numbers of intestinal cell population with organised orientation, which would collectively increase the ability of coculture preparations to respond to the stimulus applied. Moreover, it was believed that with the substrate flexibility, the traction force originated by cells on the soft surface and the availability of large number of cells in the substrate, these would create the optimum parameters for detecting cellular contractile tension induced by intestinal cell population in response to the application of drug.

Primarily, silicone elastomer mixture was prepared at a dilution of (9.75 base: 0.25 curing agent) and allowed to set for approximately 24h into a glass petri dish. Such mixture dilution was selected to further increase the flexibility of the substrate. Subsequently, the 12.5micron masterstamp was inverted onto the silicone elastomer mixture and allowed to set for an additional 24h. The 12.5 pattern size was chosen as it revealed to promote the best alignment for the coculture of ISMC and neurones (Figure 3.4.26f). The masterstamp was gently peeled from the silicone mixture and the set silicone mixture consequently peeled from the petri dish. The latter was cut within the areas where patterns were visible, creating small patterned constructs of approximately 0.5 x 1.0cm (Figure 3.4.28). The constructs were dipped onto a mixture of 20µl fribronectin plus 80µl sterile water (1µg/ml), the excess was wiped off, and allowed to air dry. Coculture of ISMC and neurones taken from segments of the ileum as described in section 3.3.2 and purified as described in section 3.3.6 were plated on the sylgard substrates using a 1:2 dilution (cell suspension: medium) and incubated overnight in a sealed petri dish at 37°C. High cell density was chosen to promote a high cell number attaching to silicone pattern substrates. Subsequently, when cells spread and displayed a satisfactory degree of alignment, preparations were exposed to a sub-maximal dose of KCl (6mM). The changes in tension of the cells were measured using the optical microscope images elaborated with image processing software (NIH scion image). The data was then used to construct profile plot and surface plot. The surface profile displays a 3-dimensional imitation of the selected image's surface, whereas the profile plot allows summing up and averaging all the pixels data along the row or column parallel to the shorter axis, which can then be used to plot all the averaged intensities against the longer axis. This will allow accessing the surface deformations induced by cells in response to the application of the drug.

3.3.14 INTESTINAL CELLS SEEDING ONTO LIQUID CRYSTAL COATED SURFACES

In additional attempts to develop a flexible matrix able to display surface deformation in response to contractile activity exerted by the cells, shear sensitive cholesteric liquid crystal was used. Shear sensitive cholesteric liquid crystal was produced from a mixture of three components: cholesteryl chloride (0.25g), cholesteryl pelargonate (0.38g), and cholesteryl oleyl carbonate (0.38g). The melting points for these liquid crystal compounds are 94°C-96°C, 74°C-77°C, and 113°C respectively. The mixed compounds were transferred into a vial and heated up on a hot plate to their highest melting point of 113°C. As the temperature increased, the solid material gradually melted into a more turbid liquid-crystalline phase at 70°C and subsequently to a clear isotropic phase at the highest melting point of 113°C. When the mixtures reached this liquid phase, 20µl were transferred onto one cover slide and another cover slide was used to gently spread the liquid crystal material into a smooth layer and allowed to set for 5 min under sterile conditions. Coculture of ISMC and neurones taken from segments of the ileum as described in section 3.3.2 and purified as described in section 3.3.6 were plated onto the cholesteric liquid crystal coating using a 1:2 dilution (cell suspension: medium) and incubated overnight in a sealed petri dish at 37°C. Intestinal cells were primarily assessed for their viability on liquid crystal using the dye exclusion assay. In separate experiments, cells were plated on liquid crystal and monitored for 12-24h until they displayed satisfactory proliferation. The preparations were then exposed to a single application of KCl (6mM). In another series of experiments, cells were plated in the liquid crystal for 12-24h and preparations were pre-contracted with a single application of KCl (6mM) before the application of a single dose of noradrenaline (40µM). The responses were measured using Scion Image software as explained in section 3.2.13.

3.3.15 STATISTICAL ANALYSIS

Comparative results were given as means ± standard error of the mean. Following tests for normality, all the significance of the differences were tested by one-way ANOVA followed by the Bonferroni adjustment test with the exception of single cell contractility studies where significance of the differences was tested using paired t-test.

Table 3.1 Chemicals/drugs used in the present study, their solvent and suppliers.

Chemicals/drugs	**Solvents**	**Suppliers**
Agarose	Distilled water	Agar Scientific
Anti mouse α-smooth muscle actin antibody	BSA	Sigma
Anti mouse α-actinin antibody	BSA	Sigma
Anti rabbit 5-HT_3 receptor antibody	BSA	Sigma
Anti mouse TRITC conjugated labeled secondary antibody	BSA	Sigma
Anti rabbit FITC conjugated labeled secondary antibody	BSA	Sigma
Atropine sulphate	Distilled water	Sigma
Bovine albumin serum (BSA)	HBSS	Sigma
Carbachol	Distilled water	Sigma
Cholesteryl chloride	Distilled water	Sigma
Cholesteryl pelargonate	Distilled water	Sigma
Cholesteryl oleyl carbonate	Distilled water	Sigma
Cytosine Arabinoside	Distilled water	Sigma
DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride)	HBSS	Sigma
EDTA (Ethylene diamine tetraacetic acid)	Distilled water	Sigma
Ethidium Bromide	Distilled water	Sigma
Fibronectin	Distilled water	Sigma
Hanks balanced salt solution (HBSS)	-	Sigma
Noradrenaline	Distilled water	Sigma
KCl (Potassium Chloride)	Distilled water	Sigma
Scorpion venom (*maurus palmatus*)	Distilled water	Sigma
Sodium chloride	Distilled water	Sigma
Sodium hydroxide	Distilled water	Sigma
Tris (hydroxymethyl) aminomethane	Distilled water	Sigma

3.4 RESULTS

3.4.1 ENZYME TREATMENTS AND DISSOCIATION

Intestinal cell preparations harvested from segments of both the jejunum and the ileum yielded significantly ($p<0.05$ and $p<0.001$ respectively) higher number of cultures reaching confluence compared to the duodenum (Figure 3.4.1a). Lower trypsinisation times of 30, 45 and 60 min yielded higher number of confluent cultures in segments of the duodenum, jejunum and ileum as compared to the longer trypsinisation times of 75 and 90 min (Figure 3.4.1b). The cell viability assay also demonstrated that segments taken from both the jejunum and the ileum yielded a significantly ($p<0.05$ and $p<0.01$, respectively) higher percentage of viable cells compared to the duodenum (Figure 3.4.2a). Similarly, Lower trypsin incubation times of 30, 45, and 60 min resulted in a higher percentage of viable cells. Further dissociations at 75 and 90 min led to a significant ($p<0.05$ and $p<0.001$ respectively) reduction in the percentage of viable cells in the segments taken from the jejunum, and significant ($p=0.001$ and $p<0.001$ respectively) reduction in viability of cells harvested from the ileum (Figure 3.4.2b). However, there were no significant differences in cell viability or number of cultures reaching confluence in relation to the age range of neonates (Figure 3.4.1c and 3.4.2c). Results obtained in the different intestinal regions at varying trypsin dissociation times and age range of neonates, clearly demonstrate that extraction of cells from the ileum gives better yields and higher viabilities compared to cell preparations isolated from both the jejunum and the duodenum.

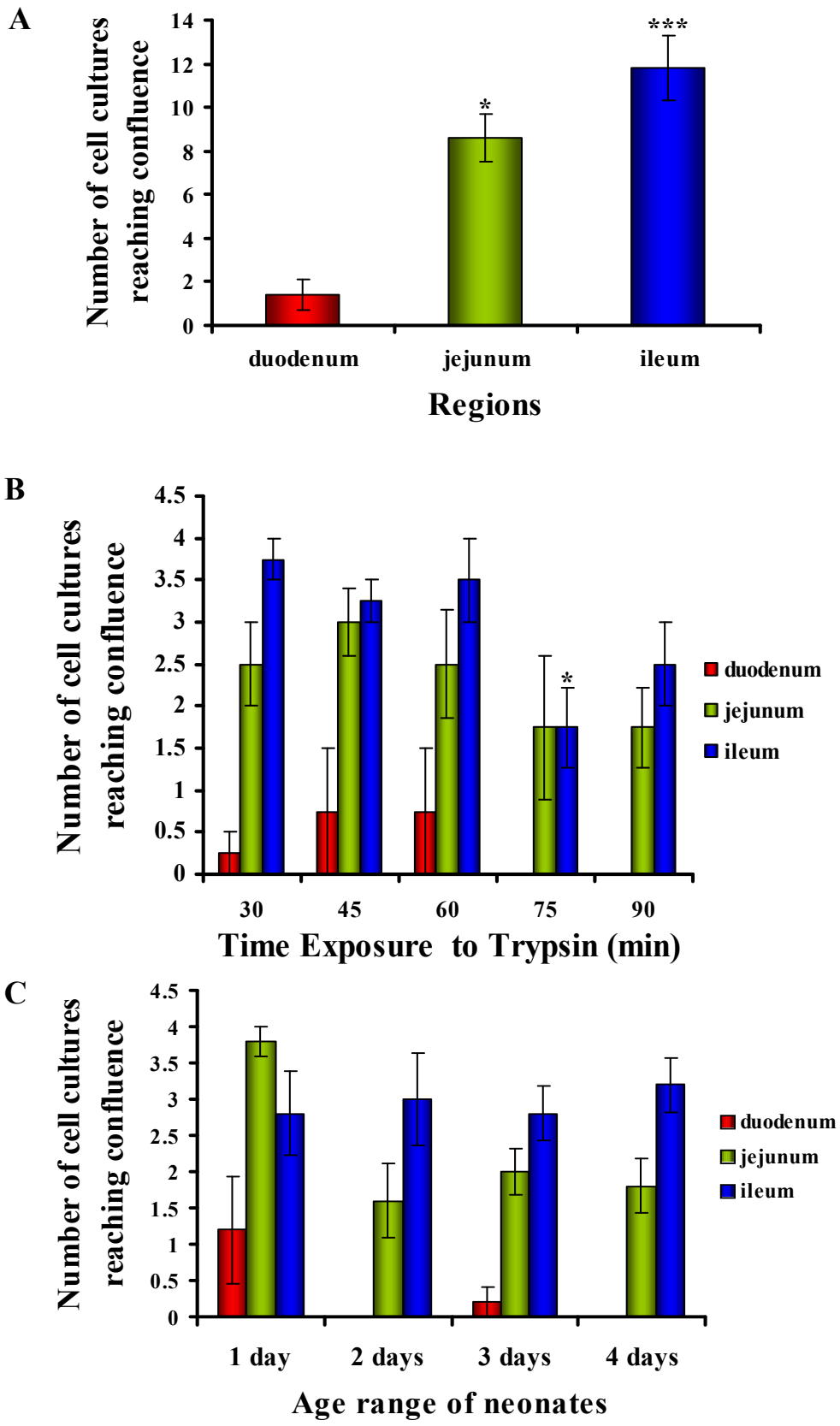


*Figure 3.4.1: Intestinal cell cultures obtained from segments taken from the duodenum, jejunum, and ileum reaching confluence (A). Intestinal cell cultures harvested at varying trypsin dissociation times taken from different intestinal regions (B). Intestinal cell cultures taken from the intestine of different age range of neonates (C). Each point represents the mean ± s. e. mean; n=16 (A) n= 4 (B, C).*p<0.05, **p<0.01 and ***p<0.001 taken as significant differences in the group.*

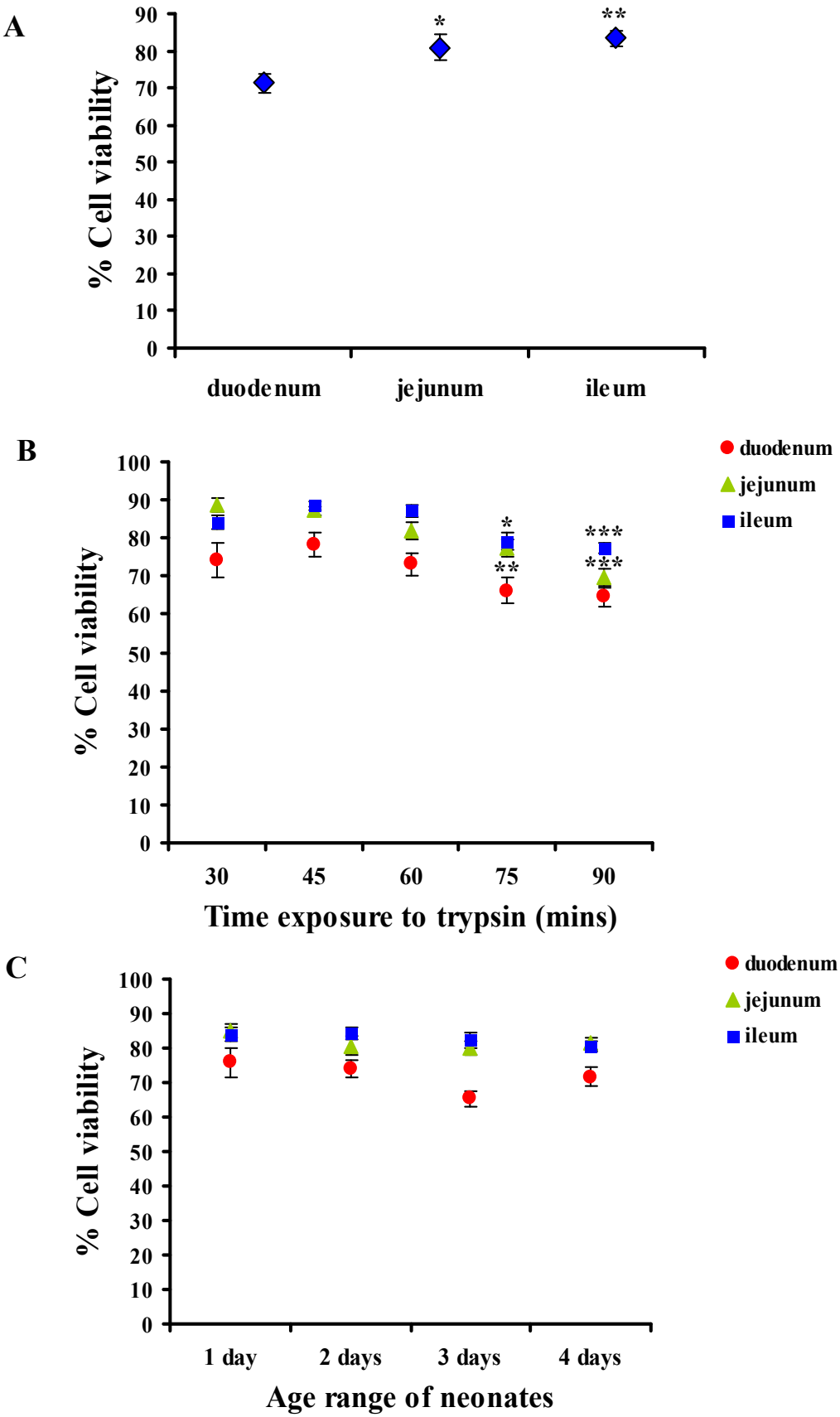


*Figure 3.4.2: Percentage of viable cells obtained from the duodenum, jejunum, and ileum (A). Percentage of viable cells harvested at varying trypsin dissociation times taken from different intestinal regions (B). Percentage of viable cells taken from different age range of neonates (C). Each point represents the mean ± s. e. mean; n=16 (A; B) n= 4 (C). *p<0.05, **p<0.01 and ***p<0.001 taken as significant differences in the group.*

3.4.2 HISTOLOGY

Histological experiments were carried out on the intestinal segments (Figure 3.4.3) to investigate which layer of intestine have been removed following progressive exposure time to the dissociation enzyme trypsin (15, 30, 45, 60, 75, and 90 min). Segments from different regions of the intestine were taken and 15 min incubation in trypsin the intestinal segments of the duodenum, the jejunum, and the ileum revealed very minor cell dissociation from the longitudinal muscle layer (Figure 3.4.4a1-3). After 30 min exposure to trypsin part of the muscularis externae including the muscle layers together with the myenteric plexus were removed in segments taken from the jejunum and the ileum, but not in segments taken from the duodenum (Figure 3.4.4b1-3). A major part of the muscularis externae was dissociated following a 45 min exposure to trypsin in segments taken from the duodenum, jejunum and the ileum (Figure 3.4.4c1-3). Following 60 min exposure to trypsin, the muscularis externae was completely removed in all intestinal segments (Figure 3.4.4d1-3). Further exposure to trypsin for 75 and 90 min led to complete dissociation of the intestine submucosal layers in all segments taken from the intestine (Figure 3.4.4e1-3 and 3.4.4f1-3). Thus, for subsequent experiments a trypsinisation time of 30 min were chosen for cell isolation from segments taken from the ileum as it was the shortest of the trypsinisation times that enabled the maximum acquisition of viable ISMC and myenteric neurones from the muscularis externae.

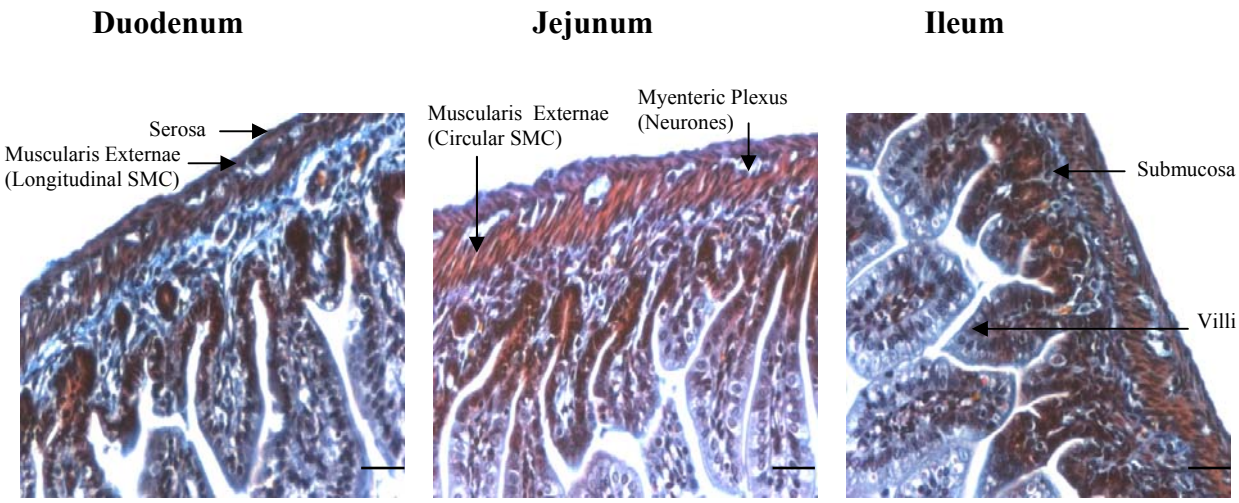
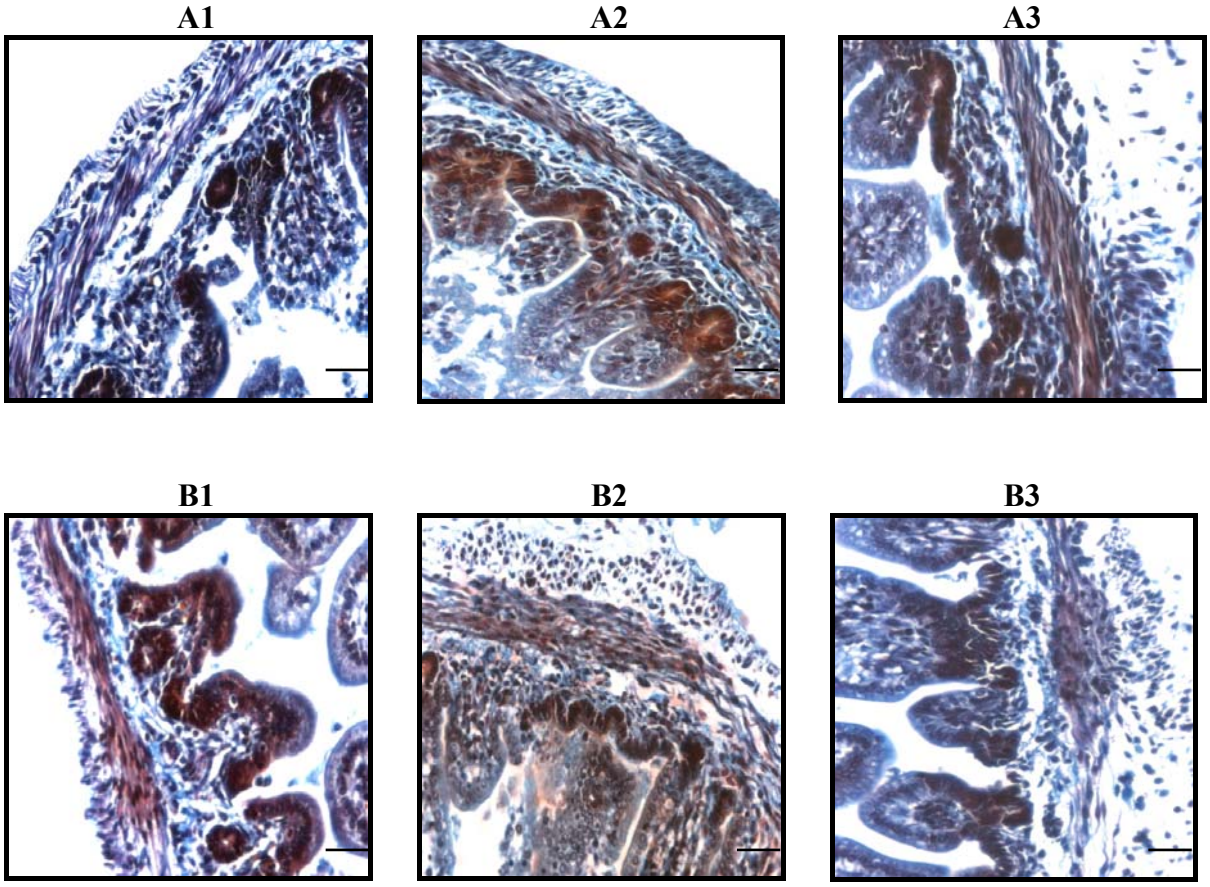


Figure 3.4.3: Representative micrographs illustrating histological observations of intestinal segments taken from the intact duodenum, jejunum and ileum. Nuclei, cytoplasm, and connective tissues as stained in black, yellow-green, and red-blue, respectively (scale bar=100 μm).



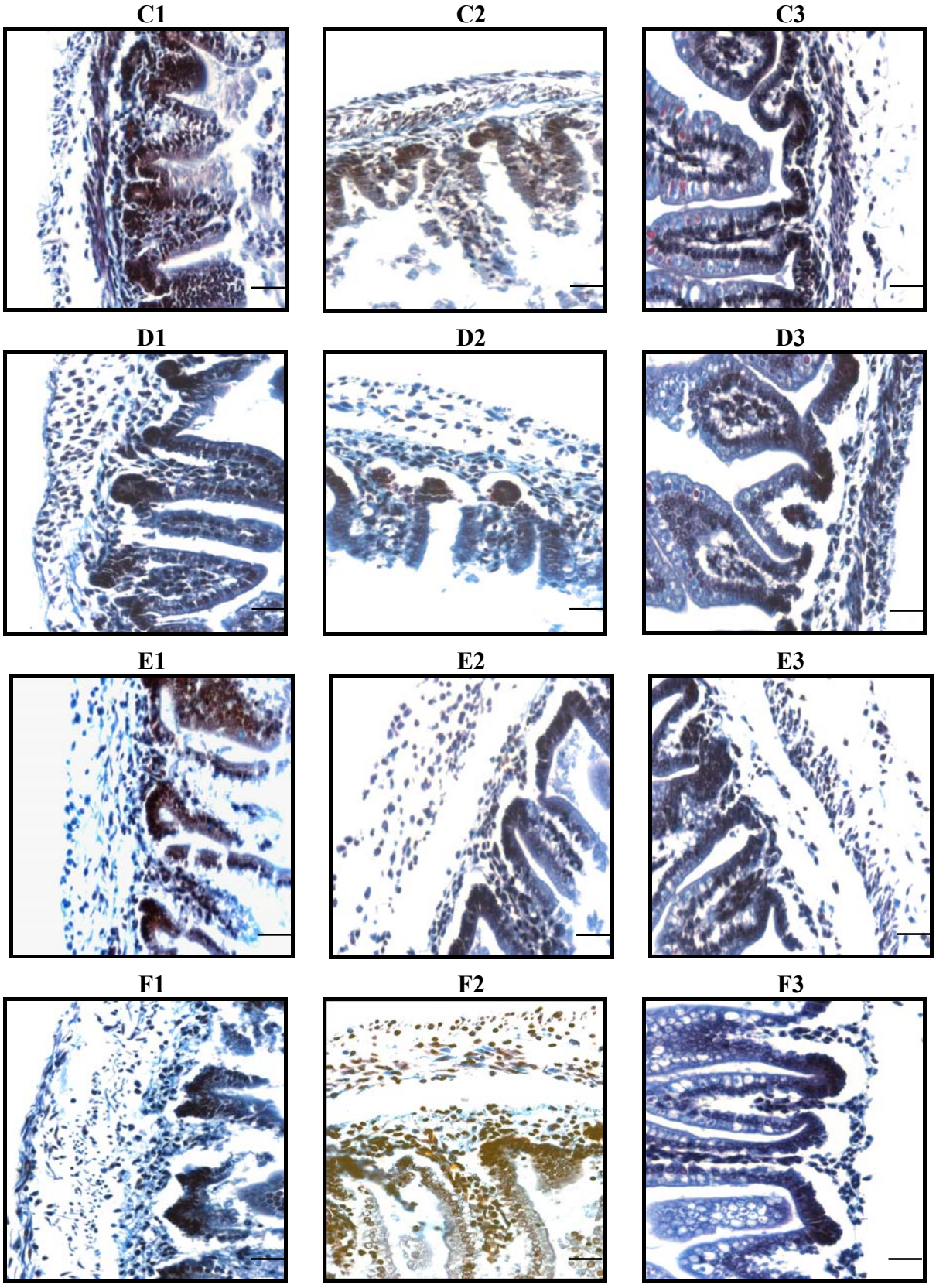


Figure 3.4.4: Representative micrographs illustrating histological images in different intestinal segments taken from the duodenum (1), the jejunum (2), and the ileum (3), following incubation with trypsin for 15 min (A), 30 min (B), 45 min (C), 60 min (D), 75 min (E), and 90 min (F). Scale bar = 100 µm.

3.4.3 DIFFERENTIAL ADHESION – MORPHOLOGICAL DESCRIPTION OF CELL TYPES

Morphologically, the cell population isolated from segments taken from the ileum of the neonates as described in section 3.3.2 consisted of ISMC, neurones and fibroblasts. Thus, techniques for purifying cells were adopted in order to separate different cell types and yield pure cultures of individual cell types.

The differential adhesion technique was primarily applied to minimise the number of fibroblasts in culture as explained in section 3.3.6. Cell attachment times were determined via time-lapse video microscopy, in which a frame was captured every 10 min. Monitoring of these cultures showed that fibroblasts attached first to the surface (after approximately 30 min), followed by smooth muscle cells (1-5h), and lastly, a major number of clusters of neurones started adhering after 5 h. Differential adhesion technique for separation of fibroblasts resulted in cocultures consisting of ISMC and neurones with uniform distribution of myenteric neurones among the ISMC (Figure 3.4.5a-c).

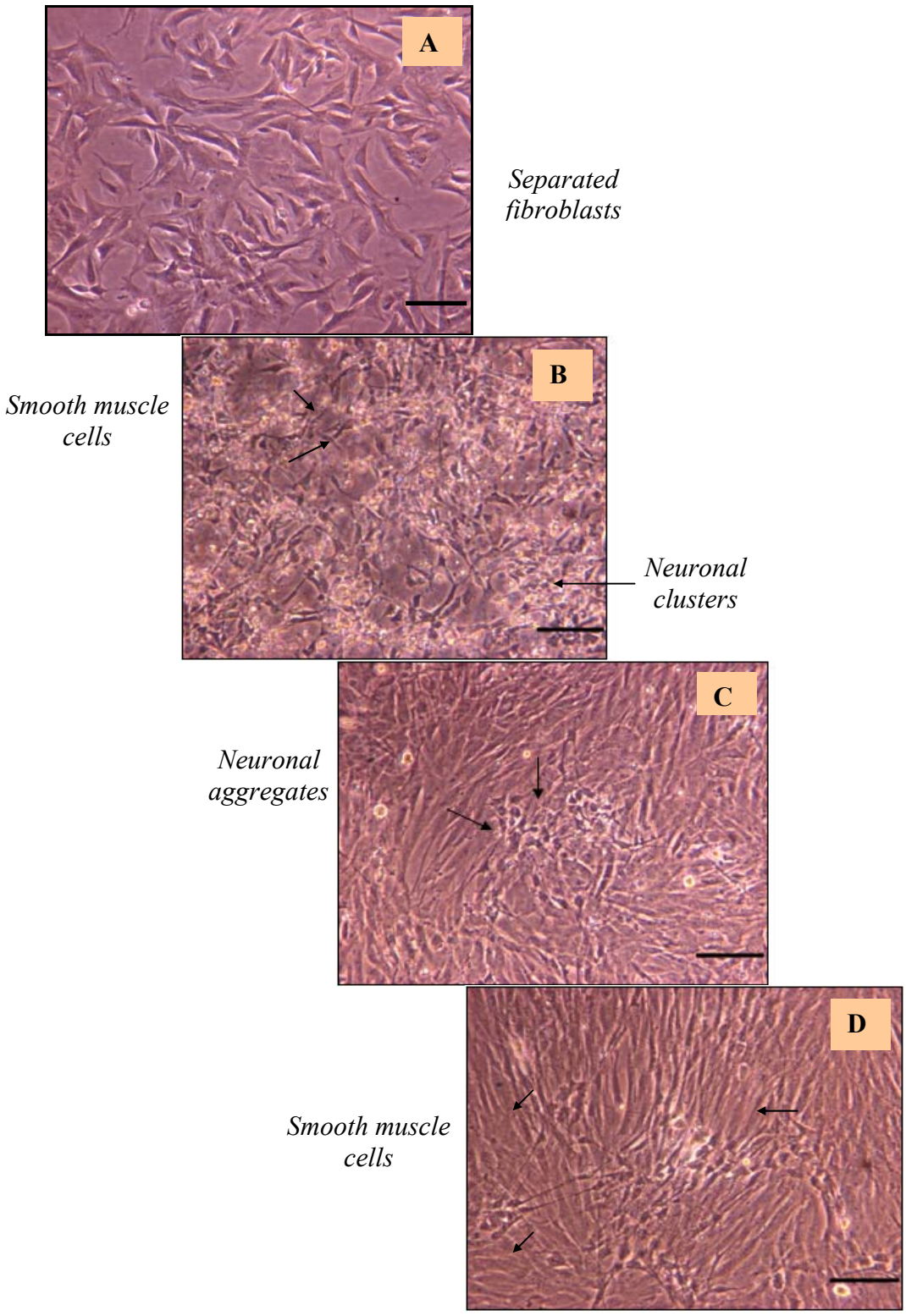


Figure 3.4.5: Representative micrographs illustrating the coculture of intestinal cells isolated from segments taken from the ileum of rat neonates. At day 1, after the separation of fibroblasts (A) through differential adhesion ISMC-like cells and rounded clusters of neurones (B) were observed. By day 2, ISMC developed into a relatively uniform layer throughout culture with intervening areas of characteristically developed myenteric neurones (C). By day 5, ISMC were arranged in multilayered regions with intervening neurones showing extending neurites (D). Scale bar=100μm.

3.4.4 PURIFIED ISMC

In the experiments that followed attempts were made to obtain pure ISMC preparations. Cells were obtained from segments taken from regions of the ileum and exposed to trypsin for 30 min. To obtain pure ISMC cultures, differential adhesion technique was employed primarily for removing fibroblasts. This was followed by incubation for 5h allowing attachment of sufficient numbers of ISMC to the surface of the flask (Figure 3.4.6a). The differential adhesion technique considerably decreased numbers of neurones in culture, although sparse clusters of neurones were observed throughout the culture of ISMC (Figure 3.4.6b). Morphological monitoring clearly demonstrated that treatment with scorpion venom (30 μg/ml) for 24h resulted in an increase in the proportion of ISMC in cultures (Figure 3.4.6c) compared to untreated cultures. Furthermore, treatment with scorpion venom (30 μg/ml) induced a selective neurotoxicity without affecting the ISMC. It was also observed that single dose treatment following the first cell cultivation was not sufficient to completely abolish the presence of neurones. It was hypothesised that the presence of neurones in cultures obtained from neonatal intestine is inevitable, since neuronal replication rates are extremely high at this stage of tissue development. Thus, upon confluence, cells were passaged and exposed to a second dose of scorpion venom (30 μg/ml) for 24h, which subsequently led to multilayered regions of spindle shaped ISMC with no apparent presence of neurones (Figure 3.4.6d).

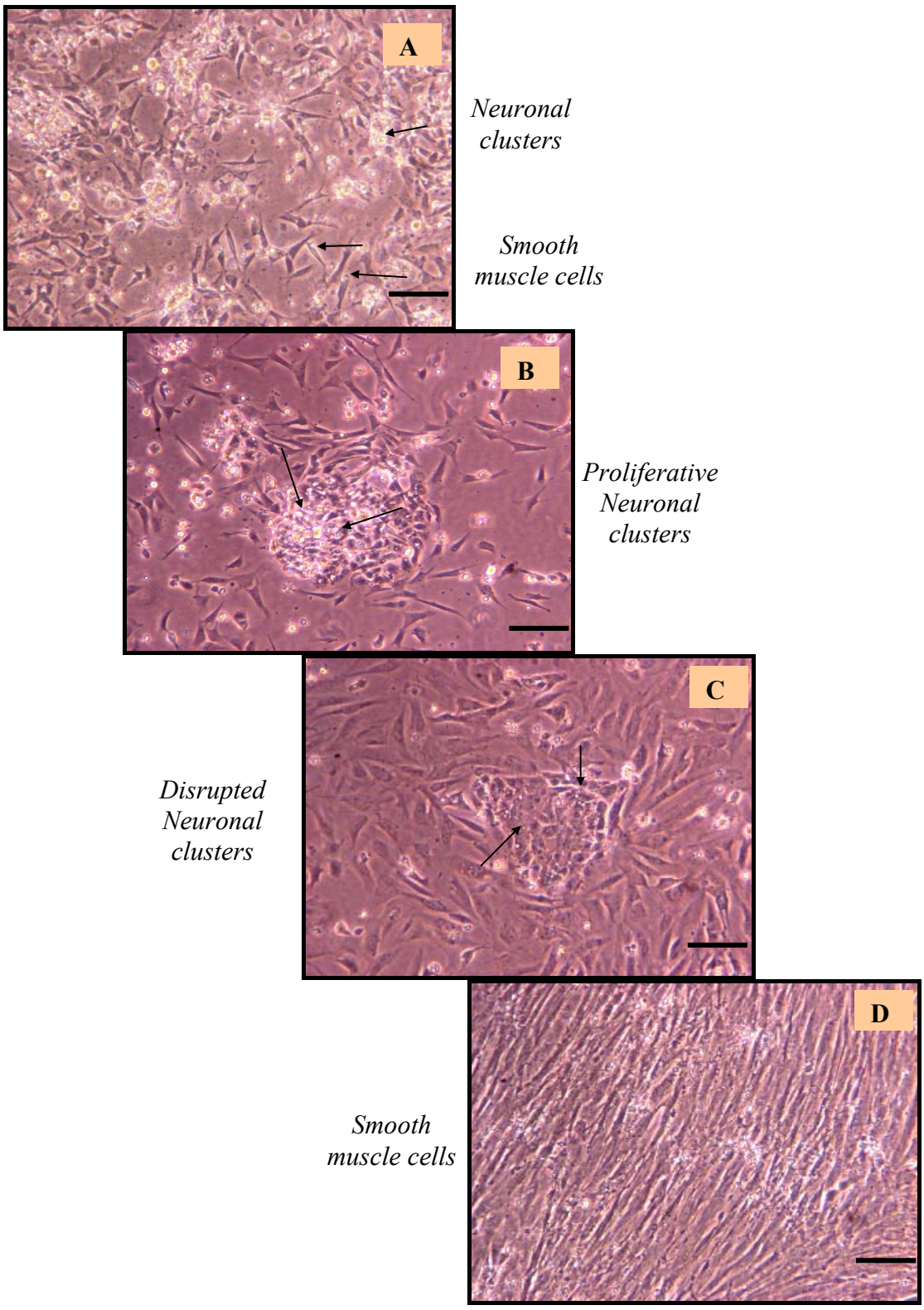


Figure 3.4.6: Representative micrographs showing cells isolated from segments taken from the ileum of rat neonates at day 1 (A) and following differential adhesion to minimise numbers of neurones and fibroblasts resulted in cultures of ISMC-like and few clusters of neurones (B). At day 2, following pre-treatment with scorpion venom (30 μg/ml) there was reduction in the number of neurones and increased proliferation of ISMC (C). Incubation with second dose of scorpion venom resulted in cultures displaying multilayered spindle-shaped cells, which were uniform throughout the culture (D). Scale bar=100μm.

3.4.5 PURIFIED MYENTERIC NEURONES

Attempts were made to obtain pure cultures consisting of neurones. Segments were taken from the ileum of rat neonates and trypsinised for 30 min. For purified neuronal cultures, remaining cells in culture (accessory cells) were significantly removed by a single treatment with antimitotic cytosine arabinoside (6 μM) for 72h resulting in the formation of complex network of neurones by day 7 (Figure 3.4.7a-c).

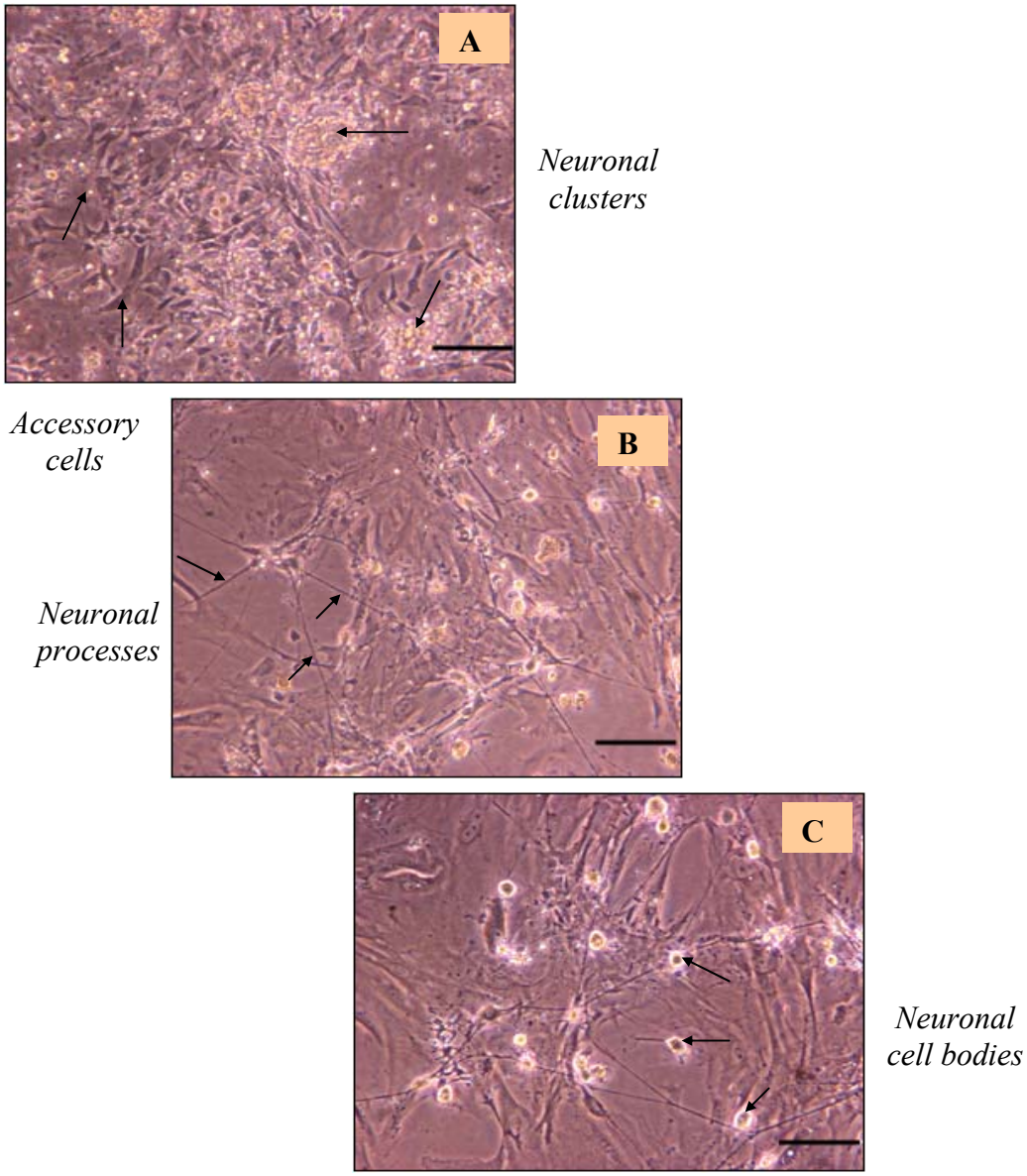


Figure 3.4.7: Representative micrographs showing mixed population of cells isolated segments taken from the ileum of rat neonates at day (A). Following a single treatment with cytosine arabinoside (6 μM) non-neuronal cells growth was significantly reduced and neurones start to develop processes (B). By day 7, the neuronal cells had enlarged cell bodies with processes that extended throughout the culture (C). Scale bar=100μm.

3.4.6 IMMUNOCYTOCHEMISTRY

Experiments were carried out to further validate the purity of the cultured cells. Dual-label immunocytochemistry on cocultures showed clusters of myenteric neurones adjacent to ISMC, which were identified by positive staining with antibodies to serotonin-$5HT_3$ receptors and α-smooth muscle actin, respectively (Figure 3.4.8a). Purified ISMC cultures stained positive to α-smooth muscle actin displaying multilayered regions of actin filaments and negative to antibodies raised against $5HT_3$ receptors confirming the absence of neurones (Figure 3.4.9a-b). Similarly, purified myenteric neurones stained positively to anti $5HT_3$ receptors antibodies in the large cell bodies and extended neurites, but negatively to α-smooth muscle actin (Figure 3.4.10a). Additional immunocytochemistry included staining with anti α-actinin antibodies, which also stained positive on the ISMC cultures, equally showing multilayered regions of actinin filaments (Figure 3.4.11a-b).

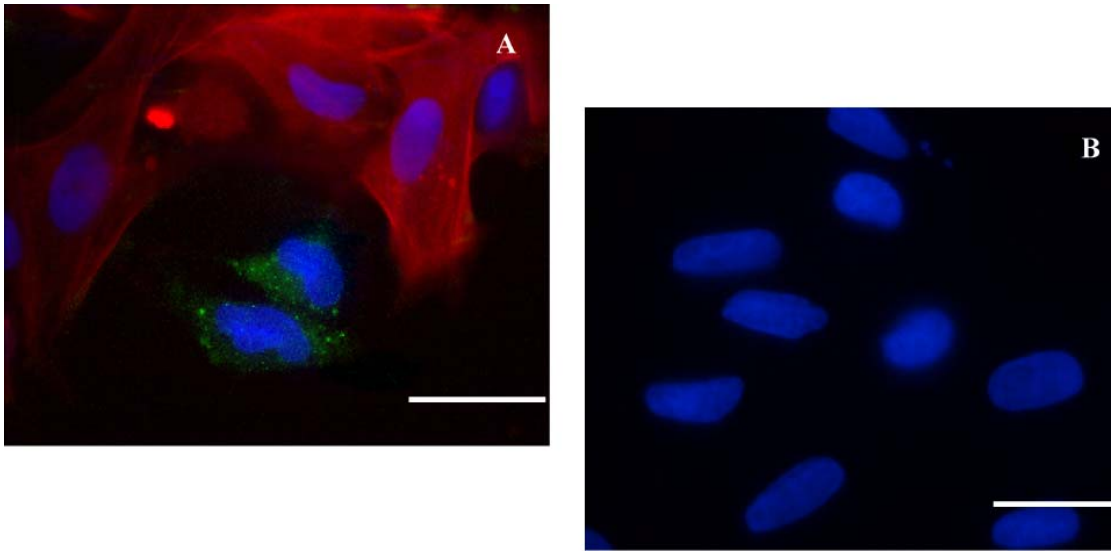


Figure 3.4.8: Representative micrographs of coculture preparations isolated from segments taken from the ileum of rat neonates with dual-label staining directed to $5HT_3$ receptors (green) and α-smooth muscle actin (red) antibodies for characterization of neurones and ISMC respectively. DAPI mount was used for labeling cell nucleus (blue). Positive diffusion of the $5HT_3$ receptors antibody within the neuronal cell bodies opposed by neighbouring cells identified as ISMC (A). Negative controls showed no staining to both primary antibodies (B). Scale bar=25μm.

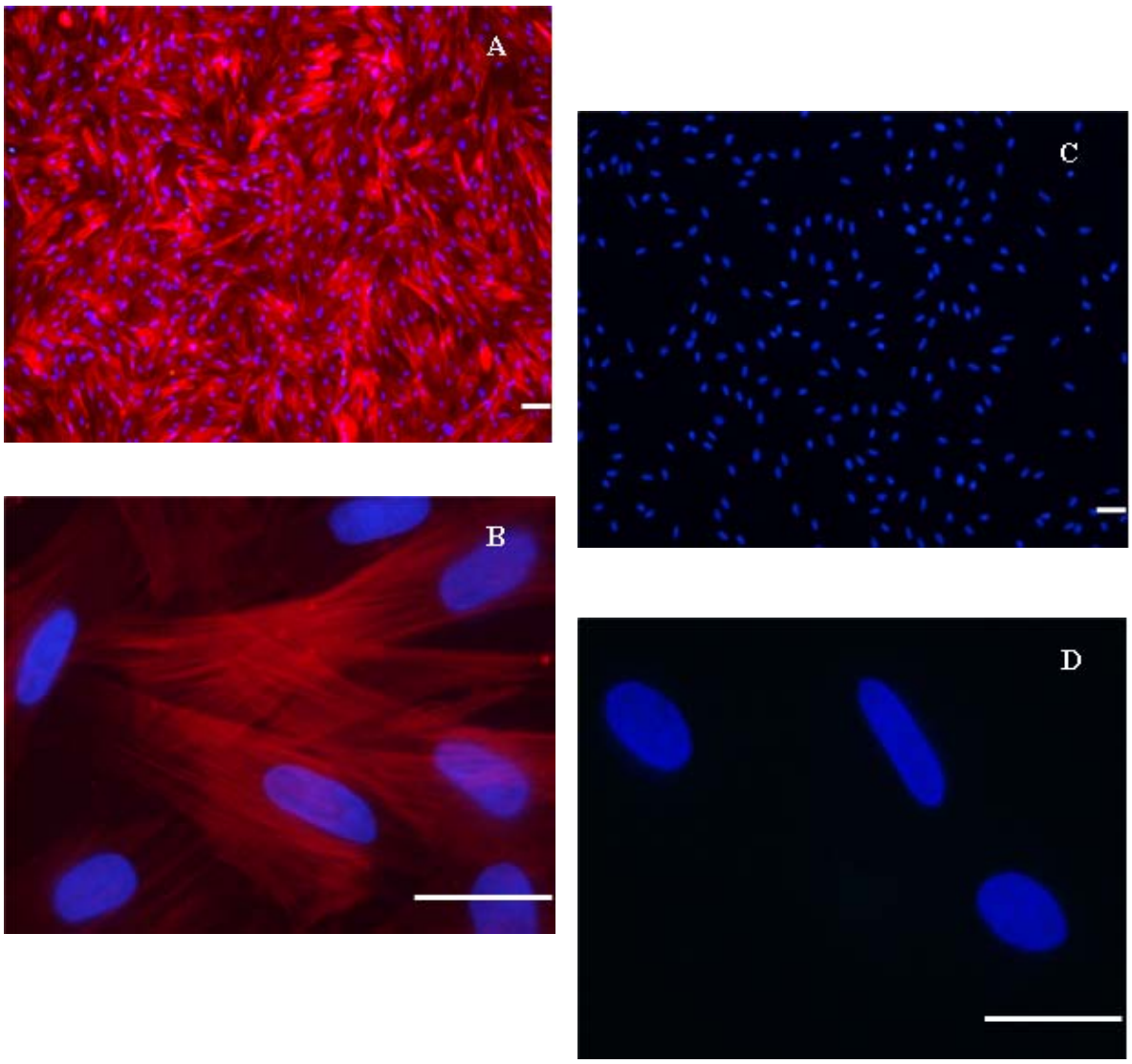


Figure 3.4.9: Representative micrographs of ISMC preparations isolated from segments taken from the ileum of rat neonates with dual-label staining directed to $5HT_3$ receptors (green) and α-smooth muscle actin (red) antibodies for confirmation of the nature of the purified ISMC. DAPI mount was used for labeling cell nucleus (blue). Uniform ISMC actin filaments stained positive throughout the culture (A, B). Negative labeling to $5HT_3$ receptors antibody confirmed the absence of the neurones in the preparation (A, B). Negative controls showed no staining to both primary antibodies (C, D). Scale bar=25μm

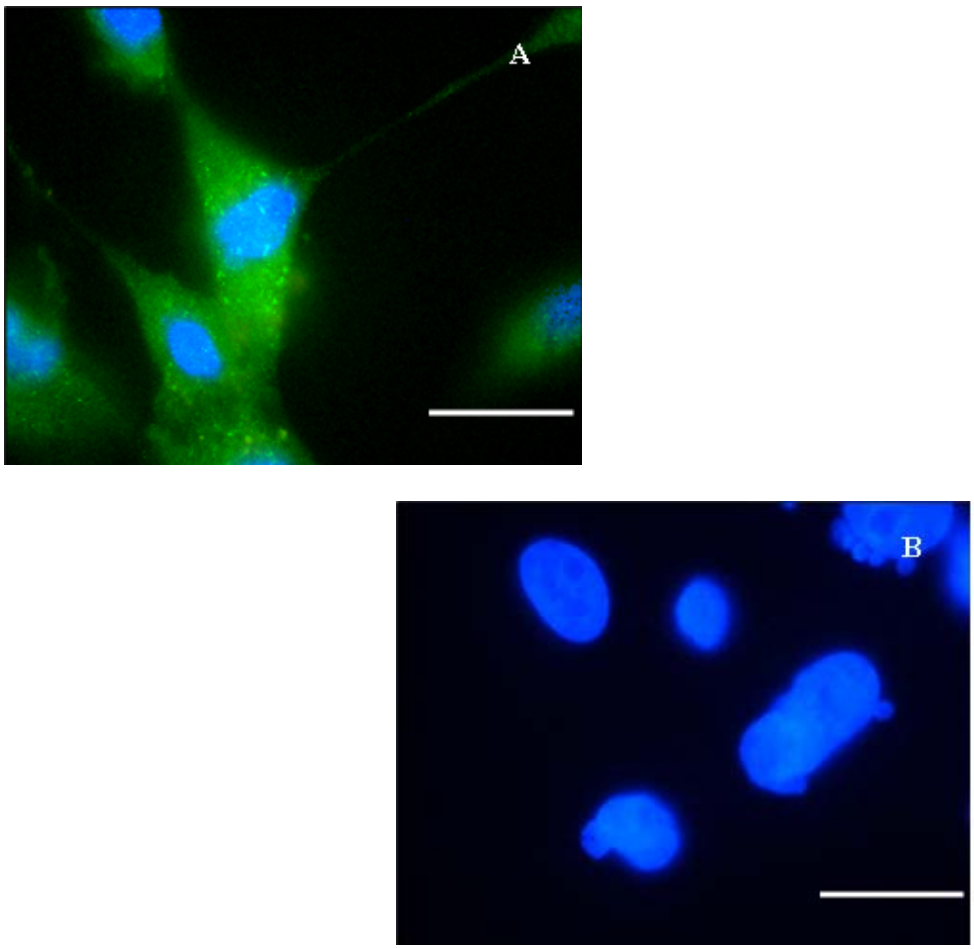


Figure 3.4.10: Representative micrographs of neuronal preparations isolated from segments taken from the ileum of rat neonates with dual-label staining directed to $5HT_3$ receptors (green) and α-smooth muscle actin (red) antibodies for confirmation of the nature of the purified neurones. DAPI mount was used for labeling cell nucleus (blue). Neuronal cell bodies and extended neurites stained positive to $5HT_3$ receptors antibody (A). Negative immunoreactivity to α-smooth muscle actin antibody confirms the absence of the ISMC in the preparation (A). Negative controls showed no staining to both primary antibodies (B). Scale bar=25μm.

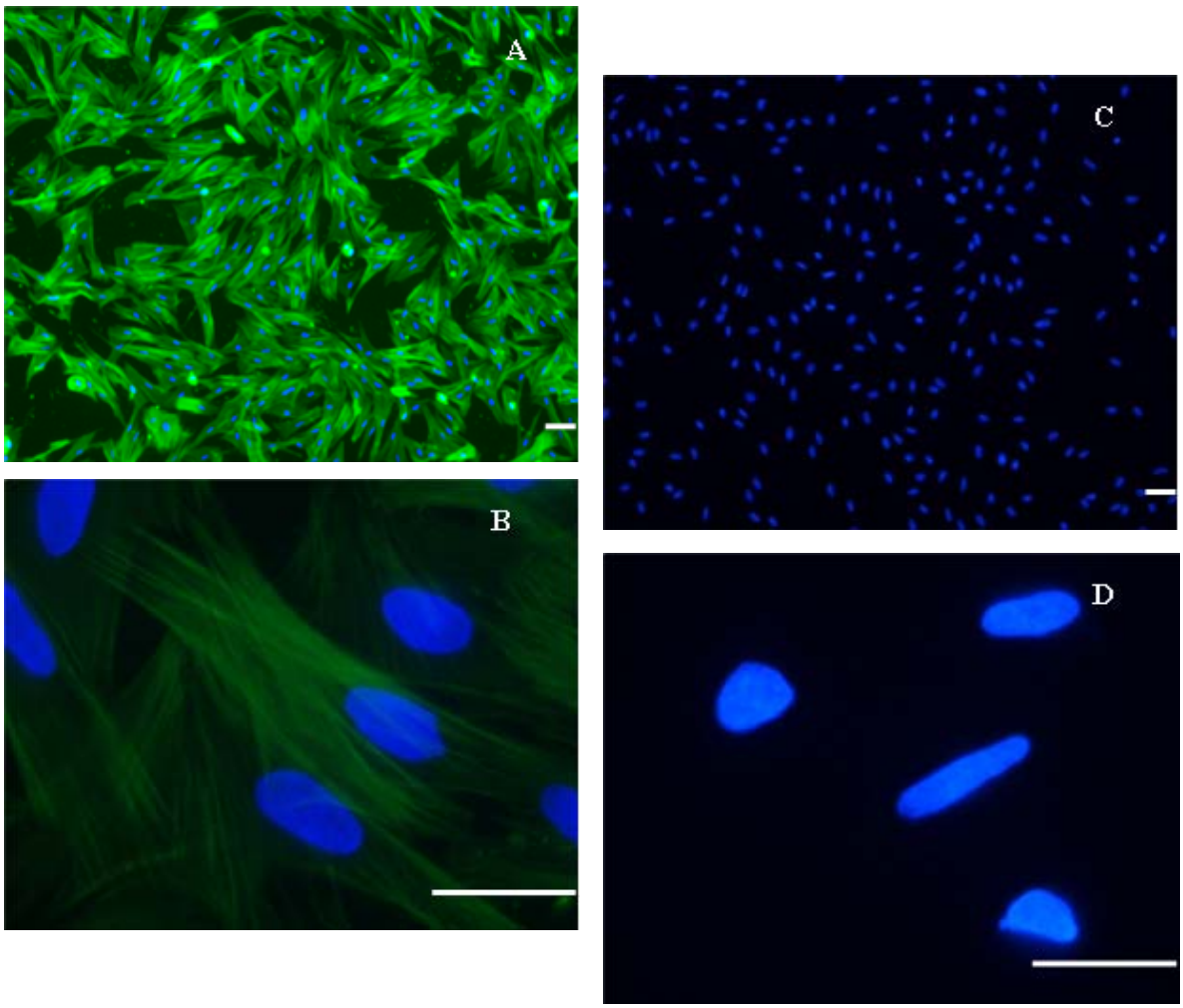


Figure 3.4.11: Representative micrographs of ISMC preparations isolated from segments taken from the ileum of rat neonates stained with smooth muscle actinin antibodies (green) for confirmation of the nature of the purified ISMC. DAPI mount was used for labeling of cell nucleus (blue). Uniform ISMC actinin filaments stained positive throughout the culture (A, B). Negative controls showed no staining to primary antibodies (C, D). Scale bar=25μm.

3.4.7 CRYOPRESERVATION

The aim of these experiments was to investigate cell viability following different cryopreservation procedures. The trypan Blue dye exclusion assay indicated that cell viability in all different groups was >70%. However, although cells are viable after freezing, this does not guarantee the genomic functionality, which was subsequently assessed by determining the degree of genomic damage induced by freezing using the comet assay. The comet assay revealed that some cells displayed a uniform rounded shape with no apparent genomic damage since no migration of DNA fragments from the nucleus was visible (Figure 3.4.12a). These observations were made in fresh cell preparations (control) and cells cryopreserved using a slow cooling protocol. However, when cells were rapidly frozen, upon thawing the comet assay revealed cells resembling a 'comet' with a distinct head and tail. The head was composed of intact DNA, while the long comet tails were indicative of significant genomic damage, which migrated from the nucleus following electrophoresis (Figure 3.4.12b). The percentage of damaged DNA was calculated by comparing the tail moment of migrated DNA tail in those controls and cryopreserved samples.

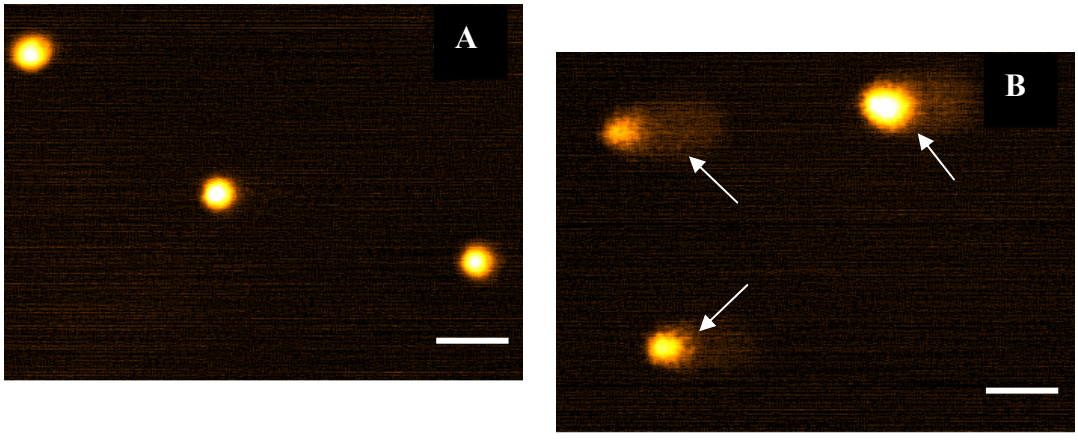


Figure 3.4.12: Micrographs representing images obtained from the comet assay. Unfrozen cell preparations (control) isolated from segments taken from the ileum of rat neonates revealed intact cell nuclei with no evidence of DNA damage (A). Images obtained from cryopreserved ISMC via rapid cooling are clear evidence of a comet with a head composed by intact DNA, while the extent of DNA liberated referred as the 'tail' (arrows) demonstrates the degree of DNA damage displayed by cells (B). Scale bar = 25 µm.

In the unfrozen fresh samples (control), the degree of DNA damaged assessed in the ISMC was 2.34±0.35 (mean olive tail moment for DNA damage±s.e.mean). This was significantly ($p<0.001$) less than the extent of damage in the groups that underwent cryopreservation using protocol 1, where vials containing the cells were directly placed in a -80ºC freezer followed by liquid nitrogen storage, which showed an overall mean of 10.15±0.72 damaged cells. However, ISMC that were cryopreserved via protocol 2, where cells were gradually cooled by incubations at room temperature (15 min), on ice (20 min), -80 freezer (overnight) and liquid nitrogen showed a degree of DNA damage (2.62±0.36), which was not significantly different compared to the controls (2.34±0.35) (Figure 3.4.13a). Thus, protocol 2 was subsequently used to evaluate the effect of long-term freezing on ISMC and revealed that cryopreservation for 1 week (2.62±0.36) and 1 month (3.81±0.72) results in minimal DNA damage compared to the control (2.34±0.35). Moreover, cryopreservation for 4 months (5.1±0.9) induced a significant ($p<0.05$) increase in the degree of DNA damage exhibited by ISMC as compared to the control (2.34±0.35) (Figure 3.4.13b).

These observations demonstrated a strong positive linear correlation ($R=0.977$) between the time length of cryopreservation and the genomic damage exhibited by cells. Thus, as length of cryopreservation increases a very slow but gradual genomic damage is imposed on ISMC.

Qualitative analysis of the effects of freezing on ISMC in culture was generated by micrographs comparing the morphology of cultured ISMC from controls (Figure 3.4.14a) and cultured ISMC following cryopreservation (Figure 3.4.14b). Both cultures showed distinctive ISMC characteristics of spindle-like cells, which grew on top of each other to form multiple layers throughout the culture. No apparent morphological differences were observed.

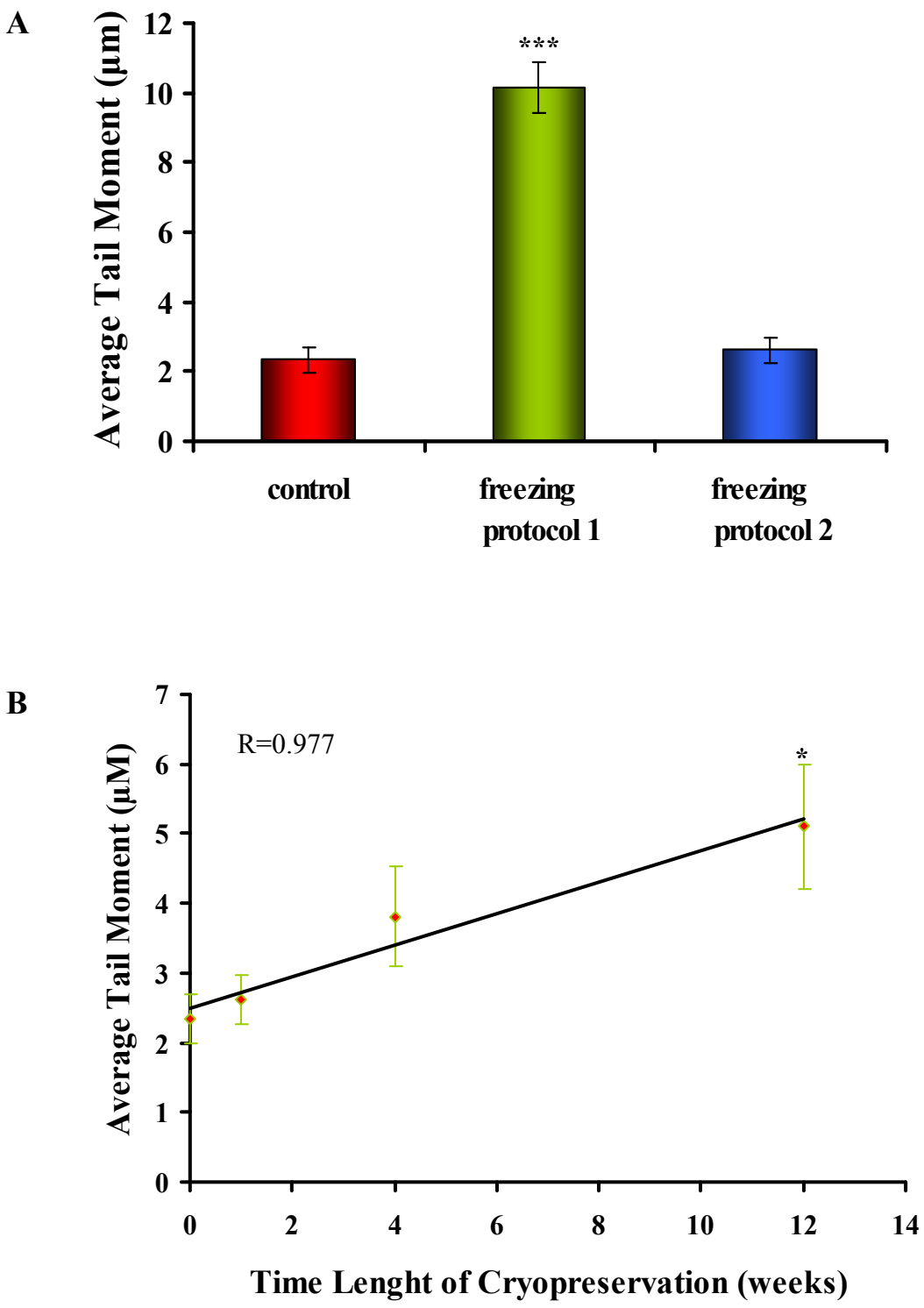


*Figure 3.4.13: Mean average tail moment correspondent to the genomic damage exhibited by freshly isolated ISMC from segments taken from the ileum of rat neonates (control) and ISMC exposed to different cooling rates via protocol 1 and 2 (A). The genomic damage induced by long-term cryopreservation on ISMC (0 weeks, 1 week, 1-month, and 4-months). The intercept indicating a linear relationship between length of cryopreservation and genomic damage exhibited (B). Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, and p***<0.001 taken as significant differences in the group.*

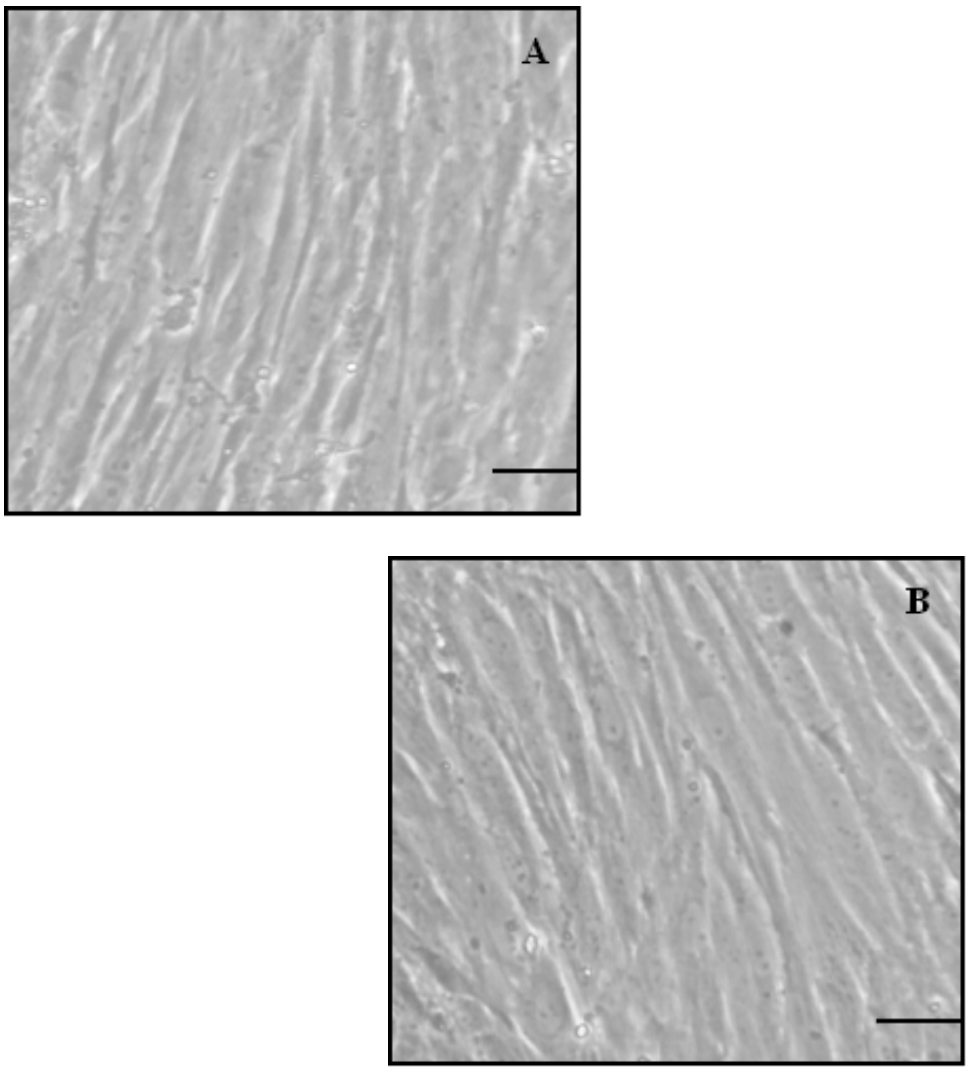


Figure 3.4.14: Micrographs showing comparative images of unfrozen cultured ISMC isolated from segments taken from the ileum of rat neonates (A) and cultured ISMC following cryopreservation procedure for 1-week (B). Both cultures displayed spindle shaped cells characteristic of ISMC, with no apparent morphological changes. Scale bar = 25μm.

3.4.8 THE EFFECT OF KCl, 5-HT, AND CARBACHOL ON THE ISMC PREPARATIONS CULTURED ON PLASTIC SURFACE

Experiments were carried out to investigate functionality of the ISMC cultured models. The non-cumulative addition of KCl (0.03-30mM), 5-HT (0.03-100nM) and carbachol (0.003-3μM) induced concentration dependent contractions at the varying contact times of 0-300 sec (Figure 3.4.16a, 3.4.17a, 3.4.18a). The contractile responses or changes in tension of the cells were characterised as changes in the cell dimensions and were quantified via gray-scale measurements captured by the ten-pixel points distributed around the inner border of the cell over the different time frames (Figure 3.4.15). Maximum contractions were observed at 10mM, 100nM, 1μM for KCl, 5-HT, and carbachol respectively. The maximum contraction observed in single ISMC in response to KCl, 5-HT, and carbachol produced responses in the range of 14±2.0 (gray-scale units±s.e.mean), 7.0±1.0, and 3.0±1.0 respectively.

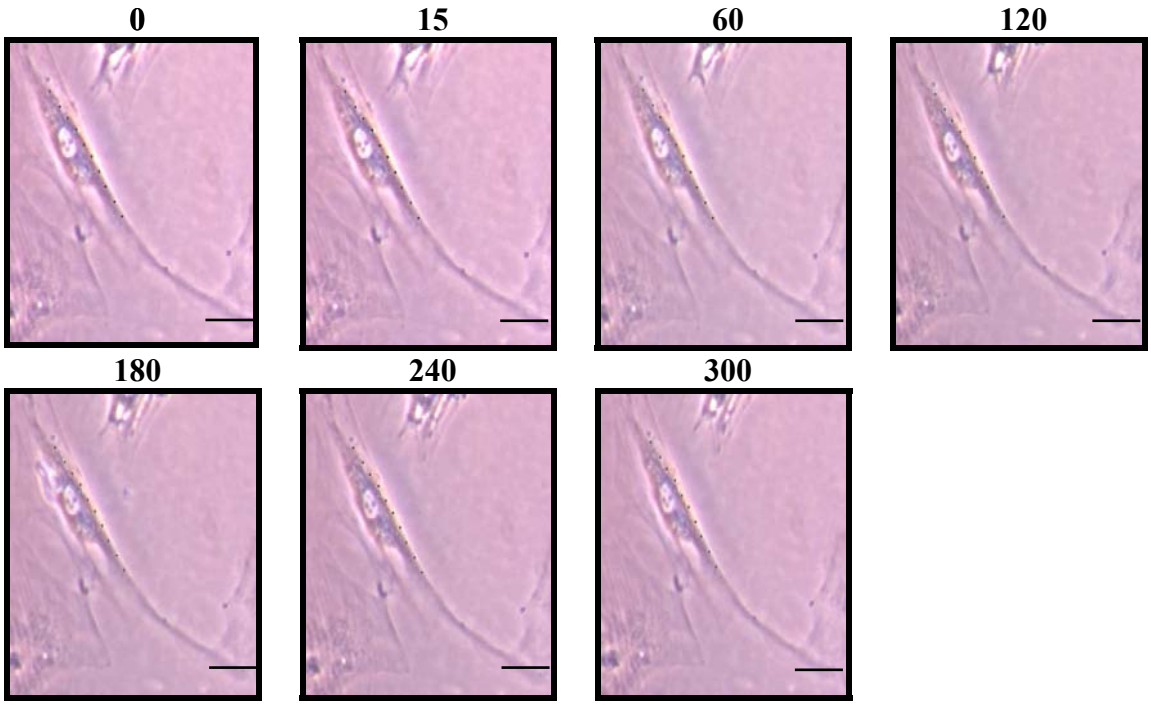


Figure 3.4.15: Representative micrographs illustrating responses induced by (carbachol (300nM) on ISMC isolated from segments taken from the intestine of rat neonates. The response was measured from before, 15, 60, 120, 180, 240, and 300 sec respectively, after application of the drug. Scale bar = 50μm.

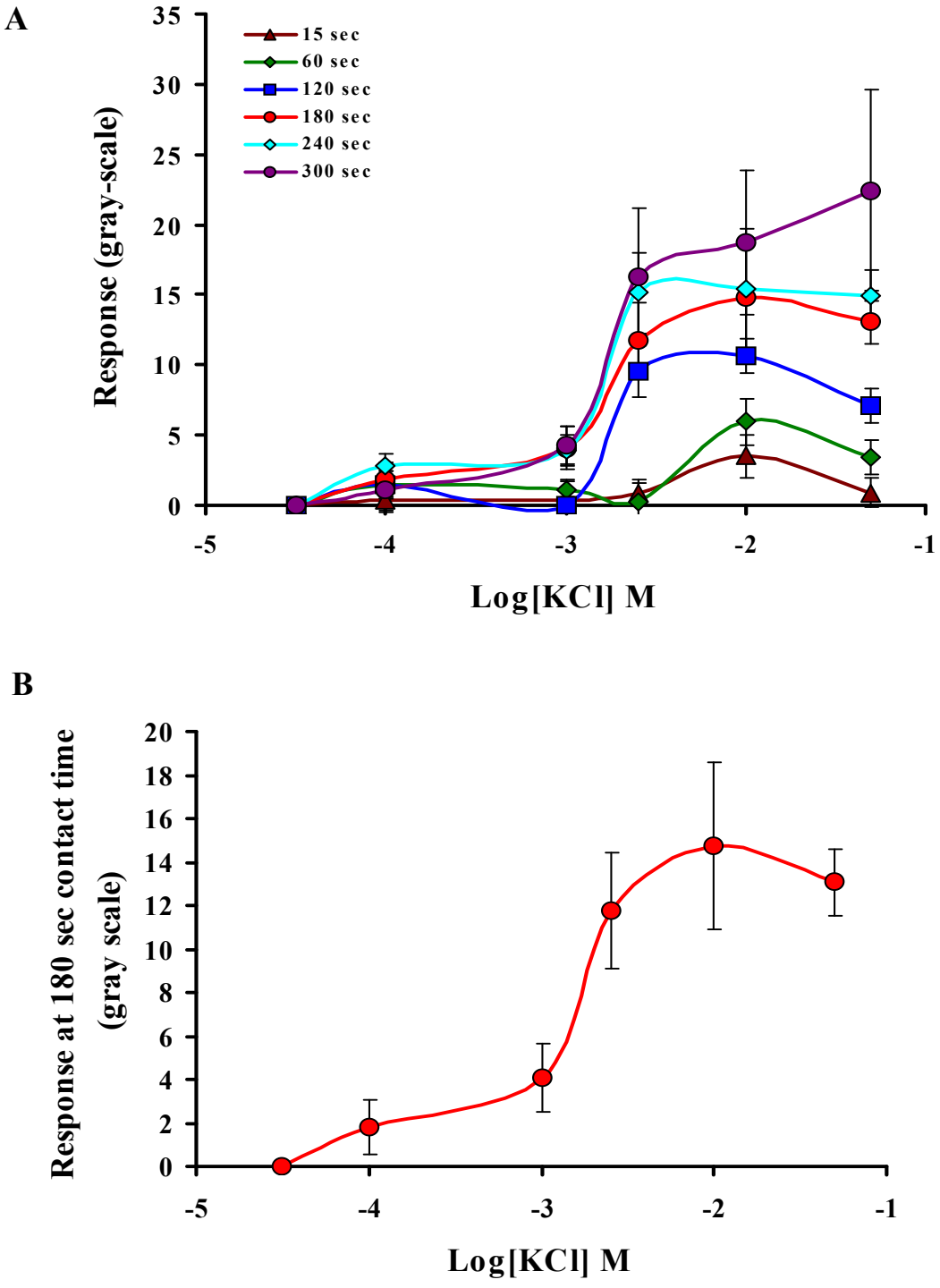


Figure 3.4.16: Contraction responses induced by KCl (0.03mM - 30mM) in the single ISMC preparations isolated from segments taken from the ileum of rat neonates. ISMC were exposed to KCl at different contact times (A). The concentration-response curve to KCl (0.03mM - 30mM) at 180 sec contact time (B). Each point represents the mean ± s. e. mean; n=4.

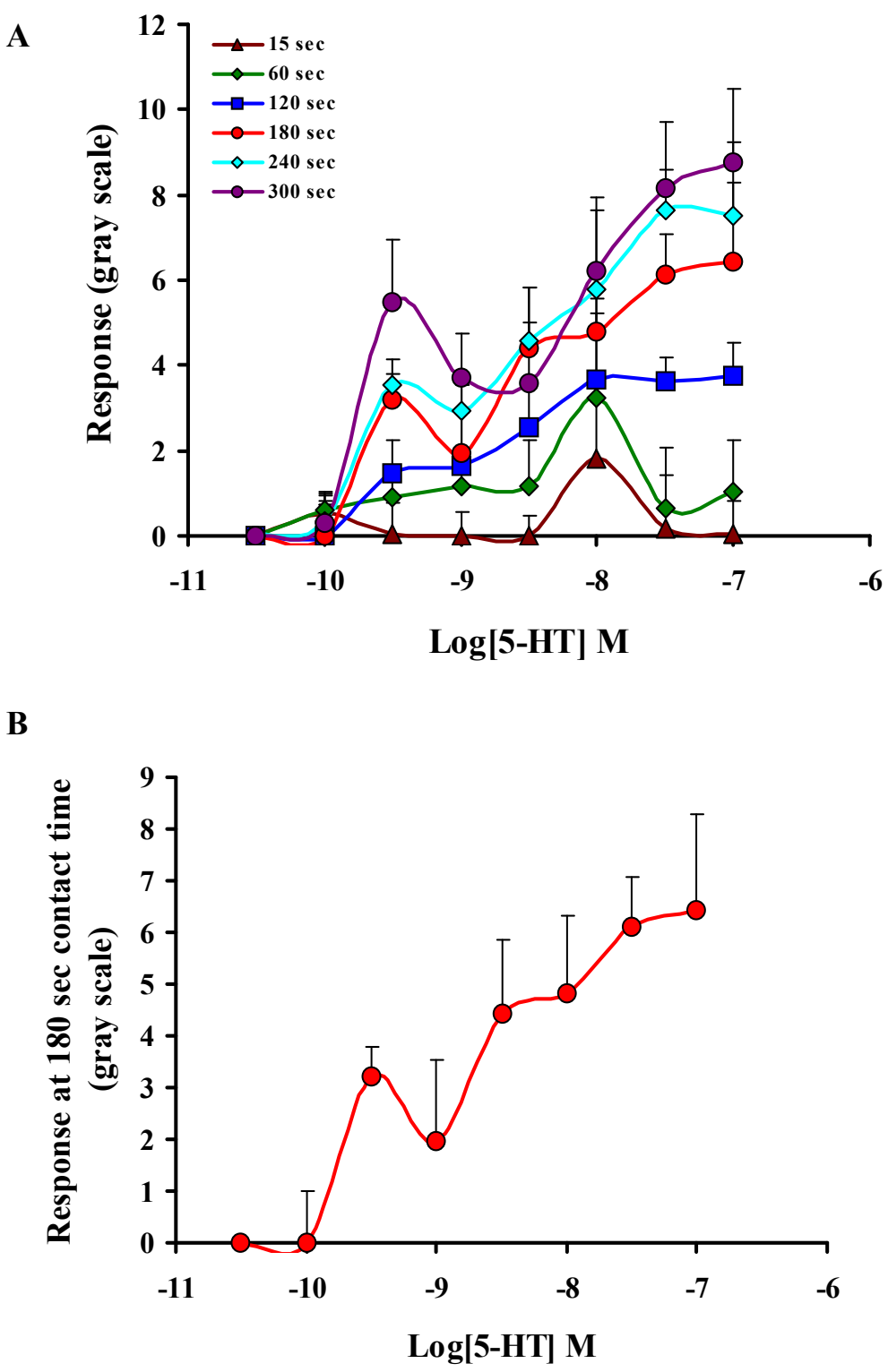


Figure 3.4.17: Contraction responses induced by 5-HT (0.03nM - 100nM) in the ISMC preparations isolated from segments taken from the intestine of rat neonates. ISMC were exposed to 5-HT at different contact times (A). The concentration-response curve to 5-HT (0.03nM - 100nM) at 180 sec contact time (B). Each point represents the mean ± s. e. mean; n=4.

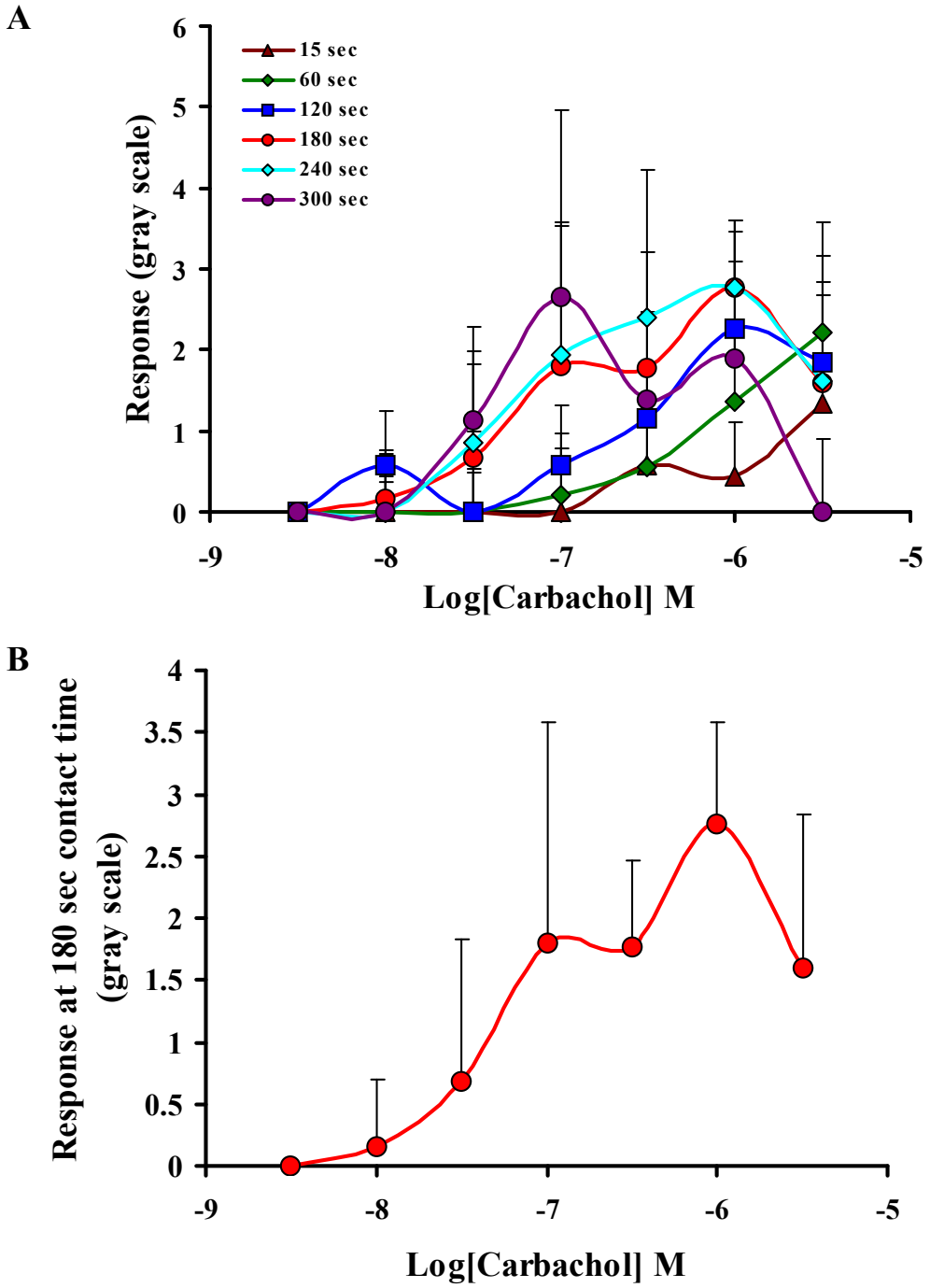


Figure 3.4.18: Contraction responses induced by carbachol (0.003µM- 3µM) in the ISMC preparations isolated from segments taken from the ileum of rat neonates. ISMC were exposed to carbachol at different contact times (A). Concentration- response curve to carbachol (0.003µM- 3µM) at 180 sec contact time (B). Each point represents the mean ± s. e. mean; n=4.

3.4.9 THE EFFECTS OF KCL, 5-HT, AND CARBACHOL ON THE ISMC PREPARATIONS CULTURED ON SYLGARD SURFACE.

Experiments were carried out to investigate functionality of IMSC on softer substrates. Results illustrated that in the silicone elastomer surface single ISMC were able to attach and proliferate in a similar manner as in the plastic surface. The flexibility of the silicone elastomer substrate caused cells to display a slightly shorter and rounded appearance compared to those seeded on plastic surfaces (Figure 3.4.19). Cells were isolated from segments taken from the ileum of rat neonates according to section 3.3.2 and non-cumulative concentration-response curves to KCl (1μM-3mM), 5-HT (0.03nM-30nM), and carbachol (0.1nM-100nM) were established. (Figure 3.4.20a, 3.4.21a, and 3.4.22a). In these experiments 120 sec was chosen for the representation of each drug dose cycle as it was repetitively the lowest contact time at which significant changes in cell responses was observed (Figure 3.4.20b, 3.4.21b, 3.4.22b). Maximum contractions were observed at 1mM, 100nM, 0.1μM for KCl, 5-HT, and carbachol respectively. The maximum contraction changes produced responses in the range of 10±2.0 (gray-scale units±s.e.mean), 4.0±1.0, and 13.0±5.0 respectively.

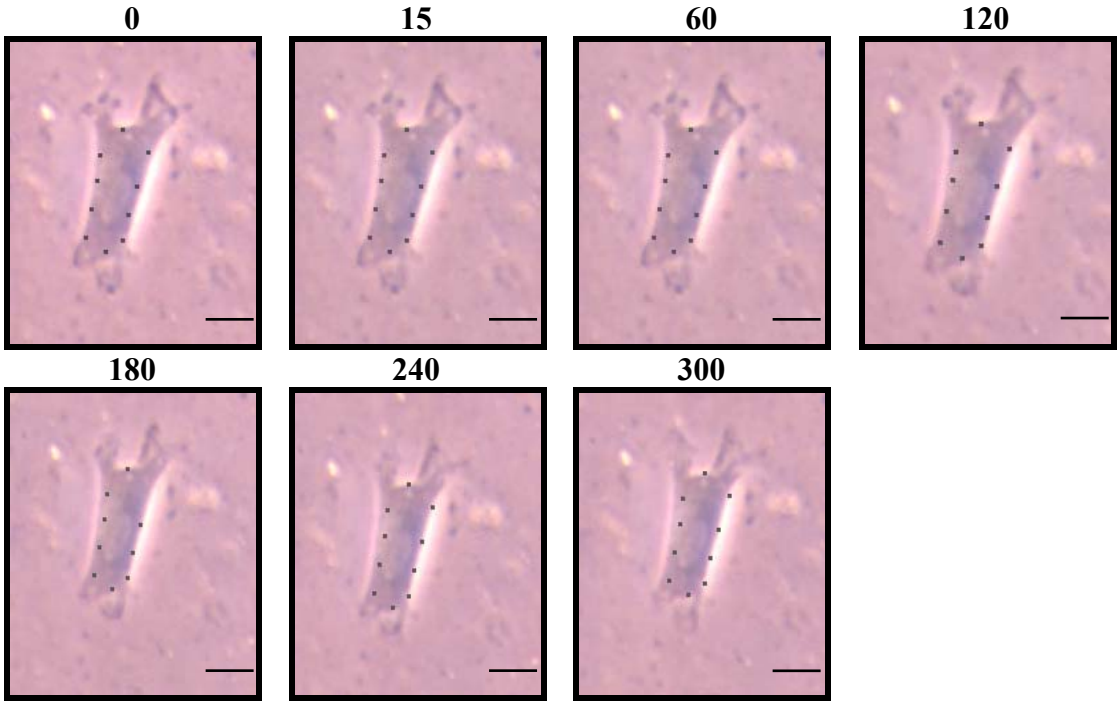


Figure 3.4.19: Representative micrographs illustrating responses induced by KCl (500µM) on ISMC isolated from segments taken from the intestine of rat neonates. The response was measured from before, 15, 60, 120, 180, 240, and 300 sec respectively, after application of the drug. Scale bar = 50µm.

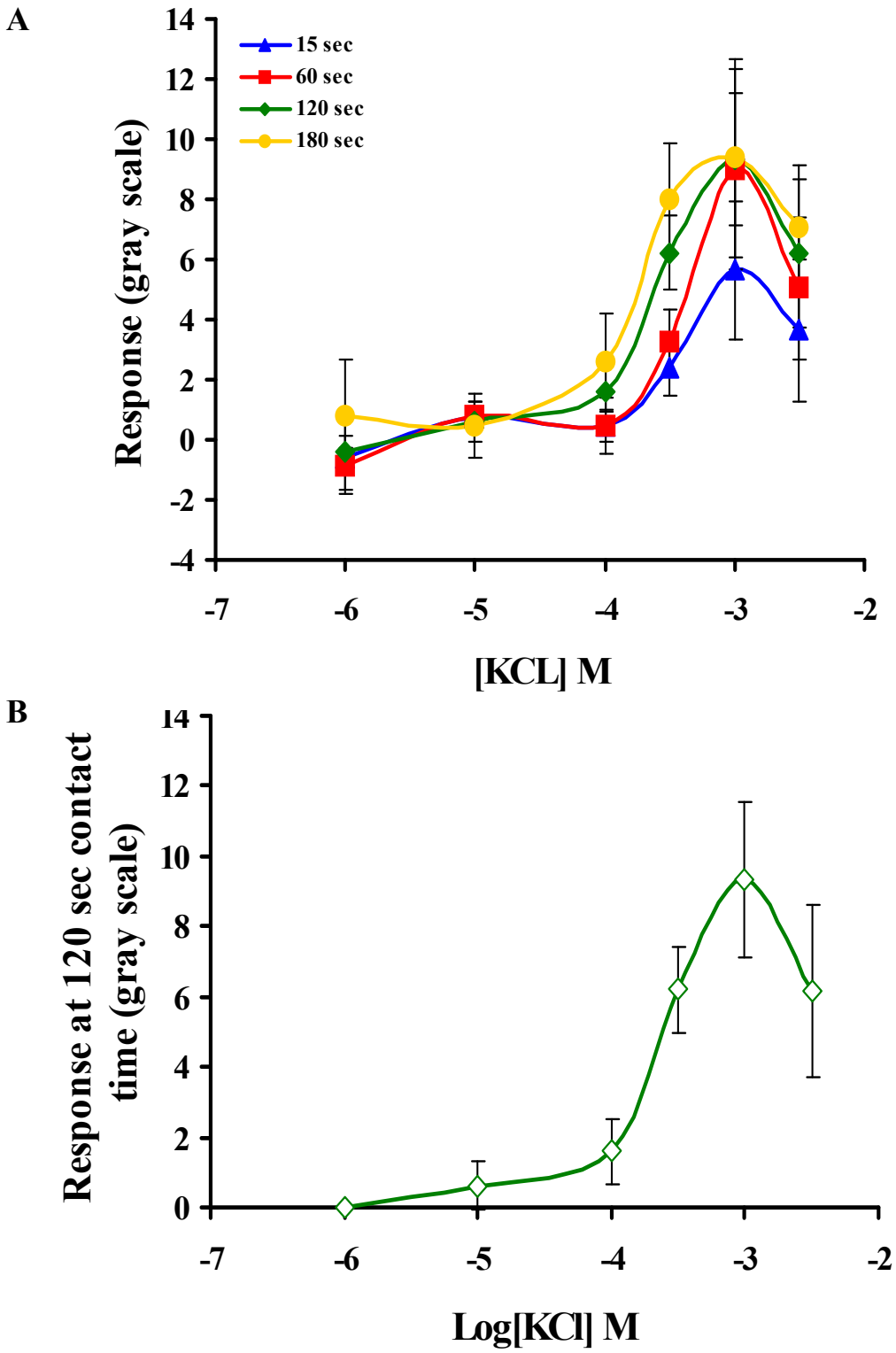


Figure 3.4.20: Contraction responses induced by KCl (1µM - 3mM) in the single ISMC preparations isolated from segments taken from the ileum of rat neonates. ISMC were exposed to KCl at different contact times (A). The concentration-response curve to KCl (1µM - 3mM) at 120 sec contact time (B). Each point represents the mean ± s. e. mean; n=4.

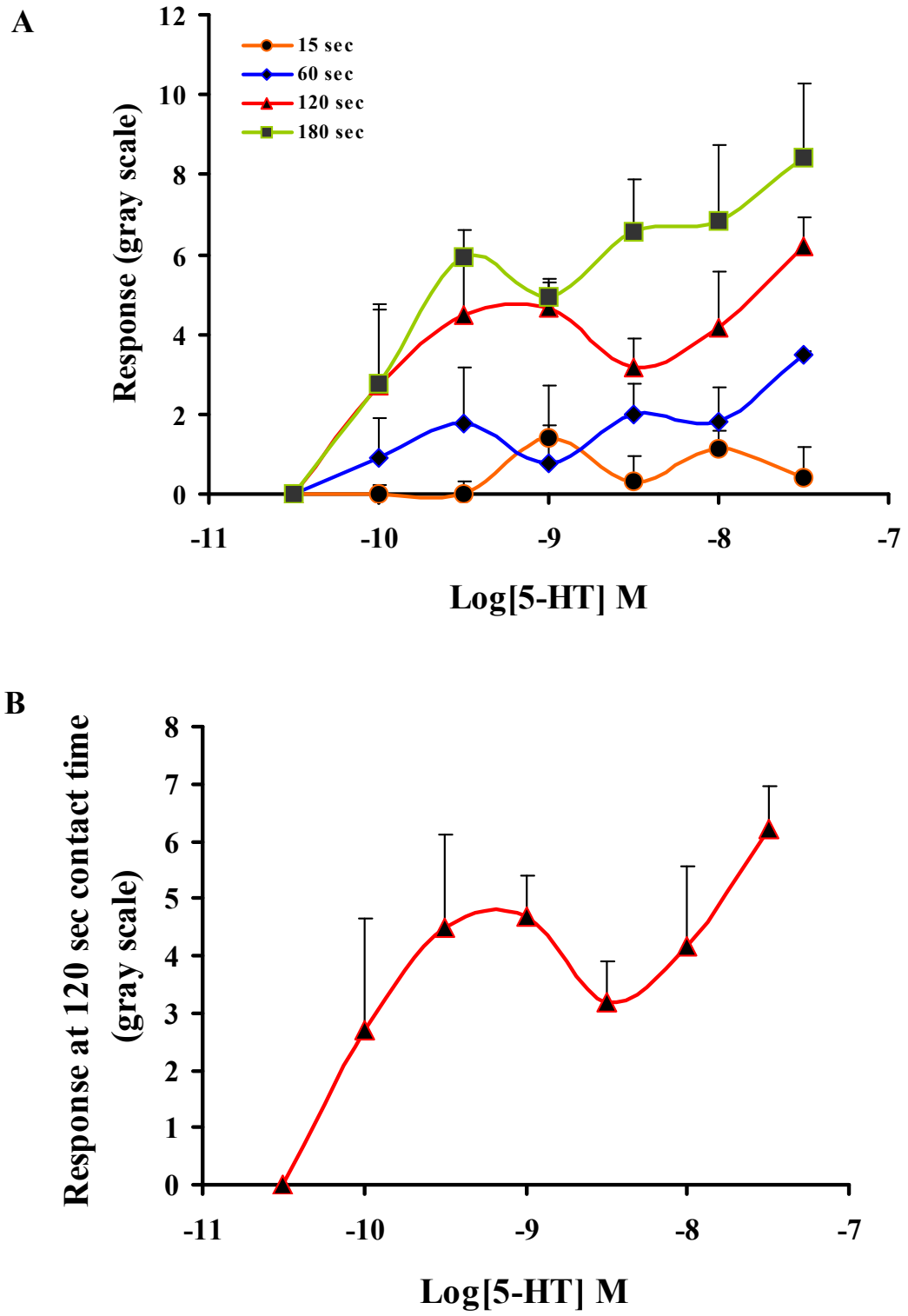


Figure 3.4.21: Contraction responses induced by 5-HT (0.03nM - 30 nM) in the ISMC preparations isolated from segments take from the ileum of rat neonates. ISMC were exposed to 5-HT at different contact times (A). The concentration-response curve to 5-HT curve to 5-HT (0.03nM - 30nM) at 120 sec contact time (B). Each point represents the mean ± s. e. mean; n=4.

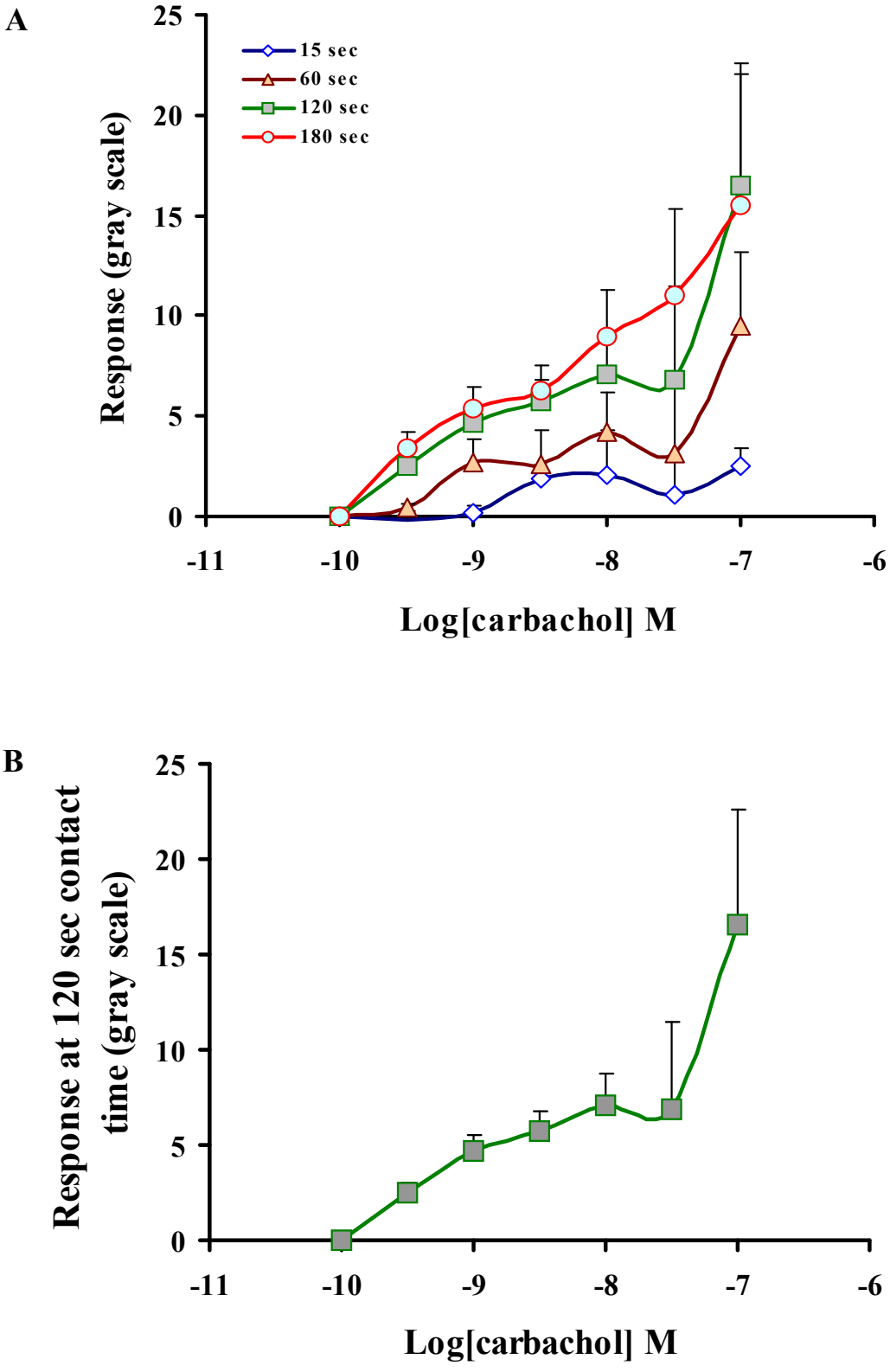


Figure 3.4.22: Contraction responses induced by carbachol (0.1nM- 100nM) in the ISMC preparations isolated from segments taken from the ileum of rat neonates. ISMC were exposed to carbachol at different contact times (A). The concentration-response curve to carbachol (0.1nM- 100nM) at 120 sec contact time (B). Each point represents the mean ± s. e. mean; n=4.

3.4.10 THE EFFECT OF NORADRENALINE ON THE RELAXATION OF ISMC CULTURED ON SYLGARD SURFACES

Separate experiments were carried out to investigate the relaxation effects of noradrenaline (1nM-10μM) on single ISMC isolated from segments of the ileum of rat neonates. The administration of noradrenaline caused the cells to display an 'expanded and swollen' appearance (Figure 3.4.23). The response to non-cumulative doses of noradrenaline produced concentration dependent relaxations (Figure 3.4.24a) that were equally visible following 120 sec contact time with the cell (Figure 3.4.24b).

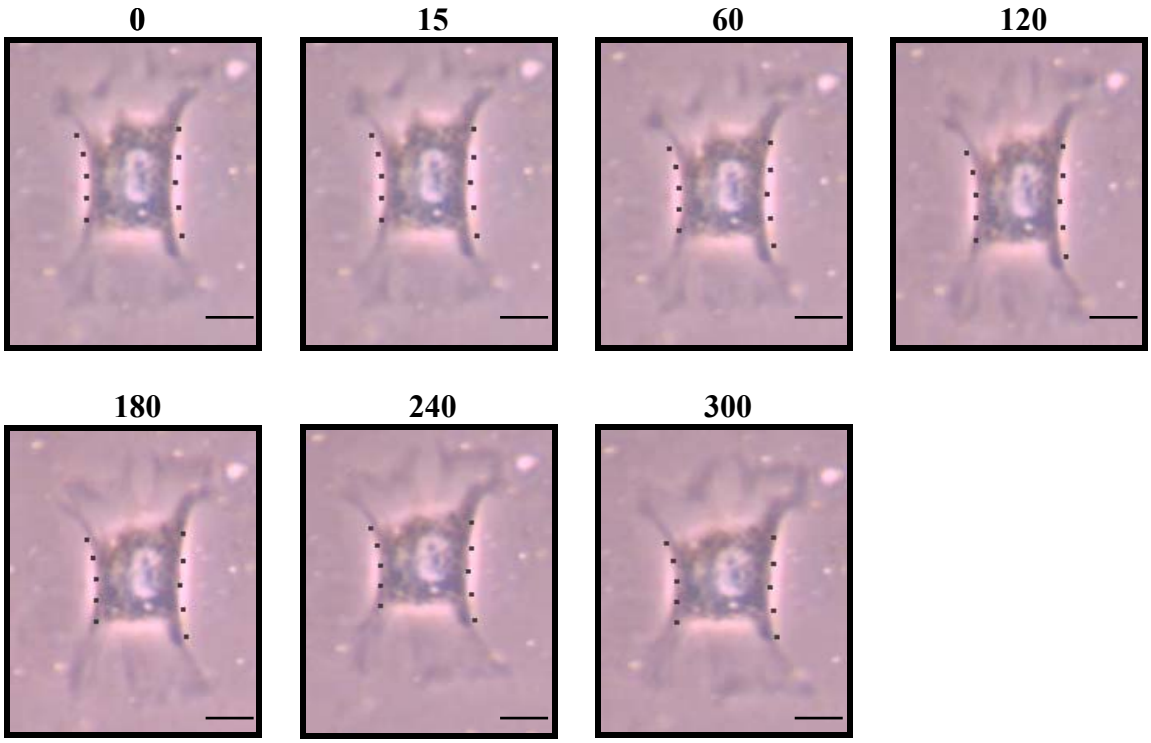


Figure 3.4.23: Representative micrographs illustrating responses induced by noradrenaline (300nM) on ISMC isolated from segments taken from the intestine of rat neonates. The response was measured from before, 15, 60, 120, 180, 240, and 300 sec respectively, after application of the drug. Scale bar = 50μm.

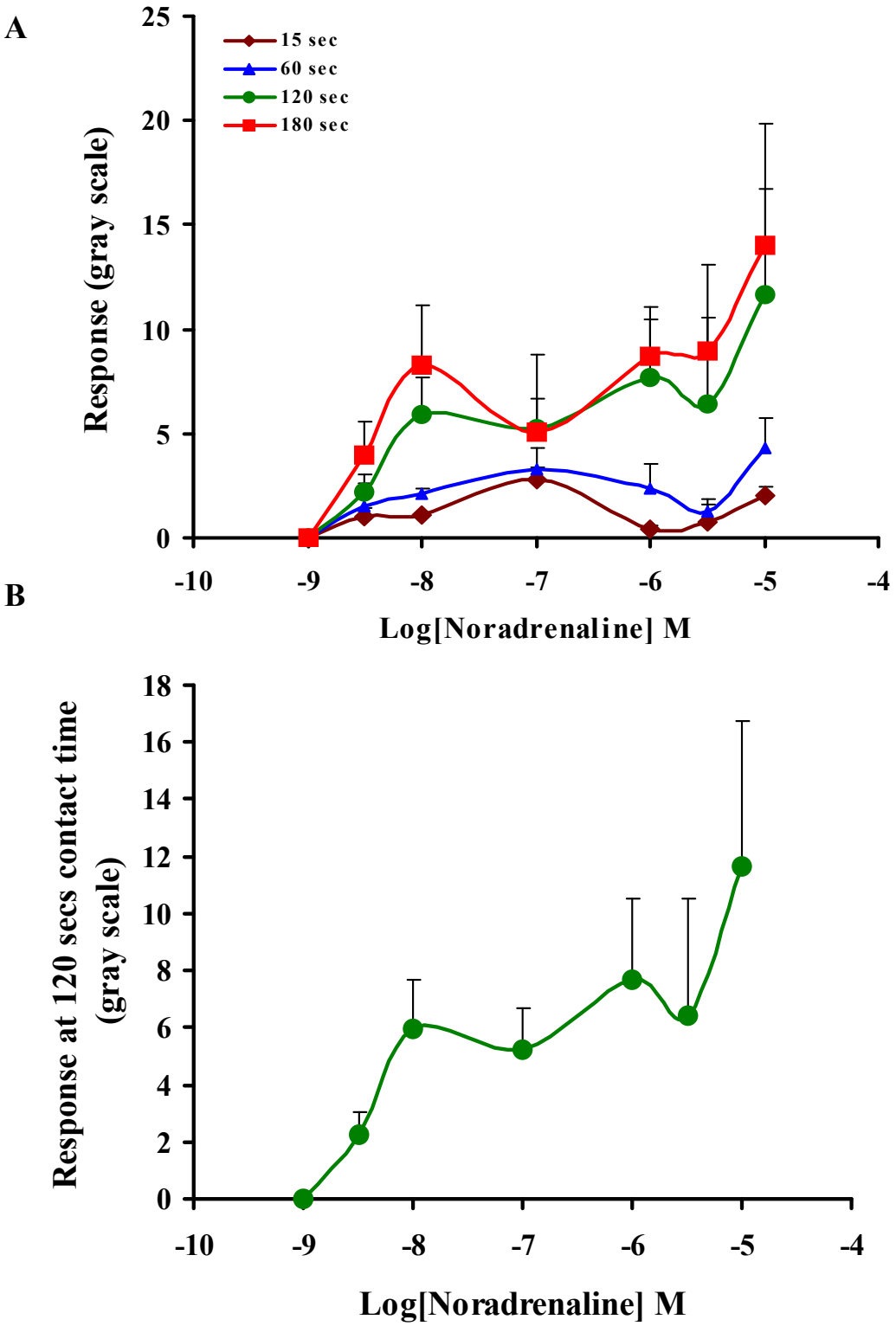


Figure 3.4.24: Relaxation responses induced by noradrenaline (1nM- 10μM) in the ISMC preparations isolated from segments taken from the ileum of rat neonates. ISMC were exposed to noradrenaline at different contact times (A) varying drug contact time during the cycle (A). The concentration-response curve to noradrenaline (1nM- 10μM) at 120 sec contact time (B).Each point represents the mean ± s.e. mean; n=4.

3.4.11 THE ABILITY OF ATROPINE TO MODIFY CARBACHOL INDUCED CONTRACTION ON ISMC PREPARATIONS CULTURED ON SYLGARD SURFACE

Experiments were carried out to investigate the ability of the cholinergic antagonist, atropine to modify the contractile responses induced by carbachol on ISMC. The contractile responses to carbachol (0.1nM-100nM) were constructed in the absence and presence of atropine (0.1μM). Results demonstrated that atropine (0.1μM) significantly ($p<0.05$) reduced the contraction induced by carbachol on ISMC at concentrations of 0.3nM and 3nM. Higher concentrations of carbachol in the presence of atropine induced maximal contractile responses that were comparable to those induced by carbachol alone (Figure 3.4.25a).

3.4.12 COMPARISON BETWEEN CUMULATIVE AND NON-CUMULATIVE DOSE-RESPONSE CURVES TO CARBACHOL IN ISMC PREPARATIONS CULTURED ON SYLGARD SURFACE

Non-cumulative doses of carbachol produced dose-related responses to carbachol (0.3nM-100nM). Maximum contractions of 13.0±5.0 were observed at a concentration 0.1 μM. However, cumulative addition of carbachol produced a rapid response, where the maximum contraction of 10.5±5.0 was observed at a concentration of 3nM (Figure 3.4.25b).

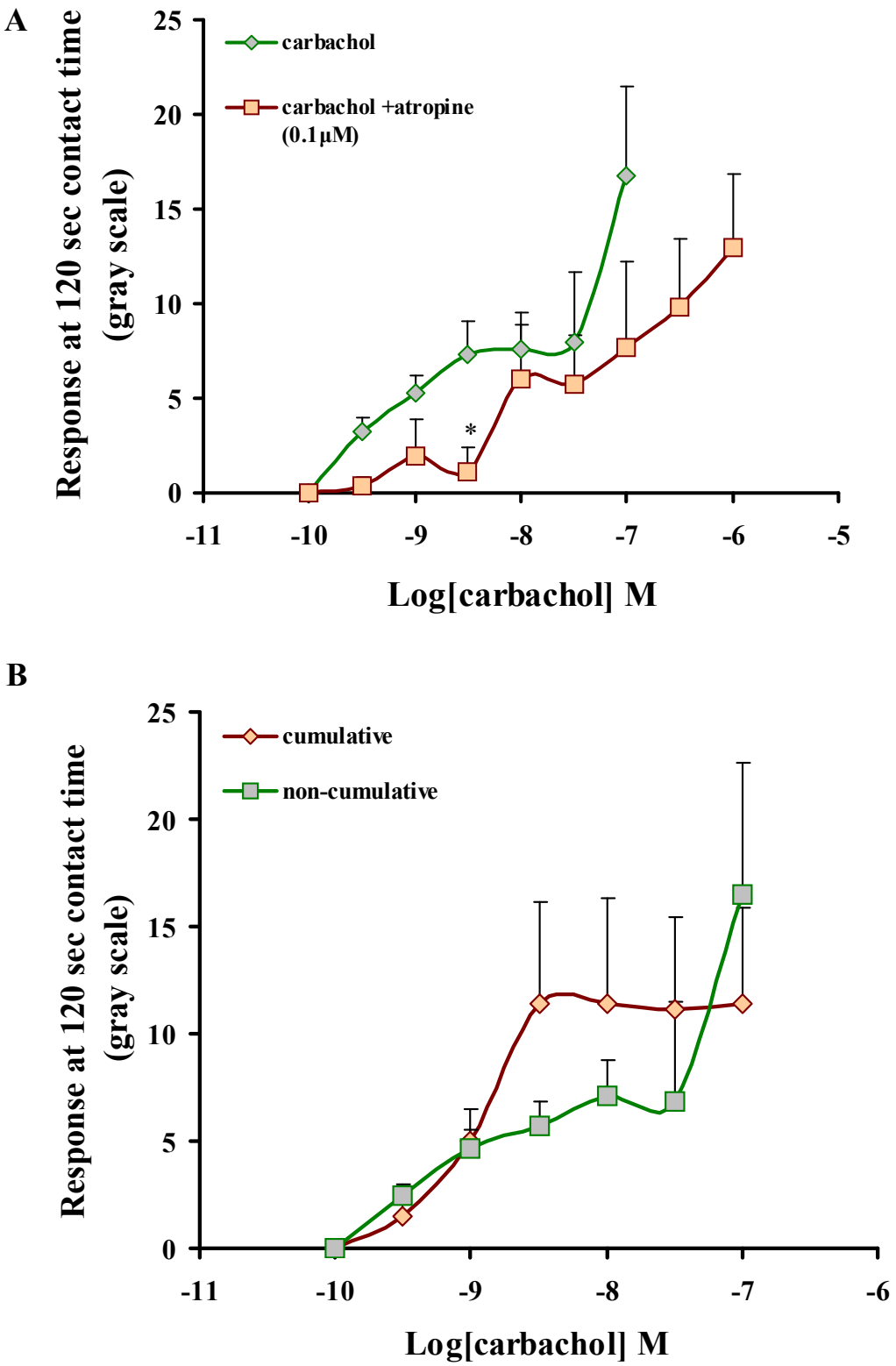


*Figure 3.4.25: Contraction responses induced by carbachol (0. nM- 100nM) in absence and presence of cholinergic antagonist atropine (0.1µM) in the ISMC preparations isolated from segments taken from the ileum of rat neonates at 120 sec contact time (A). Contraction responses induced by cumulative and non-cumulative carbachol applications on ISMC preparations at 120 sec contact time (B). Each point represents the mean ± s.e. mean; n=4.*p<0.05, **p<0.01, ***p<0.001were taken as significant differences in the group.*

3.4.13 INTESTINAL CELLS RESPONSE TO GUIDANCE CUES ON GLASS AND THIN SYLGARD PATTERNED CONSTRUCTS

Cultured ISMC and enteric neurones, normally results in layers of spindle- like shaped cells, which grow continuously on top of each other topped by projections of nerve processes. Thus, attempts were made to assess if the use of micro-contact printing aimed to re-orientate cells in culture, would allow these cells to align in an organised manner as in the intact tissue.

The guidance ques assay, in which cells isolated from segments taken from the ileum of rat neonates were exposed to different size patterns of 3.8, 5, 12.5, and 25 micron (Figure 3.4.26a-d) revealed that the intestinal cells aligned more precisely to the 12.5 micron fibronectin pattern producing an angle alignment of 10.81±4.17 (mean cell alignment angle to patterns ± s.e.mean). Additional stamp sizes of 5 micron and 25 micron also produced a satisfactory degree of cell alignment of 13.31±2.8 and 12.91±4.73 respectively. 3.8 micron patterned produced a less aligned re-orientation of intestinal cells (17.9±2.9). (Figure 3.4.27). In all instance, the fibronectin patterns promoted cell spreading and caused a marked alignment (Figure 3.4.26f). Additionally, the fibronectin patterns resembled natural components of the extracellular matrix indicating that they may be suitable for the orientation of large number of cells on patterned constructs.

When the intestinal cells were plated onto the thin sylgard patterned constructs with a pattern size of 12.5 micron (established from preceding experiments), the cell population displayed an aligned and organised distribution, which resembled the nature of the sectioned intact intestine (Figure: 3.4.28-30).

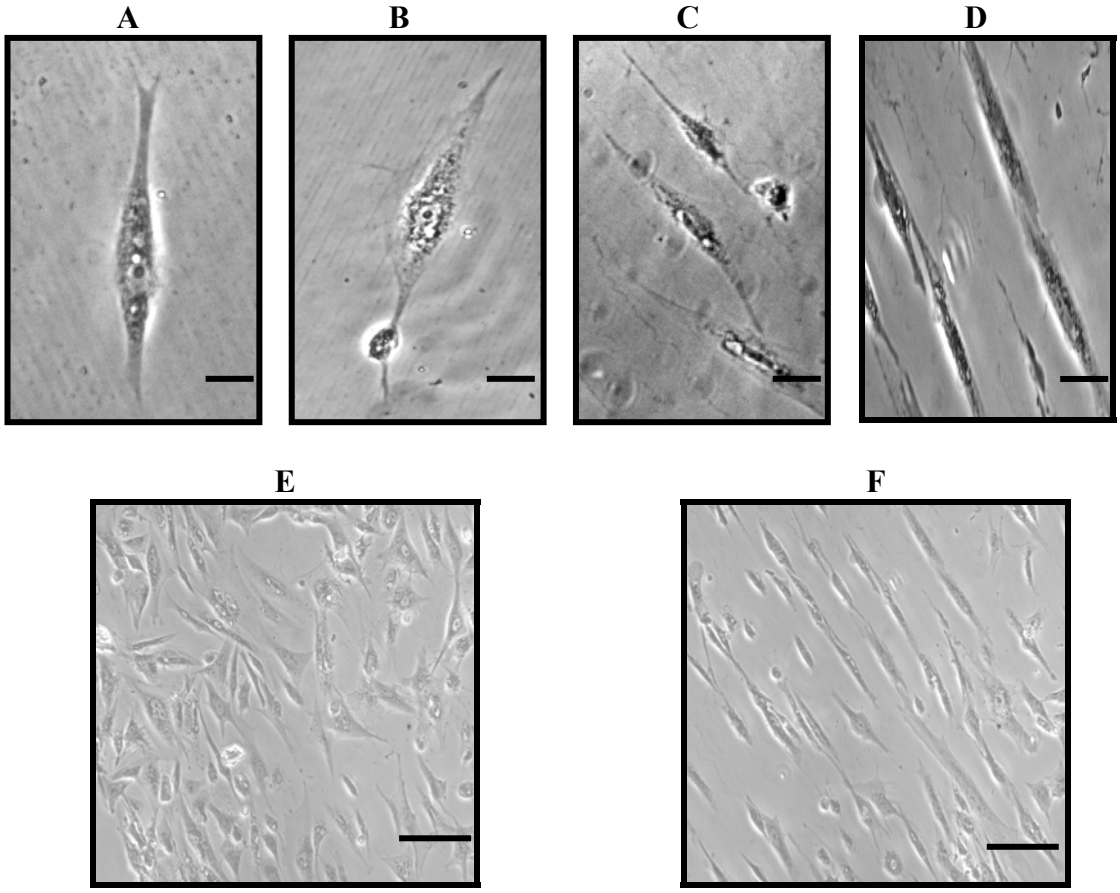


Figure 3.4.26: Intestinal cells isolated from segments taken from the ileum of rat neonates' response to different sized patterns of 3.8 micron (A); 5.0 micron (B); 12.5 micron (C); and 25 micron (D) stamped with fibronectin. Scale bar = 50 µm. The differences in morphology and cell orientation in the control (E) and fibronectin patterned surface at 12.5 micron (F). Scale bar = 100 µm.

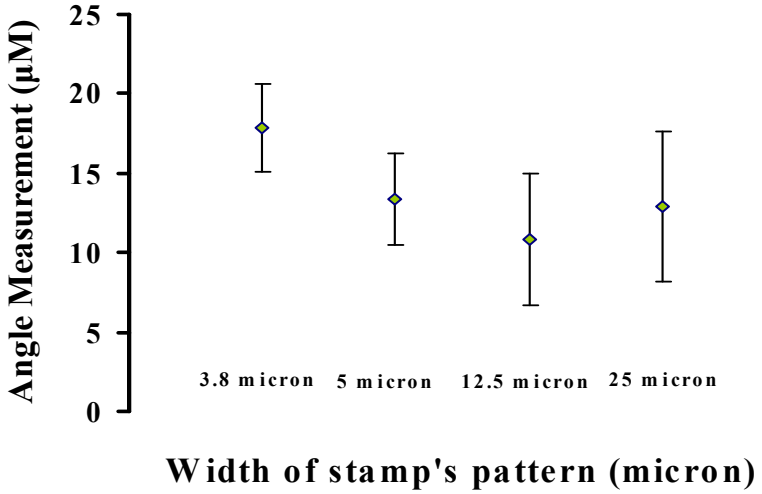


Figure: 3.4.27: The degree of alignment of cocultures of ISMC and neurones exposed to varying patterns of 3.8, 5, 12.5, 25micron. Each point represents the mean ± s.e.mean; n=7.

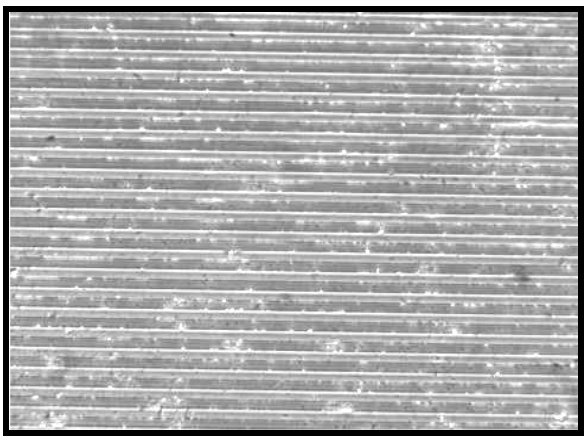


Figure 3.4.28: Micrograph representation of the thin sylgard constructs with 12.5micron patterned surface and fibronectin coated. Scale bar=50μm.

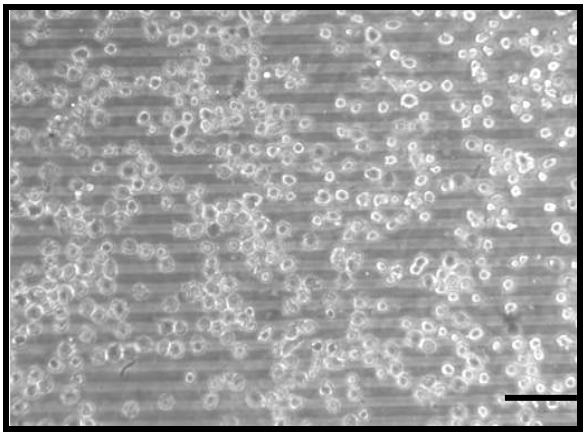


Figure 3.4.29: Micrographs representing the cell seeding onto the thin sylgard substrates. The combination of ISMC and neuronal cell population isolated from segments of the ileum of rat neonates were plated onto the thin sylgard substrates previously coated with fibronectin. Scale bar=50μm.

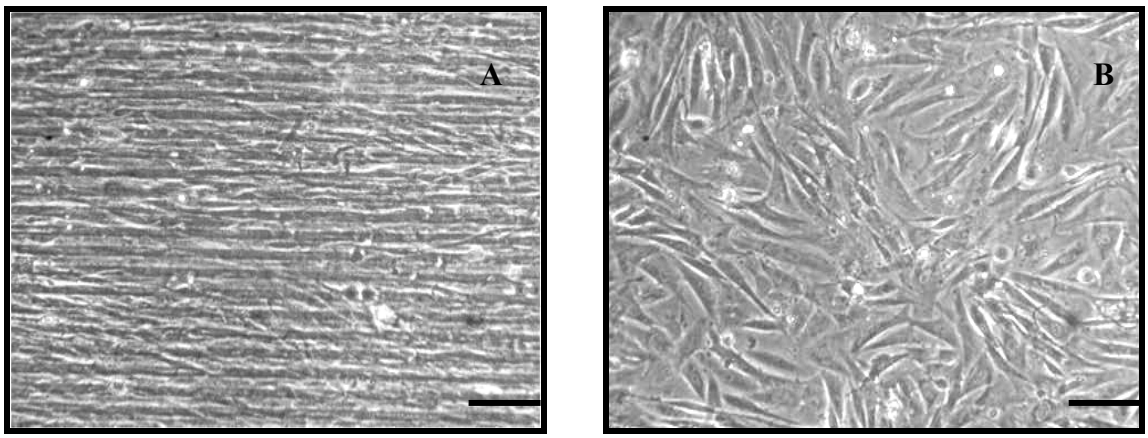


Figure 3.4.30: Micrographs representing the cell proliferation and reorientation in the thin sylgard patterned constructs (A) as opposed to cells seeded on unpatterned sylgard constructs (B) following 24 hours incubation at 37℃. Scale bar=50μm.

3.4.14 THE ABILITY OF INTESTINAL CELL POPULATION TO RESPOND TO KCl-INDUCED CONTRACTIONS ON THIN PATTERNED SYLGARD SUBSTRATES

The population of aligned intestinal cells when exposed to KCl (6mM) failed to induce contractions with sufficient force to cause visible deformation of the sylgard substrates. The plot profile (Figure 3.4.31a) and surface plot (Figure 3.4.33b) analysis revealed no significant changes to the sylgard surface prior and subsequent to the KCl applications.

3.4.15 THE ABILITY OF INTESTINAL CELL POPULATION TO RESPOND TO KCl-INDUCED CONTRACTIONS ON SHEAR SENSITIVE LIQUID CRYSTAL COATED SURFACES

The failure of ISMC and neurones to respond to KCl-induced contractions on the thin sylgard pattern substrates, prompt the investigation of ISMC and neurones functionality on other materials with additional elasticity, namely cholesteric liquid crystals. The percentage viability of cells exposed to the liquid crystal material relative to the control was higher than 98%. Cocultures isolated from segments taken from the ileum of rat neonates and grown on the liquid crystal coating displayed firm attachment to the substrate, which generated distinct traction forces on the liquid crystal coating resembling those of a wrinkled surface. Furthermore, cells appeared round in shape and re-arranged themselves into clusters. The cell clusters on liquid crystal when exposed to KCl (6mM) induced contractions with sufficient force to cause visible deformation of the liquid crystal substrate. This was monitored for varying contact times of 0-180 sec. The surface profile analysis revealed displacements on the liquid crystal surface subsequent to the KCl application (Figure 3.4.32). Similarly, the plot profile analysis indicated sustained surface deformations with increased contact time in KCl (Figure 3.4.33b).

3.4.15 THE ABILITY OF INTESTINAL CELL POPULATION TO RESPOND TO NORADRENALINE-INDUCED RELAXATION ON SHEAR SENSITIVE LIQUID CRYSTAL COATED SURFACES

The ability of intestinal cell population seeded on liquid crystal to produce contractile responses to KCl prompt the investigation of the suitability of the liquid crystal material for studies of relaxation of intestinal cell population. This was achieved by pre-contracting the cells with KCl (6mM) and subsequently analysing the relaxation responses induced by noradrenaline (40μM). The cell seeded on liquid crystal when exposed to KCl induced contractions with sufficient force to cause visible deformation of the liquid crystal substrate. On its contracted form cells were subsequently exposed to noradrenaline. The effect on noradrenaline on pre-contracted cells displayed a flatten appearance, which was sufficient to loosen the deformations caused by pre-contraction with KCl (Figure 3.4.34). When analysing the plot profile analysis, the deformations to the surface induced by cells in response to KCl displayed an increase in the mean contrast intensities compared to the control. The mean contrast intensity was reduced once cells were exposed to noradrenaline and gradually returned to values observed during KCl application (Figure 3.4.35).

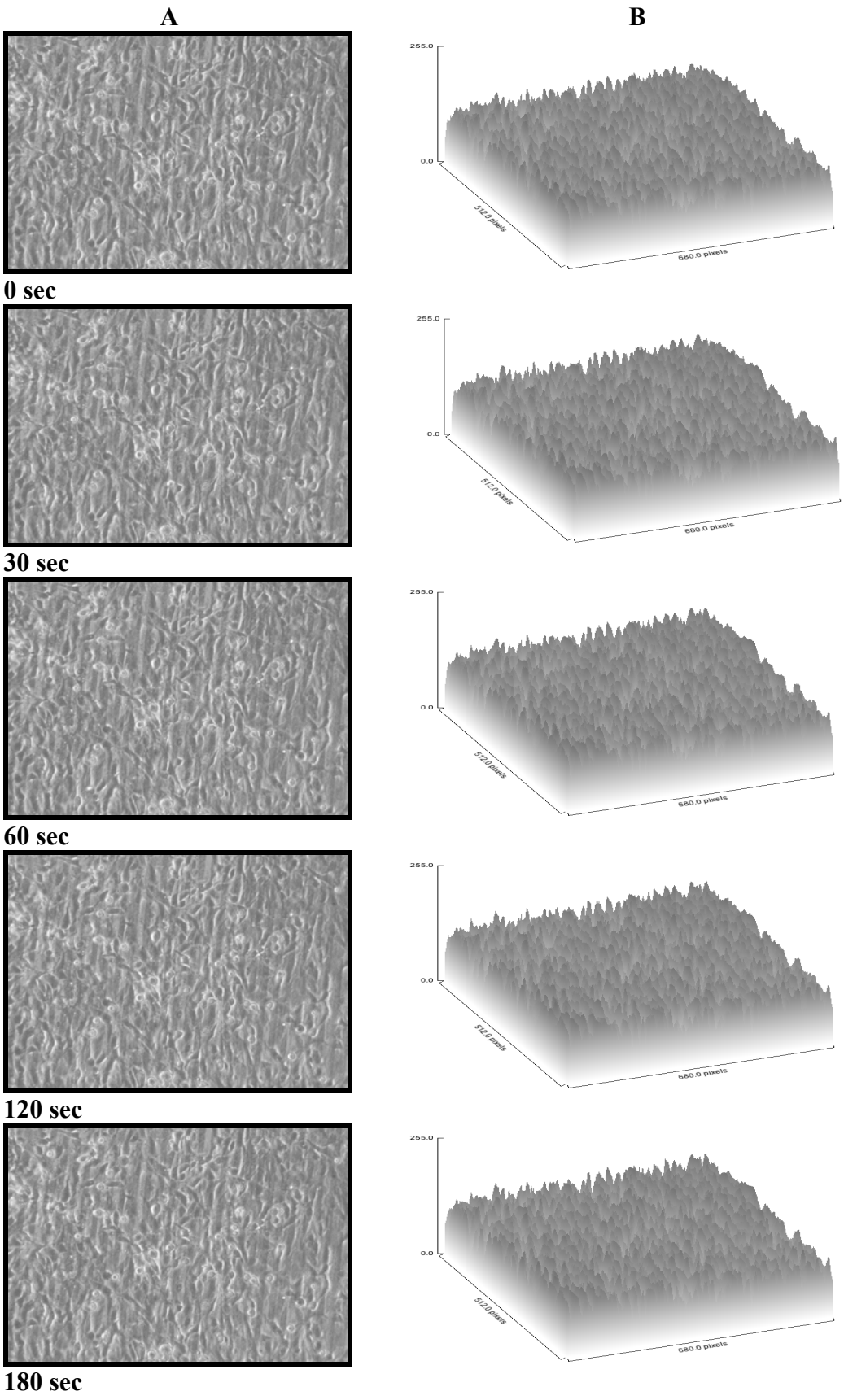


Figure 3.4.31: Micrographs representing intestinal cell population isolated from segments taken from the ileum of rat neonates embedded on patterned sylgard constructs (A) and their respective surface profiles (B). Following applications of KCL (30 sec) the organised cell population of ISMC and neurones failed to respond since no changes in cell shape or substrate displacement were observed on the micrographs or surface profiles.

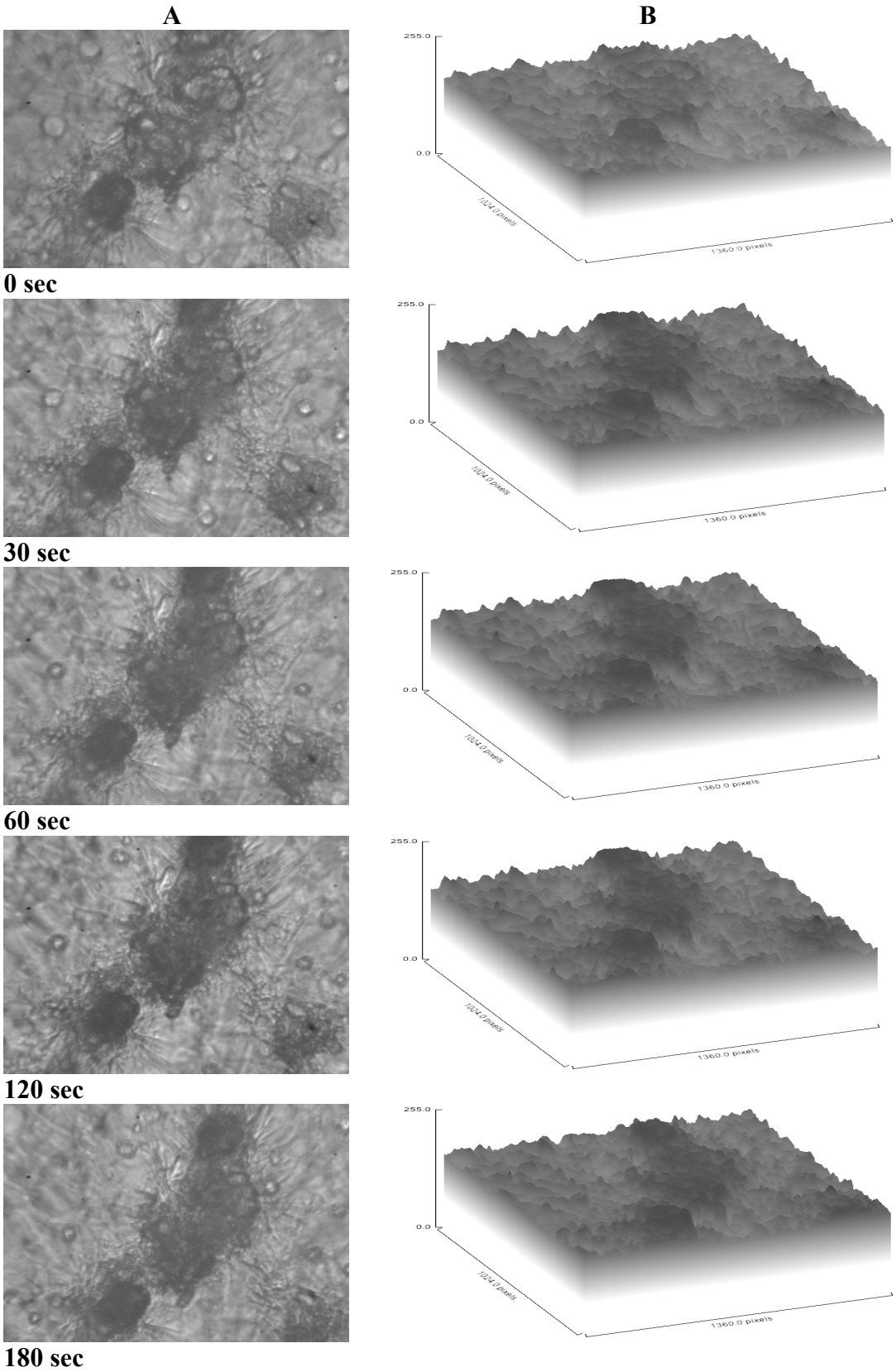


Figure 3.4.32: Micrographs representing intestinal cell population embedded on cholesteric liquid crystals coating (A) and their respective surface profiles (B). Following applications of KCL (30 sec) the aggregate of cells contracted consequently pulling the substrate. By 120-180 sec there is clear displacement of cell aggregates on the liquid crystal substrate in response to the drug application.

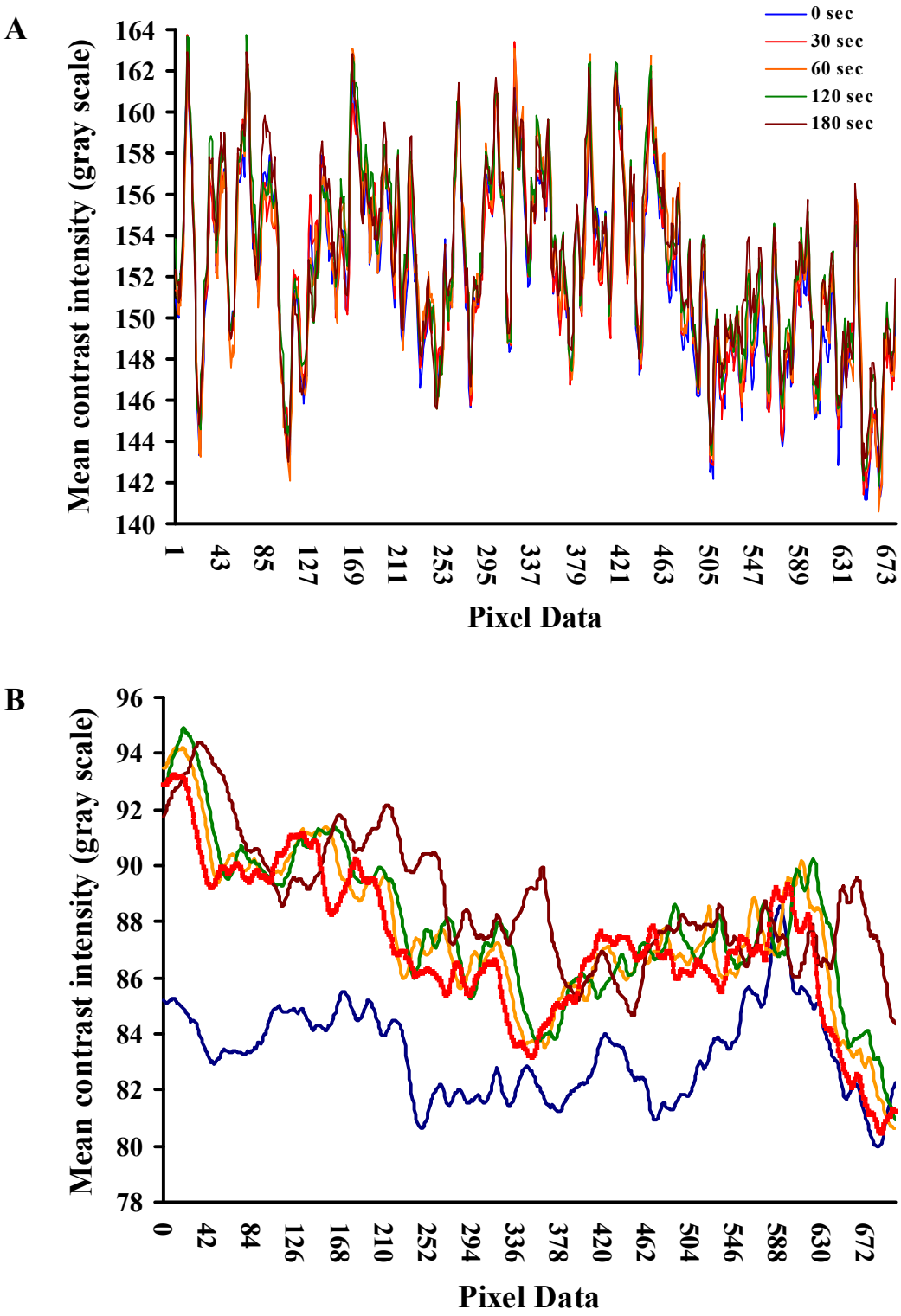


Figure 3.4.33: Graphs comparing the plot profile on the effect induced by KCL on the coculture of ISMC and neurones seeded on sylgard patterned substrates (A) and cholesteric liquid crystals coated substrates (B). The profile plot was measured and average gray-scale intensities displayed were analysed. Combined cell population response to KCL application on cells seeded on the sylgard patterned substrates induced little effect as there was a continuous overlap of the averaged intensities along the different time frames of 0, 30, 60, 120, and 180 sec (A). In the liquid crystals substrates, the cell population response to KCl application was translated into deformations to the substrate surface displaying clear differences in the profile plots from 0-180 sec (B).

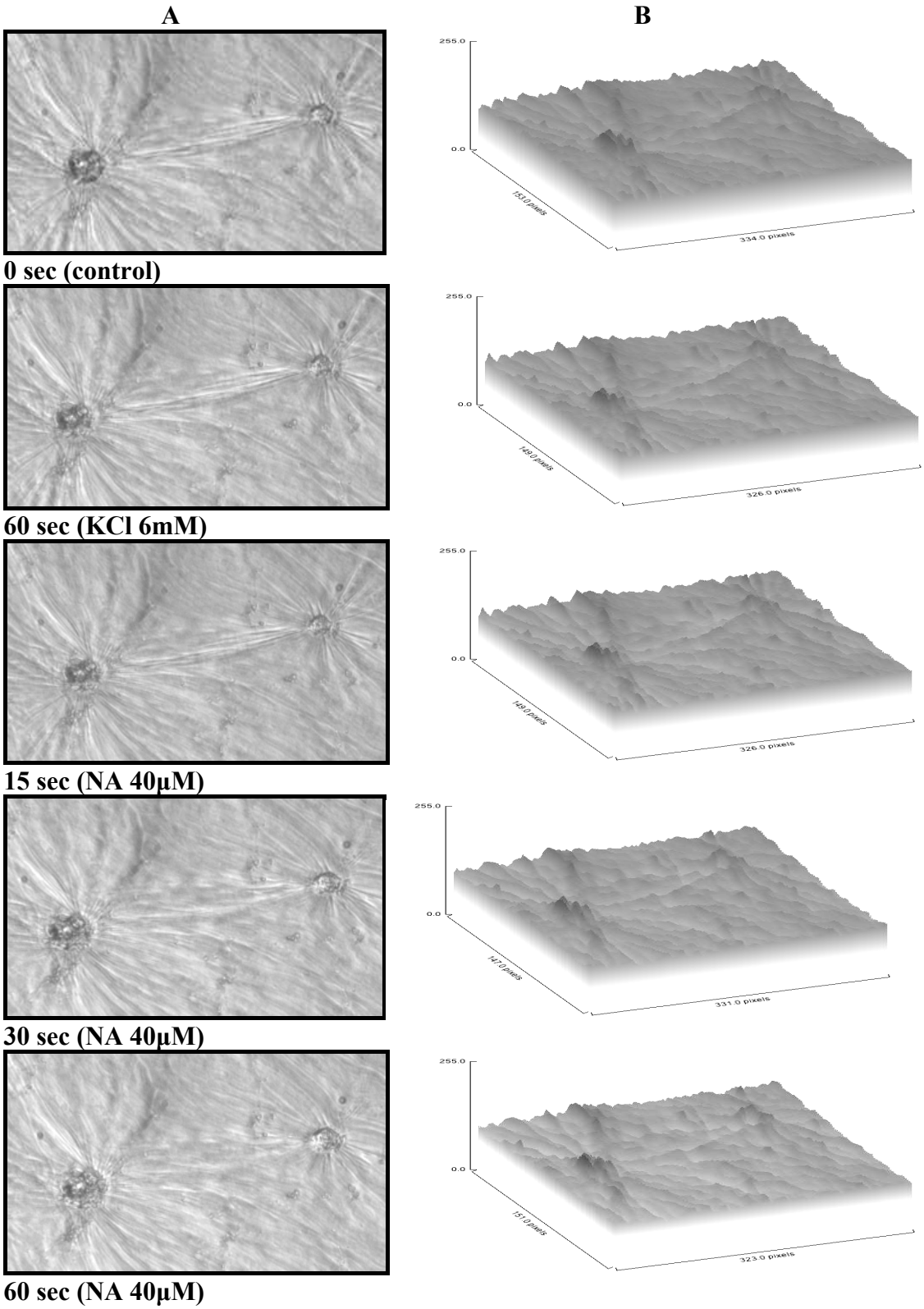


Figure 3.4.34: Micrographs representing intestinal cell population embedded on cholesteric liquid crystals coating (A) and their respective surface profiles (B). Cells were pre-contracted with KCl 6mM (60 sec) and subsequently a single dose of NA (40µM) was applied. The effects of KCl application caused the cells to contract and pull the substrate. Subsequently, the application of NA on the pre-contracted cells induced a relaxation response resulting in loosen substrate appearance.

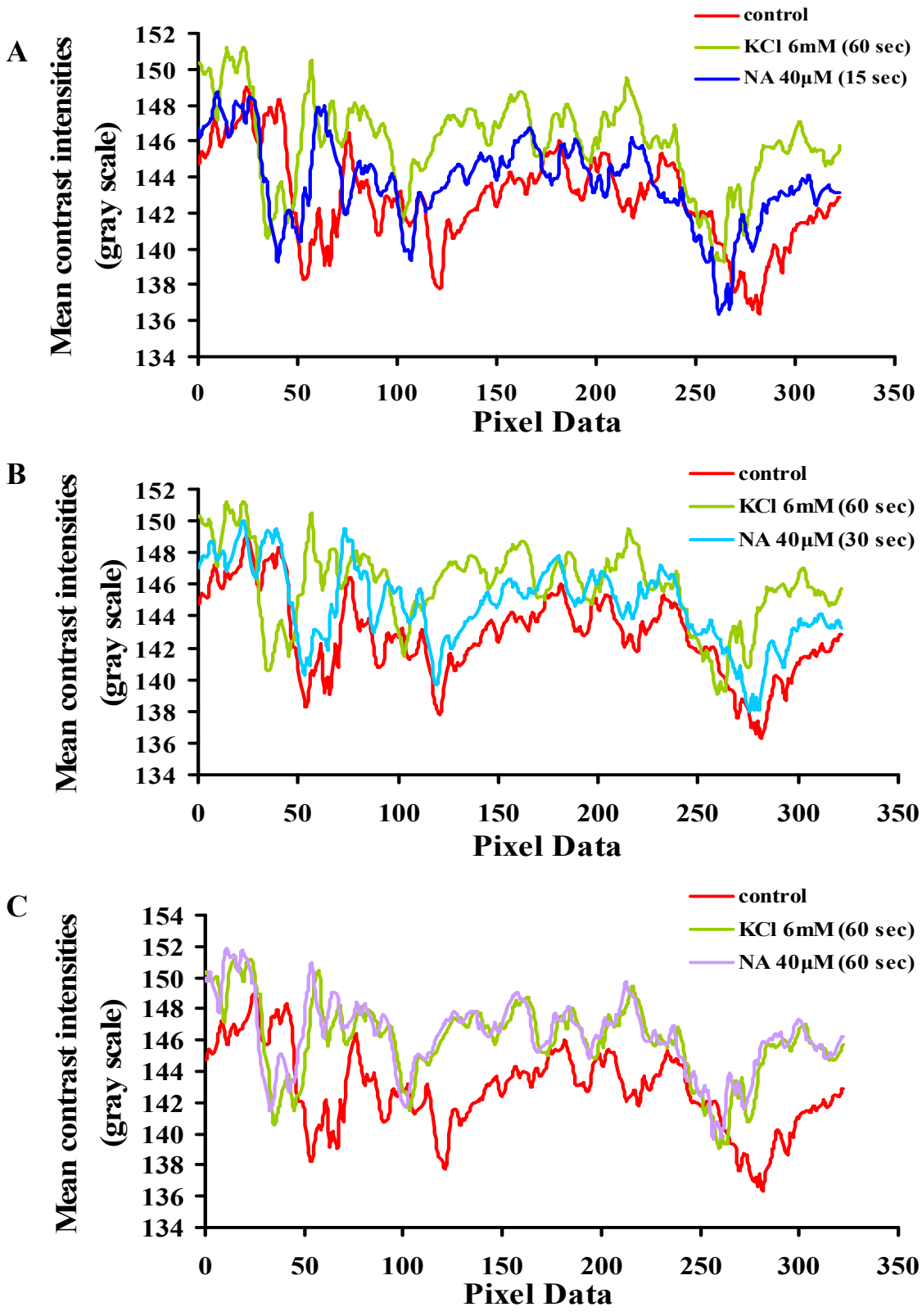


Figure 3.4.35: The effect induced by NA on KCl pre-contracted coculture of ISMC and neurones seeded on cholesteric liquid crystal coated substrates. The profile plot was measured and the average intensities displayed were analysed. The combined cell population response to the application of KCl (6mM) induced contractile responses. The KCl pre-contracted cell population were consequently exposed to a single dose of NA (40µM), which induced clear relaxation responses observed at 15 (A), 30 (B), 60 sec (C). These responses were translated into deformations to the substrate surface displaying clear differences in the profile plot from the control.

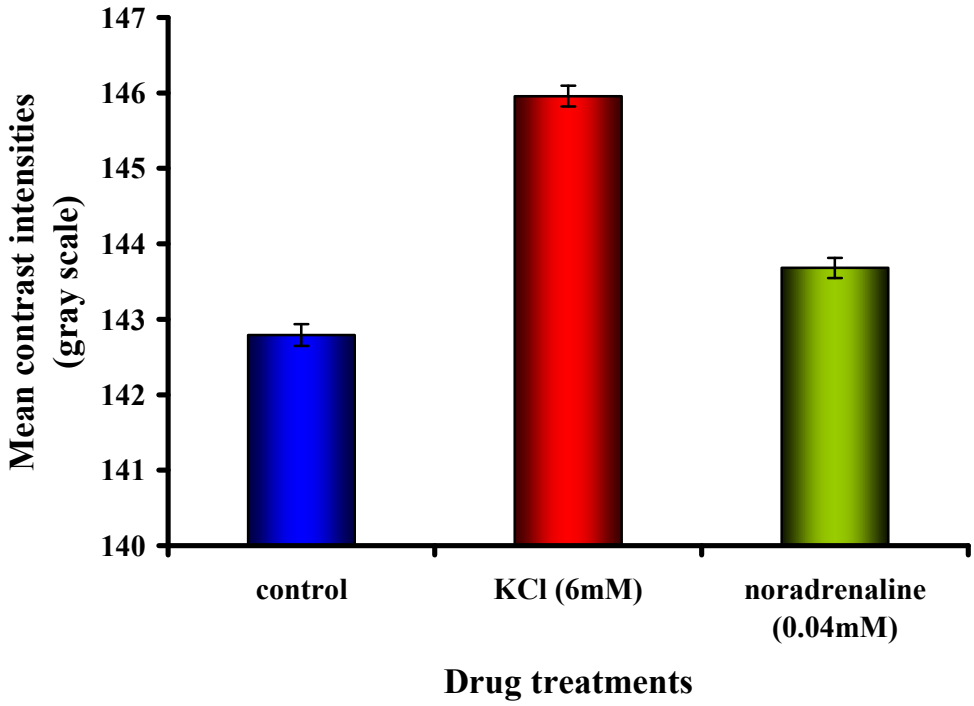


Figure 3.4.36: Graph representing the average of all point pixels per image following the applications of KCl (6mM) for 1 min and consequently the application of noradrenaline (0.04mM) for 30 sec on cocultures of ISMC and neurones seeded on liquid crystal surface. The application of KCl caused cells to contract at a force sufficient to cause deformations to the liquid crystal surface. Subsequently the application of noradrenaline caused cells to relax and loosened the deformations induced by KCl. These displacements of the liquid crystal surface were measured by averaging the variation in gray scale intensities following each drug application. Each point represents the mean±s.e.mean.

3.4.16 THE EFFECTS OF SUBSTRATE STIFFNESS ON CELL MORPHOLOGY AND ADHESION

Experiments were carried out to compare the effect of substrate stiffness on intestinal cell population morphology and adhesion. When comparing the results for cell morphology displayed by the single ISMC isolated from segments taken from the ileum of rat neonates and seeded on different surfaces, the micrographs show visible differences in cell shape. The spreading of cells is enhanced as substrate stiffness increases. Hence, the intestinal cells plated on the softer substrate - shear sensitive liquid crystal coating revealed that although cells are clearly attached, the cell shape remains relatively round throughout the entire viewing period. However, a more spread morphology is observed in cells plated on patterned sylgard constructs and plastic surfaces (Figure3.4.34a-c).

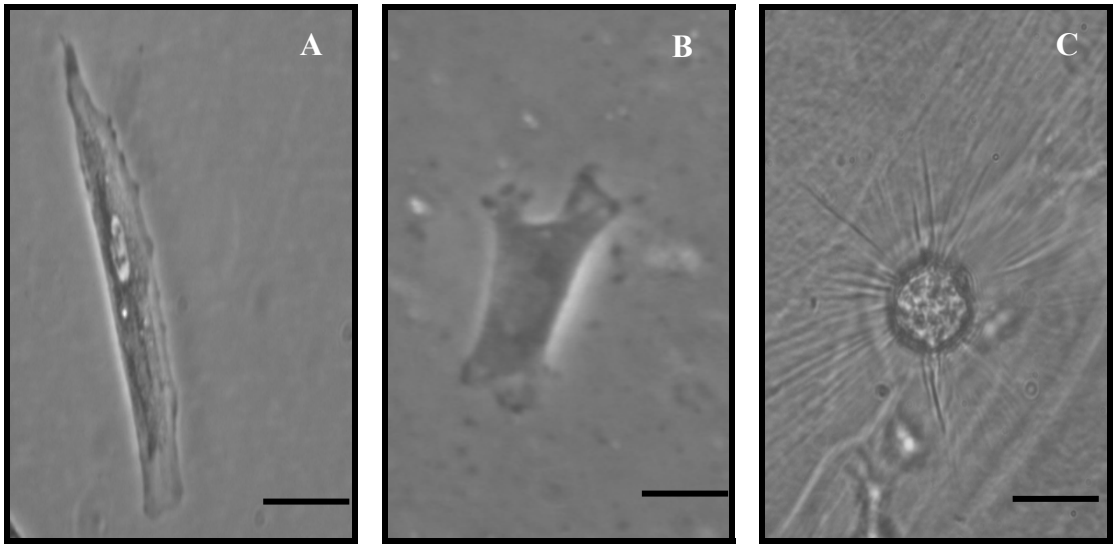


Figure 3.4.36: Micrographs representing the effects of substrate stiffness on cell morphology. Cells isolated from segments taken from the ileum of rat neonates and seeded on plastic surface clearly attach and spread well (A). Cells on sylgard substrate attach well, however, spreads relatively less than those attached to plastic substrate (B). By contrast, cells plated on shear sensitive liquid crystal coating show clear attachment reflected by the wrinkling of the surface at the indentation points and a relative round shape comparing to the more rigid substrates (C). Scale bar=25μm

3.5 DISCUSSION

Harrison and Carrel were the first to develop a cell culture method for studying the behaviour of animal cells free from systemic variation that might arise due to the complexity of the tissue (Harrison, 1907, Carrel, 1912). In more recent times with the advances in cell culture methods and the development of new dissection techniques, this has enabled the isolation of various cell types from the stomach, intestine, and bladder from both adult and neonatal animals (Bitar & Makhlouf, 1982b, Collins & Gardner, 1982, Seidel & Johnson, 1983, Frings et al., 2000). In the intestine, however, cell dispersion from neonatal source has proven challenging because of the difficulties in obtaining pure and functional preparations for experimental testing.

Thus, the aim of the present studies was to develop a cell culture model of intestinal cell populations including ISMC, myenteric neurones, and a coculture of ISMC and myenteric neurones from neonates. Furthermore, to characterise the functionality of the cell culture models by using pharmacological active agonists and antagonists capable of activating specific receptor systems to induce a contraction or relaxation responses.

The cell dissociation method reported in the present study provided evidence that the regions of the intestine from which cells are isolated, as well as the length of time allowed for trypsinisation influences the viability and growth rate of cells in culture. To support these studies, histological procedures also established that increase in trypsinisation times lead to gradual dissociation from the outer-most layers into the inner layers of the intestine. This suggests that this method can be used to determine the estimated time required for isolation of single cell types from specific intestine layers.

Previous studies performed on the isolation of ISMC from neonatal tissue reported the use of dissection under sophisticated optical control. This involved the removal of the entire muscularis externae layer with the support of an advanced microscope and fine forceps (Frings et al., 2000) or specific removal of the longitudinal muscle layer with the help of

stereomicroscopes (Smirnov et al., 1992). The method developed here, however, consisting simply of tying both ends of the intestine prior to dissociation provides an inexpensive technique for isolation of intestinal cells from neonatal tissues, which can be adjusted for isolation of specific intestinal layers. Although this may also be achieved via microdissection techniques, microdissection systems are expensive, unavailable to many researchers, and isolation of large numbers of intestinal cells such as presented here, would prove extremely time consuming or even impracticable in complex tissues (Kwapiszewska et al., 2004).

In an attempt to separate and culture different cell types, the differential adhesion technique was used, which minimised the number of fibroblastic cells in culture as well as allowing the separation of neurones from ISMC. The differential adhesion technique has already been shown to be a useful method of separating fibroblasts and myogenic cells (Richler & Yafee, 1970, Gareth et al., 1990, Rando & Blau, 1994, Qu et al., 1998).

Further selective purification of ISMC was achieved through treatment with scorpion venom *maurus palmatus*. According to numerous morphological (Vachon, 1952) as well as some biochemical (Goyffon & Kovoor, 1978) characteristics, scorpion venoms are divided into two groups: buthoids and chactoids. The buttoids, because of their relevant medical importance associated with severe envenoming and death have been widely studied and their toxicity has been demonstrated to target the excitation of axonal membranes by the modification of their ionic conductance (Romey et al., 1975, Catterall, 1980, Pelhate & Zlotkin, 1982). The Chactoids, including scorpio *maurus palmatus* were poorly investigated since they were thought to be medically non-significant (Goyffon & Kovoor, 1978). On the basis of this information, scorpion venom *maurus palmatus* was assessed for purity and the most prominent characteristic present in the toxin components was the cooperativity between the two toxin units (paralytic fraction and the lethal factor), which when investigated through neurophysiological studies showed interaction with insect

axonal preparations and extremely low toxicity compared to other buthoid venoms. Results also revealed that scorpion *maurus palmatus* possesses cooperative interaction and blocking/suppressing effects on the ionic conductance for both potassium and sodium ions. It seems that the disturbance of the electrolyte balance in both ions affect cell membrane integrity and nerve transmission though interferences with the conductance of axons (Lazarovici et al., 1982, Possani et al., 1999). Thus, treatment in culture with this toxin caused selective abolition of neurones, which when compared with untreated cultures contained relatively higher number of ISMC but otherwise identical cultures (Blennerhassett & Lourenssen, 2000) and no disturbance to ISMC population was observed. Moreover, it has been postulated that the presence of neurones '*in vitro'* might correlate with the decrease in serum availability in culture resulting in decreased proliferation of ISMC (Blennerhassett & Lourenssen, 2000). Hence, removal of the neurones may increase the availability of serum proteins and promote an increase in the proliferation of ISMC.

Myenteric neurones were purified through antimitotic treatment with cytosine arabinoside. Pre-treatment with this drug has been primarily used to purify neurones derived from dorsal root or superior cervical ganglia (Blennerhassett & Lourenssen, 2000, A.Eccleston et al., 1989, Eccleston et al., 1989) since it inhibits DNA replication of non-neuronal cells (Harrington & Perrino, 1995). In the present study a single dose with cytosine arabinoside treatment resulted in the removal of accessory cells (ISMC and fibroblast) and rapid proliferation of myenteric neuronal cells bodies with extended neurites.

To examine the purity and nature of the different population of cells in culture immunocytochemistry was used. These experiments on the combined cell population of enteric neurones and ISMC referred as cocultures confirmed the presence of both ISMC and neuronal cell populations. Antibodies to α-smooth muscle actin displayed a uniform distribution of ISMC among positively stained myenteric neurones to antibodies raised

against 5-HT_3 receptors. Anti-smooth muscle α-actin antibodies represent a useful tool for the study and characterisation of smooth muscle in culture (Skalli et al., 1986). Receptors to $5HT_3$, on the other hand, are expressed in the enteric nervous system (Johnson & Heinemann, 1995, Mawe et al., 1986, Richardson et al., 1985) and play important roles in the enteric physiology stimulating neuronal activity. Recent studies on isolated myenteric neurones obtained from the mouse small intestine have shown immunoreactivity to antibodies raised against $5HT_3$ receptors in neurones, labeling both neurites and cell bodies (Liu et al., 2002). Thus, in the present studies $5HT_3$ receptors antibodies were used as a marker for myenteric neurones. In such experiments the purified myenteric neurones showed uniform expression of $5HT_3$ receptors within the cell bodies extending to the processes and no immunoreactivity to α-smooth muscle actin. Furthermore, purified ISMC cultures showed uniform expression and distribution of α-smooth muscle actin and no expression of $5HT_3$ receptors. Additionally, purified ISMC also displayed intense diffuse labeling in the cytoskeleton of majority of cells following staining with antibodies raised against α-actinin. The distribution of α-actinin in single isolated muscle cells filaments has been shown in previous studies, where it displayed an high expression throughout the cytoplasm (Fay et al., 1983).

Although this work shows that ISMC and myenteric neurones can be readily dissociated from the neonatal intestine, the continuous use and availability of these cells in culture is nonetheless limited. This is primarily attributed to ISMC and the number of phenotypic changes that they experience, causing impairment in their ability to contract. It has been reported that smooth muscle expression of actin isoforms decrease during proliferative phase after initial cell seeding (Halayko et al., 1996). While they may partially recover once cells become confluent, the actin levels do not return to those present in freshly isolated smooth muscle cells. There have been additional studies showing the decrease in

smooth muscle specific contractile proteins during proliferation and with each subsequent passage (Panettieri et al., 1989, Tom-Moy et al., 1987).

This has generated an ever-increasing need to develop cryopreservation techniques, which would enable a constant availability of cells and enhanced ability to study their functional characteristics '*in vitro'*. Furthermore, limitations that are normally observed in primary cultures due to limited growth potential and the absence of continuous lines of healthy cells would be avoided.

The basic principle of cryopreservation of cells comprises bringing cells into contact with the cryoprotectant agent and subsequently reducing the temperature of the cells to a cryopreservation temperature. However, these are thought to be often dependent on the type of cells being frozen (Fuller et al., 2004). Because cells differ cytologically, this also means that there is a cell type-related variance in the way in which solutes enters the cell and form ice crystals. Thus optimum freezing for each individual cell type can only be predicted by the rate of freezing they are exposed. On the basis of this information, we have developed a simple controlled rate-freezing method without the rate-controlled programmed freezer. Results revealed that a three-steps cooling reflecting gradual freezing of the cells in protocol 2 was successful for cryopreservation of ISMC with minimal DNA damage compared to the controls. This result suggests that the use of sophisticated and expensive equipments is not entirely necessary to achieve rates of cooling sufficiently uniform to ensure the viability of ISMC.

To assess the DNA damage associated with freezing the comet assay was employed. This method detects double stranded DNA breaks in individual cells due to oxidative stress and it has been used for human monitoring studies looking at the DNA damage in lymphocytes related blood sample storage (Anderson et al., 1997) and to evaluate DNA integrity of frozen-thawed on cryopreserved spermatozoa (Evenson et al., 1991). This assay has proven effective at detecting low levels of DNA damage in the cells and there is evidence that

integrated measure of the tail moment is a more sensitive measure of the freezing-induced DNA damage comparing with the length of the comet (Olive et al., 1991).

When looking at the long-term storage, results revealed that cells cryopreserved for up to 1-month showed no substantial damage compared to unfrozen samples, however, cryopreservation of ISMC for 4 months induced a small but significant increase in the genomic damage to ISMC. Although the are no evidences from the present studies, the fluctuations in genomic damage may not to be due to cryoinjury but a possible variation of the physiological state at the time ISMC were acquired, essentially with regards to the cell dissociation procedure. When establishing cell culture models the optimisation of the physical dissociation, which comprises the use of proteolytic enzymes including trypsin to cleave cellular bonds and mechanical dissociation through which cells are dispersed are imperative to give a good yield of viable cells. However, this does not eliminate the presence of aggregates (cell clusters) in the culture. Thus, the problem rises when these cells are used for storage since these aggregates will allow a good diffusion of cryoprotectant to the cell in the periphery, but poor diffusion to the inner most cells, thereby making them more susceptible to oxidative stress during freezing (Zalzmann, 1999). Therefore, although the viability of cells does not substantially differ, more sensitive techniques such as comet assay clearly detects the oscillations in the DNA damage exhibited by these cells. Additionally, there is evidence indicating that the cryopreservation technique applied presents the best possible maintenance of the integrity of cryopreserved cells, there will be no alterations at the phenotypic, biochemical, chromosomal and molecular level due to cryopreservation (Elgmann, 1997). These observations are in line with the present results since the genomic damage in unfrozen ISMC (control) was comparable to those of ISMC cryopreserved for 1-week using the slow cooling protocol. On the other hand, the linear relationship between the time length of cryopreservation and the genomic damage exhibited by ISMC suggests that although

optimised cryopreservation methods protect cells from instant genomic damage, the long-term storage, although minimal, does interfere with genomic integrity of cryopreserved cells. In support of these observations, previous studies investigating the effects of short and long-term cryopreservation of peripheral blood monoclonal cells has shown that the effect of cryopreservation becomes more significant over time resulting in reduced responses by cytokines (Owen et al., 2007).

Therefore, the cryopreservation technique here employed demonstrates that ISMC isolated from segments taken from the intestine of rat neonates can be readily stored with reduced genomic damage. The stability of cells in the cryo-state is adequate and this technique will indeed aid cell availability and phenotypic stability for experimental testing.

The contractile ability of isolated ISMC was investigated through a series of pharmacological studies, in which single cells were exposed to selective agonists and antagonists. ISMC functionality was assessed on two distinct surfaces: rigid (plastic) and soft (silicone elastomer). ISMC responses to KCL, 5-HT, and carbachol were measured by microscopically assessing the changes in cell dimension on the distinct surfaces in response to the drug treatments. In all cases, maximum contractile responses observed on the soft surfaces were evoked during a shorter drug contact time and using the same or lower concentrations of the drugs compared to the experiments carried out on the rigid surface. In relation to this, several studies on the cell behaviour on different substrates have shown that substrate elasticity significantly modifies cell behaviour including applied traction forces (Pelham & Wang, 1999, Beningo & Wang, 2002). This explains the rounder and less spread morphology of ISMC plated on the soft surfaces. Furthermore, this elucidates why the contractions elicited by ISMC in response to selective drugs produced a more immediate response on softer materials other than plastic surfaces. This is because in the normal status of the mammalian tissue such as the intact intestine the most common

sites of attachment for a cell is another similar cell or the extracellular matrix, and these materials possess significant elasticity.

According to this relationship between substrate adequate properties and contractile cell behaviour, the results suggest that silicone elastomer substrate could be considered as a relevant substrate to study contractile properties of ISMC.

In the series of experiments that followed single ISMC were exposed to noradrenaline and the relaxation responses were measured. In such experiments, the changes in cell dimension were visibly distinct from those observed while assessing contractile responses. The swollen feature displayed by ISMC in response to noradrenaline treatment allowed isometric measurement of single cell relaxation. Previous studies have been reported on the measurement of smooth muscle relaxation by measuring the changes of cell length in suspension. In such studies computerised system permitted the direct measurement of the length of single cells. This was achieved by incubating cell suspension with a selective drug, stopping the reaction and subsequently proceeding to a computerised measurement of cell length (Moummi & Woodford, 1992). This technique, however, failed to provide the true kinetics and reversibility of the response and to identify the natural properties possessed by individual cells. The present study enabled the construction of time-course and dose-response curves on the same cell acting as its own control.

To assess the contractility of the cells, series of experiments were carried out. In such experiments the effect of carbachol was investigated in the absence and presence of the cholinergic antagonist, atropine (0.1μM). The results demonstrated that the non-cumulative addition of carbachol induced contractile responses, which were antagonised by atropine. This effect was surmountable and/or competitive, with no alteration of the maximum response induced by carbachol. Atropine is the standard muscarinic antagonist that has demonstrated similar effects in the inhibition of carbachol-induced contraction on isolated rat ileal preparations in '*in-vitro*' organ bath experiments (Nkeh et al., 1993).

However, when carbachol was added cumulatively, the contractile responses were reduced. One possible explanation could be the development of desensitisation following repeated exposures to carbachol. Previous studies have demonstrated that carbachol causes non-specific desensitisation in segments taken from the guinea pig ileum (Himpens et al., 1991). In many smooth muscle cells it has been reported that prolonged stimulation initially induced contraction, but the muscle eventually relaxes in spite of the continue presence of the drug (Eglen et al., 1991). In line with this information, it appears that the decline observed in response to cumulative carbachol application is a result of to the repeated carbachol applications.

The results obtained in this functionality study, together with previous studies reported by Bitar and Makhlouf on the characterisation of ISMC receptors (Bitar & Makhlouf, 1982a), suggest that isolated ISMC offer a valuable biological tool for drug screening to the pharmaceutical industry. This is primarily advantageous as in the single cell experiments the drug distribution is more evenly achieved, it allows the characterisation of receptors and their postsynaptic interaction, and finally this experimental approach provides a useful tool for investigating the mode of action of drugs at the cellular level.

Nonetheless, limitations include the small data sampling population and cell-to-cell variability, which fails to outline a conclusive drug action within a particular population of cells.

In response to the outlined limitations, attempts were made to create highly flexible substrates that would allow detection of mechanical forces exerted by cells in response to applied stimulus.

The control of cell mechanics and shape is crucial for many cellular functions including cell growth, proliferation and migration (Chen et al., 1997). To better understand the mechanisms involved in cellular behaviour and function in the '*in vitro'* environment, cells

are often attached to various micropatterned surfaces (Whitesides et al., 2001). This ability to spatially cell population into a complex differentiated structure has been linked to better stimulus sensing (Kane et al., 1999). This is particularly important in the native intestine, as individual cell population exist in an orientated architecture, which is crucial for the proper electrochemical coupling between smooth muscle and neurones to stimulate the transmission of directed contraction over long distances. In contrast, cultured intestinal cells spread to form a sheet of over-layered cells with disorganised orientation, bearing little similarities to the normal intestinal morphology.

Previous attempts have been made to align cardiac myocytes '*in vitro'*. These studies have shown that conduction velocities and action potentials were faster in cultures of aligned cardiac myocytes (Lieberman et al., 1972, Thomas et al., 2000). Thus, attempts in organising isolated cells into more natural structures should provide new opportunities for studying their mechanical force.

In order to examine the mechanical properties amenable for practical applications, an efficient and reliable method for fabricating cell-based actuators and controlling their mechanical and dynamical properties in the cellular microenvironment has to be developed.

In line with this information, the motivation of the study was to develop a highly flexible sylgard patterned thin substrate with high mechanical properties and the ability to promote intestinal cell alignment. According to this experimental design, it was hypothesised that if cells could align in an organised manner resembling that of the intact intestine, the combined cell response to the drug application would produce detectable mechanical forces. The generated forced in turn would be captured by microscopy imaging as the highly flexible substrates displayed displacement field of the surface.

Hence, cell preparations were first assessed for the most adequate pattern size. The combined population of neurones and ISMC responded best to guidance cues of

12.5micron. In experiments that followed the 12.5micron templates were used to produce the thin sylgard patterned constructs onto which the combined population of neurones and ISMC were plated. The organised distribution displayed by the intestinal cells resembled that of the intact intestine and furthermore reassured the possibility that they would equally respond to drug applications causing displacement of the sylgard surface. However, the force exerted by intestinal cells on patterned sylgard substrates in response to KCl failed to induce contractions that were translated into displacements in the surface.

It is well established that most cells in multicellular organisms are attached to softer materials than glass or plastic surfaces. These include other similar cells or the extracellular matrix, both of which have elastic moduli on the order of 10-10,000 Pa (Bao & Suresh, 2003, Wakatsuki et al., 2000). Forces generated by the contractile cells applied to the membrane attachment sites can deform materials with the same range of stiffness but cannot move an attachment site on a rigid surface. The results of the present study implied that the elastic property of the patterned sylgard substrates were not within the elastic modulus range suitable to study mechanical forces at a cellular level. Results also indicated that in order to be able to study forces generated by contractile cells on artificial substrates, one must primarily find a material with a close elastic modulus to that of the extracellular matrix that surrounds these cells. However, the challenge of finding a stimulus responsive biomaterial with matching elastic modulus to that of the intact intestine cytoskeleton is indeed immense.

In line with this evidence, the intensive search into biomaterials with known responsive properties for the detection of mechanical forces has led to the examination of shear sensitive cholesteric liquid crystals. The characteristic feature of liquid crystal is their exceptional responsiveness to excitations.

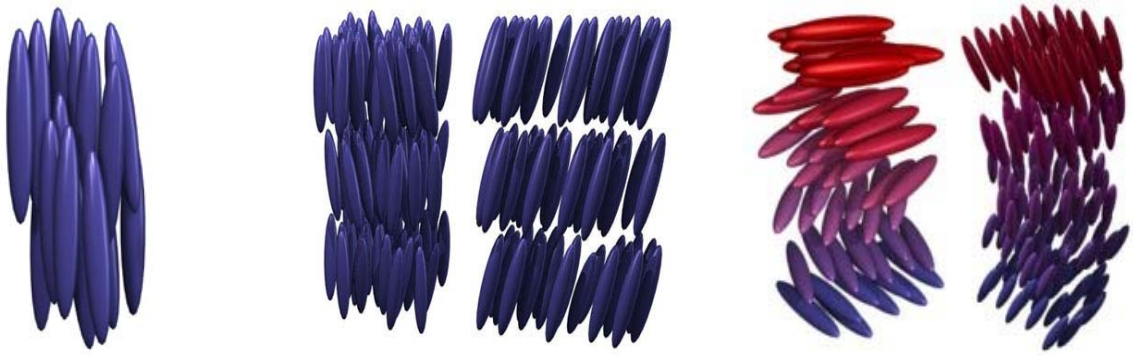
Liquid crystals are organic compounds that exist in a state between a liquid and a solid form. They are viscous, jelly-like materials that may flow like liquids but resemble crystals

due to the light scattering and reflection. Their unique responsivity arises as a result of symmetry as geometrically they must have different optical properties in all directions (anisotropic) and revert to the same optical property in all directions (isotropic) via thermal action or the influence of solvent (Fergason, 1964).

Liquid crystal phases can be characterised by the type of ordering they display. One can distinguish the liquid crystal molecules through their positional order, orientational order, and moreover by the short and long range ordering they may exhibit. Most liquid crystals when exposed to heating will be driven into a conventional liquid phase, characterised by random and isotropic molecular ordering with short-range order. However, at lower temperatures liquid crystals might exhibit one or more phases with significant orientational structure and long-range orientational order while still having the ability to flow.

One of the most common liquid crystal phases is the nematic, where molecules or mesogens possess a high degree of long-range orientational order, but are randomly distributed as in a liquid and all point in the same direction. The smectic phase is one where in addition to orientational order, the mesogens are grouped into layers enforcing long-range positional order in one direction. If chiral molecules are added to the nematic phase, the end product is a cholesteric phase liquid crystal in which the single directional order rotates around a space direction called the helical axis. These spatial variations in the orientation continuous symmetry are responsible for the softness of the liquid crystals and for their remarkable responsivity to excitations (Leslie, 1968). Cholesteric liquid crystals assemble into a helical molecular arrangement that selectively scatters white light in a three-dimensional spectrum. This feature is attributed to the helical field length, and hence the wavelength of the scattered light, is influenced by shear stress normal to the helical axis. Thus, the weakly ordered liquid crystal phase together with its unique optical property reflects the way in which they respond to detection of heat, light, sound,

mechanical pressure, and chemical environment with immense sensitivity (Fergason & Brown, 1968).



Mesogens ordering in a nematic phase | *Mesogens ordering in a smectic phase* | *Mesogens ordering in a chiral nematic or cholesteric phase*

Figure 3.5.1: Schematic illustrations of the liquid crystal mesogens ordering adapted from (Palffy-Muhoray, 2007).

Therefore, cholesteric liquid crystals were considered for studying the contractile activity in isolated cell populations of the intestine. In fact, the idea that liquid crystals may be involved in cell structure is not recent. This idea was initially proposed by Lehmann, one of the first to investigate liquid crystals in 1900 (Lehmann, 1900). At the time, however, there seemed to be no advantage in associating liquid crystals with cell formation. The lack of utility and knowledge of the structure and properties of liquid crystals resulted in its disappearance from biological literature until recent years.

At present the role of liquid crystals in biological systems has only began to be explored, especially in their role in sensory systems, in cellular shape and transmission of information (Fergason & Brown, 1968).

In the present study the cellular shape and the potential use of the liquid crystals material as transducer to capture cell responses was analysed. The coculture of ISMC and neurones seeded onto the cholesteric liquid crystal coating displayed a significantly rounder shape. Although rounder, the cells were firmly attached to the liquid crystal coated surface. This was displayed by the visible indentation lines exerted by firmly attached cells on the liquid

crystal surface. When the coculture of ISMC and neurones were exposed to the application of KCl, a decrease in cell size was observed in response to KCl. Moreover, due to the softness, elasticity and responsiveness of the liquid crystal coating the combined cell population response resulted in shear force being applied onto the substrate, which translated in the displacement field of the surface. In subsequent experiments, when the coculture of ISMC and neurones were pre-contracted with KCl and then exposed to noradrenaline, an ISMC muscle relaxant, the cell population response resulted by initially contracting and increasing the shear force being applied onto the substrate in response to KCl. This was then followed by the cells relaxing and decreasing the shear force applied to the surface in response to noradrenaline. These changes could be closely observed by changes in gray scale intensities. These results suggest that the liquid crystal material may be a suitable choice for studies of cell contraction in response to different drugs. More importantly, the liquid crystal substrate may also be suitable for studies of relaxation responses on pre-contracted cells.

Although, it was previously documented that most cells are attached to another cell or an extracellular matrix with an elastic modulus in the order of 10-10,000Pa (Bao & Suresh, 2003, Wakatsuki et al., 2000). A more recent review has indicated that myoblasts, the developmental precursor of myotubes, striated and muscle cells grow on substrates with compliances comparable to mature muscle tissue with an elastic modulus in the range of 0.012MPa (Engler et al., 2004).

In line with this information, although, the elastic modulus of the liquid crystal material is not documented, there are reports that when liquid crystals exist in the three-dimensional order, their elastic modulus is in the order of 0.5Mpa (Nozières et al., 2001). Thus, the literature suggests liquid crystal materials have an elasticity that is similar to that of the tissue. In support of the present study, the cell preparation when seeded in a compliant matrix of appropriate stiffness was more responsive to the drug application. This is

believed to occur as the artificial mechanical components of the tissue, in this case the cholesteric liquid crystal coating possesses the mechanical elasticity, which mostly resembles those surrounding mammalian cells.

In conclusion, we developed a cell culture model where individual cell populations or in combination were successfully extracted from the different layers of the intestine, purified and individually characterised. The results were reproducible and reliable. The genomic integrity of cells remained unchanged following cryopreservation for 1-month and demonstrated minimal DNA damage when cryopreserved for up to 4-months. The above conditions would guarantee viable cell cultures with limited or minor DNA damage. Liquid crystals as extracellular matrices would provide a potential candidate for studying the contractile and relaxant properties of intestinal cells to selective drugs.

The possibility of using intestinal cell populations isolated from segments of the intestine of rat neonates as a tool for the pharmacological characterisation of receptors capable of activating receptors in individual cell population or in cocultures is indeed desirable. Creating biological actuators from cholesteric liquid crystal materials is possible due to the biocompatibility and responsiveness of the liquid crystal material here investigated. However, it yet remains to evaluate whether full concentration-response curves can be performed and yield reproducible results. Furthermore, there are limitations in the quantitative evaluation of the data from mechanical force microscopy to make it relevant to the field of pharmacology. This would perhaps imply the development of computer modeling to convert the surface deformations induced by cells in response to drug application into measurable forces at a cellular level.

CHAPTER FOUR

DEVELOPMENT OF AN INTESTINAL CELL CULTURE MODEL OF INFLAMMATION

4.1 INTRODUCTION

The GI tract is unique among organ systems since it possesses autonomic function in isolation and is equally influenced by the central nervous system. The GI tract is organised in layers and has a highly complex enteric nervous systems that regulate and coordinate the GI absorption, secretion, motility, and sensation (Gershon & Tack, 2007). The enteric nervous system consists of submucosal and myenteric ganglia and an intricate network of intrinsic and extrinsic afferent neurones, ascending and descending interneurones, inhibitory and excitatory motor neurones that interact with the longitudinal and circular smooth muscle, mucosal, and endocrine cells (Beatie & Smith, 2008). Considering the complexity in regulating the GI function it is not surprising that when GI dysregulation occurs, the impact exerted on health is significant. Functional GI disorders emerge from inflammation as a defense mechanism that the body employs to fight the constant challenges originated from the external environment. Because of its enormous mucosal surface, which is continuously exposed to antigen, mitogenic, mutagenic, toxic stimuli, the GI tract is clearly susceptible to such inflammatory responses. The consequences of the onset of the inflammatory insult is an immune response that targets potential pathogens by activation of mucosal immune cells including neutrophils, macrophages, and cytotoxic T cells that attack and destroy nearby cells either directly or indirectly through the release of soluble factors such as reactive oxygen and nitrogen metabolites, cytotoxic proteins, lytic enzymes, and cytokines (Fiocchi et al., 1994, Grisham & Yamada, 1992).

The mounting effect of the immune response against antigens within the intestinal mucosa produces local tissue injury (Figure 4.1.1).

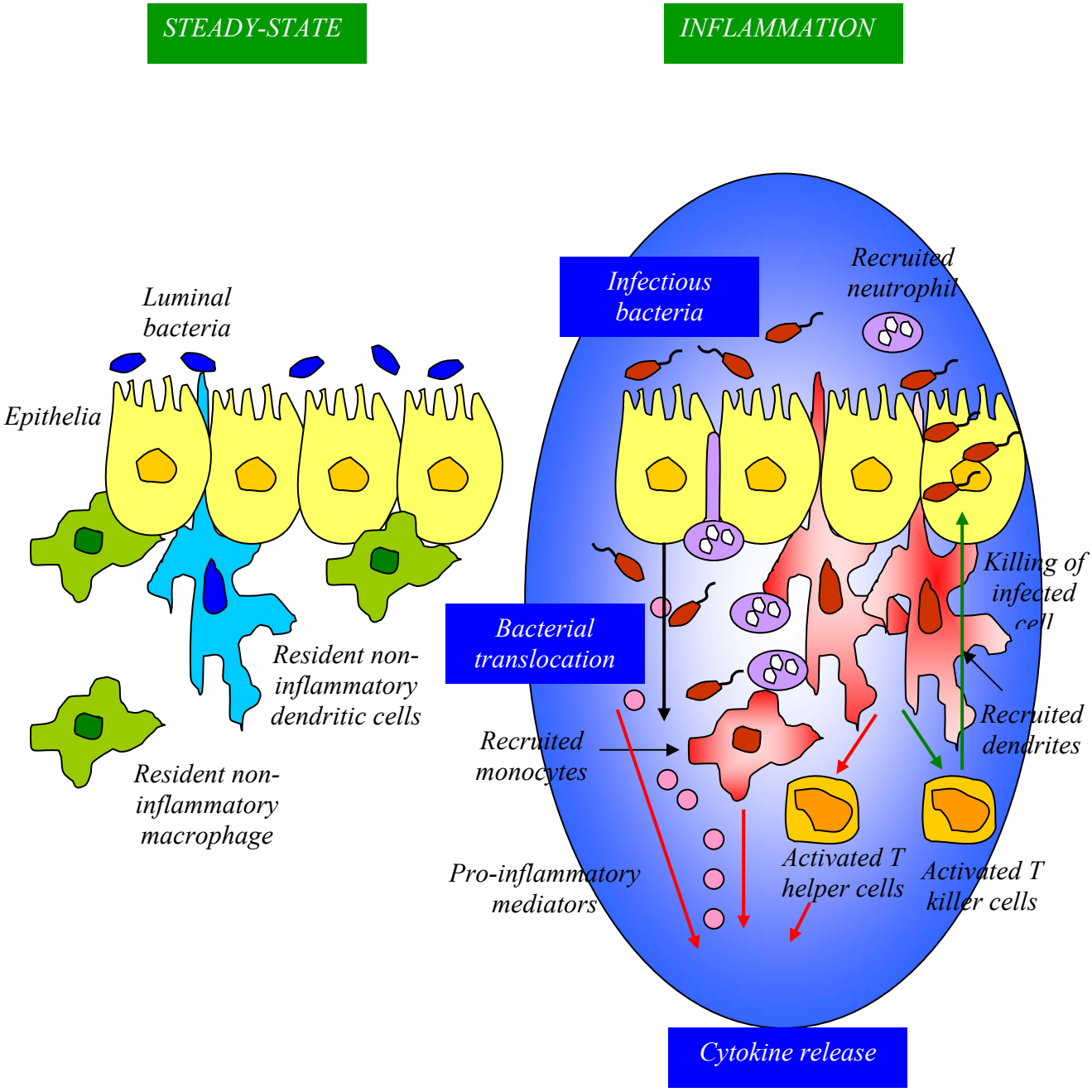


Figure 4.1.1: Diagram representing the mucosal surface interactions with microflora bacteria and infectious bacteria. In the steady state the luminal bacteria that colonises the gut epithelial are targeted by resident immune cells, which immune cells that keep the homeostasis of epithelia. State of inflammation commences with interaction of endotoxin with the epithelial cells, which leads to translocation of the bacteria across the intestinal barrier and recruitment of immune cells that release inflammatory mediators.

In newborn infants, the most commonly documented episode of the onset of GI inflammation occurs after the first feed. This results in infection that attacks predominantly premature infants due to the fragility of the tiny host (Anand et al., 2007). As a result the continuous inflammation attacks the intestine from within inducing the development of necrotising enterocolitis (Anand et al., 2007). Necrotising enterocolitis is the leading cause of death from GI disease in preterm neonates (Anand et al., 2007, Nanthakumar et al., 2000). The precise aetiology of the chronic intestinal inflammation, however, still remains elusive despite the intensive research that has been carried out over the past few years. More recently, intestinal cell culture models of inflammation derived from fetal epithelium have been developed to use as tools to examine the action of proinflammatory mediators in the model of necrotising enterocolitis (Nanthakumar et al., 2000). The data acquired have been useful in assessing the enhanced cytokine responses and may in part explain the factors that may contribute to the excessive inflammatory response in necrotising enterocolitis (Nanthakumar et al., 2000). The epithelial models of inflammation, however, are restricted exclusively to the investigation of epithelium responses to inflammatory insult (Liboni et al., 2004). Such models of inflammation were primarily developed since it is an established notion that when the inflammation response occurs in the intestine, the effector immune cells located in the epithelial lining of the gut dominate such a response causing destruction of other mucosal cells (Fiocchi et al., 1994). Progress in understanding the process of intestinal inflammation has led to further evidence of the involvement of epithelial, endothelial, muscle, and nerve cells as effector and regulatory elements of the immune response (Fiocchi, 1997). Therefore, the development of an intestinal model of inflammation, representative of all intestinal cell layers would be desirable to assess the complex interplay between conventional immune cells and newly proposed non-immune cell interactions. It is hypothesised that the information derived from such an‘*in-vitro’*

approach will provide evidence of the pro-inflammatory mediators and cytokines implicated in the process of GI inflammation in neonates.

4.2 AIM

The aim of the present study was to first develop an intestinal cell culture model of inflammation including all cellular components representative of the intact intestine. The inflammation status was achieved by lipopolysaccharide (LPS)-induced inflammation *'in vitro*'.

Secondly, following confirmation of the stimulation of pro-inflammatory cytokines and mediators, the model was used to investigate the effects of granisetron, a 5-HT_3 receptor antagonist, L-NAME, a nitric oxide (NO) synthase inhibitor, and Chaga mushroom extract, a wood-rotting fungus with reported anti-inflammatory properties on inflammation induced by LPS.

4.3 METHODS

4.3.1 CELL CULTURE AND LPS STIMULATION

The animal housing and conditions were followed as described in section 2.3.1. Rat neonates were killed by dislocation of the neck. The whole intestine was subsequently removed and emptied of its contents by gently flushing with HBSS. The length of the intestine was approximately 15cm in its contracted form. One segment (2cm) was taken 12-14 cm distal to the pyloric region, that was considered as the ileum as explained in section 3.3.1. The segments were then enzymatically dissociated in 1ml of 0.25% trypsin/EDTA for 30 min at 37ºC. The preparations were then triturated by gently pipetting the contents up and down for approximately 5 min until a homogenous mixture was achieved. The mixture was then transferred to a clean centrifuge tube and 1ml media added: Dulbecco modified eagle's medium (DMEM) with HEPES modification (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and supplemented with 10% fetal calf serum (FCS), 100U ml^{-1} penicillin and 100µg ml^{-1} streptomycin solution, 2mM L-glutamine, and 0.2% amphotericin B.

The cell suspensions were centrifuged for 5 min at 450xg. Supernatant was discarded and 5ml media was added. The cell suspensions were triturated so as to obtain a suspension of single cells and then transferred to $25cm^2$ cell culture flask and incubated at 37°C. Cells were routinely monitored and medium was changed at 48h intervals. On day 7 as cells reached confluence, the cell culture media was replaced by FCS-free media supplemented with 2Mm glutamine. In separate experiments investigating the effect of low glutamine supplementation on intestinal inflammation, the cell culture media was replaced by FCS-free media supplemented with 0.5mM glutamine. After 24h, intestinal cell culture preparations were treated with 0, 10, 50, and 100µg/ml of LPS. Following incubation for 24h with the varying treatments with LPS, cell culture supernatant were collected,

centrifuged at 450xg, and stored at -20°C for subsequent analysis of IL-8 and nitric oxide (NO) production.

4.3.2 DETERMINATION OF NITRATE AND IL-8 CONCENTRATION IN CELL SUPERNATANT

The ability of LPS to stimulate IL-8 secretion was quantified by an ELISA assay and expressed as pg/mg of total cellular protein. IL-8 was quantified in each supernatant in triplicate. Colorimetric results were read on a Titertek Multiscan II 96-well plate reader at a wavelength of 450nm.

The biological activities of NO were measured as its end product, nitrite using the Griess reagent. Since NO is a gaseous free radical with a short half-life *'in vivo'* of a few seconds, the levels of their metabolites (nitrate and nitrite) are more stable for the measurement of NO in biological fluids (Marletta et al., 1993). The Griess assay determines NO concentration based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The Griess Reaction is based on the two-step diazotization reaction in which acidified nitrite produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N- (1-naphthyl) ethylenediamine to form the chromophoric azo-derivative, which absorbs light at 540 - 570nm. Briefly, the cell culture supernatant (100µl) was mixed with 100µl Griess reagent and incubated for 10 min before absorbance was read at 570nm using the plate reader. The concentration of nitrite in the samples was determined with reference to a sodium nitrite standard curve. NO was quantified in each treatment in triplicates.

4.3.3 CELL VIABILITY, PROLIFERATION AND ADHESION STUDIES

To assess cell dynamics following exposure to varying LPS treatments cell viability, cell proliferation, and cell adhesion studies were carried out. Following cell dispersion from segments taken from the ileum of rat neonates, cells were seeded onto cell culture flasks and allowed to reach confluence before they were exposed to the varying LPS treatments. As cell culture supernatants were collected for IL-8 and NO measurements, the intestinal cell monolayer in the cell culture flaks were subsequently used for cell viability, proliferation and adhesion studies. Cell viability and cell proliferation were performed as described in section 3.3.3. The only exception being that unlike cell viability studies, in cell proliferation studies the cell counting involved count of both stained and unstained cells hence enabling the determination of the total cell number in the cultures. In the cell adhesion studies, media was removed from the cell culture preparations and the cell culture surface washed with HBSS. HBSS was removed and replaced by 1ml of 0.25% trypsin/EDTA and microscope images were taken every 30 sec until cells were completely detached from the cell culture surface.

4.3.4 IMMUNOCYTOCHEMISTRY

To assess the phenotypic properties of ISMC exposed to the LPS treatment, immunocytochemistry was carried out. This technique was used to qualitatively assess the amount of α-smooth muscle actin fibers between LPS-treated and untreated preparations.

Cells dispersed from segments taken from the ileum of rat neonates, seeded on cell culture flasks and treated with LPS (50μg/ml) were subsequently purified for ISMC as described in section 3.3.6. The LPS concentration of 50μg/ml was selected, as it was the lowest LPS concentration necessary to induce the release of pro-inflammatory mediators. The ISMC

were subsequently grown on glass cover slides overnight, fixed in 2% formaldehyde for 5 min and kept in PBS until used for immunostaining experiments.

Immunocytochemistry on purified ISMC was carried out with mouse anti-α-smooth muscle actin antibody. Briefly, cells were made permeable by incubating in PBS containing 0.1% Triton X-100 for 15 min. Slides were then bathed in PBS containing 2% goat serum for 30 min before incubation for 60 min with α-smooth muscle actin (1:100 in PBS-1%BSA) at room temperature. This was followed by incubation with mouse TRITC-labeled secondary antibody (1:30 in PBS-1%BSA) for 60 min at room temperature. Both incubations were preceded by 3 washes with PBS. Negative controls were carried out, in which primary antibodies were replaced with PBS. Preparations were mounted using DAPI. Stained samples were observed with a Nikon Eclipse 80i Fluorescence, and images were digitally captured with SOP (ACT-2U) software. Digital recombination of images using Adobe Photoshop was applied for the combined display of cytoplasmic and nucleus staining.

4.3.5 PHARMACOLOGICAL ASSAYS

Following confirmation that LPS stimulation promotes pro-inflammatory responses on the entire intestinal cell population isolated from segments taken from the ileum of rat neonates, experiments were carried out using this intestinal cell culture model to examine the role of 5-HT in the inflammatory process using granisetron (0.01, 0.1, 1μM) a 5-HT_3 receptor antagonist. In separate experiments the anti-inflammatory potential of L-NAME (0.1μM) a partial NO synthase inhibitor was also investigated. Intestinal cell culture preparations were pre-treated for 1h with media containing granisetron, L-NAME, or vehicle. Subsequently, media was replaced by FCS-free media supplemented with 2mM glutamine and containing 50μg/ml of LPS for 24h. This approach was sought to assess the potential anti-inflammatory action of granisetron and L-NAME in the management of

intestinal inflammation in neonates. In separate experiments, intestinal cell culture preparations were pre-treated primarily for 1h with media containing granisetron, L-NAME, or vehicle, after which LPS (50μg/ml) was concurrently added to the media and incubated for additional 24h. This continual drug treatment for 25h was sought to assess the effect of sustained anti-inflammatory drug management. The supernatant was collected and centrifuged for 5 min at 450xg and subsequently assayed for NO production as described in section 4.3.2. In separate experiments cell culture preparations were treated solely with granisetron (1μM) or L-NAME (1μM). The culture supernatant was then assayed for NO production.

In another series of experiments the anti-inflammatory potential of the Chaga mushroom extract was investigated. Chaga mushroom extract was obtained from Fungi Perfecti's Mycomedicinals, Chicago USA. According to the manufacturer's description, 30 drops of this alcohol-based extract are equivalent to 1g, which was further diluted in water to yield a concentration of 180μg/ml. Intestinal cell culture preparations were pre-treated for 1h with media containing Chaga mushroom extract (180μg/ml) or vehicle. Subsequently, media was replaced by FCS-free media supplemented with 2mM glutamine and containing 50μg/ml of LPS for 24h. The culture supernatant was then assayed for NO production.

4.3.6 STATISTICAL ANALYSIS

Comparative results were presented as means ± standard error of the mean. Following normality tests, the significance of the differences was tested by one-way ANOVA followed by Bonferroni adjustment test.

Table 4.1: Chemicals/drugs used in the present study, their solvent and suppliers.

Chemicals/drugs	**Solvents**	**Suppliers**
Chaga mushroom ethanolic extract	Distilled water	Fungi Perfecti's Myocomedicinals
Lipopolysaccharide (LPS)	HBSS	Sigma
Griess Reagent	Distilled water	Sigma
Scorpion venom (*maurus palmatus*)	Distilled water	Sigma
Granisetron HCL $2H_20$	Distilled water	Sigma
Trypan blue	Distilled water	Sigma
L-NAME (L-Nitro-Arginine Methyl Ester)	Distilled water	Sigma

4.4 RESULTS

4.4.1 THE EFFECTS OF LPS AND GLUTAMINE TREATMENTS ON IL-8 AND NO PRODUCTION IN INTESTINAL CELL CULTURE

The extracted cells showed a basal level of NO of 12.5μM±0.5 and a basal level of IL-8 of 7.5pg/ml±1.5 in the absence of any inflammatory promoter.

The intestinal cell cultures treated with varying concentrations of LPS (10, 50, 100μg/ml) and supplemented with 0.5mM or 2mM glutamine were assessed for the levels of pro-inflammatory cytokines and mediators by measuring IL-8 and NO levels in the cell culture supernatants.

Pre-treatments with LPS plus 2mM glutamine induced the release of NO in the intestinal cell preparations, which achieved significance at 50 and 100μg/ml of LPS ($p<0.001$). However, when cells were treated with LPS plus 0.5 mM glutamine, NO production was only significantly ($p<0.001$) elevated at 100μg/ml (Figure 4.4.1a).

Pre-treatments with LPS (10, 50, 100μg/ml) plus 2mM glutamine induced the production of IL-8 in a concentration–dependent manner, which achieved significance ($P<0.05$) at 100μg/ml. The intestinal cell culture pre-treatment with LPS (10, 50, 100μg/ml) plus 0.5 mM glutamine also induced the release of IL-8, however, the levels of IL-8 were comparable to those in the control cell preparations (Figure 4.4.1b).

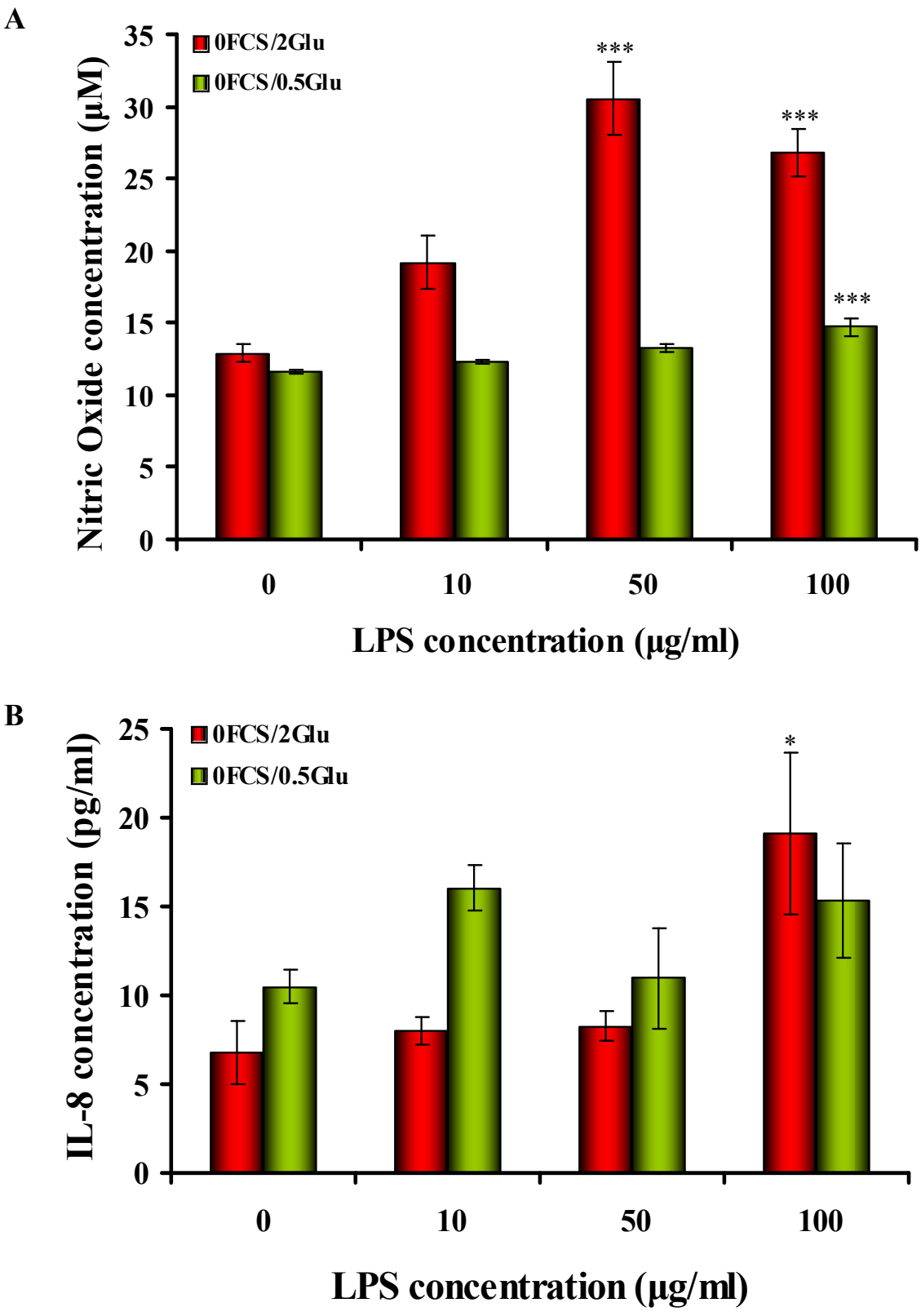


*Figure 4.4.1: The effect of increasing concentrations of LPS (0, 10, 50, 100 µg/ml) plus glutamine 2 mM or 0.5mM treatments on the production of pro-inflammatory agents NO (A) and cytokine IL-8 (B) in intestinal cell preparations. Each point represents the mean±s.e.mean; n=4. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

4.4.2 THE EFFECT OF LPS TREATMENTS ON CELL VIABILITY, CELL ADHESION AND CELL PROLIFERATION

To address the effects LPS-induced inflammation on the cellular dynamics of intestinal cell preparations, cell viability, adhesion and proliferation were examined. The intestinal cell culture preparations treated with LPS at the varying concentrations of 10, 50, 100μg/ml showed no significant differences in the cell viability when compared with untreated cell culture preparations (Figure 4.4.2a).

The cell proliferation analysis showed that LPS treatments enhanced cell numbers. Intestinal cells isolated from segments of the ileum produce cell yields of $2x10^5$. Following cell seeding for 7 days until cultures reached confluence, treatments with LPS at concentrations of 10, 50, 100μg/ml produced cell numbers of $4x10^5$, $5.5x10^5$, $6x10^5$, respectively. Although, increased cell proliferation was observed in LPS treated cell preparations, these were not significantly different compared to the untreated cell cultures ($3x10^5$) (Figure 4.4.2b).

In separate studies assessing the cell adhesion properties of intestinal cell preparations following LPS treatments, results revealed that LPS challenge at any given concentration had no effect on the rate of intestinal cell detachment (Figure 4.4.3). Time-lapse micrographs demonstrated progressive cell detachment that was completed following 180 sec incubation with trypsin/EDTA. These were observed in untreated and LPS-treated intestinal cell cultures (Figure 4.4.4).

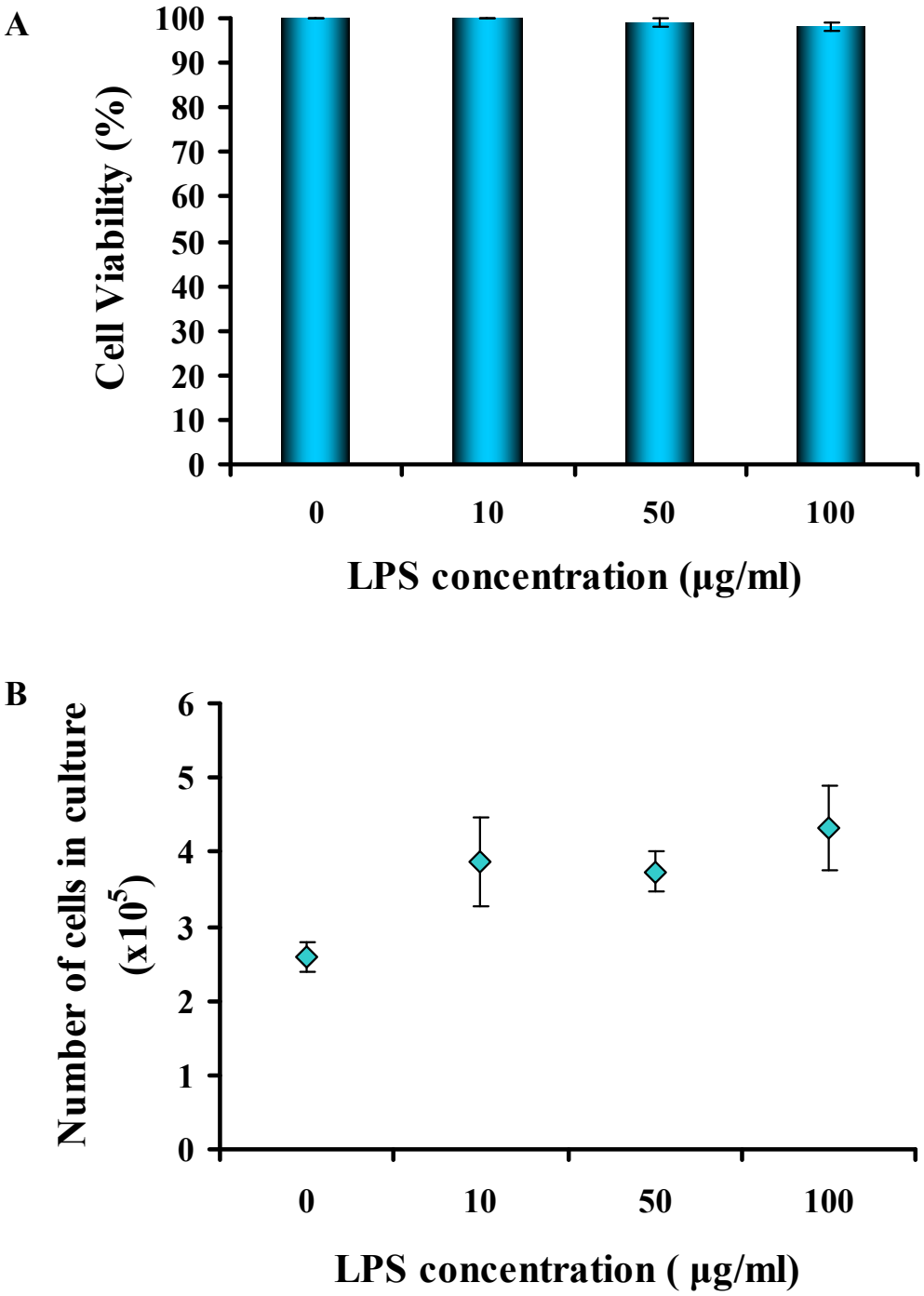


*Figure 4.4.2: Intestinal cell viability following treatment with increasing concentrations of LPS (10, 50, 100µg/ml). LPS treatments had no effect on intestinal cell viability when compared to untreated intestinal cell preparations (A).Intestinal cell proliferation following treatment with increasing concentrations of LPS (10, 50, 100µg/ml). LPS treatments demonstrated dose dependent increase in intestinal cell proliferation (B).Each point represents the mean±s.e.mean; n=3. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control values.*

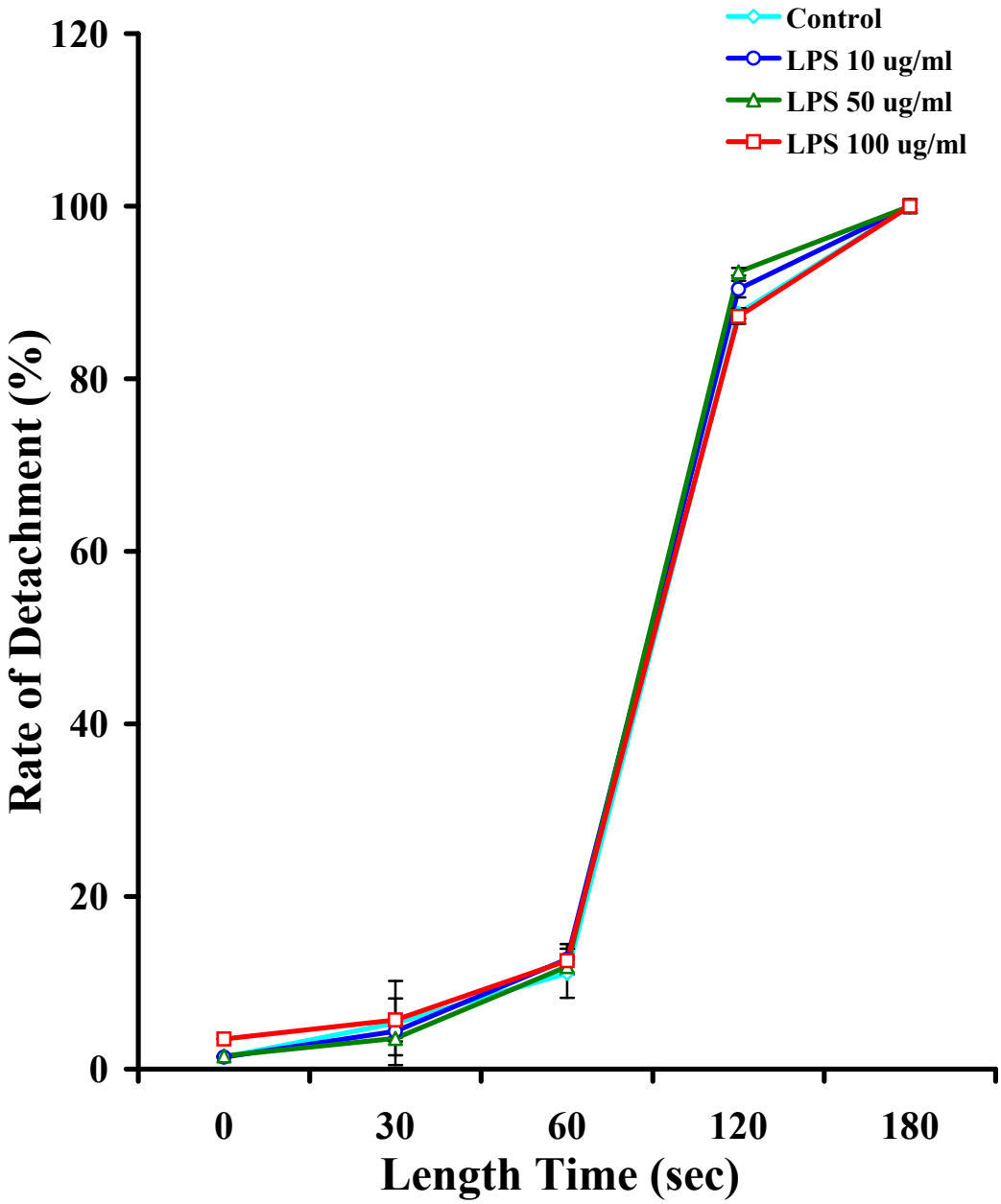


Figure 4.4.3: The effect of LPS treatment on the cell adhesion ability of intestinal cells. Intestinal cell culture preparations were exposed to increasing concentrations of LPS (0, 10, 50, 100 um/ml) and once cells reached confluence, proteolytic enzyme trypsin was used to cleave the bound between cells and the surface promoting cell detachment. This was monitored for each LPS treatment and the percentage of cell detachment rate calculated at 0, 30, 60, 120, and 180 sec.

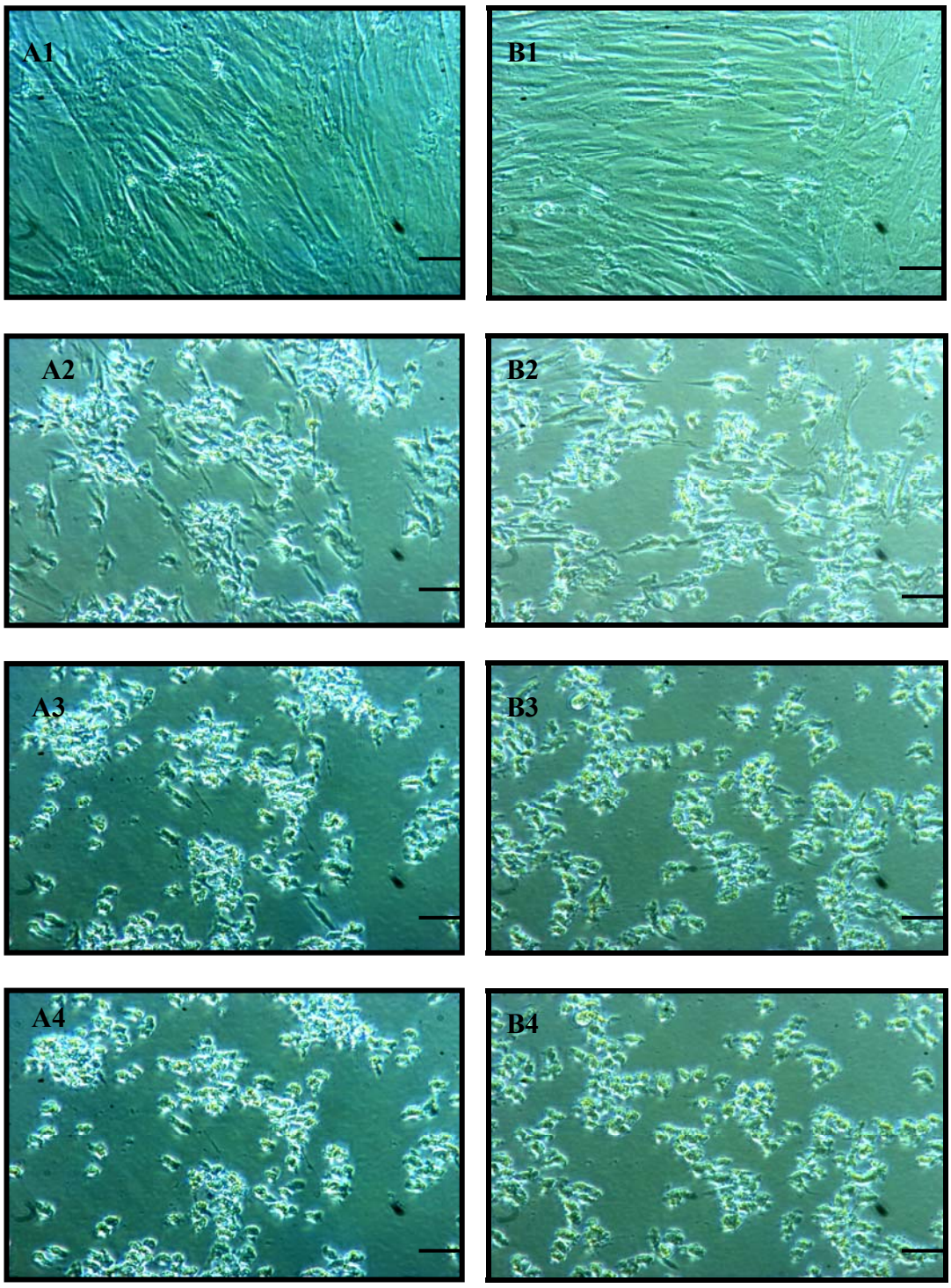


*Figure 4.4.4: Micrographs illustrating the rate of cell detachment on LPS-treated at 50*μg/ml *(A) and untreated (B) intestinal cell culture preparations at 0 (1), 60 (2), 120 (3), 180 sec (4). Following exposure to proteolytic enzyme trypsin, intestinal cell preparations demonstrated gradual and coordinated rate of cell detachment between the differently treated cell cultures. Scale bar=50 μm.*

4.3.3 IMMUNOCYTOCHEMISTRY

Immunocytochemistry was carried out to investigate the phenotypic changes induced by LPS (50μg/ml) treatment on ISMC. Antibody to α-smooth muscle actin indicated that LPS treatment caused a decrease in the expression of actin fibers compared to untreated intestinal cell preparations. Moreover, the actin fibers of ISMC from LPS-treated preparations displayed an impaired distribution compared to ISMC of untreated intestinal cell preparations (Figure 4.4.5).

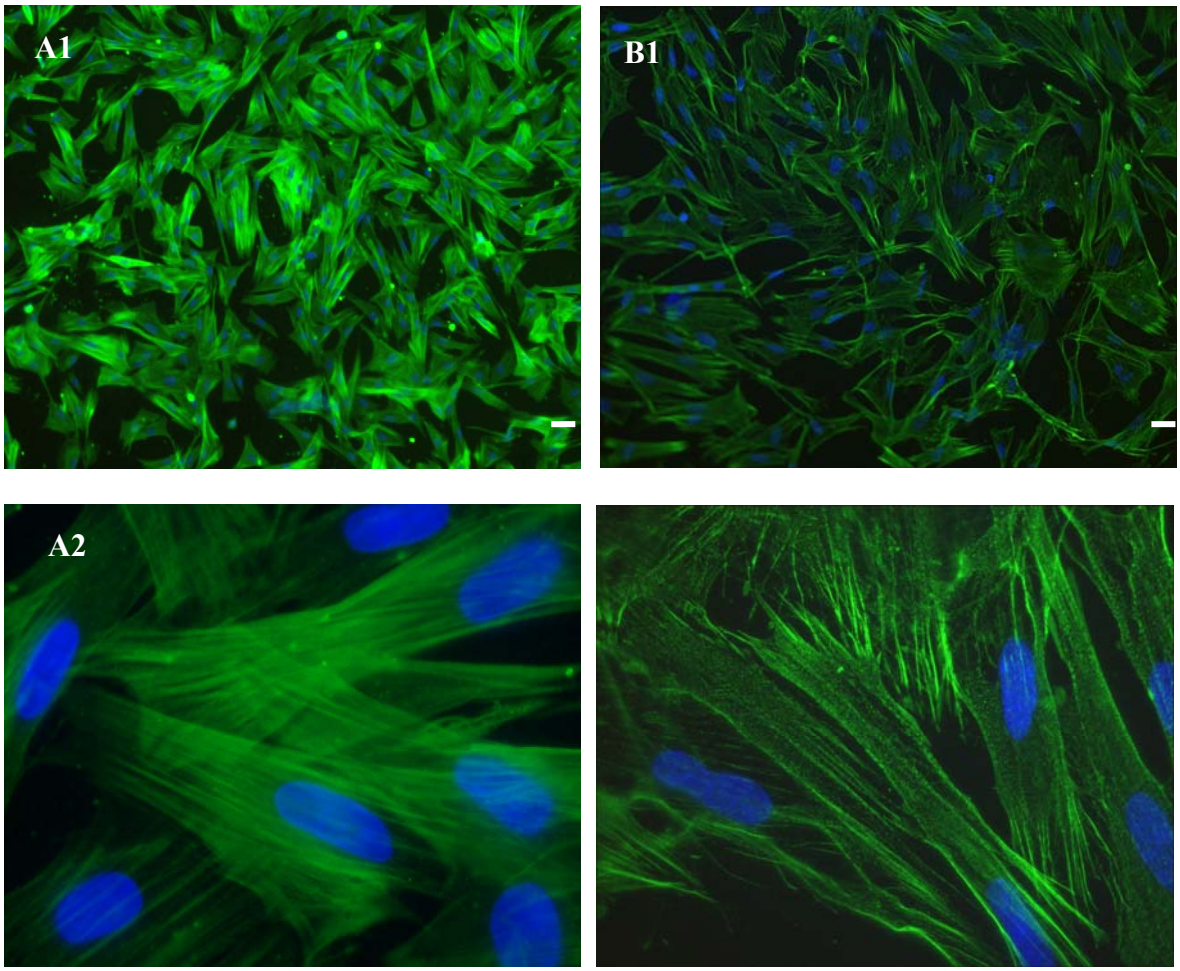


Figure 4.4.5: Representative micrographs showing a comparison of the α-actin fibers expression and distribution between purified ISMC from LPS-treated (B1-2) and untreated (A1-2) intestinal cell preparations. ISMC isolated from segments taken from the intestinal rat ileum stained with α-smooth muscle actin antibody (green) for confirmation of the nature of the purified ISMC. DAPI mount was used for labeling of cell nucleus (blue). Scale bar=25μm.

4.4.4 THE EFFECT OF GRANISETRON AND L-NAME ON LPS-INDUCED INFLAMMATION IN THE INTESTINAL CELL CULTURE PREPARATIONS

The extracted cells from the intestine showed a basal level of NO of 10μM±0.5 in the absence of any inflammatory promoter (Figure 4.4.6a). The cell preparations treated with LPS (50μg/ml) alone induced a significant ($p<0.05$) increase in the level of NO production compared to controls. The level of NO was still significantly ($p<0.05$) high when cells were treated for 1h with the 5-HT_3 receptor antagonists, granisetron at a concentration of 0.01μM prior to the application of LPS as compared to the level of NO in non-treated cells. Pre-treatment for 1h with granisetron at concentrations of 0.1μM and 1μM showed an increase in the level of NO, however, these were not significant as compared to control cell preparations (Fig. 4.4.6a). In addition, the level of NO was significantly ($p<0.05$-$p<0.001$) high when cells were treated for 1h with granisetron (0.01, 0.1, and 1μM) and concurrently with LPS (50μg/ml) for 24h (Figure 4.4.6a).

Pre-treatment for 1h or 25h with the NO synthase inhibitor, L-NAME, prior to the application of LPS caused a significant ($p<0.05$-$p<0.01$) increase in the level of NO production compared to non-treated cells (control) (Figure 4.4.6a).

In separate studies the effect of 1h treatment with granisetron (1μM) or L-NAME (1μM) was investigated on intestinal cell preparations in the absence of LPS. Following 1h treatments with granisetron or L-NAME (1μM), results revealed no influence of these drugs on the levels of NO production in the intestinal cell preparations as compared to the basal level of NO in control cells. However, longer incubation times of 25h with granisetron or L-NAME caused a significant ($p<0.001$) increase in the levels of NO production compared to controls (Figure 4.4.6b).

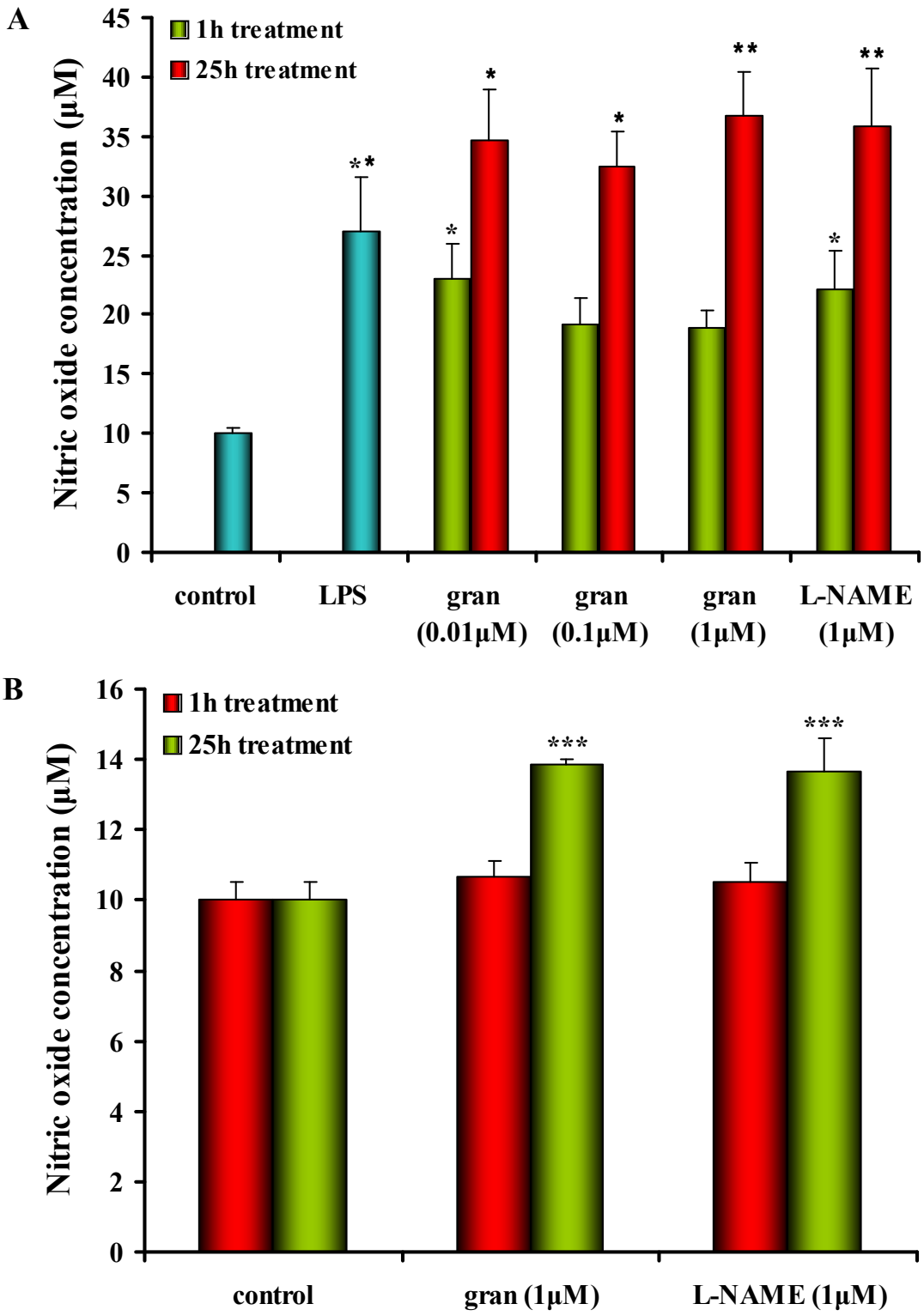


*Figure 4.4.6: The effects of 1h and 25h pre-treatment with granisetron (0.01, 0.1, 1µM) or L-NAME (0.1µM) on LPS (50µg/ml)-treated intestinal cell cultures (A). The effects of 1h and 25h treatment with granisetron (1µM) or L-NAME (0.1µM) on intestinal cell preparations (B). Each point represents the mean±s.e.mean; n=3. *p<0.05, **p<0.01, ***p<0.001 taken as a significant difference compared to the control.*

4.4.5 THE EFFECT OF CHAGA MUSHROOM EXTRACT ON LPS-INDUCED INFLAMMATION IN THE INTESTINAL CELL CULTURE PREPARATIONS

The extracted cells from the intestine showed a basal level of NO of 9.5μM±0.5 in the absence of LPS stimulation (Figure 4.4.7). The cell preparations treated with LPS (50μg/ml) alone induced a significant ($p<0.05$) increase in the level of NO production compared to controls. Pre-treatment for 1h with Chaga mushroom extract at a concentration of 180μg/ml prior to the application of LPS (50μg/ml) did not significantly change the level of NO production in comparison to the controls. Thus, there were no significant differences in NO production between LPS plus Chaga-treated preparations and controls. Moreover, the intestinal cell preparations pre-treated for 1h with Chaga mushroom extract prior to the application of LPS (50μg/ml) caused a significant ($p<0.05$) reduction in the level of NO production when compared to the LPS-treated preparations (Figure 4.4.7).

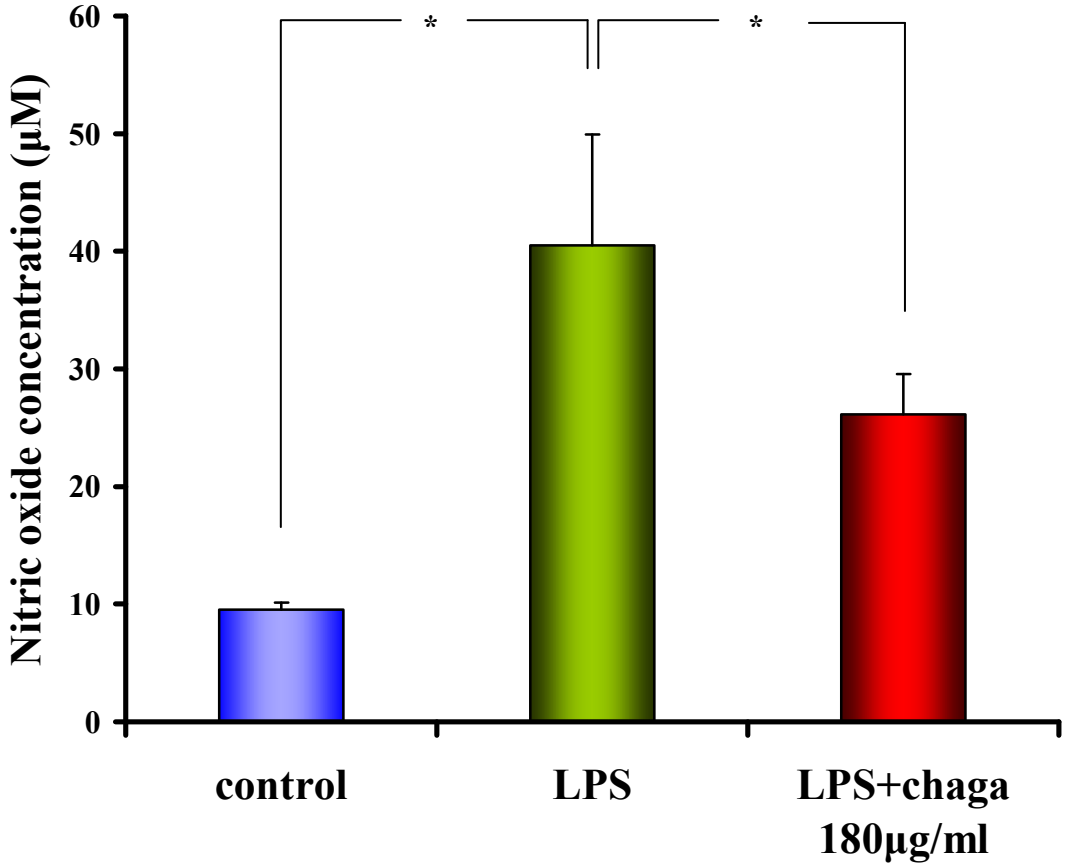


*Figure 4.4.7: The effects of Chaga mushroom extract (180µg/ml) treatments on LPS (50µg/ml)-treated intestinal cell cultures. Each point represents the mean±s.e.mean; n=4. *p<0.05 taken as a significant differences compared to the control.*

4.5 DISCUSSION

Respiratory distress syndrome has been over many years the major medical challenge in premature newborns. With the development of surfactant to reduce the incidence of respiratory disease, necrotising enterocolitis has become the leading case of death in these patients. Necrotising enterocolitis is diagnosed in between 0.19 to 2.4 per 1000 live births (Feng et al., 2005, Henry & Lawrence, 2005). The disease is characterised initially by intestinal inflammation, which may progress into intestinal necrosis. Several theories have been proposed to explain the development of necrotising enterocolitis and they are all centered on the role of stressed premature intestine in association with bacteria. It is thought that the presence of gas within the walls of the intestine from enteric bacteria is the pathogenic cause of necrotising enterocolitis (Foglia, 1995, Anand et al., 2007). The study of inflammatory response *'in vitro'* is limited as the commercially available cell lines only represent one cell type. However, the progression of local intestinal injury to a diffused multisystem process suggests the involvement of circulating pro-inflammatory cytokines in different layers of the intestine and the involvement of different cell types in the pathogenesis of necrotising enterocolitis (Anand et al., 2007).

In the present study attempts were made to develop an intestinal cell culture model of inflammation with cells isolated from the neonatal rat ileum. The developed model consisted of cells extracted from different layers of intestine and was used to investigate the effects of LPS in the absence and presence of some pharmacologically active compounds such as granisetron, a 5-HT_3 receptor antagonist, L-NAME, a NO synthase inhibitor. Additionally, the model was used to investigate the potential anti-inflammatory properties of the Chaga mushroom extract.

The extracted cells when assayed for NO and IL-8 showed a basal levels of 12.9μM±0.5 and 6.75pg/ml±1.7, respectively. The application of LPS (10, 50, and 100μg/ml) induced

an increase in the levels of NO production (19.15±1.8, 30.55±2.4, and 26.8±1.6 respectively). The application of LPS (10, 50, and 100μg/ml) also induced an increase in the levels of IL-8 production (8.0±0.7, 8.25±0.9, and 19.1±4.5 respectively). NO and IL-8 have been shown to be markers of GI inflammation in epithelial cell models (Eckmann et al., 1993) and animal models (Jourd'Hevil et al., 1997).

Recent research studies have suggested that pathologic microorganisms, particularly Gram-negative bacteria, communicate with the intestinal epithelium. Through this communication, these pathologic microorganisms interact with the epithelial cells to stimulate the activation of pro-inflammatory cytokines (Ulevitch & Tobias, 1995). During this period of antigen insult, the bacteria may cross the intestinal epithelium resulting in the widespread activation of the mucosal immune system. This is initiated by the activation of T cells (Ford et al., 1996) producing pro-inflammatory cytokines that may cause direct epithelial damage (Homan et al., 1989). This results in the stimulated release of inflammatory mediators by neighboring recruited cells (Rowe et al., 1994) or recruitment of additional blood-borne inflammatory cells, which in turn activate additional cytokines. This continuous status of inflammation may become established resulting in further tissue invasion by bacteria and the onset of necrotising enterocolitis (Ford, 2006).

To examine this, previous models of intestinal inflammation have been achieved through exposure to cell wall Gram-negative bacteria, namely endotoxin LPS. Studies have shown that treatments with LPS stimulates cellular inflammatory insult and releases immune related factors such as NO (Chen et al., 2001), and cytokines including tumour necrosis factor (TNF-α) (Tracey & Ceremi, 1994), interleukin 6 (IL-6) and IL-8 (Ulevitch & Tobias, 1995, DeFranco et al., 1998) that promote inflammatory responses.

Some of the studies reported on cell models of GI inflammation include RAW 264.7 macrophage cell line (Ohgami et al., 2003), H2 primary crypt-like human fetal enterocytes cell line (Nanthakumar et al., 2000), Caco-2 a widely studied human adult intestinal

epithelial cell line (Nanthakumar et al., 2000, Liboni et al., 2004), and H4 a recently developed human fetal non transformed primary small intestine epithelial cell model (Liboni et al., 2004). Data obtained from such studies have contributed enormously to our understanding the mechanisms of activation of the immune system. These studies also provide supporting evidence on the role of various cytokines and immune mediators in onset of GI inflammation and demonstrate their potential use as a tool for pharmacological analysis of candidate drug therapeutics in GI inflammation (Ohgami et al., 2003).

Although, the aforementioned models of GI inflammation are representative of cell populations with well-documented involvement in the mechanisms inflammatory injury, however as indicated before, results obtained from such models following the onset of inflammatory insult are limited to the individual cell population being analysed and not to the overall host responses. These unidirectional models are based on the traditional understanding that intestinal inflammation is a process in which the mucosal immune compartments, namely the epithelium lining, chooses the appropriate effector functions necessary to deal with each encountered antigen. This decision-making process represents the coordinated response of several cell types present in the epithelial mucosa, which mediate the immune response (Laroux et al., 2001). However, emerging evidence showing that all cell types populating the mucosa have an active role in the intestinal immunity and inflammation has increasingly challenged this unidirectional model (Fiocchi, 1997). Apart from epithelial cells, it has been reported that endothelium, mesenchymal, nerve cells, muscle cells and even acellular components of the extracellular matrix appear to be involved in the immunoregulatory activity under normal and inflammation conditions (Fiocchi, 1997, Raghow, 1994).

Therefore, in the present study it was hypothesised that by creating a model of intestinal inflammation, which was representative of all cellular and acellular components of the intestine, would produce a model of the intestine where overall host responses could be

assessed. Such a model was sought to investigate the scale of inflammatory challenges encountered by neonates and furthermore contribute to the pathophysiology of necrotising enterocolitis.

The experiments were carried out by exposing the intestinal cell preparations with increasing concentrations of LPS (10, 50, 100 μg/ml) and the cell culture supernatants were measured for the levels of NO and IL-8 production in response to inflammatory challenge. The results demonstrated that inflammatory stimuli by LPS on intestinal preparations caused a significant increase in NO levels at concentrations of 50 and 100 μg/ml compared to untreated intestinal cells. The IL-8 production was also significantly increased in response to LPS treatment at 100μg/ml. These results correlate with studies by Nanthakumar et al, who observed a significant increase in the level of IL-8 after following the exposure to LPS in the fetal enterocytes cell line (Nanthakumar et al., 2000). This was confirmed by Liboni et al, who reported increased level of IL-8 after treatment with LPS in human fetal non-transformed primary small intestine epithelial cell model (Liboni et al., 2004). Increased NO production in response to LPS exposure was also reported in RAW 264.7 macrophage cell line (Ohgami et al., 2003).

In recent studies by Haung et al, and Liboni et al, it was reported that reduction in the level of glutamine in cells treated with LPS led to an increase in the level of IL-8 production (Huang et al., 2003, Liboni et al., 2004). However, results in the present study showed that reduction in the level of glutamine from 2mM to 0.5mM in cells treated with LPS did not have any significant effect on the level of IL-8 production. Although, the level of NO production was significantly higher in preparations treated with low glutamine compared to controls, this was only observed in intestinal preparations exposed to 100μg/ml LPS. The NO levels observed at each given concentration of LPS failed to produce excessive release of NO comparable to those observed under commonly used '*in vitro'* glutamine supplementation of 2mM. Data derived from the present study show discrepancies from

those presented by Liboni et al, in which glutamine deprivation upregulated pro-inflammatory mediators in fetal non transformed primary small intestine epithelial cell model (Liboni et al., 2004). However, findings from the present study are in line with '*in vitro'* observations of increasing IL-8 production in macrophages and monocytes inflammation models with increased glutamine concentration (Wallace & Keast, 1992, Murphy & Newsholme, 1999). The models of inflammation derived from macrophages, monocytes, and epithelial cells are unidirectional models consisting of cell cultures of a single immune effector cell type that are still challenged by evidence that other cells from the mucosa may be implicated in intestinal immune response to inflammation (Fiocchi, 1997). However, the intestinal cell culture model of inflammation developed in the present study offers a multidirectional model comprising the various cell populations harvested from segments taken from the ileum of rat neonates. Such model combines the interaction between effector cells promoting the release of the pro-inflammatory mediators and cells affected by these mediators in normal and inflamed mucosa, which makes practical sense and possibly more closely reflects reality.

To elucidate the effects of LPS treatments on intestinal cell function, preparations were assessed for cell viability, proliferation, and adhesion. Treatments with varying LPS concentrations did not influence cell viability significantly compared to the untreated cell preparations. These were in line with reports by Mochida et al, while investigating the inflammatory responses to LPS in murine macrophages (Mochida et al., 2007). Similarly, treatments with varying LPS concentrations did not alter cell adhesion properties compared to the untreated cell preparations. Mucosal damage is a characteristic feature during inflammatory injury, which requires efficient wound healing action. The response of the epithelial cells to injury is a complex process, where cell-matrix interactions play important roles in the wound healing process (Kolachala et al., 2007). Studies investigating the levels of expression of the binding protein, fibronectin in the regulation of

intestinal inflammation has revealed that fibronectin was abundantly expressed and synthesised by intestinal epithelial cells. Fibronectin was also demonstrated to be transcriptionally upregulated in epithelial cells during the onset of inflammatory response and during recovery phase. The epithelial derived fibronectin appeared to potentiate cell attachment through epithelial-matrix interactions and fibronectin expression. This observation suggests that during the onset of intestinal inflammation, fibronectin expression is upregulated in the epithelial cells to support the inflammatory injury tolerated by cells and maintain normal epithelial integrity including epithelial-matrix interactions (Kolachala et al., 2007). Thus, this explains why the rate of cell detachment remains the same between LPS treated and untreated cell preparations.

In separate experiments investigating the effect of LPS treatments on intestinal cell proliferation, results revealed an increase in the intestinal cell number with increasing concentrations of LPS. Although the onset of inflammation insult through LPS exposure increased cell numbers in culture, this was not significantly different as compared to control preparations. Nonetheless, the results correlate with characteristic morphology of the inflamed intestine, where inflammation results in thickening of the tissue layers with excessive deposition of extracellular matrix including collagen (Graham et al., 1988) and thickening of the tissue layers that have been associated with the expansion of all intestinal layers and enhanced ISMC proliferation (Graham et al., 1988). The extensive literature shows that the onset of smooth muscle division is associated with the modulation of cellular phenotype towards reduced expression of contractile proteins (Owens et al., 2004). In line with this information, the experiments that followed focused on the investigation of ISMC contractile proteins expression in the LPS treated and untreated preparations. The immunocytochemistry procedure using antibodies directed to α-smooth muscle actin revealed that ISMC from LPS treated cell preparations displayed a downregulated expression of actin filaments, with a disorganised orientation and rough appearance.

Whereas ISMC from untreated cell preparations displayed a high expression of actin filaments that aligned across the cytoskeleton and produced a smooth appearance. These results correlated with previous studies, where phenotype modulation of smooth muscle through reduction in actin expression was associated with various pathological conditions including inflammation (Johansson & Persson, 2004, Owens et al., 2004). However, the factors that may be involved in the increased cell proliferation through stimulation of smooth muscle growth and modulation of smooth muscle phenotype through reduction in actin expression remain to be identified.

One important factor that has been related to regulatory role of ISMC proliferation is the intestinal innervations. In the animal model of colitis where ISMC hyperplasia was described, there was a 40% loss in intrinsic neurones, which occurred early in the time course following the induction of colitis (Sanovic et al., 1999). Related studies showed that innervations of ISMC by myenteric neurones in vitro maintained the high expression of α-smooth muscle specific actin and reduced the sensitivity to growth stimuli (Blennerhassett & Lourenssen, 2000). Thus, there seems to be a correlation between the effect of inflammation on the enteric nervous system and the extent of ISMC hyperplasia in affected tissues.

Another factor that may play a regulatory role in ISMC proliferation '*in vitro'* is the production of pro-inflammatory cytokines. These include the inducible nitric oxide synthase (iNOS), which is induced in response to pro-inflammatory stimulus (Marlow & Blennerhassett, 2006). In other studies, the smooth muscle phenotype was investigated in correlation to iNOS expression in the vasculature. The intimal smooth muscle exhibited a different morphological phenotype from normal cells in the media and showed higher activity of the iNOS promoter in response to inflammatory response (Yan & Hansson, 1998). Interestingly, iNOS expression has also been associated with ISMC cytoskeleton changes through disruption of actin polymerization (Zeng & Morrison, 2001). Such

findings in the impairment of the actin cytoskeleton formation are believed to be potentiated by cytokine formation in response to inflammatory stimulus through increase of NO production during bladder inflammation (Johansson & Persson, 2004). This was subsequently confirmed in observation where bladder smooth muscle were stimulated with various cytokines including TNF-α and IL-1β and stained for iNOS and α-actin production. The bladder smooth muscle cells that expressed iNOS displayed low actin expression (Johansson & Persson, 2004).

Therefore, the increase in the cell proliferation would affect the ISMC phenotype, which in turn would lead to impairment of the actin fibers. Furthermore, the excessive production of pro-inflammatory cytokines and mediators are characteristic occurrences during intestinal inflammation. Such occurrences were efficiently reproduced in the present *'in vitro'* model of intestinal inflammation. This study is the first to report the development of a coherent model of inflammation, which may be used for pharmacological screening of potential compounds in the treatment of intestinal inflammation.

Various inflammatory mediators, including TNF-α, IL-1, IL-6, IL-8, and IL-8 have been implicated in the pathogenesis of necrotising enterocolitis (Ford et al., 1996). Ford et al, have also shown that cytokine activation can lead to persistent, local production of NO through activation of inducible iNOS, which leads to damage to the intestine (Ford et al., 1996). During the last decade, it became increasingly clear that NO can be produced by almost all mammalian cells, including endothelium lining and vasculature, neurones of the central and enteric nervous system, and cells of the immune system (Moncada, 1992, Nathan & Xie, 1994). NO is produced by distinct a enzyme systems: a constitutive enzyme that is observed primarily in endothelium lining the vasculature (eNOS), a neural associated constitutive NO found in neurones of the brain and ENS (nNOS) and the inducible enzyme (iNOS) whose expression in endothelium, epithelium, and inflammatory

cells requires protein synthesis that is induced by cytokines as a response inflammatory conditions (Nathan & Xie, 1994). In the gut, NO synthase activity has been found within the intestinal wall in the enteric neurones (Aimi et al., 1993), enteric blood vessels (Nichols et al., 1993), and enterocytes (M'Rabet-Touil et al., 1993). Such localisation of NO releasing sites are associated with constitutive NO production (eNOS and nNOS) and have been deemed beneficial, contributing to enhanced mucosal blood flow and sustained vascular integrity (Kubes, 2000). However, intestinal NO produced by neutrophils (McCall et al., 1989), macrophages (Hibbs et al., 1988) and mast cells (Salvemini et al., 1990) are associated with inducible NO production (iNOS) and are deleterious to the intestine (Hogaboam et al., 1995). Sustained overproduction of NO resulting from iNOS upregulation can have profound effects on the gut epithelium (Ford, 2006). Since NO is produced in large quantities by enterocytes in the intestinal walls of patients with necrotising enterocolitis transforming the intestinal villi into necrotic tissue, one could argue that any selective NO inhibitor could be used to reduce such effect. However, this would lead to a reduction in the production of both inducible iNOS and constitutive enteric nNOS. Such deficiency in constitutive nNOS may in turn also predispose the intestines to necrotising enterocolitis. Thus, maintaining the homeostasis of the internal GI tract, especially with regards to NO, is essential. The selective inhibition of iNOS, which does not affect the constitutive types is desirable and could have therapeutic values in treating intestinal inflammation, particularly with regards to necrotising enterocolitis.

In the present study, pre-treatment of intestinal cell preparations for 1h with L-NAME (1μM), a NO synthase inhibitor, prior to exposure to LPS (50μg/ml) for 24h caused a slight reduction of the NO levels, however, these were still significantly high when compared to cell preparations with no LPS treatment. Surprisingly, treatment with L-NAME (1μM), for 1h followed by concurrent application of LPS (50μg/ml) for 24h caused a significant increase in NO production compared to controls.

The findings from the present study demonstrated that the time of treatment with NO synthase inhibitor has a critical bearing on its action on intestinal injury following challenge with the endotoxin LPS. Thus, L-NAME may either increase or decrease the intestinal injury. These divergences may reflect the dual function of NO with beneficial and detrimental effects in the intestine. The reduced NO production observed over a shorter contact time with L-NAME may possibly reflect the beneficial effects of NOS inhibitor in the present experimental inflammatory model. The reduction of NO production by L-NAME suggests that L-NAME is able to reduce the exacerbated NO levels following LPS challenge (Miller et al, 1993). L-NAME, although potent is not a selective inhibitor of the INOS isoforms (Vallance and Leiper, 2002). Thus, the effects of inhibiting iNOS as well as the constitutive isoforms (nNOS and eNOS) for a longer duration may lead to the detrimental effects as observed in the present study, when longer incubation with L-NAME caused an increase in NO production.

The anti-inflammatory and immune modulatory properties of 5-HT_3 receptor antagonists have also been demonstrated '*in vitro*' and '*in vivo'* (Fiebich et al., 2004, Vega et al., 2005, Seide et al., 2004). Tropisetron, a selective 5-HT_3 receptor antagonist was found to inhibit LPS-stimulated secretion of TNF-α and IL-1β in monocytes (Fiebich et al., 2004).

The beneficial effects the 5-HT_3 receptor antagonist granisetron have also been reported in the treatment of diarrhoea-predominant irritable bowel syndrome (DePonti & Tonini, 2001, Camilleri et al., 2007). The important role attributed to 5-HT_3 in intestinal immunomodulation prompted the investigation of the anti-inflammatory effects of granisetron, a 5-HT_3 receptor antagonist (Tamura et al., 1996) on NO production in the present '*in vitro'* model of intestinal inflammation.

Results revealed that treatments with granisetron at 0.01μM for 1h contact time followed by application of LPS (50μg/ml) for 24h did not modify the increased level of NO induced by LPS, thus showing no differences between LPS-treated preparations and granisetron

plus LPS treated preparations. The level of NO was significantly high when compared to cell preparations with no LPS treatment. In addition, treatments with granisetron at 0.1μM and 1μM showed an increase in the level of NO, however, these were not significant. Pre-treatment with granisetron was also able to increase the basal level of NO indicating the involvement of endogenous 5-HT in the production of NO. The 5-HT_3 receptor activation might inhibit the increase in the level of NO as in the present experiments antagonism of these receptors increased the level of NO.

Further support for the involvement of 5-HT and its receptors in inflammation come from studies by Cloez-Tayarani et al, where the pre-treatment of LPS- stimulated blood monoclonal cells with 5-HT and its associated antagonists such as ketanserin, a 5-HT_2 receptor antagonist inhibited TNF-α production and increased IL-1β production (Cloez-Tayarani et al., 2003).

The study also demonstrated that there were no differences observed when blood mononuclear cells were incubated with the testing agent for a shorter or longer contact times or when the testing agent and LPS were added concurrently (Cloez-Tayarani et al., 2003). Thus, in the present study attempts were made to investigate the effect of a short pre-treatment time with the anti-inflammatory drugs in the management of intestinal inflammation. This was achieved by incubating cells for 1h with the test agent such as granisetron prior to the exposure to LPS. Whereas in separate experiments the concurrent incubation with the test agent plus the stimulant was the approach adopted to investigate the effect of sustained anti-inflammatory drug treatment in the management of intestinal inflammation. The ability of the short-term treatment with granisetron to reduce the levels of NO production during the inflammation challenge induced by LPS and to separately induce a significant increase in the level of NO production during sustained treatment suggests the possible involvement of 5-HT in inflammation response through 5-HT_3 receptors.

Further experiments are required to investigate if activation of 5-HT_3 receptors would result in a reduction in the level of NO.

In line with this finding, results have revealed an interesting correlation between the 5-HT_3 receptor antagonist granisetron and NOS inhibitor L-NAME treatments on NO production. Such findings showing similar profile to the drug treatments following LPS challenge, suggests that like NO, 5-HT appears to participate in mucosal function associated with intestinal inflammation. (Sanders & Ward, 1992, Briejer et al., 1992).

In addition, studies investigating the effect of 5-HT on NO production in human vascular endothelial cells revealed that treatments with 5-HT increased the production of constitutive eNOS (Fukatsua et al., 2006).

It is well documented that complex interactions of the immune system, nervous, and epithelial systems occur to provide integrated responses to physiological and pathophysiological stimuli. Although the pathways by which these interactions occur are not clear, intensive research is beginning to unravel these interactions. The enteric nervous circuit has been reported to regulate the concentration of luminal antigen or bacterial products by evoking protective events including the initiation of mast cell degranulation or activation phagocytes and other auxiliary immune cells. On the other hand, antigenic challenge sensitised by the tissue during mucosal injury leads to release of inflammatory mediators that act locally to influence neural and epithelial functions while simultaneously recruiting additional auxiliary immune cells. Most of the regulatory molecules released from immune cells include histamine, 5-HT and prostaglandins that influence local enteric circuits while concurrently exciting submucosal neurones (Cooke, 1994). Furthermore, in infectious disease caused by endotoxin LPS, the inflammatory response may also activate the 5-HT containing platelets (Timmons & Hawiger, 1986). In this direct mechanism, LPS action also leads in the translocation of platelets to the inflamed tissue and a consequent

increase in the local concentration of 5-HT (Endo et al., 1997). Such observations possibly indicate that 5-HT may interact with the inflamed tissue macrophages and contribute to the activation of various cytokines mediating infections and inflammatory disease (Devoino et al., 1988). Thus, the presence of 5-HT at inflammatory sites suggests its possible involvement in the control of inflammatory modulation. The effect of 5-HT on production of cytokines has been the subject of a few studies. It has been shown that 5-HT promotes IL-16 (Laberge et al., 1996) IL-1β (Cloez-Tayarani et al., 2003). Moreover, because 5-HT_3 receptors have been implicated in the treatment of women suffering from IBS with diarrhoea (Humphrey et al., 1999), it was postulated that a 5-HT_3 receptors antagonist such as granisetron might be a desirable candidate in the reduction of excessive 5-HT activation that it is believed to contribute to the augmentation inflammatory response.

In the present study, when assessing the effect of granisetron or L-NAME on the *'in vitro'* intestinal models in the absence of LPS challenge, non-significant changes in NO production were observed over 1h treatment compared to the control. However, the 25h treatment caused a significant increase in NO production. Treatments with NOS inhibitors have been reported to cause increased neutrophil recruitment, increased stress, increased microvascular and epithelial permeability (Kubes & Granger, 1994, Suematsu et al., 1994). Additionally, chronic treatments with such inhibitors have been associated with intestinal inflammation (Miller et al., 1993, Miller et al., 1994).

Further experiments were carried out looking at the anti-inflammatory properties of the Chaga extract. The Chaga mushroom, *Inonotus obliquus*, is a wood-rotting fungus that has been reported to have natural beneficial properties in humans including anti-bacterial, anti-allergic, anti-inflammatory and anti-oxidant activities protecting the tissues against free radicals such as NO (Naba, 1994). The Chaga mushroom is used in East-European countries to treat gastritis and cancers and traditionally used in some Asian countries as an

herbal extract for the treatment of inflammatory diseases. The pharmacological importance of the Chaga mushroom extract as been ascribed with a number of biologically active immunomodulatory properties, which seem to protect against free radicals such as NO (Allgaher, 1991).

In the present study the pre-treatment of intestinal preparations with ethanolic Chaga mushroom extract (180μg/ml) significantly reduced the levels of NO production following the exposure to LPS.

The activity of NO synthase has been reported to be the main factor for NO production during functional GI disorders such as Crohn's disease and Ulcerative Colitis (Boughton-Smith et al., 1993). The result suggests that Chaga mushroom extract may possess anti-inflammatory properties, which appear to protect cells by inhibiting the release of free radicals such as NO. In line with the present findings, studies investigating the anti-inflammatory properties of the Chaga mushroom extract have revealed that it inhibits iNOS expression via the down-regulation of nuclear factor (NF)-kappa B binding activity (Park et al., 2005). At the molecular levels the stimuli of inflammatory molecules, such as LPS have been reported to act through receptors initiating a cascade of NF-kappa B-dependent events that culminate with the transcription of and translation of pro-inflammatory cytokines and other mediators (Nanthakumar et al., 2000). Further experiments are needed to investigate the effect of the Chaga mushroom extract on cytokines and perhaps using longer contact times and varying concentrations.

In the present study a multidirectional model of intestinal inflammation was developed to investigate key aspects of intestinal inflammation *'in vitro'*. These include aspects related to tissue hyperplasia and enteric innervations, which were demonstrated through the investigation of intestinal cell proliferation and immunocytochemistry. In line with previous studies, the present studies on the onset of inflammation showed an increase in

cell proliferation and a decrease in the contractile protein expression in the smooth muscle cells, which have been associated with loss of intestinal innervations (Blennerhassett & Lourenssen, 2000). Nutrients supplementation was another aspect that was explored by assessing the effect of glutamine supplementation on the model of intestinal inflammation. It was observed that commonly used glutamine supplementation in the cell culture environment was more suitable for stimulation of inflammatory responses and the release of pro-inflammatory mediators compared to lower glutamine supplementation. The level of IL-8 production and NO production were significantly elevated in the present model of intestinal inflammation confirming that LPS treatment is effective in inducing intestinal inflammation *'in vitro'*. Finally, attempts were made to reduce the levels of pro-inflammatory mediators NO using granisetron, L-NAME and the Chaga mushroom extract. The results revealed that short-term treatment with granisetron (0.1μM and 1μM) induces reduction of NO production, which showed no significant differences compared to the controls. This suggests the possible role of 5-HT_3 receptors during the inflammation response. The treatments with the Chaga extract induced a significant reduction of the level of NO production suggesting that such compounds might also be valuable in management of intestinal inflammation since it significantly reduced the level of NO production.

Thus, the model of intestinal inflammation may contribute greatly in future work examining more selective drugs, namely iNOS inhibitors. It would also be interesting to further investigate the anti-inflammatory properties of the Chaga mushroom extract at higher concentrations and perhaps at longer contact times. The model may also be a valuable tool for the investigation of other pro-inflammatory mediators and cytokines that may be involved in the pathology of intestinal inflammation in neonates. This may offer

potential therapeutic benefits in the management of inflammation contributing to the pathogenesis of necrotising enterocolitis.

CONCLUSION

The aims of this study were first to investigate the role of 5-HT receptors mediating contractile responses in the intact intestinal segments taken from the ileum of rat neonates. This investigation focused on the '*in vitro*' measurement of changes in the tension of the intact intestinal segments to exogenously added ACh, atropine, a cholinergic antagonist, 5-HT and 5-HT receptor agonists and antagonists.

Secondly, the study focused on the development of an intestinal cell culture model to obtain purified ISMC, myenteric neurones, and a coculture of ISMC and myenteric neurones. This was achieved by various purification procedures and characterised by immunocytochemistry. A method for the long-term storage of ISMC was developed through cryopreservation. The functionality of isolated cells from segments taken from the ileum of rat neonates was assessed by isometric measurements of single cell contraction and relaxation responses to specific agonists. Cell functionality was further assessed in large cell populations of ISMC and myenteric neurones using substrates of different tensile strength.

Thirdly, attempts were made to develop an intestinal cell culture model of inflammation from segments taken from the ileum of rat neonates. Inflammation was induced by treatments with the endotoxin LPS and the mounting inflammatory response were assessed by measuring the levels of the pro-inflammatory mediators such as IL-8 and NO, cell proliferation and the profile of ISMC contractile proteins. Furthermore, the effects of granisetron, a 5-HT_3 receptor antagonist, L-NAME, a NO synthase inhibitor, and the Chaga mushroom extract, a wood-rooting fungus with reported anti-inflammatory properties, were assessed for their ability to reduce the augmentation of the NO production in response to the induced inflammation by LPS.

The results of the experiments reported in the thesis have revealed that *'in vitro'* organ bath experiments are suitable for the investigation of the role of 5-HT receptors mediating

contractile responses in the rat neonatal ileum. 5-HT induced contractile responses in the neonatal and adult intact rat ileum. These contractile responses were significantly reduced by atropine in the neonatal rat ileum but not in the adult rat ileum. Such observations highlighted the differences related to the expression of 5-HT receptors during GI tract postnatal maturation. Therefore, the studies that followed were focused on the characterisation of 5-HT receptors that may be involved in mediating contractile responses to 5-HT in the segments taken from the neonatal rat ileum. Pre-treatments with methysergide (5-$HT_{1/2/5\text{-}7}$ receptor antagonists), ritanserin (5-HT_2 receptor antagonist), or RS 23597 190 (5-HT_4 receptor antagonist) at 1μM significantly reduced contractile responses induced by 5-HT at concentrations lower than 3μM. The application of granisetron (5-HT_3 receptor antagonist) at 1μM significantly reduced contractile responses induced by 5-HT at concentrations higher than 30μM. Furthermore, the combined treatments with ritanserin, granisetron, plus RS 23597 190 at 1μM reduced or abolished contractile responses induced by 5-HT. This indicated the involvement of 5-HT_2, 5-HT_3, and 5-HT_4 receptors mediating 5-HT-iduced contractions in the neonatal rat ileum. However, pre-treatments with WAY 100635 (5-HT_{1A} receptors antagonist) or SB 267790A (5-HT_7 receptor antagonist) at 1μM had no influence on the contractile responses induced by exogenously applied 5-HT. Pre-treatments with WAY 100635 and SB 267790A also had no influence on the contractile responses induced by 5-$HT_{1A/7}$ receptor agonists, 5-CT (0.3nM-0.1mM) and 5-HT_{1A} receptor agonists, 8-OH-DPAT (0.3nM-0.1mM), which itself failed to induce a measurable response. These observations indicated the unlikely involvement of 5-HT_{1A} and 5-HT_7 in mediating 5-HT contractile responses in the segments taken from the neonatal rat ileum.

Further experiments are required to explore the role of 5-HT receptors mediating 5-HT relaxation responses in the neonatal rat ileum. For example, it would be interesting to

investigate if 8-OH-DPAT applications would induce a relaxation response in pre-contracted tissues.

In an attempt to reduce the number of animal usage in pharmacological research, efforts were made to extract cells from the intestine, which were viable following storage to be used for subsequent experiments. In such studies the ligation of both ends of a segment taken from the neonatal intestine allowed gradual separation of the intestinal layers following the exposure to trypsin. This was confirmed through histological procedures that demonstrated that increased incubation times with the proteolytic enzyme, trypsin causes gradual cell dissociation from the outer most layers towards the deepest layers of the intestine. The combination of histological procedure with viability and confluence studies support that isolation of cells from segments taken from the ileum and a trypsinisation time of 30 min provided the most adequate parameters for the isolation of a mixed population of fibroblasts, ISMC and myenteric neurones from the neonatal (1-4 days old) intestine. Cell mixtures were purified using differential adhesion technique and selective cytotoxicity. The purified ISMC, myenteric neurones and co-culture of ISMC and myenteric neurones were characterised by immunocytochemistry using antibodies to α-smooth muscle actin, labeling ISMC cytoskeleton and 5-HT_3 receptor antibody labeling myenteric neurones. A cryopreservation technique for the long-term storage of ISMC was established through a gradual cooling process, which led to minimal genomic damage. Functionality of single ISMC in culture was assessed in plastic and silicone elastomer surfaces using specific agonists known to induce contraction and relaxation responses in the ISMC. The application of KCl, 5-HT, carbachol, induced contractile responses on ISMC plated on plastic and silicone elastomer substrates. As cells displayed changes in dimension in response to the drug application, this caused a variation in gray scale intensities, which were captured by the peripherally located pixel points. The application of noradrenaline

induced relaxation responses in the silicone elastomer substrate. Atropine significantly reduced contractile responses induced by carbachol in the silicone elastomer substrate. Silicone elastomer provided a more suitable substrate for the single cell functionality studies compared to the plastic substrate and revealed contraction and relaxation patterns comparable to '*in vitro'* organ bath set up. Thus, as an attempt to produce a suitable substrate for functionality studies of large population large cell populations, cocultures of ISMC and myenteric neurones were seeded onto patterned silicone elastomer substrate and cholesteric liquid crystal substrate, and single dose application of KCl analysed. Results revealed that cholesteric liquid crystal materials provide a more suitable substrate for cell functionality studies as responses induced by cells following drug application was translated into deformation in the surface of the liquid crystal. In the pattern silicone substrate, however, cell responses to KCl were not translated into surface deformation. This may suggest that the elastic modulus of liquid crystal material is more closely related to that of the extracellular matrix that surrounds these cells in the intact intestine. In subsequent experiments the cholesteric liquid crystal material was used to investigate the effect of noradrenaline on KCl pre-contracted cells. The ability of the coculture of neurones and ISMC to produce relaxation responses following the application noradrenaline suggests that the liquid crystal material is suitable in studies investigating contraction and relaxation responses induced by cells following the application of pharmacologically active compounds.

Although cholesteric liquid crystal materials display the biocompatibility and the elastic properties necessary to promote substrate displacement as a result of contractile responses induced by the cells following drug application, it remains to determine if full concentration response curves could be performed and yield reproducible results. Additionally, it remains to evaluate if data derived from time-lapse miscroscopy may be relevant to the field of pharmacology.

Stimulation of ileal cells with increasing concentration of the endotoxin LPS (10, 50, 100μg/ml) induced inflammation responses. This was confirmed through the measurement of pro-inflammatory mediators such as IL-8 and NO, which caused a significant increase in the level of IL-8 production following LPS challenge at 100μg/ml, and NO following LPS challenge at 50μg/ml and 100μg/ml. The use of normal glutamine supplementation during cell culture (2mM) enhanced the intestinal inflammation response compared to low glutamine supplementation (0.5Mm). The supplementation of cell cultures with the normal glutamine levels (2mM) induced higher level of NO and IL-8 production. In the presence of LPS the cell viability and cell adhesion remained unchanged, indicating the possible modulatory role of extracellular matrix proteins in maintaining homeostasis in normal and inflamed intestine. Cell proliferation was increased in response to LPS stimulation, suggesting that during intestinal inflammation the enteric innervations are partially affected and this leads to decreased ability of nerves to efficiently coordinate intestinal cell growth resulting in increased cell proliferation. This observation concurred with immunocytochemistry analysis assessing the α-smooth muscle actin expression in the ISMC cytoskeleton during normal and diseased conditions. The significant reduction in the expression of actin fibers indicated an increase in the cell proliferation through reduced nerve coordination.

The effect of pre-treatments with granisetron and L-NAME on the level of NO production was investigated during intestinal inflammation induced by LPS. A 1h treatment with L-NAME (0.1μM) showed an increase in NO production. An increase in the level of NO was also observed following 1h treatment with granisetron (0.01μM). The treatments with granisetron at 0.1μM and 1μM showed an increase in the NO production, however these were not significant. The prolonged treatments for 25h caused an increase in the level of NO production. This may be related to the fact that although the level of NO production increases during the activation of the inflammatory response, this consists of only the

inducible isoform of NO, namely iNOS. However, other constitutive isoforms are also present in the intestine and may play a vital role in the GI tract physiology. Thus, sustained treatment with L-NAME, which is not specific to the NO isoforms may inhibit all isorforms leading to further augmentation of the inflammatory response. The increase in the level of NO induced by pre-treatments with granisetron indicates the involvement of endogenous 5-HT in the inflammatory responses via $5HT_3$ receptors. The endogenous 5-HT might reduce the inflammatory response by activating the $5\text{-}HT_3$ receptors.

In separate experiments the effect of the Chaga mushroom extract, a wood-rotting fungus that has been reported to have natural anti-inflammatory properties in protecting the tissues against free radicals such as NO was investigated. Pre-treatment of intestinal preparations with ethanolic Chaga mushroom extract (180μg.ml) significantly reduced the levels of NO production following the exposure to LPS.

The present study indicates that the developed cell culture model of intestine offers a useful tool for *'in vitro'* analysis of potential therapeutics in the treatment of intestinal inflammation.

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APPENDIX A: PUBLISHED JOUNAL PAPERS, CONFERENCE PAPERS AND MEETING ABSTRACTS