EXPLORING NOVEL APPLICATIONS FOR URSODEOXYCHOLIC ACID IN THE TREATMENT OF CARDIOVASCULAR CONDITIONS

OLADIPUPO ADEYEMI
NATIONAL HEART AND LUNG INSTITUTE, IMPERIAL COLLEGE
A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Declaration

I, Oladipupo Adeyemi, hereby declare that the work presented in this thesis is my own. Information derived from other sources and work done in collaboration has been appropriately cited.

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Dedication

To Vivian, my darling wife and ally through the many chapters of this work.

Acknowledgements

I have been immensely helped through the period of this work by a number of people:

Above all I would like to thank my Lord and Saviour Jesus Christ for giving me the grace and fortitude to see this work through.

I am very thankful to my father, Dr. Adebola Adeyemi and mother, Mrs Adebowale Adeyemi, who have stood by me in many ways throughout this period.

I am truly grateful to Professor Julia Gorelik for giving me the opportunity to work with her and develop as a scientist and person. Apart from science, your advice on tenacity and presenting will stay with me for life.

In reality, the contributions of Professor Alexey Glukhov to this work are immeasurable. I couldn't have got better help. Thank you for your wisdom and patience with a number of difficult techniques…and for coming to my wedding!

I would like to thank my second supervisor, Professor Catherine Williamson, for her regular reviews of my work and advice on a field that was totally new to me. Also I am thankful to Professor Nick Peters for allowing me to use his equipment and providing resources.

Without the help of Dr Anita Alvarez Laviada with patch-clamp work and analysis, I will have been left with a significant gap in my thesis. Dr Jose Alonso Mardonez was very helpful with initial work on patch clamp work and throughout my thesis. I am really blessed to have worked with a selfless colleague like you. The instructions of Dr Navaratnam Naveenan on western blotting helped to answer an important question.

I must also thank members of the Gorelik group and NHLI: Dr Francisca Schultz, Dr Ivan Diakonov, Mr Peter O'Gara, Dr Rasheda Chowdury, Dr Sophie Schobesberger, Mr Phil Rawson, Dr Peter Wright, Navneet Bhogal, Dr Claire Poulet and Alveera Hasan for help with various aspects of the project.

Abstract

As a bile acid, ursodeoxycholic acid (UDCA) is found endogenously formed in humans and other mammals where it is primarily involved in lipid metabolism. It is approved under a number of different brand names for the treatment of gallstones and Primary Billiary Cholangitis (PBC). Previous studies in cardiac cell models of Intrahepatic Cholestasis of Pregnancy (ICP) have demonstrated that UDCA can also be protective against the conduction slowing effects of the bile acid, taurocholic acid (TC).

Therefore, this thesis aimed to identify novel applications for UDCA in the treatment of fetal arrhythmias in ICP. It describes the development of a novel isolated-perfused neonatal rat heart model as a model of the fetal heart (FH) and an adult female rat heart model as a model of the maternal heart (MH) for evaluating the mechanism of bile acid induced arrhythmias. Using optical mapping and electrocardiogram (ECG) recording techniques, it separately evaluated TC effects in the atria and ventricles of the FH and compared them to effects in the MH model. It also evaluated the effects of UDCA in ischemia in a pilot study.

It was observed that TC treatment induced significant conduction slowing in both the atria (ECG PR interval prolongation) and ventricles of the FH, but as occurs in ICP, these effects were absent or less pronounced in the MH model. Interestingly, co-administration of UDCA with TC was protective as it inhibited the observed conduction slowing effects. Using selective cardiac calcium channel blockers, it demonstrated that the conduction slowing effects of TC were mediated by blockade of the T-type calcium channel subtype. This was confirmed using the patch clamp technique that demonstrated that TC reduces the calcium current amplitude in neonatal rat and adult human fetal cardiomyocytes and this effect was inhibited with UDCA treatment. No beneficial effects were identified for UDCA in the treatment of acute global ischemia.

Publications and abstracts

Abstracts

Ursodeoxycholic acid protects against fetal arrhythmias in Intrahepatic Cholestasis of Pregnancy by modulation of calcium channels. *Oladipupo Adeyemi, Alexey V Glukhov, Anita A Laviada, Jose L. Sanchez-Alonso, Catherine Williamson, Julia Gorelik FALK Symposium Freiburg 2014*

Role of TGR5 in the heart *J. Gorelik, D. Papoutsis, I. Diakonov, O. Adeyemi, C. Williamson FALK Symposium Freiburg 2014*

Ursodiol protects against bile acid induced arrhythmias by inhibition of calcium channel blockade *Oladipupo Adeyemi, Alexey V Glukhov, Anita Alvarez-Laviada, Jose L. Sanchez-Alonso, Catherine Williamson, Julia Gorelik NHLI post-graduate day London 2014*

T-type calcium channels as a novel target for the treatment of bile acid induced arrhythmias in fetuses *Oladipupo Adeyemi, Alexey V Glukhov, Anita Alvarez-Laviada, Jose L Sanchez-Alonso, Catherine Williamson, Julia Gorelik Gordon Research Conference Pisa 2015*

Papers

Ursodiol protects against bile acid induced arrhythmias by inhibition of t-type calcium channel blockade. *O. Adeyemi, Alexey V Glukhov, Anita Alvarez-Laviada, Jose L. Sanchez-Alonso, Catherine Williamson, Julia Gorelik Draft ready for submission*

List of abbreviations

4-AP	4-aminopyridine
α-SMA	Alpha-smooth muscle actin
μM	Micro molar
ABCG5/8	Sterolin 1 and Sterolin 2
ANOVA	Analysis of Variance
APD_{70}	Action Potential Duration at 70% repolarisation
AV	Atrio-ventricular
b.p.m	Beats per minute
BASIC	Bile Acid-Sensitive Ion Channel
BCRP	Breast Cancer Resistance Protein
BDM	2,3-butanedione monoxime
BSEP	Bile Salt Export Pump
CAMK	Ca^{2+}/Calmodulin-dependent protein kinase
CDCA	Chenodeoxyholic Acid
CO_2	Carbon dioxide
CV	Conduction velocity
CYP	Cytochrome P450
DCA	Deoxycholic Acid
Di-4-ANEPPS	4-(2-(6-(Dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)pyridinium hydroxide inner salt
DMSO	Di-methyl Sulfoxide
ECG	Electrocardiogram
ECL	Enhanced chemiluminescence
FGF	Fibroblast Growth Factor

FH	Fetal heart model
FLUO-4	Fluorescent calcium dye
FOV	Field of View
FXR	Farnesoid X Receptor
G-protein	GTP-binding protein
GSH	Glutathione
Hz	Hertz
I_{Ca}	calcium current
I_{Na}	sodium current
ICP	Intrahepatic Cholestasis of Pregnancy
IVC	Inferior Vena Cava
LA	Left atrium
LCA	Lithocholic Acid
Lid	Lidocaine
LV	Left ventricle
M199	Cell culture medium
M_2	Muscarinic 2-receptor
MDR	Multidrug-resistance-1 P-glycoprotein
MFB	Myofibroblast
MH	Maternal heart model
MIB	Mibefradil
MRP	Multi-drug Resistance associated Protein
mm	Millimetre
ms	Millisecond
n	sample size
NASH	Non-alcoholic Steatohepatitis

Nif	Nifedipine
ns	Not significant value
NTCP	Sodium Taurocholate co-transport Protein
***nor*UDCA**	24-*nor*ursodeoxcholic acid
OATP	Organic Anion Transporting Polypeptide
OCT	Organic Cation Transporter
O_2	Oxygen
p	P value (calculated probability)
pA	picoAmpere
PBC	Primary Biliary Cholangitis
PPARα	Peroxisome Proliferator-Activated Receptor alpha
PKA	Protein Kinase A
PKC	Protein Kinase C
PR interval	Period from beginning of the P wave until the beginning of the QRS complex
PSC	Primary Sclerosing Cholangitis
PXR	Pregnane X Receptor
QT interval	Period from beginning of the Q wave until the end of the T wave
RA	Right atrium
RIPA	Radio Immuno Precipitation Assay buffer
RPM	Revolutions per minute
RV	Right ventricle
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
s.e.m	Standard Error of the Mean
SVC	Superior Vena Cava
TBST	Tris-Buffered Saline and Tween 20
TC	Taurocholic acid

TEMED	Tetramethylethylenediamine
TGR5	G-protein coupled bile acid receptor 1
T_{max}	Time of maximum plasma exposure
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
VDR	Vitamin D Receptor
Ver	Verapamil

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1 General Introduction

1.1 BILE

1.1.1 History

"The body of man has in itself blood, phlegm, yellow bile, and black bile; these make up the nature of the body, and through these he feels pain or enjoys health. Now, he enjoys the most perfect health when these elements are duly proportioned to one another in respect to compounding, power and bulk, and when they are perfectly mingled. Pain is felt when one of these elements is in defect or excess, or is isolated in the body without being compounded with all the others."

***Hippocratic Corpus** 4th Century BC*

Bile is formed in a variety of mammals such as bears and humans but also in other vertebrates including sharks and reptiles. It has been known from antiquity to play an important but obscure role in body function. Bile referred to as Yellow bile and Black bile were said to be part of the Four Humours, the other two being blood and phlegm, basic substances that have to be balanced in the body for health. In ancient Greece, excess "black bile" in the body was associated with melancholia (Greek: *melas kholé* literally black bile), one of four human temperaments (Hippocrates 2002). In traditional medicine, bile extracted from animals particularly the Black bear has been applied in China for the treatment of ailments for thousands of years (Feng et al. 2009). However, only more recently has its mechanism of formation, nature and composition been elucidated (Boyer 2013).

In the middle of the 19th century the active constituents of bile, originally referred to as bile salts, were first described by Strecker (Strecker 1848). A review of articles published in the early 20th century show that the study of bile was mainly focused on the physiological (Osterhout 1919; Whipple & Christman 1914) and pathophysiological roles of bile and its bile acids constituents (Pearce et al. 1912; Elman & Mcmaster 1925; Brooks 1921). Not until the mid-20th century did the mechanisms involved in bile production begin to be clearly (Brauer et al. 1954; Sperber 1959). Then following this period the beneficial properties and application of individual bile acids became more widely used in conventional

medicine for the treatment of gallstones and other liver and bile duct diseases (Bell, Sutor, et al. 1972; Palma 1992).

1.1.2 Biosynthesis and secretion of bile

Bile is a lipid-rich fluid that is synthesised in the liver hepatocytes (FIGURE 1-1) and aids in the elimination of cholesterol and xenobiotics from the body, as well as the dispersion and efficient absorption of digested dietary lipid in the upper small intestine (Crawford et al. 1995). It is primarily formed in the liver before being transported to the intrahepatic bile ducts, where it is modified by bile duct cholangiocytes through a process of secretion and absorption(Reshetnyak 2013). Bile salts secreted by the liver pass into the intestine, are absorbed in large part by the ileum, and return to the liver by way of the portal vein, thus completing a portal enterohepatic circulation (FIGURE 1-2) (Small et al. 1972). The bulk of bile is stored in the gallbladder where it can be concentrated or secreted into the intestinal lumen following meals.

The process of bile synthesis is under the regulation of a system of complex molecular mechanisms, with nuclear receptors acting as key inhibitory feedback controls (Chiang 2009). Bile acids which are the major organic component of bile are formed in a multistep cascade from cholesterol (discussed below) involving several enzymes. As bile moves through the enterohepatic circulation, bile acids act as signalling molecules to activate the nuclear receptor, farnesoid X receptor (FXR), leading to negative feedback of bile synthesis by inhibiting transcription of genes for the key enzyme, e.g. cholesterol 7α-hydroxylase (CYP7A1). FXR can also induce an intestinal hormone, fibroblast growth factor 15 (FGF15; or FGF19 in human), which activates hepatic FGF receptor 4 (FGFR4) signalling to inhibit bile acid synthesis (Chiang 2009).

The primary secretory unit in the liver consists of a canalicular network which is formed by the apical membrane of adjacent hepatocytes and sealed by tight junctions (Boyer 2013). These tight junctions help to create a physical barrier between the bile lumen and blood preventing diffusion of bile constituents into the bloodstream but allow the passage of small ions. The apical surface area is

modified to create microvilli with transport proteins, a number of which are ATP dependent, that move bile acids and other bile constituents from the hepatocyte into the bile lumen followed by the passive movement of water through tight junctions (Arrese & Trauner 2003). Transport proteins include the organic anion transporter protein (OATP), Organic cation transporter (OCT), canalicular multidrug resistance associated protein (MRP2) (Alrefai & Gill 2007).

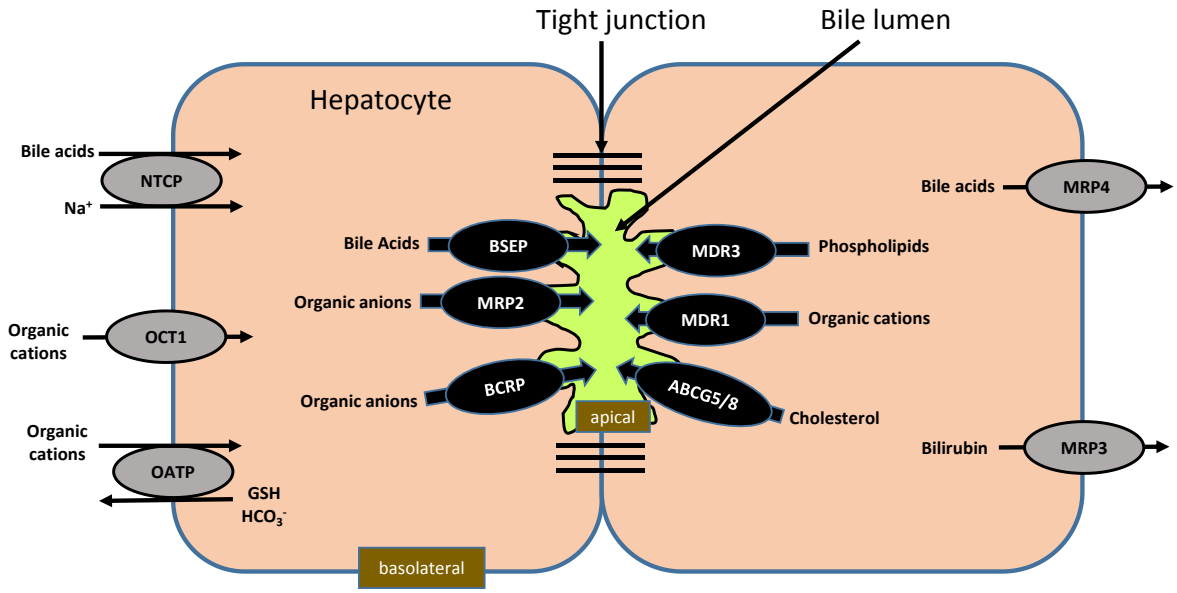


Figure 1-1 Bile secretion in hepatocytes.

The primary secretory unit of bile made up of two hepatocytes, indicating apical and basolateral regions. ***NTCP (Gene name: SLC10A1)*** *– Sodium Taurocholate transport Protein;* ***OATP (SLCO1B1)*** *– Organic Anion Transporting Polypeptide;* ***MRP (ABCC2)*** *– Multi-drug Resistance associated Protein;* ***ABCG5/8*** *– Sterolin 1 and Sterolin 2;* ***BSEP (ABCB11)*** *– Bile Salt Export Pump;* ***BCRP (ABCG2)*** *– Breast Cancer Resistance Protein;* ***OCT (SLC22A5)*** *– Organic Cation Transporter;* ***MDR (ABCC1)*** *– Multidrug-resistance-1 P-glycoprotein;* ***GSH*** *– Glutathione (modified from* (Geier et al. 2006)*).*

The constitution of bile undergoes further modification in the bile duct. Cholangiocytes act to alkalinize canalicular bile and make it more fluid, through a number of secretory and absorptive functions of these cells(Boyer 2013). As with hepatocytes in the liver, cholangiocytes also possess a number of transporter proteins, however, these are highly regulated by meal-induced hormone release and their specific receptors (Franchitto et al. 2013). Fluidization is accomplished by increasing bile water while alkalinisation involves the active transport of bicarbonate anions. The secretion of

bicarbonate is under the influence of neural and hormonal factors such as acetylcholine, Vasoactive Intestinal Polypeptide (VIP) and bombesin (Alvaro et al. 1997; Won Kyoo Cho & Boyer 1999). This process of alkalinizing bile, prevents lipophilic weak acids from being protonized and reabsorbed by diffusion across the biliary epithelium(Boyer 2013).

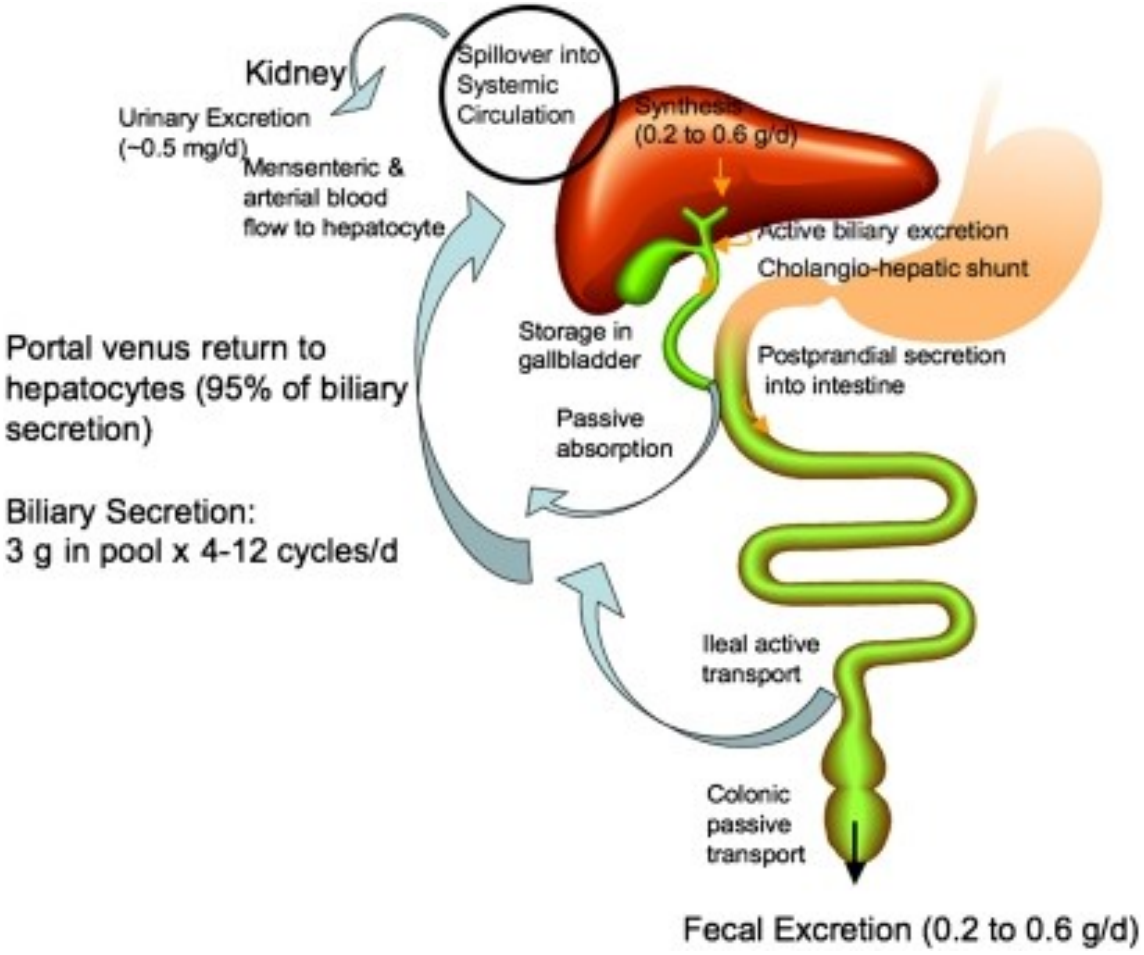


Figure 1-2 Enterohepatic circulation of bile.

Only a small proportion (5%) of bile salts that are formed everyday are lost in faeces, with the majority undergoing hepatic conjugation and subsequent bacterial deconjugation in the intestine prior to recirculation (from (Chiang, 2009))

1.1.3 Bile Composition

The volume and composition of bile is tightly regulated as changes in the relative concentration of specific parameters, e.g. bile salts, cholesterol or phospholipids, has long been known to result in diseases including cholestasis and gallstone formation (Reinhold et al. 1937; Denbesten et al. 1974).

The greater part of bile is composed of water (~95%) with the rest of it being made of a number of organic and inorganic solutes (Boyer 2013). The concentration of inorganic solutes is similar to that found in plasma (Boyer 2013). The presence of different pigments gives it a characteristic yellow-green colour following synthesis however; as it passes through the gastrointestinal tract it is modified by enzymes to a brown colour. The chemical composition of bile is as follows: bile acids (61%), fatty acids (12%), cholesterol (9%), proteins (7%), phospholipids (3%), bilirubin (3%) and others (5%; including elements such as phosphorus, magnesium, iodine, iron, and copper) (Barrett & Ganong 2010).

Bile serves as the major route of elimination of cholesterol from the body. It is also used to deliver folic acid, vitamin D, pyridoxine, and transcobalamin to the intestine (Boyer 2013). Other prominent constituents of bile are glutathione (GSH) and oxidized GSH (GSSG). Studies have shown that most of the substances found in the body can also be found in bile (Reshetnyak 2013).

1.2 THE BILE ACIDS

As the major organic solutes in bile, bile acids play a significant role in the properties and function of bile. They are classified as either primary or secondary bile acids (FIGURE 1-3) and can occur conjugated with taurine or glycine and to lesser extent with sulphates and glucuronides (Hofmann & Hagey 2008). In different species the proportion of individual bile acids as a percentage of the total bile acid pool can vary. Similarly, the specific bile acids found in different species can also differ depending on intestinal metabolism, presence of conjugation molecules and specific enzyme expression (Monte et al. 2009).

1.2.1 Nomenclature

The term bile acids is used to describe the water-soluble, amphipathic end-metabolites of cholesterol that facilitate intestinal absorption of lipids, enhance proteolytic cleavage of dietary proteins, and exert potent antimicrobial activity in the small intestine (Hofmann & Hagey 2008). However use of this term is generic as it actually describes distinct chemical classes with a carboxyl group and does not

refer to their state of ionization. There has been no satisfactory collective term to describe all the members of the group (Hofmann & Hagey 2008). Bile acids with 24 carbon atoms (C_{24}) are referred to as cholanoic acids while those with 27 carbon atoms (C_{27}) are termed cholestanoic acids (Hofmann et al. 1992). There is complexity in the nomenclature of these molecules as they were named long before their structures were described (Hofmann & Hagey 2008). Although attempts have been made to develop nomenclature that accurately describes the group and its sub-classes, it appears that acceptance has been limited (Haslewood 1976; Hofmann et al. 1992).

1.2.2 Chemical properties

Most bile acids can be divided into 3 classes of molecules: 1.) C_{27} bile alcohols 2.) C_{27} bile acids and 3.) C_{24} bile acids (Hagey et al. 2010). The two common structural components of all three classes are the 19-carbon (C_{19}) steroid nucleus and the presence of a side-chain (Hagey et al. 2010). The steroid ring is composed of three cyclohexanes and one cyclopentane fused together and is fully saturated in all molecules. Differences can be found in the sites and planar orientation of hydroxyl groups, length of the side chain and location of the carboxyl group.

Bile acids are ampiphiles with different polarities on the surfaces of the molecule, resulting in hydrophobic (little or no affinity for water/hydroxyl groups) and hydrophilic (affinity for water/hydroxyl groups) surfaces. Changes in the stereochemistry of the A/B ring juncture results in changes in the polarity of the molecule making the α face hydrophilic and the β face hydrophobic. The hydrophobicity-hydrophilicity of bile acids can be determined using reverse phased high performance liquid chromatography (HPLC) (Heuman 1989).

Furthermore, the number of hydroxyl groups can also affect the binding of bile acids to calcium which can affect biliary calcium excretion, calcium salt precipitation and gallstone formation (Hofmann & Mysels 1992; Gleeson et al. 1990). Unconjugated forms of bile acids tend to have a greater affinity for calcium compared to conjugated forms.

1.2.3 Species differences

Species differences in bile acids can be categorised into three groups: (1) side-chain structure (2) stereochemistry of the A/B ring fusion and (3) the distribution of the number, position and stereochemistry of hydroxyl groups in the steroid nucleus (Monte et al. 2009). Generally, 24-carbon atom bile acids are usually found in bile of humans, mammals and other higher animals while 25 to 27 carbon atoms predominate in lower species.

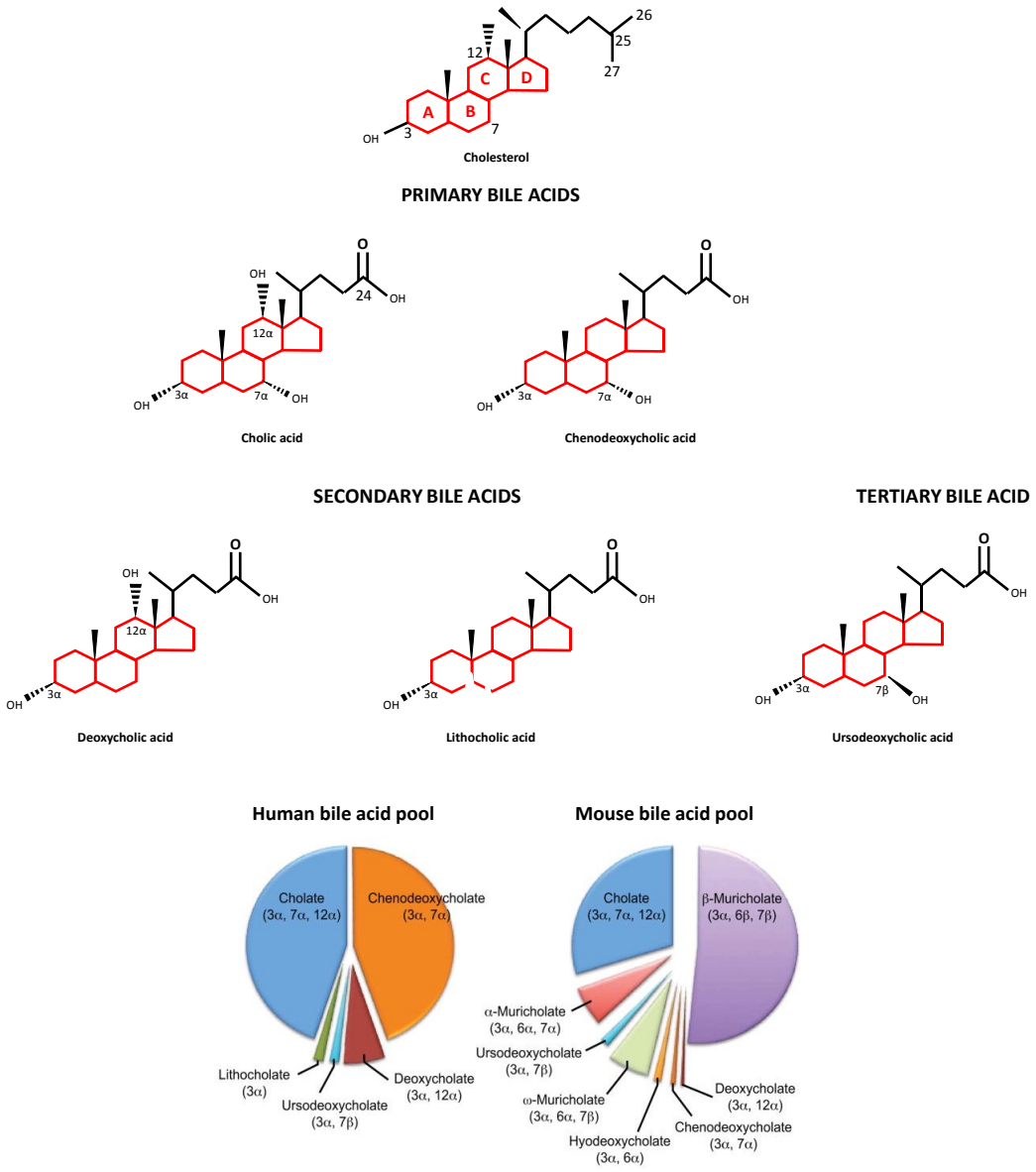


Figure 1-3 Bile acid structure and composition.

Chemical structures of 27 carbon cholesterol and the 24 carbon primary, secondary and tertiary bile acids found in humans. Highlighted in red is the conserved steroidal ring. Numbering indicates positions

of carbon atoms. Charts show the relative proportions of the key bile acids in the bile acid pools of humans and mice – from (De Aguiar Vallim et al. 2013).

Evaluation of the types of bile acids found in different species shows that bile acid profiles are similar within taxonomy families independent of diet, however, at the species level significant variations can be found (Hagey et al. 2010). As shown in Figure 1-3, in humans, cholic acid and chenodeoxycholic acid are the primary bile acids, but in mice cholic acid and β-muricholic acid predominate (De Aguiar Vallim et al. 2013), while in pigs cholic acid and hyodeoxycholic acid are the primary bile acids (Kevresan et al. 2006). Individual bile acids can make up a greater proportion of the total bile acid pool in some species compared to others. An example is seen in bears where ursodeoxycholic acid – a tertiary bile acid in humans – is present as a primary bile acid and can make up to 70% of the total bile acid pool in some species (Hagey et al. 1993).

In a separate study by Hofmann and colleagues (Hofmann et al. 2010), key differences were identified in the composition of bile acids found in mammals compared to non-mammals. These include: differences in the hydroxyl group configuration of the default bile acid, chenodeoxycholic acid (CDCA), a preferred conjugation with glycine rather than taurine in mammals, as well as a much higher number of mammalian species with ≥10% deoxycholic acid (DCA). Amphibian species were found to have some of the most complex bile acid profiles found in vertebrates with some species having significant amounts of $C_{27,}$ C_{26}, C_{28} or other side chain length bile alcohols as well as C_{27} bile acids and C_{24} bile acids. By comparison, in reptiles the majority of lizard species analysed had solely C_{24} bile acids and a minority had solely C_{27} bile acids. The importance of these differences to the physiological function of each species has been proposed but is not fully understood (Hagey et al. 1993; Sola et al. 2006).

1.2.4 Biological Synthesis

Bile acids formed in the liver from the direct metabolism of cholesterol are referred to as primary bile acids while those formed from the further metabolism of these primary bile acids by intestinal bacteria are referred to as secondary bile acids (Russell 2003). Significant conjugation with taurine and glycine

also occurs with the primary and secondary bile acids, which serves to increase their solubility and excretion but can also alter their physiological roles.

At least 14 enzymes are involved in the process of bile acid synthesis and the liver is the only organ where all of these enzymes are present (Russell, 2003, Chiang, 2009). In man, bile acid synthesis occurs through two pathways: the classic (neutral) pathway or the alternative (acidic) pathway (FIGURE 1-4) (Ferdinandusse & Houten 2006).The classic bile acid biosynthetic pathway is initiated by enzyme cholesterol-7α-hydroxylase (CYP7A1), while the alternative (acidic) pathway is initiated by CYP27A1 (Chiang, 2009). Synthesis of bile acids can be divided into four stages: (a) Initiation of synthesis by 7α-hydroxylation of sterol precursors (b) Further modifications to the ring structures (c) Oxidation and shortening of the side chain and (d) conjugation of the bile acid with an amino acid (Russell, 2003).

The final step of conjugation is dependent on the relative abundance of either amino acid – glycine or taurine, in the body. However, the ratio of free to conjugated bile acids can be regulated by the nuclear receptor protein, PPARα (Russell, 2003). The rapid conjugation process leads to the bulk of bile acids occurring in the conjugated form under physiological conditions in humans (Reshetnyak 2013). This is the case with endogenously synthesised bile acids or with exogenous bile acid formulations. Conjugation occurs in parenchymal cells of the liver, and the resulting conjugates are re-excreted in the gall bladder (Kevresan et al. 2006). It serves to increase the amphipathicity and enhance the solubility of the molecules making them impermeable to cell membranes (Russell, 2003).

Bile acids synthesized in the liver are secreted into bile, which flows through the bile duct to the intestine. Up to 95% of bile acids are efficiently absorbed from the intestine, returned to the liver, and resecreted into bile in the enterohepatic circulation process (Pauli-Magnus & Meier 2005). Unabsorbed bile acids are passed into the large intestine where they undergo bacterial transformation, including de-amidation and dihydroxylation(Hofmann & Hagey 2008). They can also undergo conjugation catalysed by the enzyme sulfotransferase-2A1, to aid detoxification and elimination in urine or faeces (Alnouti 2009). Due to their detergent nature, bile acids are cytotoxic

at high concentrations and so it is necessary that their levels are tightly regulated, and that they are detoxified through conjugation or reabsorption(Pauli-Magnus & Meier 2005) .

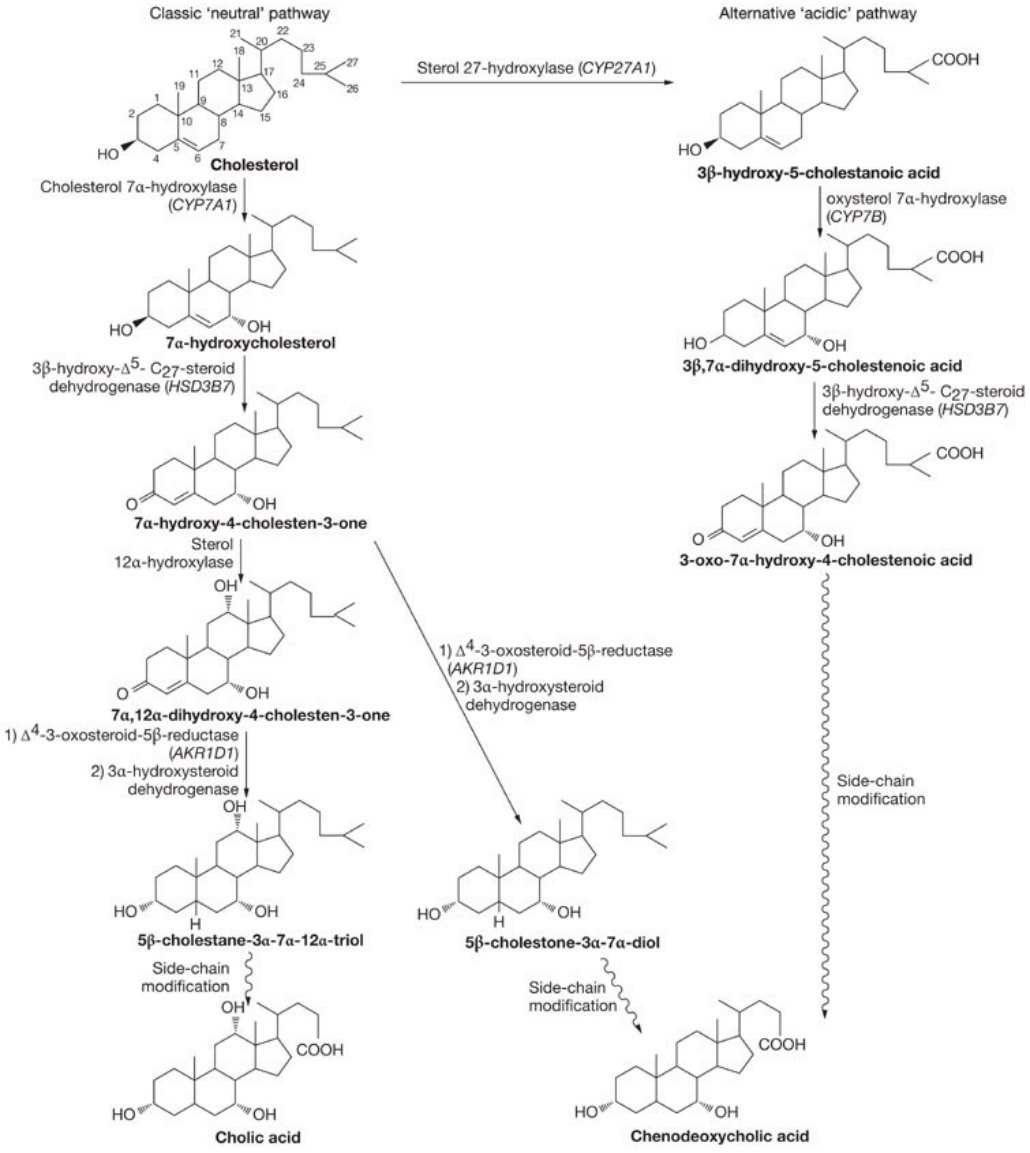


Figure 1-4 Enzymatic regulation in the Classic and Alternative pathways of primary bile acid formation.

Cholesterol acts as the substrate for both pathways. Cholic acid and chenodeoxycholic acid are the end products – from (Sundaram et al. 2008).

1.2.5 Physiological roles

Bile acids represent the primary pathway for cholesterol catabolism(Monte et al. 2009). The ampiphatic nature of bile acids allows them to act as physiological detergents that aid in the intestinal

digestion and transportation of lipids(Monte et al. 2009). They are able to form mixed micelles together with biliary phospholipids, which allows the solubilization in bile of cholesterol and other lipohilic compounds including dietary fat and lipid soluble vitamins(Monte et al. 2009). The micelles, which are roughly 4-7nm, are made up of a core with the hydrophobic lipids and the hydroxyl groups of the bile acids on the outside and so are readily absorbed across the microvilli.

Bile also acts as a medium for the elimination of drugs and their metabolites (Chiang 2013). Following entry into the liver, drugs can be transported into bile either unchanged or as more hydrophilic metabolites after phase I and/or phase II biotransformations, or can be excreted into blood by basolateral transport proteins (Ghibellini et al. 2006). Excretion into bile can actually increase their duration of action through the cycle of enterohepatic circulation (Levine 1978). Some drugs are conjugated with glucuronides in the liver, however once secreted into bile they are transported to the intestines where they can then be hydrolysed by gut bacteria and reabsorbed (Levine 1978). The molecular weight, polarity and even the route of administration of the drugs can determine if they are excreted in bile or by other routes, such as in urine(Levine 1978).

By activating G-protein coupled receptors and nuclear receptors, bile acids act as signalling molecules to regulate their synthesis(Chiang 2013), in inflammation (Miyake et al. 2000) or in the cardiovascular system (Kida et al. 2013; Desai et al. 2010), and the development of arrhythmias (Gorelik et al. 2002; Sheikh Abdul Kadir et al. 2010; Rainer et al. 2013) have also been described. Receptors that have been described for bile acids include the Farnesoid X receptor, Pregnane X receptor (PXR), vitamin D3 receptor (VDR), G-protein coupled bile acid receptor 1 (TGR5) and constitutive androstane receptor (CAR)(Schaap et al. 2014). By activation of the FXR and TGR5 receptors, bile acids have also been shown to regulate glucose, lipid and energy metabolism (Kuipers et al. 2014; Kida et al. 2013).

1.2.6 Bile acids as drugs in modern medicine

Based on their demonstrated efficacy in traditional medicine and serendipitous discoveries, bile acids – in particular CDCA and UDCA – became widely-used therapeutic agents in conventional medicine.

Some of the earliest proposed uses of bile acid were as therapeutic agents for the dissolution of gallstones(Hofmann 2009), although these were not widely applied. In 1938, it was observed that symptoms of rheumatoid arthritis were reduced in patients who also developed jaundice, and it was thought that bile acids might confer some clinical benefit in these patients (Hench 1938).

Decades later a series of clinical trials highlighted the efficacy of CDCA in the dissolution of gallstones (Bell, Hitney, et al. 1972; Coyne et al. 1975). These studies showed that treatment of patients with CDCA alone or in combination with phenobarbital improved cholesterol solubility and caused a reduction or complete disappearance of gallstones in about half of patients compared to the placebo group. However, by the next decade evidence appeared showing a number of side effects (including diarrhoea and elevated plasma cholesterol) for CDCA and that UDCA was more efficacious and better tolerated (Fromm et al. 1983; Erlinger et al. 1984). This has resulted in a decline in CDCA use.

Presently, UDCA is used not only in the treatment of gallstones but also for the treatment of different types of cholestatic liver diseases including Primary Sclerosing Cholangitis (PSC)(Mendes & Lindor 2010), Primary Biliary Cholangitis (PBC) (Kaplan & Gershwin 2005) and Intrahepatic Cholestasis of Pregnancy (ICP)(Geenes & Williamson 2009). In these diseases, UDCA has been shown to be efficacious with few side effects (Kondrackiene et al. 2005; Hempfling et al. 2003). Beyond its efficacy in hepato-billiary diseases, potential applications of the drug have also been proposed for the treatment of cardiovascular diseases based on laboratory and clinical findings (Bahrle et al. 1998; Von Haehling et al. 2012). These unique properties and potential benefits of UDCA have led to increased interest in the study of this bile acid as a therapeutic agent beyond its current use in hepato-billiary diseases (Rajesh et al. 2005; Miragoli et al. 2011).

1.3 URSODEOXYCHOLIC ACID

Ursodeoxycholic acid (UDCA) was first identified following an analysis of bear bile and only later was it found to be a component of human bile (Hagey et al. 1993). The name *urso*-deoxycholic acid is derived from the familial name of bears, *Ursidae*. In most vertebrates, it generally constitutes <1-5% of the total human bile acid pool but levels in bears tend to be much higher. The biological advantage for bears of having a high proportion of UDCA is unclear, although levels in black bears have been shown to vary during winter supporting a possible role in cellular protection during periods of hibernation (Sola et al. 2006). UDCA might also protect bears from gut atrophy, lithogenesis, and bacterial overgrowth during hibernation (Rivard et al. 2007). Another possible explanation might be that taurine conjugated UDCA is more effective at digesting the high percentage of fat in the diet of these animals (Hagey et al. 1993).

1.3.1 Physicochemical properties

UDCA ($C_{24}H_{40}O_4$; IUPAC: 3α,7β-dihydroxy-5β-cholan-24-oic acid) is a white or almost white powder with a melting point between 200 – 204°C. It is readily soluble in alcohol, sparingly soluble in acetone, in chloroform and in ether (Ursofalk 2015). As with other bile acids UDCA has a bitter unpleasant taste.

UDCA is more hydrophilic compared to other dominant bile acids including CDCA and deoxycholic acid (DCA) (Lazaridis et al. 2001). This is due to the orientation of the hydroxyl groups present in the molecule i.e. 7β position rather than 7α found in CDCA). As a dihydroxy bile acid it is also more hydrophilic than monohydroxy bile acids, but less so compared to trihydoxy molecules (Lazaridis et al. 2001). Despite this it is practically insoluble in water (Hofmann 1994).

In an *in vitro* analysis of calcium binding properties of different bile acids, Gleeson and colleagues (Gleeson et al. 1990) found that glycine conjugated UDCA had the highest affinity of the ten conjugated bile acids compared. Unconjugated UDCA affinity was similar to that of other dihydroxy bile acids, CDCA and DCA, and greater than that of the trihidroxy compounds.

The ability of UDCA to solubilize cholesterol is lower than CDCA, this is likely related to its higher critical micellization concentration (CMC) – a measure of its ability to form micelles (Hofmann, 1994).

1.3.2 Biological synthesis

In humans, UDCA only makes up about 4% of the total human bile acid pool(Lazaridis et al. 2001). It is present as a tertiary bile acid, suggested to be formed by transformation mediated by the bacteria *Clostridium baratii* found present in the colon (Lepercq et al. 2004). It is formed from the 7β-epimerization of CDCA in two steps: oxidation of the 7α-hydroxyl group by a 7α-hydroxysteroid dehydrogenase (7α-HSDH) and stereospecific reduction of the 7-keto functionality by a 7β-hydroxysteroid dehydrogenase (7β-HSDH)(Lepercq et al. 2004). Due to its hydrophilic nature, UDCA can be passively absorbed into the circulation, although, as with other bile acids, it is rapidly conjugated with taurine and glycine following synthesis (Hofmann et al. 2005).

1.3.3 Artificial synthesis

To obtain bile from bears, a lot of traditional medicine relies on the farming of bears and crude extraction techniques (Feng et al. 2009). Therefore, it is important to mention that all the UDCA used in this project and for clinical purposes in modern medicine are derived from a number of artificial synthesis techniques (Sawada et al. 1982; Giovannini et al. 2008). As reviewed by Eggert and colleagues (Eggert et al. 2014) the techniques can be summarised as microbial and chemo-enzymatic methods. CA and CDCA are the most commonly employed biological starting materials and depending on the structure of the starting material different combinations of regio- and enantio-selective enzymes are used to catalyse the oxidative and reductive steps. Yields from purely chemical methods are usually about 30% but can be as high as 70% with whole-cell techniques(Eggert et al. 2014).

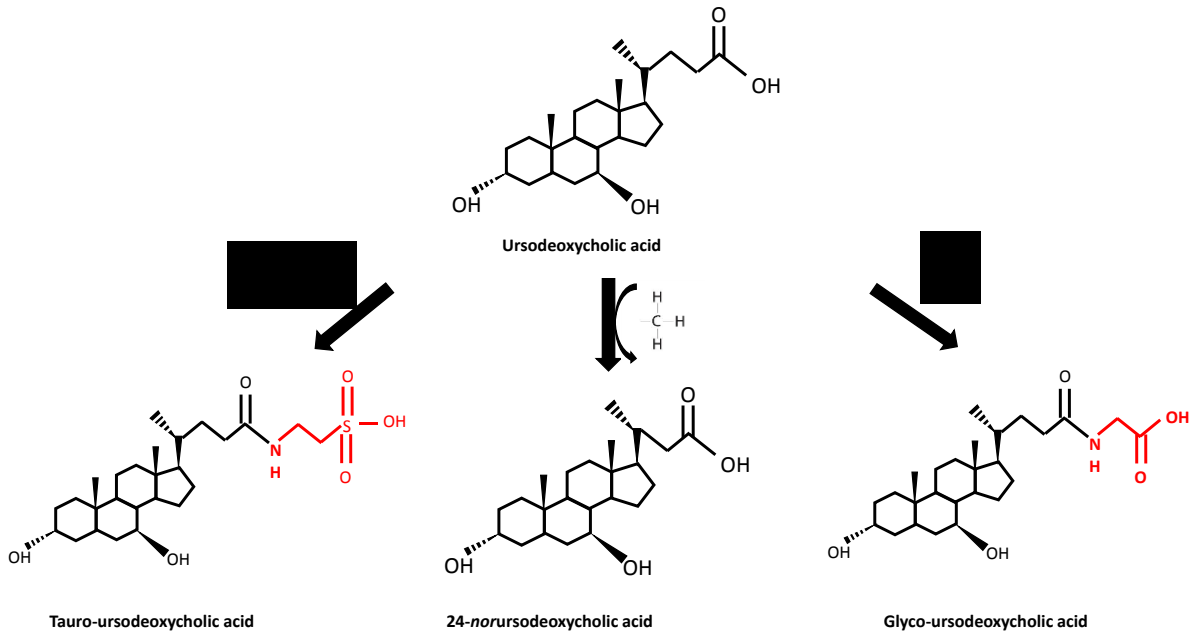


Figure 1-5 Structure of conjugated UDCA and 24-norUDCA.

Analogue is formed by the loss of a methyl group from the side-chain of UDCA

1.3.4 Receptors and ion channels

As previously mentioned bile acid function can be mediated through different receptors (see FIGURE 1-6). Bile acid receptors can be classified into two groups: Nuclear receptors (FXR, PXR and VDR) and Membrane receptors (TGR5 and acetylcholine muscarinic receptor) (Pols et al. 2011). In comparison to other bile acids, UDCA activity at the bile acid receptors is either weak or absent.

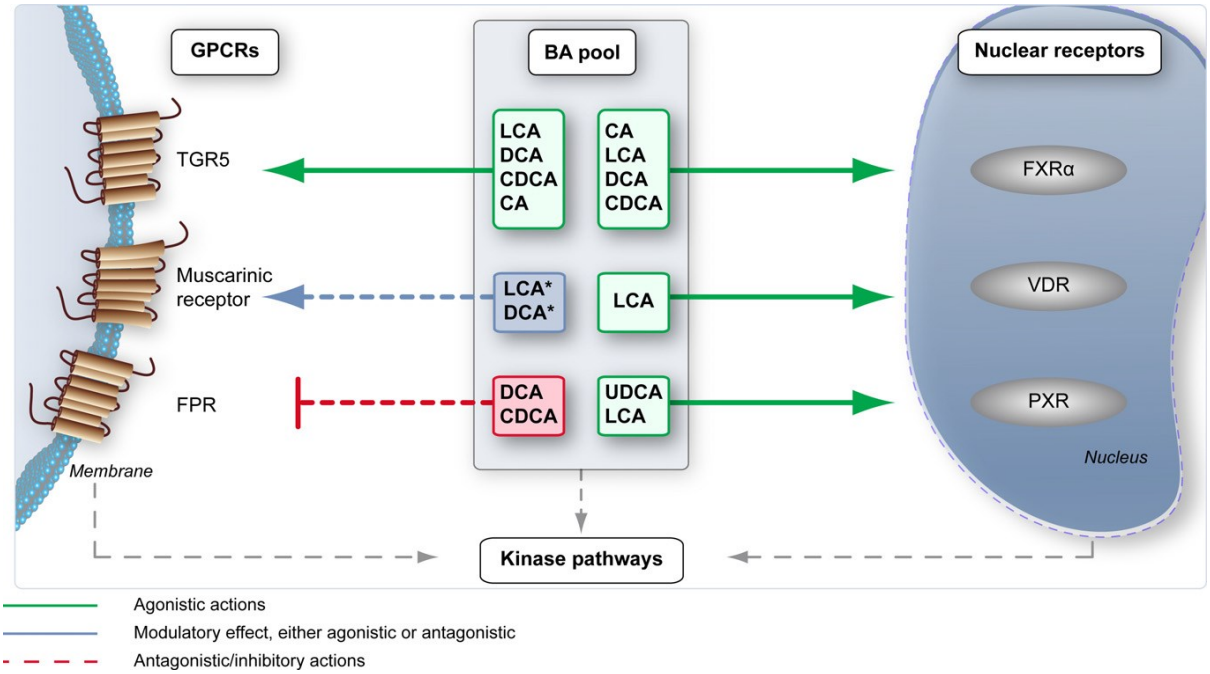


Figure 1-6 Bile acid actions are mediated by nuclear receptors and G-protein coupled receptors *(from* (Pols et al. 2011)*)*

FXR

Both UDCA and 24-nor-UDCA are not FXR ligands, despite a number of actions resembling FXR activation including bile acid homeostasis and anti-inflammatory effects (Schaap et al. 2014). However, UDCA is able to inhibit receptor activation (Howard et al. 2000) and gene repression (Chiang 2009) by other bile acids.

PXR

In human hepatocytes cultured for 48 hours, UDCA was shown to be an activator of PXR and was more effective than other bile acids at inducing the enzyme CYP3A4 (Schuetz et al. 2001).

VDR

Although UDCA is not a ligand for the VDR receptor (Makishima et al. 2002), it is able to induce VDR expression in biliary epithelial cells of human patients (D'Aldebert et al. 2009).

TGR5

The TGR5 receptor is the first known G-protein coupled receptor specific for bile acids(Maruyama et al. 2002). UDCA has been reported either to not activate TGR5 or to have weak agonistic activity at the receptor (Alemi et al. 2013). Analogues of UDCA, created by 7α-methylation resulted in improved agonism for the receptor, however it also reduced affinity for the nuclear receptor FXR (Iguchi et al. 2011).

Muscarinic receptor

The bile acid taurocholic acid (TCA) has been shown to be a partial agonist at the muscarinic (M2) receptor while deoxycholic acid (DCA) is an antagonist (Raufman et al. 2003). UDCA binding has not been described.

Bile Acid-Sensitive Ion Channel (BASIC)

Recently UDCA has been shown to be an activator of the Bile Acid-Sensitive Ion Channel (BASIC) with an EC_{50} of approximately 2.5 mM (Wiemuth et al. 2013). Although the physiological role BASIC is not known, it has been described as an epithelial sodium channel that is predominantly expressed in the liver and intestinal tract – regions where bile acids are primarily active.

Other

Glucocorticoid receptor

Although UDCA does not bind directly to the glucocorticoid receptor, it has been shown to activate it possibly through unspecific binding or membrane mediated effects as activation of protein kinase C (PKC) (Weitzel et al. 2005). It also functions as an important route for the nuclear translocation of UDCA for reducing apoptosis (Solá et al. 2005).

1.3.5 Transporter proteins

The entry of bile acids into cells and excretion into bile is also mediated by transport proteins, key among these are the sodium taurocholate co-transporting polypeptide (NTCP), the bile salt export

pump (BSEP), the apical sodium-dependent bile acid transporter (ASBT), organic anion-transporting polypeptide (OATP) and the organic solute transporter OSTα-OSTβ (Dawson et al. 2009).

UDCA acts as a substrate for these channels however its transportation might be limited in certain regions of the gut. For example, OATPs are a family of plasma membrane transporters that have been shown to mediate the exchange of extracellular organic anion or bile acid with intracellular HCO_3 or glutathione (GSH) (Alrefai & Gill 2007). They have low uptake rates for both UDCA and *nor*UDCA in hepatocytes and are not considered relevant for its effects in human beings (König et al. 2012).

UDCA is also able to induce mRNA or protein expression of these channels. In mice fed with UDCA there was an increase in mRNA levels of Abcb11 and Abcc2 which encode the canalicular bile-acid (salt) export pump, BSEP, and the canalicular conjugate export pump, Mrp2 respectively (Fickert et al. 2001). A similar observation is found in humans given UDCA for 3 weeks, where protein (but not mRNA) levels of BSEP, MDR3 and major basolateral bile-acid efflux pump are upregulated (Marschall et al. 2005).

1.3.6 Formulation

Ursodeoxycholic acid is sold under a variety of brand names in the UK such as Destolit®, Urdox® and Ursofalk®. It is available prescription-only in its more common preparations as 250 mg capsules, 150 – 500 mg tablets, or 250 mg/5 mL capsule formulations. Oral preparations of UDCA are poorly absorbed due to poor dissolution at gut pH (Scalia et al. 2000). As a result, different approaches have been investigated to increase its bioavailability including conjugation with phospholipids (Yue et al. 2008), cyclodextrin (Panini et al. 1995), 5-aminosalicylic acid (Batta et al. 1998) and through delivery in the pro-drug form for prior to further intestinal metabolism (Dosa et al. 2013).

Other methods include use of sustained release preparations and an enteric-coated capsule filled with UDCA and a sodium-carboxy methyl cellulose polymer system that resulted in increased intestinal absorption and a near doubling of the mean area under the plasma concentration curve from 16.24 ±

8.38 µM h to 27.60 ± 10.11 µM h (Scalia et al. 2000). The use of sustained release preparations by different manufacturers has been shown to result in varied pharmacokinetic outcomes, therefore it is important to obtain data for each brand prior to prescription (Simoni et al. 2002).

1.3.7 Dosage

The adult dosage of UDCA varies depending on the disease state. In PBC the recommended dosage is 12-15 mg UDCA per kg per day (Kaplan & Gershwin 2005). Perhaps because UDCA was originally used in traditional medicine before integration in modern medicine, ideal doses are not clearly defined and therapy can be empiric. In a meta-analysis of 9 randomised controlled trials that assessed UDCA in ICP(Bacq et al. 2012), the total dose given to patients varied from 450 mg/day to a 1000 mg per day (roughly 7-15 mg/kg/day). In certain instances these high doses are contraindicated as they might not improve outcomes and can increase the rates of serious adverse events (Lindor et al. 2009; Lindström et al. 2012).

In a randomized placebo-controlled trial of high-dose UDCA (28–30 mg/kg/day), patients who received the high-dose had a significantly higher risk of developing colorectal cancer compared to placebo patients (Eaton et al. 2011). This is in contrast to low-dose UDCA (9 mg/kg/day) which although ineffective for PSC has been shown to reduce colonic colorectal cancer (Tung et al. 2001). A number of completed and ongoing clinical trials have been aimed at identifying ideal doses of the drug in different diseases (Seidensticker et al. 2014; van de Meeberg et al. 1997).

Apart from dosage, the duration of therapy is also significant and has been the subject of numerous clinical trials (Guarino et al. 2005; Poupon et al. 1994). Due to the nature of the gallstone and cholestatic diseases, patients can require long term, over a year, administration of UDCA. Generally long-term treatment with mid-dose UDCA (11-20 mg/kg) has not been associated with any adverse effects (Pares et al. 2000; Shi et al. 2006). Based on this, the dose of UDCA might be a more important consideration than the duration of treatment.

1.3.8 Pharmacokinetics

Following oral administration, approximately 30 to 60% of UDCA is absorbed in the gut, with the rest being absorbed in the small intestine and colon (Lazaridis et al. 2001). Treatment of 11 patients with PBC and 11 healthy subjects for 3 weeks with 15 mg/kg/day UDCA resulted in peak plasma concentrations of (C_{max}) of 15.5 μM of UDCA (Dilger et al. 2012). In a separate evaluation, oral administration of a single 500 mg dose of UDCA to healthy volunteers resulted in peak plasma concentrations of 7 to 16 μM (Ursofalk 2015). A multiple dose regimen is advocated for UDCA as absorption rates decrease significantly at higher single doses (van de Meeberg et al. 1997; Walker et al. 1992).

T_{max} was biphasic, occurring at 60 minutes and a 180 minutes post exposure. It has a half-life of 3.5 – 5.8 days (Ursofalk 2015). The primary route of elimination is the faeces in healthy patients but in patients with severe liver disease, excretion is via the kidneys in urine. 30 – 44% of the drug excreted in faeces within the first 3 days was found as UDCA (2 – 4%), however, a larger amount is found metabolised to lithocholic acid (37%) and 7-ketolithocholic acid (5%). The lethal dose for UDCA is greater than 10 g/kg in mice and dogs and 5 g/kg in rats.

1.3.9 Side effects

UDCA is generally well tolerated in the clinic(Hempfling et al. 2003). In early controlled clinical trials in patients with gallstone disease (Roda et al. 1994) and in long-term, large-scale, placebo-controlled trials in patients with cholestatic liver disease (Poupon et al. 1991) no serious adverse effects were reported. Overall, meta-analysis of clinical trials with UDCA treatment in different diseases have concluded it to be a safe drug with minor and rare side effects (Grand'Maison et al. 2014; Braga et al. 2009).

Diarrhoea tends to be the most common side effect reported with UDCA treatment although the incidence and severity are low(Hempfling et al. 2003). In four separate studies of patients with gallstone disease the incidence rates were between 2 – 9% of patients treated, the general incidence

is roughly 3% (Hempfling et al. 2003). Severe diarrhoea was reported in two studies of patients with PSC and inflammatory bowel disease during UDCA treatment which stopped after cessation of UDCA treatment (Beuers et al. 1992). These were in small scale trials of less than 20 patients, in a larger trial of 102 patients, no such incidents were reported (Hempfling et al. 2003).

UDCA does not induce liver toxicity like CDCA does, in fact it can actually be hepatoprotective in some cases(Hempfling et al. 2003). Allergic reactions are rare and usually linked to drug interactions rather than UDCA treatment. No incidents of carcinogenicity or embryotoxicity have been reported. UDCA is used to treat symptoms of itching in patients with Intrahepatic Cholestasis of Pregnancy and no significant adverse effects have been reported (Grand'Maison et al. 2014). Other minor side effects reported include nausea and vomiting in 3 of 111 patients during a Primary Biliary Cholangitis study (Heathcote et al. 1994).

1.3.10 Drug interactions

Drug interactions with UDCA are mainly related to absorption, although recent evidence suggests that UDCA may interact with other drugs via the induction of CYP3A isoforms (Hempfling et al. 2003). Bile acid sequestrants are used in the treatment of chronic diarrhoea caused by bile acid malabsorption. This includes such drugs as the anion exchange resins colestyramine, colestimide and colestipol and aluminium containing antacids which bind to UDCA in the gut and thereby interfere with its absorption (Rust et al. 2000). In cases where both drugs are prescribed it is advised that they are given with sufficient intervals to UDCA treatment (Hempfling et al. 2003).

Studies in human and rat cells have shown that UDCA is able to induce the expression of CYP3A messenger RNA activity (Kurosawa et al. 2009; Becquemont et al. 2006). Also as a ligand for the Pregnane X receptor (PXR), it can indirectly modulate the expression of this enzyme (Hempfling et al. 2003). This can impact on the metabolism of drugs such as midazolam and digoxin that is catalysed by the enzyme. In fact CYP3A enzymes are some of the most abundant cytochrome P450 enzymes in the

liver intestinal epithelium and are known to be involved in the metabolism of about 50% of all drugs (Hempfling et al. 2003).

In other studies, UDCA increased the systemic exposure of the cholesterol lowering drug, rosuvastatin and serum bilirubin in healthy volunteers which was linked to a decrease in inorganic Anion Transporter (OATP1B1) expression and inhibition of the transcription factor HNF1α (He et al. 2008). On the other hand, combination therapy of UDCA and another statin, simvastatin, has been shown to result in an increase in the proportion of UDCA in the bile pool following a 6 month treatment period (Lanzarotto et al. 1999). Although no alarming effects have been reported, based on these findings, it has becoming increasingly important to consider the possible drug interactions of UDCA prior to prescription especially as wider applications are being investigated for the drug (Hempfling et al. 2003).

1.3.11 Current applications

UDCA is currently approved for the treatment of gallstones and PBC (NHS 2015). Although its mechanism of action is still being investigated, it is also widely used for the treatment of other hepato-billiary conditions (Kowdley 2000).

These include:

- Gallstone dissolution
- Primary Biliary Cholangitis
- Primary Sclerosing Cholangitis
- Non-Alcoholic Steatohepatitis

Gallstone dissolution

Gallstones are crystalline deposits found in the gallbladder (Gurusamy & Davidson 2014). They can be classified into different groups based on their microscopic structure and composition, but most stones can be classified as cholesterol (37-86%), pigment (2-27%), calcium (1-17%) or mixed (4-16%). It occurs

with a prevalence is about 5 – 25% of the Western adult population with a higher incidence with increasing age, pregnancy and with obesity (Kratzer et al. 1999). Among the factors that lead to gallstone formation are excessive bile cholesterol, low bile salt levels and decreased gallbladder motility (Gurusamy & Davidson 2014). Cases are classified as asymptomatic or symptomatic with treatment recommended for symptomatic patients (Gurusamy & Davidson 2014).

Cholecystectomy (surgical removal of the gallbladder) or Extracorporeal Shock Wave Lithotripsy (ESWL; use of high-energy sound waves to break gallstones) are the common treatments for symptomatic gallstone cases. In some patients with small radiolucent cholesterol gallstones, or who do not have surgical treatment, dissolution with UDCA (600 mg/day) alone or in combination with simvastatin (10 mg/day) can be beneficial (Tazuma et al. 1998). Although success rates can be as low as 27% and recurrence rates as high as 40% (Gurusamy & Davidson 2014). In some instances combination with a very low calorie diet can improve outcomes (Shiffman et al. 1995). UDCA can also reduce the risk of complications such as acute cholecystitis and pancreatitis (Venneman & van Erpecum 2006).

Primary Biliary Cholangitis (PBC)

PBC is a slowly progressive disease of the liver characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts (Kaplan & Gershwin 2005). As reviewed by Kaplan and Gershwin(Kaplan & Gershwin 2005), the disease progresses through different stages beginning with localized inflammation, followed on by reduction in the number of bile ducts, fibrosis and finally cirrhosis with regenerative nodules. Incidence of the disease can vary between 0.004 and 0.04% of the population, with the highest incidence in northern Europe. The loss of bile ducts leads to decreased bile secretion and the retention of toxic substances within the liver, resulting in further hepatic damage, fibrosis, cirrhosis, and eventually, liver failure. It is more common in women than men (ratio 10:1) and tends to occur in people over 25 years of age.

In a two-year double-blind controlled trial, UDCA (13 – 15 mg/kg/day) was effective at reducing clinically overt disease, improved serum levels of bilirubin, alkaline phosphatase, alanine aminotransferase, and mean histologic score of liver biopsies in the treated group (n=73) compared to placebo (n=73) (Poupon et al. 1991). In a more recent trial with UDCA at the same dose, the effects of UDCA on biochemical markers were shown to be long lasting being maintained up to 15 years from start of treatment (Kuiper et al. 2011). Life expectancy in patients that respond to UDCA treatment is similar to those in healthy controls for up to 20 years (Kaplan & Gershwin 2005). Overall studies have been consistent in reporting beneficial effects for UDCA in PBC, making it the front line treatment for patients that show abnormal liver biochemistry tests.

Primary Sclerosing Cholangitis (PSC)

PSC is a chronic cholestatic liver disease that involves progressive characterised by inflammation of the bile ducts(Elman & Mcmaster 1925). As reviewed by Lutz and colleagues, even though it only occurs in about 0.01% of the population, diagnosis has risen over the last 20 years. PSC tends to occur in patients with chronic inflammatory bowel diseases and is associated with an increased risk of various types of cancer (13%–14%) most prominently cholangiocellular carcinoma (malignant new growths of epithelial cells that tend to infiltrate surrounding tissues). It is more common in men than women (62%–70% are male) and in middle age (from 35 to 51).

UDCA has long been used for the treatment of PSC however its benefit is still being debated. Studies with mid-dose UDCA (10-15 mg/kg) showed some improvements in laboratory values such as serum bilirubin, disease manifestations but no benefits on survival. When high doses (up to 30 mg/kg) were used there was a higher incidence of negative end-points such as death, liver transplantation and development of oesophageal varices (Lutz et al. 2013). This has led to complete opposition or dose restrictions being placed on UDCA use by European and American agencies.

Non-Alcoholic SteatoHepatitis (NASH)

NASH is a prevalent liver disease associated with increased morbidity and mortality and is characterized by significant lipid deposition in hepatocytes (Xiang et al. 2013). NASH occurs as a progression of Fatty Liver Disease (see FIGURE 1-7), however the mechanism by which this progression develops is not well known(Xiang et al. 2013). Important risk factors for NASH include obesity, diabetes mellitus, hypertriglyceridemia, and exposure to drugs or toxins. The prevalence of NASH has increased with the rise in obesity and diabetes and is now estimated to affect 1% of Europeans(Xiang et al. 2013).

Outcomes from clinical trials of UDCA in NASH are inconsistent. Treatment of NASH with high-dose UDCA (28 – 35 mg/kg/day) over a 12 month period in 126 patients resulted in significant reductions in liver aminotransferase levels and serum fibrosis markers (Ratziu et al. 2011). A separate study with high dose UDCA (23 – 28 mg/kg/day) over 18 months in 95 patients did not find any improvements in liver aminotransferase levels (U. F. Leuschner et al. 2010). Other trials at lower doses of UDCA (13 – 15 mg/kg/day) with durations between 6 and 24 months, reported inconsistent outcomes on liver biomarkers ranging from improvements to no observed effects (Dufour et al. 2006). Overall the benefits of UDCA in NASH are usually seen when UDCA is combined with other agents such as vitamin E rather than as a monotherapy (Xiang et al. 2013).

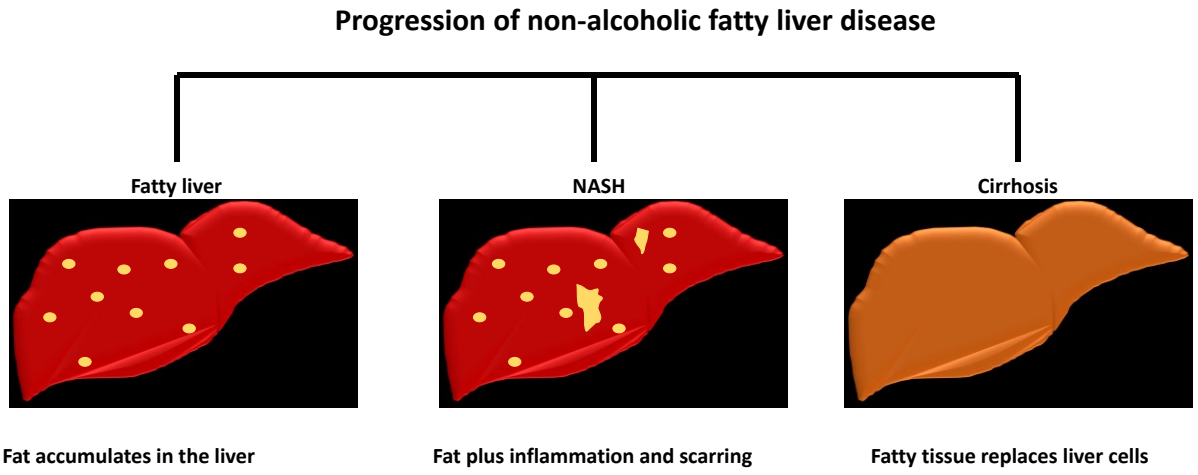


Figure 1-7 Progression of fatty liver disease.

Intrahepatic Cholestasis of Pregnancy (ICP)

ICP is a liver disorder in pregnancy that appears most often during the third trimester of pregnancy (Geenes & Williamson 2009). As reviewed by Geenes and Williamson, it is associated with a rise in maternal serum bile acid levels and/or increased concentrations of serum aminotransferase such as alanine aminotransferase (ALT). Its incidence varies with geographical location and ethnicity, and there is an increased incidence in first degree female relatives suggesting a genetic component in the disease aetiology. Other factors implicated in its pathophysiology are a rise in oestrogen levels, progesterone and environmental factors (winter months, pesticides). Current rates are as high as 4% in Chile but as low as 0.5% in Europe(Geenes & Williamson 2009). The main symptom is itching (pruritus) for the mother which tends to clear within a few weeks after birth. However, it is also associated with preterm birth, complications in the fetus and even stillbirth(Geenes & Williamson 2009).

Based on the success and safety of UDCA in other cholestatic diseases, the efficacy of UDCA has also been evaluated for ICP. In the first report by Palma and colleagues, administration of UDCA in five patients at a mean dose of 14 mg/kg/day improved pruritus scores, serum bile acid levels and glutamic pyruvic transaminase levels(Palma et al. 1992). Larger trials have since been conducted showing similar benefits. In a meta-analysis of 11 randomised controlled trials, pruritus decreased in 73% of patients while liver function tests were improved in 82% of patients (Grand'Maison et al. 2014). Although UDCA did not affect the caesarean section rate it was associated with less prematurity. The current recommended dose of UDCA for ICP treatment is 15 mg/kg/day divided into 2 or 3 doses (Grand'Maison et al. 2014). Very few side effects are reported with its use, in the rare cases where this is reported the complaints are usually diarrhoea and gastrointestinal upset (Geenes & Williamson 2009).

Monotherapy use of other agents such as dexamethasone, rifampicin and vitamin K has produced mixed results, however combination with UDCA can improve disease response (Geenes & Williamson 2009).

1.3.12 Mechanisms of action

UDCA acts at different sites and through different mechanisms to produce its beneficial effects in hepato-biliary diseases. The exact mechanisms of protection in each disease condition are still being investigated however some common mechanisms are found between them.

Some of these key protective mechanisms include:

- Alteration of the hydrophilic-hydrophobic bile acid balance.
- Protection against bile-acid induced apoptosis.
- Stimulation of hepato-biliary secretion.
- Immunomodulation.
- Membrane stabilisation.
- Hepato-biliary excretion of progesterone disulphates (specific to ICP).

A better understanding of UDCAs action in these diseases could help in discovering other applications for the drug and improve its current use.

Alteration of the hydrophilic-hydrophobic bile acid balance

Due to their detergent effects, bile acids in high concentrations can be cytotoxic. There is a correlation between the hydrophobicity of a bile-acid molecule and its detergent like properties (Attili et al. 1986). Bile is made up of a high concentration of hydrophobic bile acids which under cholestatic conditions can aggravate cholangiocellular damage by inducing cell membrane disruption and extracellular toxicity (Beuers 2006). Thus, elevations in toxic bile acids also can lead to increases in liver transaminases while prolonged exposure can lead to cirrhosis(Lazaridis et al. 2001).

Treatment with UDCA leads to an expansion in the total bile-acid pool(Beuers 2006). In particular it leads to an increase in the proportion of UDCA itself in the bile acid pool, and the displacement of other endogenous hydrophobic bile acids such as DCA and CDCA. This is partly driven by UDCA inhibition of CA and CDCA intestinal reabsorption and intrinsic hepatic clearance (Poupon & Poupon

1995). As a result UDCA becomes the most abundant circulating bile acid and the bile pool is found to be more hydrophilic and less cytotoxic. In patients receiving UDCA over a 2 year period, the proportion of UDCA compared to other bile-acids was found to increase by 42±20% in the biliary pool and 49±19% in serum bile acids(Lindor et al. 1998). This is in comparison to changes of 8±20% and -1±5% respectively in placebo patients. These significant increases in the concentration of UDCA are not harmful, as *in vitro* studies have shown UDCA to be safe up to 500 μM (Poupon & Poupon 1995).

Another benefit for the increased levels of UDCA that has been proposed is that UDCA can impair apical transporter uptake of hydrophobic bile acids into cholangiocytes(Hofmann 1994; Beuers 2006). This is linked to studies with UDCA conjugates that showed protective effects of UDCA in a rat model of cholestasis through Ca^{2+}-dependent and cPKCα-dependent mechanisms(Alpini et al. 2002). PKCα-dependent mechanisms have previously been shown to contribute to the anti-cholestatic action of UDCA conjugates in hepatocytes(Beuers et al. 2001).

Protection against bile-acid induced apoptosis

Apoptosis is a common form of cell death in cholestatic diseases such as PBC and is caused by the accumulation of hydrophobic bile acids in hepatocytes(Paumgartner & Beuers 2002). Examples of bile acids that induce apoptosis *in vitro* are the glycine conjugated Glycochenodeoxycholic acid (GCDCA) and Glycodeoxycholic acid (GDCA)(Paumgartner & Beuers 2002). This occurs via activation of the Fas death receptor a PKC-dependent and c-Jun N-terminal kinase-dependent phosphorylation of the epidermal growth factor receptor and then the back phosphorylation of the Fas receptor. This leads to increased permeability of the inner mitochondrial membrane to ions, resulting in mitochondrial swelling, the release of cytochrome c and activation of caspase receptors and apoptotic cell death(Lazaridis et al. 2001).

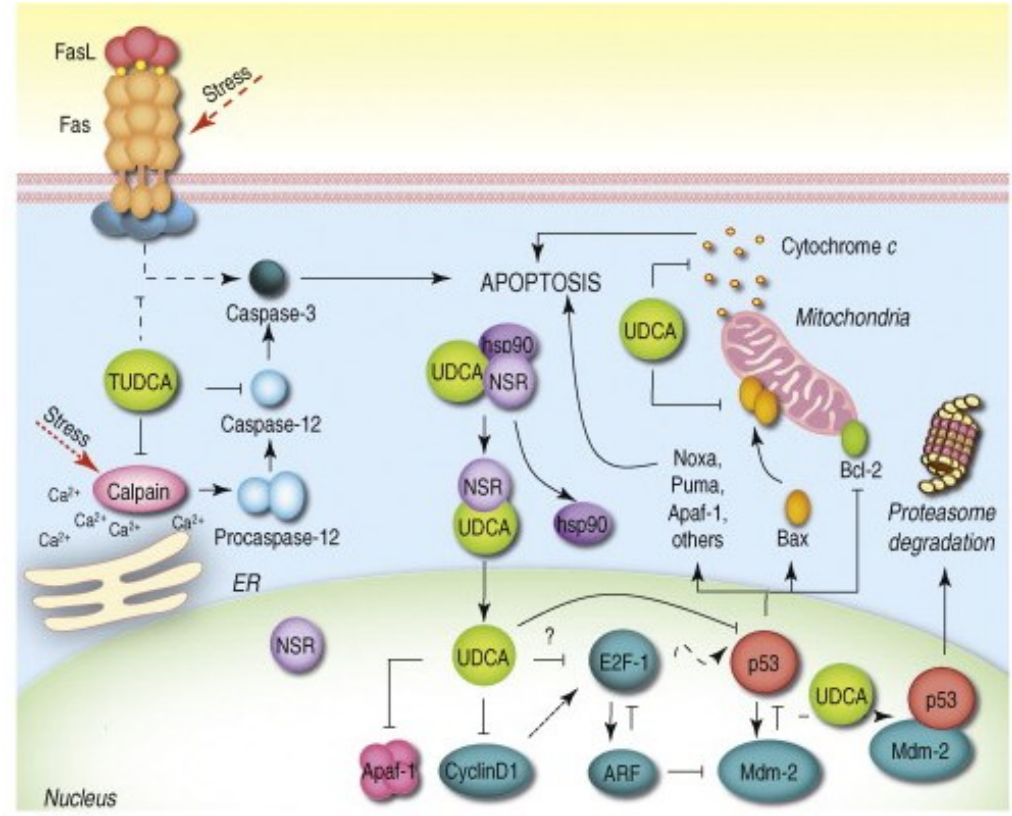


Figure 1-8 Proposed anti-apoptotic mechanisms of UDCA and TUDCA.

UDCA negatively modulates the mitochondrial pathway by inhibiting Bax translocation, ROS formation, cytochrome c release and caspase-3 activation. UDCA can also interfere with the death receptor pathway, inhibiting caspase-3 activation. Moreover, TUDCA inhibits apoptosis associated with ER stress by modulating intracellular calcium levels and inhibiting calpain and caspase-12 activation. Importantly, UDCA interacts with NSR, leading to NSR/hsp90 dissociation and the nuclear translocation of the UDCA/NSR complex. Once in the nucleus, UDCA modulates the E2F-1/p53/Bax pathway, thereby preventing apoptosis. Finally, UDCA downregulates cyclin D1 and Apaf-1, further inhibiting the mitochondrial apoptotic cascade. Cyt c, cytochrome c; Hsp90, heat shock protein 90; NSR, nuclear steroid receptor (from (Amaral et al. 2009)

Anti-apoptotic mechanisms of action for UDCA and TUDCA have been demonstrated *in vitro* and *in vivo* in rat hepatocytes (see FIGURE 1-8) (Rodrigues et al. 2003; Amaral et al. 2009). Co-administration of UDCA with DCA, inhibited the latter's ability to induce apoptosis in liver parenchyma and isolated hepatocytes(Rodrigues et al. 1998). The proposed pathway for this was by inhibition of the pro-apoptotic protein Bax stopping it from binding to the mitochondrial membrane and the subsequent

death of the cells(Lazaridis et al. 2001). With TUDCA, which is the more common form of UDCA in the body, the mechanism appears to be via activation of survival pathways involving the kinases p38, ERK MAPK and PI3K and independent of competition at the cell membrane(Beuers 2006). The differences reported in the action of UDCA and its taurine conjugate suggest that more work needs to be done to better understand its anti-apoptotic effects.

Stimulation of hepato-biliary secretion

Cholestasis is associated with a build-up of bile-acids in the liver and impairment of bile formation which leads to a cycle of injury and further impairment of liver function and necrosis(Paumgartner & Beuers 2002). The ability of liver hepatocytes to secrete bile-acids is determined by the number and activity of carrier proteins in the apical membrane and may be regulated both on a transcriptional and posttranscriptional level(Paumgartner & Beuers 2002). Therefore mutations and reduction in transporter protein mRNA expression have been linked to cholestasis progression(Keitel et al. 2006; Jansen & Sturm 2003).

UDCA is able to induce the biliary secretion of bile acids in PBC and PSC patients via a variety of pathways(Pusl & Beuers 2006). Firstly it is able to induce the expression of transporter proteins of the canalicular membrane, such as Bsep and Mrp2(Pusl & Beuers 2006). In mice fed a diet with UDCA or cholic acid, the hepatocellular levels of genes for these proteins were upregulated(Fickert et al. 2001). Outside the gut, high-dose UDCA can be protective in ICP by upregulating the expression of the placental breast cancer resistance protein (BCRP), thereby favouring bile acid efflux from the foetal compartment (Azzaroli et al. 2013).

Secondly, TUDCA is also able to stimulate the targeting and insertion of transporter molecules into the hepatocyte canalicular membrane of experimental animals (Beuers 2006). TUDCA stimulated the expression of Bsep and Mrp2 in the rat liver in experimental models of cholestasis (Beuers et al. 2001).

Immunomodulation

PBC and PSC can be classified as autoimmune diseases although the exact mechanism by which the immune system causes destruction is not fully known (Washington 2007). In PBC, inflammation is observed around the bile duct with infiltration of lymphocytes, scattered eosinophils and macrophages. The localization of these inflammatory mediators changes with disease progression. Infiltration of lymphocytes around the bile duct epithelium is also observed in PSC, with mononuclear inflammatory cells and scattered eosinophils around portal ducts (Washington 2007).

UDCA treatment in cholestatic diseases is associated with a reduction in inflammatory markers such as the immunoglobulin IgM (Leuschner et al. 1989) and HLA class I antigen expression (Calmus et al. 1990). In an experimental mouse model of hepatitis, feeding with UDCA for 4 weeks improved hepatocyte injury by suppressing IFN-γ production by liver mononuclear cells (MNC) (Takigawa et al. 2013). In the same study, glucocorticoid receptor (GR) expression was significantly upregulated in the liver MNC further suggesting immunoprotective properties for UDCA, as synthetic agonists of the receptor are very effective anti-inflammatory drugs (Bellentani 2005).

Membrane stabilisation

Hydrophobic bile acids such as chenodeoxycholate are able to induce hepatocellular damage in cholestatis patients (Güldütuna et al. 1993). As described by Guldutuna and colleagues, one of the mechanisms by which they are able to do this is by destruction of the basolateral and canalicular membrane. This is caused by solubilisation of membrane lipids due to an increase in polarity of the apolar domain of the membrane by chenodeoxycholate. UDCA is able to protect against this effect by binding to the apolar domain thereby stabilising it and preventing damage from bile acids such as chenodeoxycholate.

Hepato-biliary excretion of progesterone disulphates in ICP

Intrahepatic Cholestasis of Pregnancy (ICP) is usually observed in the third trimester of pregnancy when there is an elevation in progesterone levels. Women with ICP tend to have higher levels of the sulphated metabolite of progesterone (Meng et al. 1997). In a clinical evaluation of 8 patients treated with UDCA (14 mg/kg body weight) given in 3 doses for 10 to 21 days, the levels of sulphated progesterone metabolites reduced by up to 60% (Meng et al. 1997). In a separate study, improvements in pruritus scores were correlated with a decline in disulphated progesterone metabolites in urine that was not observed with placebo (Glantz et al. 2008). These findings suggest that one of the mechanisms by which UDCA improves outcomes in ICP is by an improvement of hepatobiliary excretion of progesterone metabolites.

1.4 POTENTIAL APPLICATIONS FOR UDCA IN CARDIOVASCULAR CONDITIONS

The efficacy and safety of UDCA in hepato-biliary diseases have made it an increasingly popular drug and have led to growing interest in understanding its mechanism of action. These include identifying potential benefits in other disease areas or for the treatment of secondary complications that might develop from the underlying disease. For example, cholestatic liver disease is associated with widespread derangements in the cardiovascular system, such as bradycardia, hypotension, QT prolongation and peripheral vasodilation (Moezi & Dehpour 2013). However, the indirect benefits of UDCA treatment on the development or progression of these disorders has not been well studied.

To date a number of i*n vitro* studies and clinical trials have been conducted to determine benefits for UDCA outside hepato-biliary diseases in neurodegenerative diseases such as Hungtington's disease, Alzheimer's disease and Parkinson's disease (Vang et al. 2014). It has also been shown to be protective in models of retinal degeneration, glaucoma and cataracts (Boatright et al. 2009). Benefits for UDCA have also been proposed in animal models for the treatment of diabetes and similar metabolic disorders(Vang et al. 2014). There is experimental and human data to suggest that UDCA can be chemopreventative in colorectal cancer although this is still a subject of controversy (Serfaty 2012;

Carey & Lindor 2012). It can also be cost-effective in diseases such as PBC as it is associated with a lower incidence of major complications, with reduced morbidity and mortality (Boberg et al. 2013; Pasha et al. 1999).

The focus of this project was to investigate new applications for UDCA in the treatment of cardiovascular conditions that develop in the presence or absence of cholestasis. The rationale behind this is based on evidence from pre-clinical and clinical studies that have shown promising results for the drug and its conjugates in treating various cardiovascular conditions. Some of this evidence is reviewed below.

1.4.1 Evidence from pre-clinical studies

UDCA treatment prevents acute transplant rejection in rats

The immunomodulatory effect of UDCA makes it an interesting therapeutic option for protecting against immune mediated organ transplant rejection. A number of studies in liver transplantation patients identified lower cases of rejection with UDCA(Friman & Svanvik 1994; Clavien et al. 1996). In an experimental heart transplantation model, the effect of UDCA against acute rejection was studied(Olausson et al. 1992). UDCA administered for a week prior to transplantation and for 2 weeks after prolonged graft survival and increased the amount of transplant tolerance in rats compared to control treated rats.

UDCA treatment protects against the development of atherosclerosis

Cholesterol and lipoproteins (Low-density lipoprotein (LDL) and High-density lipoprotein (HDL)) play significant roles in the development of plaques and atherosclerotic lesions. As the formation of bile acids is the key route of cholesterol elimination in the body, it is important to consider the role of bile acids such as UDCA on the development of atherosclerosis.

Potential benefits have been shown for UDCA in the reduction of LDL and increase in HDL. Beginning in isolated hamster hepatocytes, Bouscarel and colleagues demonstrated enhanced receptor-

dependent LDL uptake and degradation with 1 mM UDCA and its glycine and taurine conjugates (Bouscarel et al. 1991). Further *in vivo* studies in hamsters, showed that chronic feeding with UDCA can also result in a significant increase in hepatic LDL uptake (Bouscarel et al. 1995), confirming a translation of previous cellular effects. These benefits were also observed to occur in a hamster model of hypercholesterolemia induced by a high cholesterol diet with or without UDCA. When compared to animals receiving the diet supplemented with UDCA, the hepatic uptake of LDL was observed to be higher in the UDCA group and similar to levels in healthy animals (Ceryak et al. 2000).

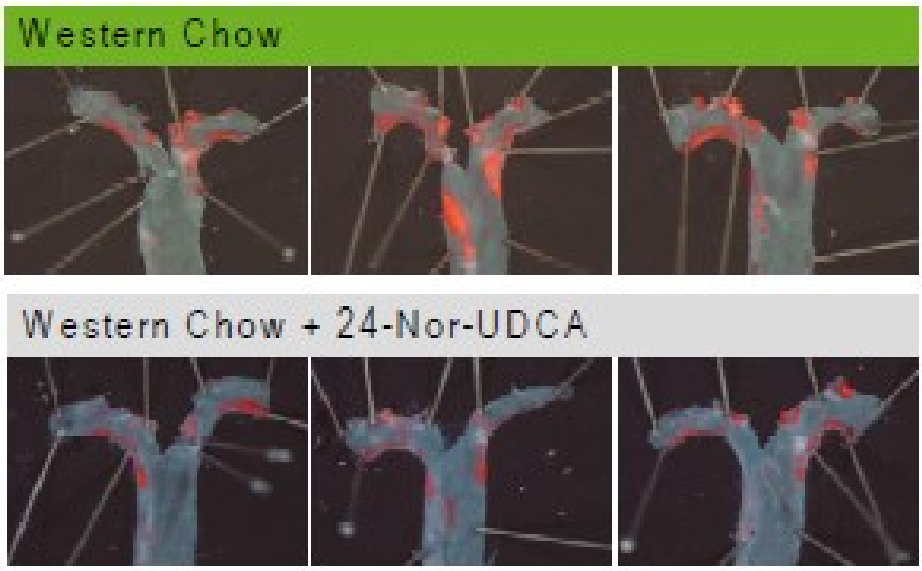


Figure 1-9 Reduction in atherosclerosis with 24 norUDCA treatment in aortic arch valves.
Sclerotic areas are red-oil stained (from (Trauner 2008)*)*

Using knockout mouse models, Trauner and colleagues showed a reduction in atherosclerosis with norUDCA feeding (see Figure 1-9) (Trauner 2008). 8 week-old LDL receptor homozygous knockout mice (n=5) were fed a cholesterol enriched diet for 8 weeks resulting in the development of severe plaques in aortic valve sections. However, in a separate group receiving the same diet supplemented with *nor*UDCA (n=5), there was a significant reduction in the development of these lesions. This effect was also seen in an ApoE knockout model of atherosclerosis.

UDCA protects against bile acid induced conduction slowing effects in neonatal cardiomyocytes

Among the fetal complications that occur in ICP is death due to cardiac arrhythmias. The exact nature and cause of these arrhythmias is still being investigated, as it appears that they occur suddenly and infrequently. Using *in vitro* models a link has been established between the elevation in maternal bile acid levels and the induction of fetal arrhythmias. Bile acids have been shown to induce adverse cardiac effects in animal models and human cells. The bile acid taurocholate (TC) caused a loss of synchronous beating, bradycardia and cessation of contraction in cultured rat cardiomyocytes (Williamson et al. 2001). Similarly in human atrial cardiomyocytes, TC has been shown to induce arrhythmias and depolarise the resting membrane potential (Rainer et al. 2013).

In a study using neonatal (1 to 2 day old) rat cardiomyocytes, previous work from our group has shown that UDCA and dexamethasone are able to protect against the arrhythmogenic effects of TC (Gorelik et al. 2003). In this study cells were pre-incubated for 16 hours prior to addition of TC at different concentrations. The rate, rhythm, amplitude of contraction and calcium dynamics were then measured using scanning ion conductance microscopy and confocal microscopy. It was found that TC induced contractile irregularities as well as fibrillation/tachycardia and loss of synchronous contraction without UDCA pre-incubation (Gorelik et al. 2002). However, in cells that were pre-incubated with UDCA the effects of TC were inhibited at lower concentrations.

Furthermore, in *in vitro* models of the fetal and maternal heart in ICP, TC also induced conduction slowing (Miragoli et al. 2011). Based on data that showed a high expression of myofibroblasts in fetal heart, an in vitro model was constructed made up of neonatal cardiomyocytes with myofibroblasts, while myofibroblasts were absent in the maternal model. TC caused a slowing of conduction velocity, re-entrant arrhythmias and induction of early after depolarizations. However, again UDCA treatment was able to protect against these changes as it hyperpolarizes the myofibroblast membrane.

UDCA protects against ischemia-reperfusion injury and cardiac infarction

Following the reperfusion of a previously occluded region of an organ, the process of re-oxygenation can contribute to further tissue damage (Carden & Granger 2000). This is referred to as ischemia-reperfusion injury. The development of damage is linked to a complex series of events including generation of reactive oxygen species and increased intracellular calcium. Myocardial infarction (the irreversible damage of heart tissue) can result from prolonged ischemia and hypoxia (Klabunde 2004). Although infarction is generally thought to be due to necrosis, apoptosis also occurs as part of the process of cell destruction (Krijnen et al. 2002). Based on the protective effects of UDCA and TUDCA in liver ischemia-reperfusion injury, its protective effect of in cardiac tissue has also been examined.

In an isolated heart perfusion model, UDCA reduced ischemia-reperfusion damage following global ischemia (Lee et al. 1999). In this study, Langendorff perfused rat hearts were treated with UDCA (20 – 160 μM) or vehicle for 10 minutes. They were then subjected to a 30 minute period without perfusion and then reperfused for 30 minutes. It was found that UDCA treatment at concentrations of 80 – 160 μM improved left-ventricular diastolic pressure (LVDP) and enhanced contractile function. It also caused a reduction in the release of lactate dehydrogenase (LDH), a marker of cellular integrity.

In an anaesthetized rat model, Rajesh and colleagues (Rajesh et al. 2005) also evaluated the effect of UDCA pre-treatment on ischemia-reperfusion *in vivo*. In the protocol animals were either treated with UDCA 40 mg/kg or vehicle for 30 minutes prior to left-coronary artery occlusion for 30 minutes followed by a 180 minute period of reperfusion. Histological evaluation of isolated hearts showed that UDCA treatment reduced the size of infarcts following reperfusion (see FIGURE 1-10). It also significantly prevented the loss of myocardial ATP compared to vehicle treatment. The mechanism of this effect was related to the anti-apoptotic effect of UDCA previously described. In this case UDCA protected against injury by inhibiting the mitochondrial permeability transition pore via activation of PI3 kinase pathways.

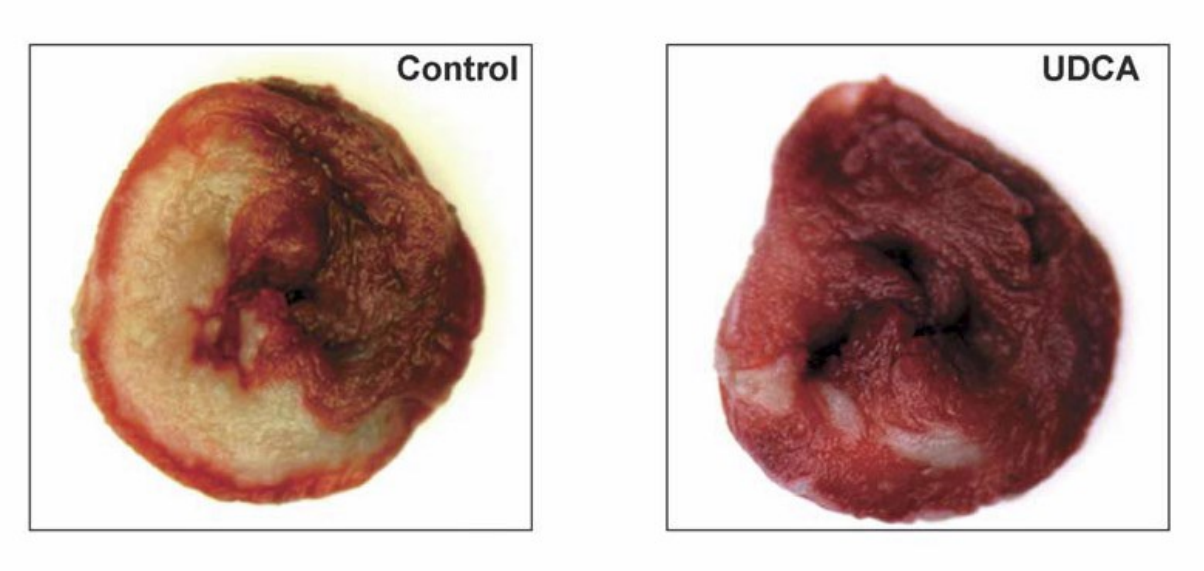


Figure 1-10 Reduction in size of infarct areas from Control and UDCA treated rat hearts.

(from (Rajesh et al. 2005)*).*

In a separate study by Rivard and colleagues, TUDCA 400 mg/kg was administered intravenously to rats to evaluate its anti-apoptotic effects (Rivard et al. 2007). The left anterior descending coronary artery was ligated and animals were sacrificed 24 hours later. Apoptosis was determined using TUNEL staining and activity of apoptosis-related enzyme caspase-3. TUNEL staining showed a reduction in apoptotic cells and caspase-3 activity in TUDCA treated cells compared to vehicle treatment. In separate rats that were not sacrificed, echocardiography was performed at 1 and 4 weeks from initial treatment and ligation. Although not significant there was a trend towards an improvement in myocardial shortening fraction.

1.4.2 Evidence from clinical studies

UDCA improves peripheral and systemic haemodynamics in patients

Bile acids have been proposed to acts as endogenous vasodilators (Bomzon & Ljubuncic 1995). A hypothesis that has been furthered by the discovery of the TGR5 receptor and its expression in the cardiovascular system (Fryer et al. 2014). This property coupled with the other cholesterol lowering and anti-inflammatory effects of UDCA, make it an attractive treatment for congestive heart failure.

In a study of the effect of 1-month therapy with UDCA (13 mg/kg/day) in patients with PBC, it was observed that UDCA significantly reduced diastolic volume without affecting systolic, diastolic or mean blood pressure (Baruch et al. 1999). In a separate study in coronary heart disease patients, 6 week therapy with UDCA (13 – 19 mg/kg) improved endothelium-dependent nitric oxide-independent vasodilatation by 161±27% compared to 83±22% in placebo (Sinisalo et al. 1999). In a double-blind study in 17 patients for 4 weeks, UDCA (~15 mg/kg) improved peak post-ischemic blood flow in the arm with a trend in improved peak post-ischemic blood flow in the leg (Von Haehling et al. 2012).

UDCA can reduce the incidence of rejection in transplant patients

The benefits of UDCA seen in animal models of transplantation have also been reviewed from clinical studies. A retrospective study was performed on 21 cardiac allograft patients that also received UDCA as part of their treatment for cholestasis and compared to 31 untreated patients (Bahrle et al. 1998). UDCA had been administered as 500 mg doses twice daily for 8 weeks. In the first 6 months following treatment, there was a significantly lower incidence acute rejection episodes requiring specific anti-rejection therapy in the UDCA group compared to control.

UDCA for the reduction of cholesterol

Contrasting results on the ability of UDCA to lower cholesterol have been reported. UDCA has been shown to decrease serum total and LDL cholesterol in hypercholesterolemic patients with PBC (Poupon et al. 1993). 13-15 mg/kg/day UDCA (n=17 patients) caused a reduction in total serum cholesterol levels compared to placebo patients (n=16), with the decrease mainly coming from a reduction in LDL levels but also a small drop in HDL. However, when assessed in non-PBC patients with hypercholesterolemia no changes in LDL or total cholesterol were observed (Braga et al. 2009). Similarly a study of the effect of UDCA 15 mg/kg/day in 12 adults did not find any improvements in cholesterol absorption or metabolism when diet and genetic differences were compared (Woollett et al. 2003).

1.5 SUMMARY

The therapeutic properties of bile have long been established. However only more recently have its composition and physiological roles been better understood. The key constituents in bile that are responsible for its endogenous function are the bile acids – a group of similar steroidal molecules with diverse physico-chemical properties that are synthesised from cholesterol. Bile acids are ampiphilic in nature allowing them to act as endogenous surfactants for the digestion of dietary lipids. They can also act as signalling molecules through nuclear and membrane receptors to regulate glucose and energy homeostasis and modulate cardiovascular function.

Ursodeoxycholic acid (UDCA) is a bile acid that forms a small proportion of the total human bile acid pool but is found in greater quantities in some hibernating species such as bears. The hydrophilic nature of UDCA gives it a unique set of properties in health and disease. In a number of clinical trials in cholestasis patients, UDCA has been shown to be protective against the effects of toxic bile acids and the rare side effects that can result when they are present in excess. This has led to its regulatory approval and wide-spread use despite its mechanism of action not being fully understood. Among other mechanisms, UDCA has been shown to protect against cell damage in cholestasis by altering bile acid composition, immunomodulation and inhibition of apoptosis.

With better understanding of the role of bile acids as signalling molecules and the demonstrated efficacy and safety of UDCA in hepato-biliary diseases, there is increasing interest to identify potential applications for this drug in the treatment of other diseases. These include cardiovascular conditions such as cardiac infarction and fetal arrhythmias linked to elevations in toxic maternal bile acids. In both conditions there is growing evidence that through its protective effect against cell damage and ion channel modulation, application of UDCA might be a novel and effective therapeutic strategy.

1.6 SCOPE OF THESIS

Using whole organ and cellular models, I evaluated the effects of UDCA and its mechanism of action in fetal arrhythmias induced by elevated bile acid levels and in ischemia reperfusion-injury and infarction. The objective of this work was to identify new uses for this clinically safe drug in the treatment of cardiovascular diseases.

1.6.1 Hypotheses

I aimed to address the following hypotheses:

- Based on the demonstrated cytoprotective effects of UDCA and its modulatory effects on MFB membrane potential, UDCA can be protective against cardiac arrhythmias associated with cardiac fibrosis.
- UDCA protects against fetal arrhythmias in ICP by modulating cardiomyocyte calcium ion channels.

1.6.2 Aims

- To develop an isolated-perfused whole-heart model of the human fetal heart for evaluating the mechanism of bile acid induced arrhythmias in ICP.
- To identify a mechanism of bile-acid induced atrial conduction slowing in ICP using ECG recordings in fetal and adult whole-heart models of ICP.
- To study the effect of UDCA on bile acid induced ventricular conduction slowing using optical mapping techniques in fetal and adult hearts.
- To evaluate the cardio-protective properties of UDCA in a model of global ischemia.

2 General Methods

2.1 Animals

All experiments were carried out in accordance with the United Kingdom Home Office Guide on the Animals (Scientific Procedures) Act 1986. All animals were housed in dedicated biomedical research husbandry facilities under the supervision of certified animal care technicians according to institutional guidelines and international laws. Regulated procedures were carried out in accordance with the specified animal allowance in our project licence (70/7399) as well as my personal licence (70/20523). Where possible I have endeavoured to share tissue from animals in order to minimise animal use in line with the '3 Rs' principles of the National Centre for the Replacement, Refinement and Reduction of animal research guidelines (NC3R's, 2015).

2.1.1 Neonatal rats

New born Sprague-Dawley rats were ordered from Harlan Laboratories (Wyton, UK). Male and female pups were ordered between 0 and 1 day old but no more than 2 days old. Animals were delivered to our premises in litters of 6 to 12 with a mother fed *ad libitum*. Average weight was approximately 7 grams. They were culled on the day of arrival for either cardiomyocyte or whole-heart isolation and not housed overnight.

2.1.2 Adult rats

Adult male and female Sprague-Dawley rats were ordered from Harlan Laboratories and Charles River (Harlow, UK) for studies. In some instances, naïve surplus or sentinel Wistar-Hans rats were also received as a kind gift from Phil Rawson at Imperial College's Central Biomedical Services (CBS). These were mainly used for model development and surgery practise.

Weights ranged between 150 and 400 grams. Animals undergoing treatment were housed in CBS facilities at a density of 2-4 animals per cage and maintained on a 12-hour light/dark cycle at approximately 21°C. Facilities are maintained at a relative humidity between 45-65%. Animals were culled for heart isolation on the day of study.

2.2 Materials

All reagents and materials were purchased from Sigma-Aldrich (Poole, UK) except otherwise stated.

2.3 Experimental models and solutions

To study drug effects in arrhythmias and infarction, I used isolated cardiomyocytes and Langendorff-perfused heart models. The specific details and perfusate solutions for each model differed and are described in the relevant sections below, however, a similar protocol for heart isolation was used for all models.

Animals were placed in an anaesthetisation chamber and anaesthetised by inhalation of isoflurane (Abbott, USA) (5% in oxygen). When a sufficient depth of anaesthetisation was achieved (confirmed by loss of righting ability and pedal pinch reflex), cervical dislocation was performed. The animal was then placed on its back and the chest cavity opened with a pair of scissors and forceps. The sternum was cut away for better access to the heart. The heart was rapidly excised with a sufficient length of aorta and surrounding tissue and placed in cold (+4°C) cardioplegic solution (described in **Section 2.3.3** below) containing porcine intestinal mucosa heparin to prevent blood clotting.

2.3.1 Cardiomyocyte isolation

To obtain neonatal rat ventricular cardiomyocytes, the atrial region of the heart was cut away. The ventricles were placed in a culture dish with HBSS and chopped into 1 mm cubes. A digestion enzyme mixture (Miltenyl, UK) is prepared according to manufacturer's instructions and warmed. HBSS was removed and enzyme mixture added to samples. The mixture was then inverted and incubated at 37°C for 15 minutes. Following this the mixture was spun on a GentleMACS^{TM} dissociator system (Miltenyi, Germany). The incubation process is then repeated twice with fresh enzyme added each time. The sample is then resuspended in 7.5 mL of cell culture medium and filtered through a 70 µm filter. It is centrifuged for 5 minutes and the supernatant removed. Pellet is resuspended in 20 mL of 10% M199

medium and placed in a T75 culture flask and incubated in 1% CO_2 for 1 hour to separate myofibroblasts from cardiomyocytes. Following this period the cells are passed through a 70 µm filter and counted before being plated on collagen coated coverslips at the required density.

2.3.2 Isolated Langendorff-perfused heart model

Background

The isolated coronary-perfused heart model developed by Oskar Langendorff in 1895 still remains an invaluable tool for studying cardiac electrophysiology. It improves on cellular models by allowing the whole heart to be studied. Furthermore, it allows the whole organ to be studied in isolation of other organ systems and exocrine control (Bell et al. 2011). It is widely applicable to hearts from humans and different animal species with protocols that could otherwise be harmful or difficult to study. More importantly it is a simple and readily reproducible model that can provide a wealth of information(Bell et al. 2011).

Although it has undergone some modifications and adaptations over the years, the basic principles still remain the same. The aorta is cannulated and perfused retrogadely, in the opposite direction to normal flow, with a perfusion buffer at physiological pH and temperature. In initial studies performed by Langendorff, blood was used for perfusion, however later studies discovered saline substituted with glucose could act as a substitute leading to the development of a bicarbonate-based physiological salt perfusion fluid by Krebs and Henseleit(Skrzypiec-Spring et al. 2007). Over the years, modified solutions have been made which more accurately reflect physiological concentrations of calcium and glucose.

Perfusion can either be done under constant pressure or constant flow, the choice of which is dependent on the study protocol. Retrograde perfusion down the aorta forces the leaflets of the aorta shut, causing the perfusate to flow through the coronary ostia and arteries into the vascular bed and thus perfusing the whole heart. Perfusate returns via the coronary veins into the coronary sinus in the right atria, freely draining into the right atrium (Bell et al. 2011). A variety of physiological parameters

can be measured using this technique including heart rate, pressure, coronary flow, myocardial contractility, temperature, electrocardiogram and optical mapping of membrane conduction velocity (Bell et al. 2011). Following treatment, hearts can be preserved to assess structural changes by immunohistological staining.

Despite its advantages it is important to be aware of key limitations of the model. As an isolated model, whole animal translation of observed effects is limited since compensation through neuronal and hormonal mechanisms cannot be studied. Deterioration of function has also been observed with prolonged perfusion limiting the duration for which the model can be used. Preparation of the heart for cannulation increases the possibility of preconditioning which can affect results (Bell et al. 2011).

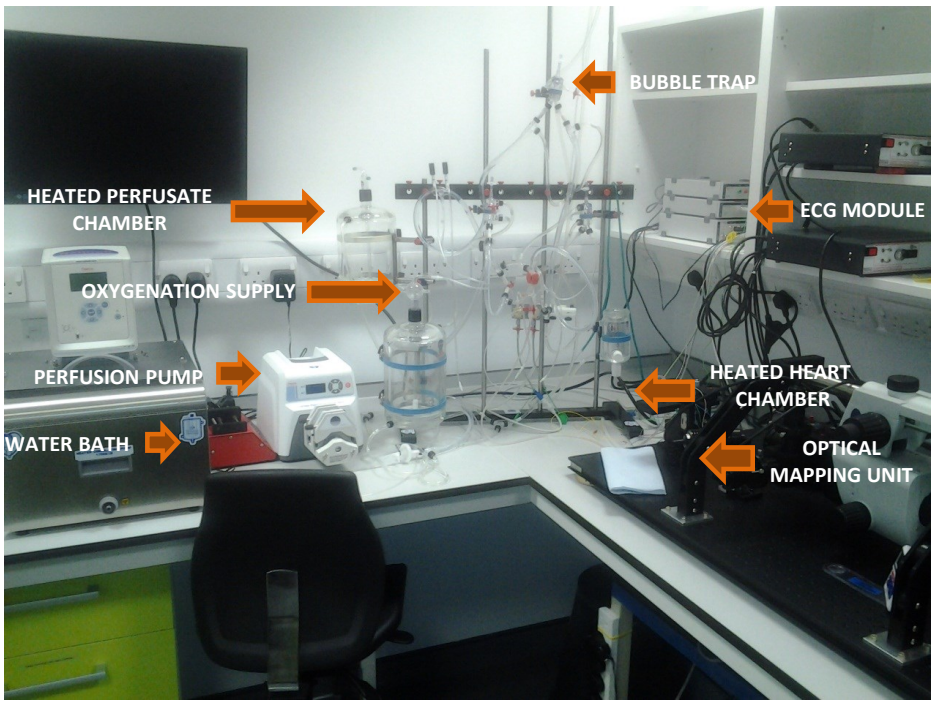


Figure 2-1 Isolated Langendorff-perfused heart set-up highlighting key apparatus.

Procedure

The Langendorff-perfused heart set-up is shown in Figure 2-1. Rapidly following isolation, an appropriate size cannula was placed in the aorta and held in place with a bull dog clip (World Precision Instruments, UK). Placement of cannula was carefully chosen to avoid occlusion of coronary arteries.

The aorta was then tied in place with non-sterile suture thread 3/0 (Fine Science Tools, Germany). Hearts were coronary perfused and superfused with Tyrode's solution (see **Section 2.3.2**) oxygenated with 95% O_2/5% CO_2 (BOC, UK) gently bubbled into the perfusate solution and maintained at 37°C. A bubble-trap and filter were connected to the perfusion set-up to avoid air bubbles and fine particles, respectively, reaching the heart. Perfusion was performed using a peristaltic pump (World Precision Instruments, UK) under constant aortic pressure. After this was done, surrounding non-heart tissue such as lung, fat and thymus tissue were cut away and discarded.

Study specific procedures used for neonatal and adult hearts and in studies of ECG and optical mapping are described in the relevant sections below.

2.3.3 Perfusate solution

This project used Tyrode's solution as the perfusion buffer. Tyrode's solution invented by Maurice Tyrode, is a modification of an earlier preparation by Ringer and Locke but contains magnesium and uses phosphate as a buffer instead of lactate. It is prepared by dissolving the following reagents in distilled water at specific concentrations (**in mM**): NaCl **128.2**, KCl **4.7**, NaH_2PO_4**1.19**, $MgCl_2$**1.05**, $CaCl_2$**1.3**, $NaHCO_3$**20.0** and Glucose **11.1**.Oxygenation with 95% O_2/5% CO_2 produces a pH of 7.4 at 37°C.

Stock solutions were prepared in two separate volumes: Stock I contained NaCl, KCl, NaH_2PO_4, $MgCl_2$ and $CaCl_2$ while Stock II contained only $NaHCO_3$. Glucose was excluded from stock formulations to avoid bacterial growth. These were stored in a fridge at the appropriate temperature. On study days they were combined in appropriate volumes with the required amount of glucose. Physiological temperature was achieved by heating solutions in a water bath and heated chambers.

2.3.4 Cardioplegia solution

Through the process of heart excision and cannulation for the Langendorff-perfused model, it is necessary to protect the heart from ischemia-reperfusion damage. Presently cardioplegic arrest

remains the gold standard for cardioprotection during this process, and involves the use of a cold hyperkalemic (elevated potassium) extracellular solution (either crystalloid or blood-based) (Chambers & Fallouh 2010). It protects the myocardium by inducing rapid and complete diastolic arrest via hyperkalemia, minimizes myocardial energy requirements through the induction of hypothermia and minimizes ischemic damage and reperfusion injury through agents that prevent or reverse unfavourable cellular changes (Donnelly & Djuric 1991).

Cardioplegic solutions contain a high concentration of potassium ions which results in depolarization of the normal resting membrane potential and calcium entry into the cell. However, due to the high extracellular potassium concentration the cell is unable to repolarize. Crystalloid solution were used for this project as a vehicle for cardioplegia solution; however, clinical studies have demonstrated favourable effects of blood-based cardioplegia solutions and are now the most commonly used clinical form of hyperkalemic cardioplegia (Donnelly & Djuric 1991; Chambers & Fallouh 2010). It is not entirely problem free especially in older patients who are more sensitive to ischemic damage and further improvements are being investigated (Chambers & Fallouh 2010).

For these studies cardioplegic solution was prepared according to the following concentrations in distilled water (**in mM**): NaCl **110**, KCl **16**, $MgCl_2$ **16**, $CaCl_2$ **1.2** and $NaHCO_3$ **10** and stored at 4°C in a fridge or kept on ice during studies. Composition is according to the widely used crystalloid formulation, St. Thomas' Hospital cardioplegic solution number 2, developed by the group of Hearse and Braimbridge (Jynge et al. 1981).

2.3.5 Rationale behind concentration of UDCA used

The concentrations of UDCA chosen for evaluation in this thesis were selected based on concentrations previously evaluated using cardiomyocytes in literature.

A previous study in our group demonstrated that UDCA at a concentration of 0.1 µM was able to inhibit TC induced conduction slowing in neonatal rat cardiomyocytes (Miragoli et al. 2011). This study

was performed in a cellular model of the fetal heart consisting of neonatal rat cardiomyocytes exposed to TC for acute (15 minutes) and chronic (12-16 hour) durations. Added to this, Miragoli and colleagues also compared UDCA effects alone at concentrations of 0.1, 1, 10 and 100 μM on conduction velocity and observed similar effects of an increase in conduction velocity at all concentrations which interestingly was slightly more pronounced at the lowest concentration of 0.1 μM. Therefore, to allow for translation to these previous studies we chose a UDCA concentration of 0.1 μM for cardiomyocyte patch-clamp studies and 1 μM for whole-heart studies on ICP. Concentrations of 1 and 5 μM were used for studies on ischemia-reperfusion models.

In clinical studies, pharmacokinetic analysis of plasma samples following 3 week treatment with UDCA (15 mg/kg/day) in adult healthy and PBC patients resulted in peak plasma concentrations of 15.5 and 0.9 μM for UDCA and TUDCA respectively (Dilger et al. 2012). However as Geenes and colleagues (Geenes et al. 2014) have pointed out, fetal bile acid concentrations are lower than maternal levels therefore it is probable that a similar assessment in fetuses will reveal peak plasma concentrations lower than 15.5 μM for UDCA. Although concentrations as high as 10 and 500 μM have also been evaluated in *in vitro* studies on hepatocytes (Bouscarel et al. 1993). We were cautious to avoid very high concentrations of UDCA since high concentrations of UDCA have been shown to cause adverse outcomes in clinical trials (see **Section 1.3.12**).

Again similar to Miragoli and colleagues, this project evaluated TC at concentrations of 40, 100 and 400 μM. Although the 400 μM is higher than levels usually reported in ICP (Geenes et al. 2014), Miragoli and colleagues also evaluated a similar concentration and reported in their study that there are occasional reports of patients with similar plasma concentrations of the bile acid (Miragoli et al. 2011).

2.4 Optical mapping of membrane voltage

2.4.1 Background

Langendorff-perfused human heart preparations were among the first models used for computerized optical mapping (Durrer et al. 1970). Optical mapping of cardiac electrical activity involves the use of high-speed imaging devices to record changes in light emission by voltage sensitive fluorescent probes bound to cell membranes in cells, whole organs or *in vivo*.

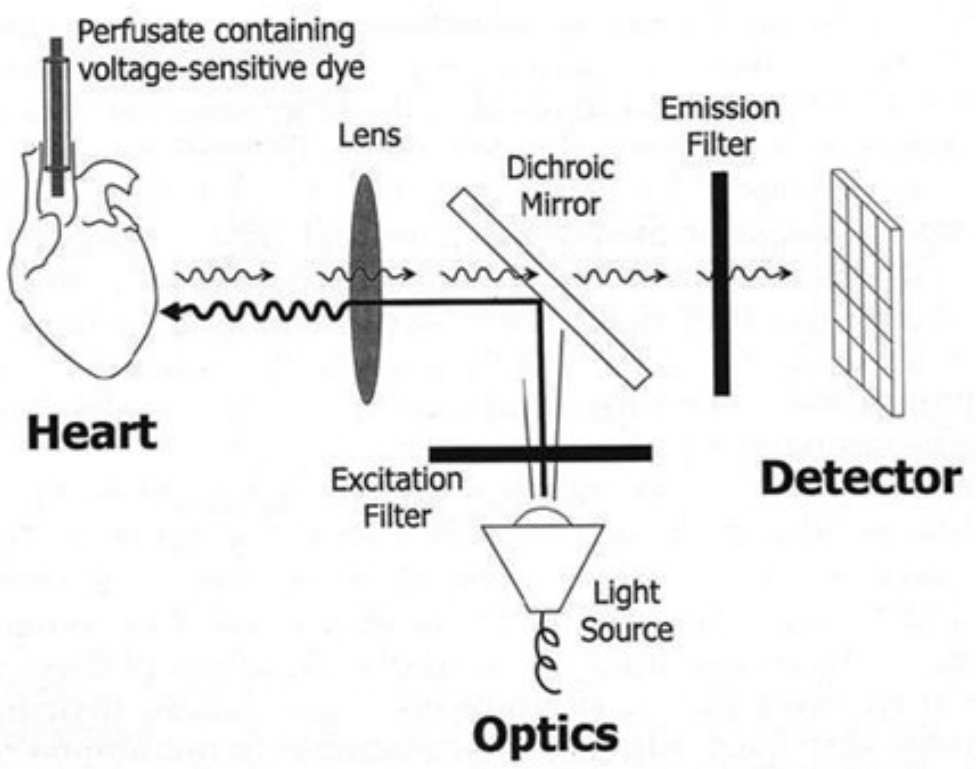


Figure 2-2 A typical optical mapping set-up.

(Image from (Arora et al. 2003))

The concept of using optical recordings to observe changes in cellular membrane potential was first demonstrated by Cohen and colleagues in nerve fibres (Cohen et al. 1968). However, the basic nature of early voltage sensitive dyes produced a low signal to noise ratio in studies limiting data interpretation. Improvements throughout the 70's and 80's helped to resolve this problem permitting investigation of arrhythmia generation and impulse propagation in regions such as the pacemaker to

be performed (Attin & Clusin 2009). A typical set-up is shown in FIGURE 2-2 above. The key components include a voltage-sensitive and/or calcium dye, light source(s), detectors and excitation and emission light filters (Attin & Clusin 2009).

The choice of dye used is dependent on focus of the study. Voltage-sensitive dyes are used to study changes in membrane potential while calcium dyes are for studying changes in intracellular calcium concentration. Voltage-sensitive dyes bind to cell membranes with high affinity and fluoresce in proportion to transmembrane voltage (Arora et al. 2003). Changes in membrane voltage cause conformational changes in the chemical orientation of dyes causing a shift in the range of light absorption and emission wavelengths. The shape of this wavelength spectra is not dependent on an absolute voltage but on relative changes in voltage. The chemical structure and absorption and emission spectra of the widely used voltage-sensitive dye, Di-4-ANNEPS, are shown in FIGURE 2-3. Other examples are Di-8-ANNEPS and RH-237. The popularity of Di-4-ANNEPS is based on its large fluorescence changes during action potential changes, coupled with low toxicity and photobleaching (Arora et al. 2003).

Calcium sensitive fluorescent dyes or indicators also work by producing emission shifts but this results from binding to calcium and not changes in voltage. They can be divided into single or dual-wavelength dyes depending on their response to calcium elevation. Single-wavelength indicators display changes in fluorescence emission when Ca^{2+} signals occur, however unlike dual-wavelength indicators, there is not a sufficient shift in either excitation and/or emission wavelengths on Ca^{2+} binding to allow ratiometric measurements (Bootman et al. 2013). Examples of single and dual-wavelength indicators are FLUO-4 and Fura-2 respectively. Both voltage- and calcium-sensitive dyes can be subject to photobleaching over time, where light exposure causes a permanent fading in fluorescence signals. Therefore it is important to limit light exposure to no more than is necessary for imaging.

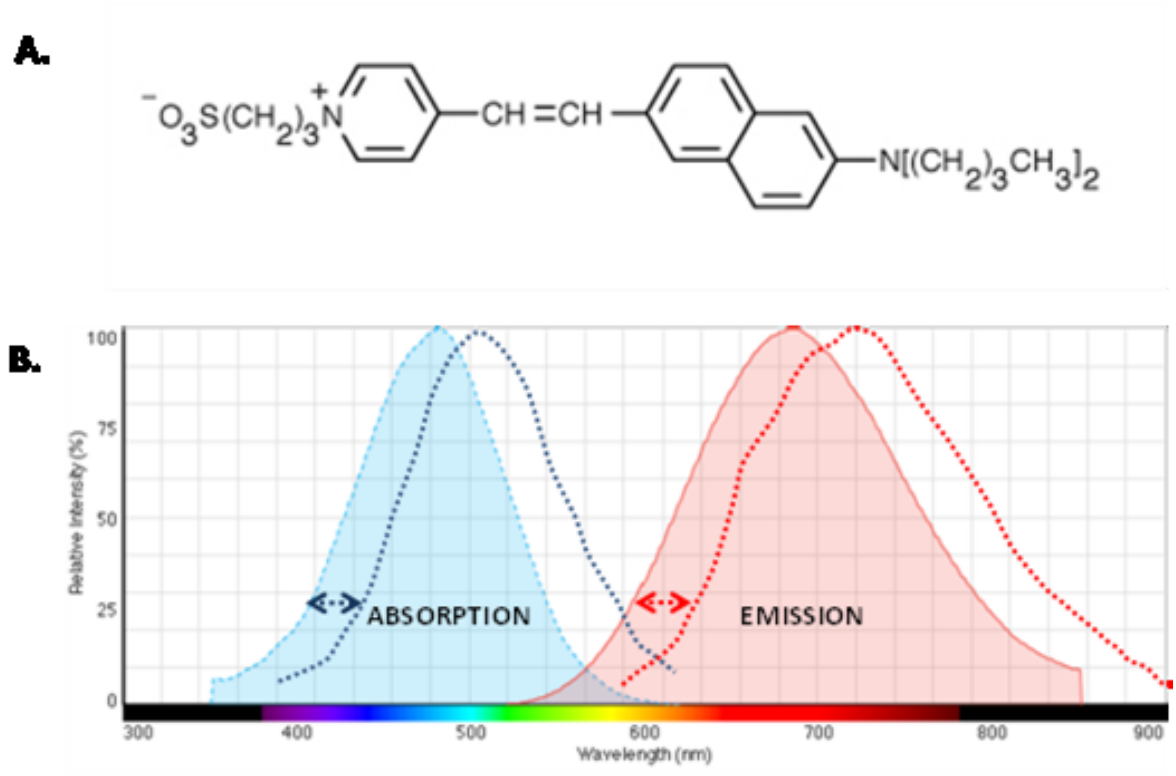


Figure 2-3 Chemical structure of Di-4-ANEPPS and its absorption and emission spectra.

A. *Chemical structure of DI-4-ANNEPS (IUPAC: 4-(2-(6-(Dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl) pyridinium* ***B.*** *Fluorescence absorption and emission spectrum of Di-4-ANEPPS. Overlaid is an example of a shift in spectra with a change in membrane potential (image modified from*(ThermoFisher 2015)*).*

In order to excite the dye, a light source of the required wavelength must be used. Common excitation light sources are tungsten-halogen lamps, mercury arc lamps and argon ion lasers (Arora et al. 2003). Tungsten light sources tend to be the most common, though not the optimal option. They emit a smooth, continuous spectrum with shorter wavelengths than other lights and are cheaper to run. Laser light emits one wavelength and is considered to be the ideal light source however this is a more expensive option with a short lifetime (Attin & Clusin 2009).

Light from the source is filtered through an optical excitation filter to allow only selected wavelengths that excite the dye to be transmitted. An emission filter transmits the emission wavelength while blocking light from at the excitation wavelength (Attin & Clusin 2009). These can be either shortpass, longpass or bandpass filters. Shortpass filters attenuate longer wavelengths and transmit shorter

wavelengths and the reverse occurs with longer wavelengths (Attin & Clusin 2009). Bandpass filters allow wavelengths, determined by the centre wavelength and bandwidth, of a particular range to pass through.

A photodetector is placed behind the emission filter to capture light from the specimen. There are of two types: photodiode arrays and charged coupled device (CCD) cameras (Attin & Clusin 2009). A photodiode is a semiconductor junction that converts light into electrical current. They are best suited to moderate or high intensity light. The CCD is a semiconductor device segmented into an array of light-sensitive recording pixels. These are activated by a photon hitting a pixel and increasing the charge on that pixel. They generally have higher spatial resolutions however this is impacted when the speed of recording is increased as it requires a reduction in the number of pixels (Attin & Clusin 2009). They are thus better suited for mapping larger preparations at low magnification (Arora et al. 2003).

A common problem with moving preparations such as the heart is the issue of motion artefacts. With each contraction the relative position of the preparation can shift from its original position below the photodetector making accurate analysis difficult. This can be overcome with the use of mechanical restraint or inhibiting the process of excitation-contraction with chemical uncouplers such as 2,3-butanedione monoxime (BDM) or cytochalasin D (Cyto-D) (Attin & Clusin 2009).

Analysis of data requires the use of a computing system with relevant software. A number of electrophysiological parameters can be derived using this technique. These include, but are not limited to, the conduction velocities of action potentials between two regions of the heart, the duration at different repolarisation times of the action potential as well as the calcium transient duration and amplitude.

2.4.2 Procedure

Following cannulation, hearts were stained with voltage-sensitive fluorescent dye by coronary perfusion. 5 to 10 uL Di-4-ANEPPS was added to perfusate and by 15 minutes of perfusion, hearts had

achieved a bright enough stain. Specimens were placed flat and pinned to the base of the chamber at the apex with a 0.1 mm insect pin (Austerlitz, Czech) to restrict movement and to allow the area for mapping to be clearly viewed. Following treatment periods, an Optosource mercury/xenon mixed gas arc lightsource (Cairn, UK) with a green excitation filter was used to illuminate the specimen. Changes in conduction velocity (CV) and action potential duration at 50% of repolarization (APD_{50}) were analysed using BV analyze software v11.08 (Brainvision Inc, Japan) and Microsoft Excel. Coronary perfused hearts were stained by perfusion with 2 mL of perfusion solution containing 20 µM Di-4-ANEPPS in Tyrode solution for 5–7 min. We observed that although this concentration was much higher than is required for staining the MH model, the optical action potentials obtained were not as sharp.

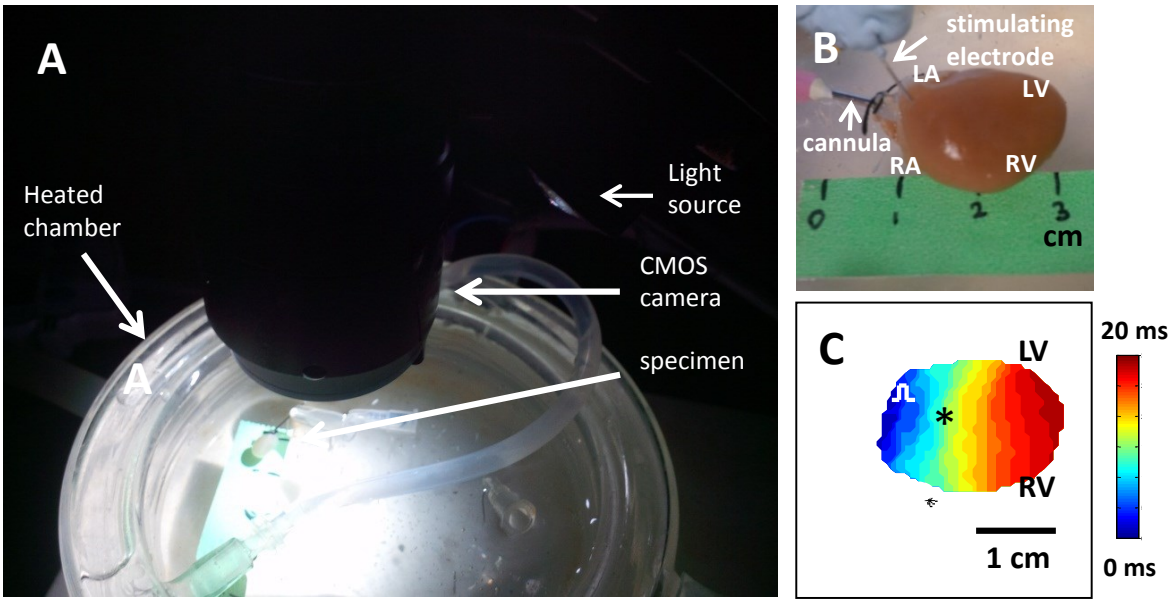


Figure 2-4 Optical mapping of isolated Langendorff-perfused heart.

A. *Set-up showing heart in heated perfusion chamber.* ***B.*** *Enlarged image showing placement of stimulation electrode and anatomical location. A scale in centimetres is shown below.* ***C.*** *Example of a reconstructed activation map with a timescale.LA – left atrium; RA – Right atrium; LV – left ventricle; RV – Right ventricle; ms - milliseconds.*

Electrical stimulation leads were placed at the base of the heart and hearts were paced with a 6 volt current at a fixed rate of 5 Hz. Simultaneously, 4 second recordings were performed with a high-speed

Complementary Metal–Oxide–Semiconductor(CMOS) camera (a variant of the CCD photodetector) using BV analyse software (BrainVision, Germany). Sampling rate was set to 1 kHz giving a temporal resolution of 1 millisecond. Using lenses of different magnifications the field-of-view (FOV) varied between 3 and 5 centimetres depending on the size of the preparation. A 600 nm emission filter (Thorlabs, Ely, UK) was placed between the lens and camera to filter light of the required wavelength. Between treatments the light source was switched off and recordings were performed in a dark room to reduce photobleaching.

Activation patterns were reconstructed using customised MATLAB (Glukhov et al. 2010) (MathWorks, Cambridge, UK), to observe changes in conduction velocity (CV) and action potential duration (APD). BV analyse and Microsoft Excel software were used to determine CV values. An example of an activation map with a timescale is shown in Figure 2-5 above. This shows the propagation of the action potential from the point of stimulation across the heart. Blue regions indicate shorter activation periods while red regions indicate longer activation periods. Conduction velocities are obtained by dividing the distance between 2 action potentials at points of interest (i.e. 1 and 2 or 2 and 3 in the figure) over the time between them.

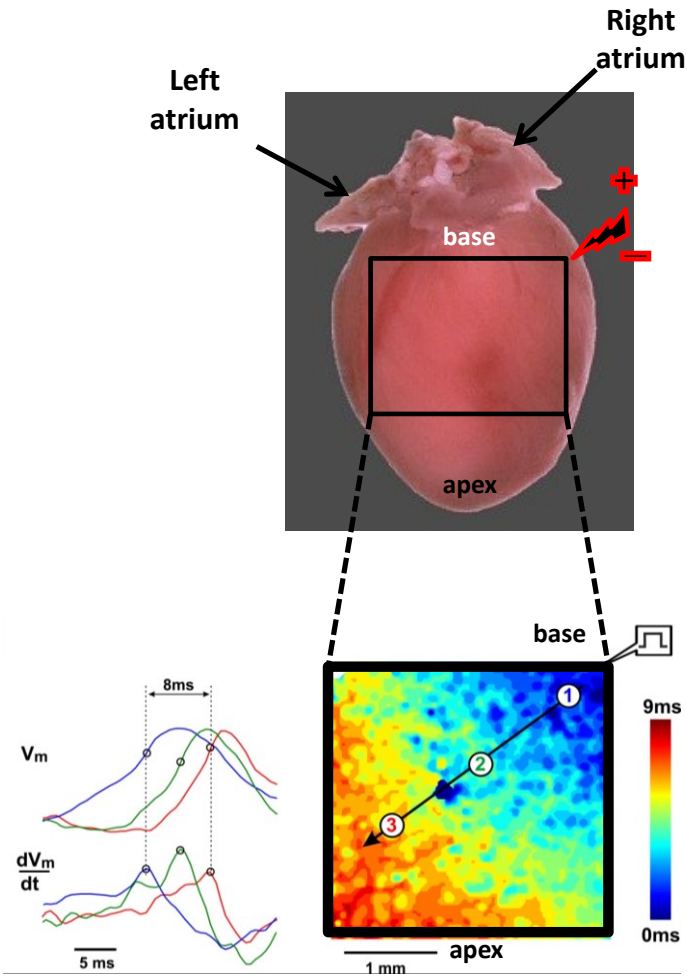


Figure 2-5 Calculation of conduction velocity and action potential duration in optical mapping.

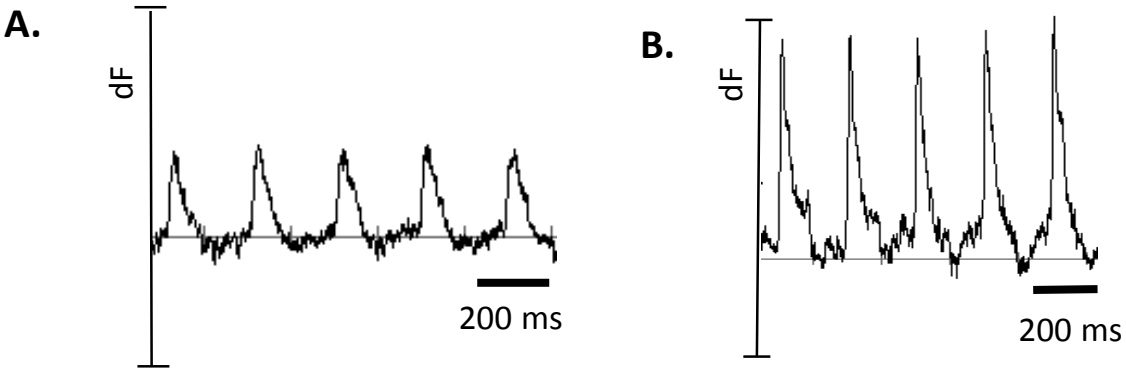


Figure 2-6 Representative optical action potentials (OAP).

A. *The fetal model and* ***B.*** *The maternal model.*

2.5 Recording of electrocardiograms (ECG)

2.5.1 Background

The ECG is a representation of the heart's electrical activity measured via electrodes placed on the body surface. It is useful clinically for diagnosing rhythm disturbances, changes in electrical conduction and myocardial ischemia or infarction (Klabunde 2004). Unlike the action potential which is derived from single cells, the ECG is a summation of electrical currents generated by all cardiomyocytes as they depolarize and repolarize.

The ECG is not a measure of absolute voltages, but voltage changes from an isoelectric baseline voltage (Klabunde 2004). It is typically divided into defined waves, intervals and segments as shown which represent periods of depolarization and repolarization in different regions of the heart (see Figure 2-7). Typically changes in ECG components can be indicative of arrhythmias and other cardiac disorders. However, due to inter-individual variability, the duration of each component is not absolute and so experience is required to accurately identify abnormalities in cardiac rhythm.

Table 2.1 ECG intervals and components

ECG COMPONENT	REPRESENTS
P wave	Atrial depolarization
QRS complex	Ventricular depolarization
T wave	Ventricular repolarization
PR interval	Atrial depolarization plus AV nodal delay
ST segment	Isoelectric period of depolarized ventricles
QT interval	Duration of ventricular depolarization and repolarization
RR interval	Duration of ventricular cardiac cycle (inverse of heart rate)

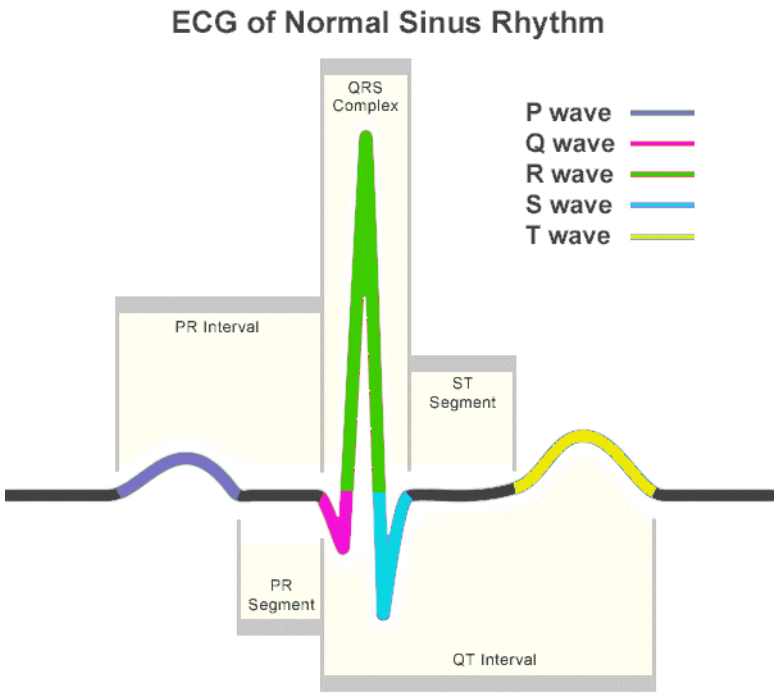


Figure 2-7 ECG of normal sinus rhythm highlighting the waves, intervals and segments.

(image from (Cardiology 2015)*)*

For example prolongation of the ECG PR interval can be indicative of AV nodal tissue damage. In certain instances this can lead to a partial or complete block in atrio-ventricular conduction, resulting in different atrial and ventricular rates of contraction and even death (Klabunde 2004). Pharmacological agents which affect key cardiac ion channels can affect the rate of depolarization or repolarization leading to ECG interval prolongation. A common example of these are drugs that inhibit the hERG potassium ion channel resulting in QT interval prolongation that can lead to the fatal arrhythmia Torsades de pointes. In incidents of hypoxia, the ST segment tends to be elevated and is a useful marker for diagnosing ischemia and infarction. In non-clinical studies where direct measurements of cardiac function cannot be obtained, components of the ECG can be used in conjugation with other cardiovascular parameters to assess cardiac function. For example the QA interval, described as the time interval from the Q wave on the ECG to point A on the aortic pressure wave, has been shown to inversely correlate with cardiac contractility (Adeyemi et al. 2009).

2.5.2 Procedure

In the present study, I obtained electrocardiogram recordings from isolated Langendorff-perfused hearts. Following the cannulation procedure described above, a unipolar biopotential lead was placed into the superfusion solution close to the heart. A ground electrode was connected to the metal cannula to reduce electrical interference. The leads are connected to a PowerLab data acquisition system and Animal Bio amplifier (AD Instruments, Oxford, UK). Sampling rate was set to 1kHz. QT interval was calculated from the onset of the Q wave deflection to the end of the T wave while PR interval was measured from the peak of the P wave to the peak of the R wave from 10 waves at the end of each baseline and drug treatment period. Recordings were performed throughout treatment periods with the final data was averaged into 30 second intervals. Analysis was performed using Microsoft excel and LabChart 7 pro software (AD Instruments, Oxford, UK).

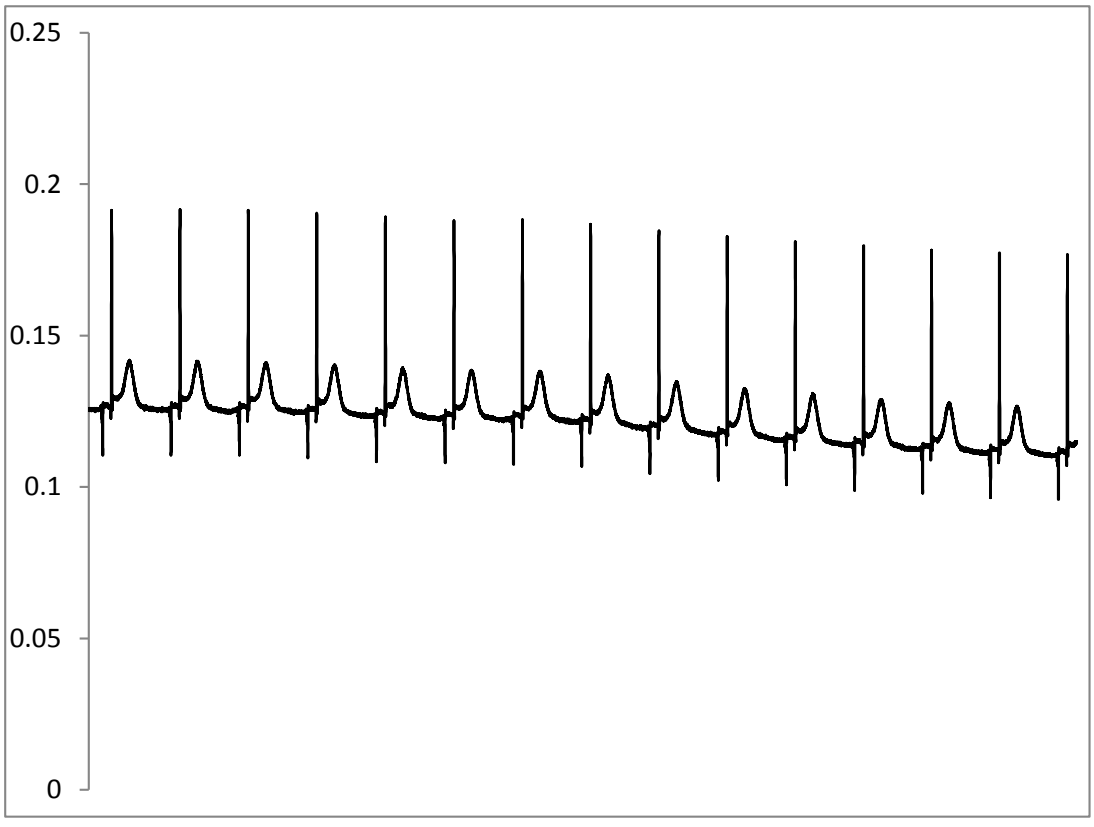


Figure 2-8 Representative ECG traces from the adult heart

2.6 Measurement of ion channel currents using Whole-cell Patch-clamp technique

2.6.1 Background

The patch-clamp technique was developed to measure ionic currents from individual ion channels in a small patch of biological membrane. It was first used by Neher and Sakmann to record currents through single acetylcholine-activated channels in cell-attached patches from membranes of denervated frog muscle fibres (Neher & Sakmann 1976) (Neher and Sakmann, 1976).Two electrodes are used in this set-up, the first is within a glass micropipette for recording and another used for reference. Both are connected to a high gain amplifier which serves to convert current-to-voltage and allows the current in the recording pipette to be fixed or altered in a step-wise fashion (Ogden & Stanfield 1981).

The principle of this technique is to isolate a patch of membrane electrically from the external solution and to record current flowing into the patch (Ogden & Stanfield 1981). This is achieved by gently pressing a glass microelectrodes filled with an electrolyte solution against the membrane and applying a light suction to get a high-resistance seal (exceeding 10 Giga-Ohms, GΩ) with less than 1 nm between the glass pipette and cell membrane (Ogden & Stanfield 1981) To achieve such a high-resistance it is necessary that the membrane area and pipette tip are clean and free of extracellular matrix, the solution free of fine particulars and debris, By having a high-resistance seal it gives a more complete electrical isolation of the membrane patch and reduces the noise of current recording (Ogden & Stanfield 1981).

Since its original application, refinements have been made to give different configurations for the placement of the pipette and cell including: cell-attached, inside-out, outside-out, perforated-patch and whole-cell. The whole-cell mode is the most often used mode of the patch clamp technique. It is achieved by rupturing the patch of membrane isolated by the patch pipette, bringing the cell interior

into contact with the pipette interior (Ogden & Stanfield 1981). Recording using this technique can either be in current or voltage-clamp modes allowing spontaneous action potentials from active or inactive cells to be recorded (Bebarova 2012). Through the use of specific ion channel inhibitors and voltage pulses of different durations and amplitude, particular ion channel currents can be derived.

The patch-clamp technique has also been adapted in other ways for studying cardiac cellular electrophysiology and structure including: AP clamp, Dynamic clamp, Cell-type transforming clamp, High-resolution scanning patch clamp and Automated patch clamp (Bebarova 2012).

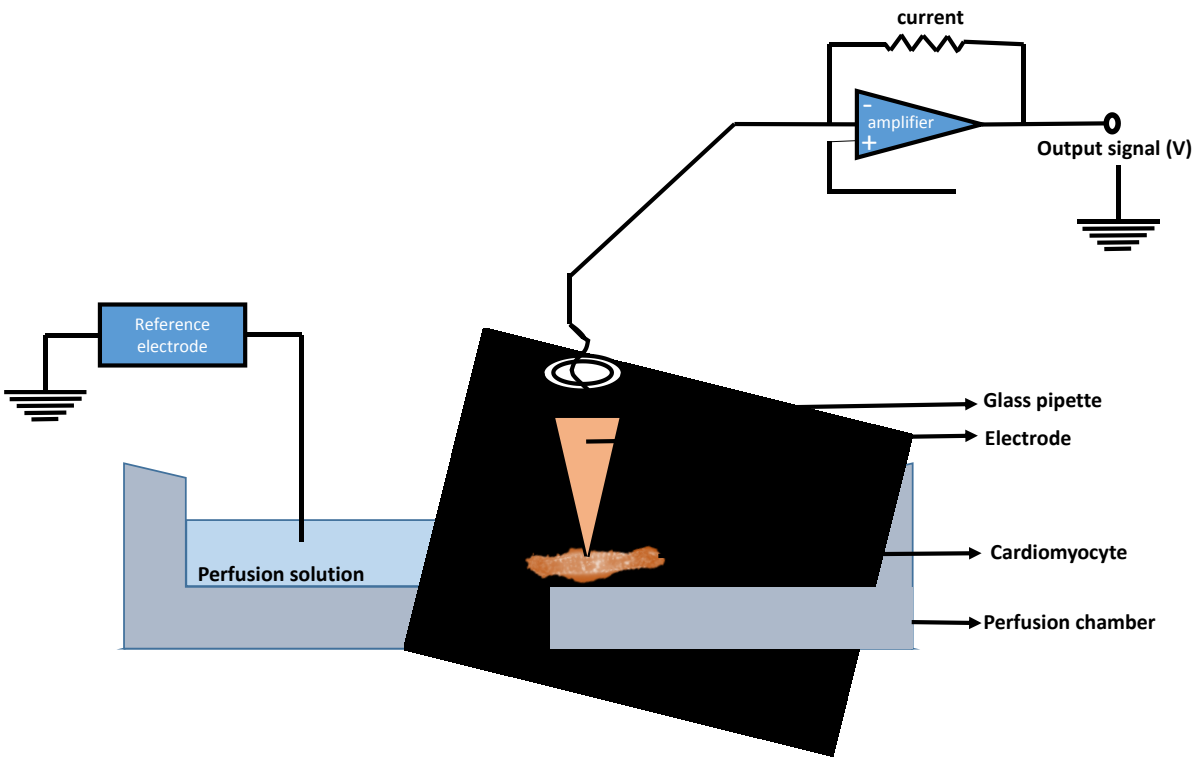


Figure 2-9 Schematic of patch-clamp set-up

2.6.2 Whole-cell calcium current protocol

This work was carried out in collaboration with Dr Anita Alvarez Laviada. Borosilicate glass micro-pipettes were pulled to give mean resistance of 3-5 MOhms. Pipettes were filled with an intracellular

solution of the following composition (**in mmol/L**): **100** Cs-methane-sulfonate, **40** CsCl, **10** HEPES, **5** EGTA, **2** MgCl2, **5** Mg-ATP, **0.75** MgCl2, pH 7.2 with CsOH.

Cardiomyocytes were perfused with the external solution composed of (**in mmol/L**): **120** Tetra-ethylammonium-chloride, **10** CsCl, **10** Glucose, **10** HEPES, **1** MgCl2, **2** CaCl2, pH 7.4 with CsOH.

L-type calcium channel blocker nifedipine (100 nM) was included in the external perfusion solution in order to selectively isolate T-type calcium current. A T-type calcium channel blocker mibefradil (1 μM) was used for isolating L-type current.

Cardiomyocytes were isolated from one day old neonatal rat hearts using enzymatic digestion. Cells were incubated in M199 medium supplemented with 5% fetal bovine serum for two days before studies. The effect of vehicle, TC (100μM) or TC (100μM) plus UDCA (100nM) on calcium channel amplitude were studied using the whole-cell patch-clamp technique. Currents were recorded using an Axopatch-1D amplifier connected to a Digidata1322A acquisition system (Axon Instruments, Foster City, USA). The bath was connected to the ground via an Ag–AgCl pellet. Data were low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier and sampled at 10 kHz.

All recordings were performed at room temperature (22-24°C). T-type calcium channel current ($I_{Ca,T}$) was recorded during 200 ms from a holding potential of -90 mV to test potentials ranging from -80 to +40 mV, with pulses applied every 2 s in 5 mV increments. Peak current amplitude was taken from in response to -40 mV test potentials. L-type currents were recorded over 200 ms from a holding potential of -40 mV to a test potential of -45 to +60 mV in 5 mV increments. Current amplitude at 0 mV was taken as peak current. Results were analysed offline using Origin and Clampfit. Peak current amplitude for each cell was divided by cell capacitance and this value was termed calcium current density. Mean value was plotted as a current-voltage (I-V) relationship.

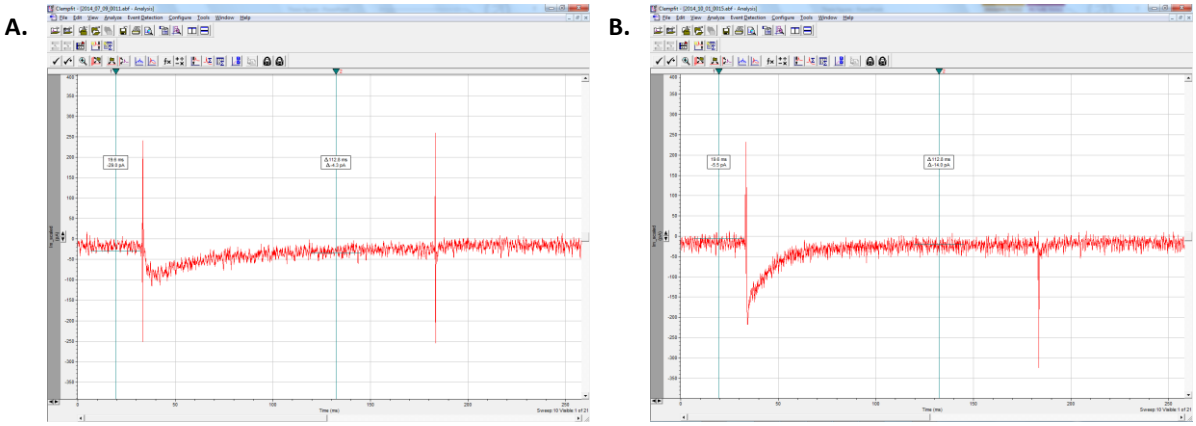


Figure 2-10 Representative calcium current traces from cardiomyocytes.

Single current traces of a ***A.****T-type current and an B****.*** *L-type current.*

2.7 Determination of protein levels by Western blotting

The Western blot technique is used in research to separate and identify proteins based on their molecular weights (Mahmood & Yang 2012). First described by Towbin and colleagues (Towbin et al. 1979), it evolved from Southern blotting technique used to detect DNA fragments and northern blotting used to detect and quantify RNA fragments. Since then modifications have been made to the technique which have served to improve the technique. The procedure used in this project can be divided into 3 steps: sample preparation, electrophoresis and protein transfer and staining.

2.7.1 Sample preparation

Liquid was removed from cells by centrifuging the sample at 2500 rpm for 5 minutes and the supernatant discarded. To wash cells of serum, cold PBS was added and using a large 5 mL pipette the mixture was pipetted up and down repeatedly and then spun at 2500 rpm for 5 minutes. The procedure was repeated twice to completely wash off medium with the supernatant discarded each time. Following this cell lysis buffer containing 1 mL of RIPA buffer, 10 µL of protease inhibitor and 1.6 µL of 1 M DTT was prepared. Between 200 and 500 µL of this mixture was added to the cells and then using a narrow pipette tip, the mixture was pipetted repeatedly in order to lyse the cells. To increase yield, the mixture was sonicated for 30 seconds at 50% pulse. The preparation was mixed gently for

15 minutes on ice and then centrifuged at 14000 rpm for a further 15 minutes to pellet cell debris. Supernatant was then aliquoted in 100 µL vials and stored at -80°C until required.

Protein content was measured using a commercial Pierce^{TM} BCA protein assay kit (Thermo Fisher, UK). In brief, the principle of the assay is in two steps based on the biuret reaction. The first involves protein reduction of Cu^{2+} to Cu^{1+}in an alkaline medium containing sodium potassium tartrate forming a light blue complex. In the second colour development step, bicinchoninic acid (BCA) reacts with the reduced copper that was formed in step one resulting in an intense purple colouration. Use of this reagent gives a more sensitive assay than the pale blue colour obtained in the first step. This BCA/copper complex has a strong linear absorbance at 562 nm with increasing protein concentrations. Absorbance of samples and protein standards of known concentrations was measured using an MRX microplate spectrophotometer (Dynex, UK) and sample protein concentration determined from a standard curve.

In order for the antibodies to bind to the particular region of the protein they target, the protein has to undergo denaturation. The required amount of protein was mixed with loading buffer containing Sodium Dodecyl Sulfate (SDS), bromophenol blue dye and vortexed. It was then heated at 95°C for 5 minutes. This serves to destroy the secondary structure of the protein.

2.7.2 Electrophoresis

A Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) gel was used as a stacking gel (pH 6.8) to separate proteins by molecular weight. This is formed from the polymerization of acrylamide and N, Nmethylenebisacrylamide which serves as a cross-linking agent (Abcam 2015). Polymerization was initiated by the addition of ammonium persulfate and TEMED. The total amount of acrylamide and crosslinker (w/v), given as %T and %C respectively, determine the size of pores and separation of molecules. Therefore, for smaller sized proteins a higher percentage of both agents is used and vice versa for larger proteins (Abcam 2015). In this study I used 10% acrylamide gels. Following mixture, the components are left for about an hour to harden into a gel. A separating gel

(pH 8.8) is added to the top of the stacking gel and individual lanes are created by inserting a lane comb.

At the end of this period, running buffer containing 10% trisglycine in water is poured into the electrophorator. The prepared gels are then placed in the electrophorator and protein samples, positive controls and molecular weight markers are loaded into individual lanes. The power supply was switched on with the voltage set to 120 volts for a minimum period of 1 hour.

2.7.3 Protein transfer and imaging

At the end of this period proteins were transferred to a polyvinylidene fluoride (PDVF) membrane moistened in ethanol. To do this the gel was carefully retrieved from glass casing in the electrophorator and sandwiched between the PDVF membrane and filter papers, while avoiding air bubbles. This was then returned to the electrophorator set to 30V and the transfer process allowed to occur for a minimum of 90 minutes or overnight. 5% non-fat milk was used as a blocking agent to prevent non-specific background binding of primary and secondary antibodies. It was prepared by mixing 5 grams with 100 mL of Tris Buffer Saline Tween20 (TBST) buffer. The PVDF membrane was placed in the milk solution and incubated for 1 hour at 4°C under constant agitation. At the end of this the membrane was rinsed twice in TBST solution.

The membrane was then placed in a 50 mL Falcon tube with the primary antibody diluted in TBST (1:1000). To enable adequate homogenous covering of the membrane and to avoid uneven binding the tube was placed on a continuous stirring roller in a cold room at 4°C overnight. At the end of this the membrane was washed thrice in TBST for 15 minute periods to remove excess antibody. The HRP-conjugated secondary antibody was then added at a dilution ratio of 1:3000 to the membrane and left to incubate under similar agitation at room temperature for 1 hour. It was then washed again three times in TBST. The membrane was dipped in enhanced chemiluminescence (ECL) mix and then placed flat in the dark chamber of an Imagequant detection unit (GE Healthcare, UK) for imaging.

2.8 Statistics

Statistical analysis was performed using GraphPad Prism 5.0 software. Results are presented as mean or percentage change from baseline values ± standard error of the mean (mean±SEM). Statistical analysis compared the mean of two treatments, indicated by a bar between treatment columns. An asterisk (*) was used to indicate statistically significant values. A p-value less than 0.05 ($p<0.05$) was considered statistically significant. 1-way ANOVA and an appropriate post-hoc test (indicated in figure legends) was applied for each data set. Bonferroni's post hoc test compares all possible pairs of means while the Dunnett's post hoc only compares drug treatment means against the control group mean.

3 Development and Characterisation of a Fetal Whole-Heart Model of ICP

3.1 Introduction

Intrahepatic Cholestasis of Pregnancy (ICP) is characterised by an elevation in maternal bile acid levels which can lead to increased pregnancy complications including fetal cardiac arrhythmias and even fetal death (Williamson & Geenes 2014). Despite the potential risks to the fetus, the observed symptoms in the mother tend to be milder with the major symptom being itching (pruritus). As such it is evident that there are significant differences in the response of the fetal heart to bile acids as compared to the maternal heart and these effects need to be investigated separately. In the treatment of ICP, the bile acid ursodeoxycholic acid (UDCA) serves as frontline treatment although its mechanism of action remains unclear (Williamson & Geenes 2014). An understanding of why fetal hearts appear more sensitive to an elevation in bile acid levels is of major importance to gain a grasp of the role of UDCA in ICP.

In order to achieve this, relevant non-human models of both the fetal and maternal heart have to be developed to more accurately define the effect of bile acids in human patients. However, to date the majority of studies on the cardiac effects of bile-acids in foetuses have been performed using cellular models. This has mainly consisted of cultured isolated ventricular rat cardiomyocytes either alone or in combination with fibroblasts (Williamson et al. 2001; Gorelik et al. 2002; Miragoli et al. 2011). Other studies on the effects of bile acids in adult cells have also made use of isolated cells from adult mice and humans, such as in the study by Rainer and colleagues (Rainer et al. 2013). There is very little information on studies performed beyond rodent cellular models, in whole-heart or *in vivo* animal models.

Although results obtained in cellular models have provided some insight into the effects of bile acids, the model is limited in a number of ways. For example, the effect of the sino-atrial and atrio-ventricular nodes in regulating intrinsic heart rate cannot be studied. Also even though a number of adverse effects can be observed in cardiomyocytes, whether or not these effects actually lead to arrhythmias cannot be determined. Furthermore, the effect of mechano-electrical changes such as

are observed in a continuously beating heart might not be observed. Therefore, it has become evident that more integrated models are required in order to determine how other components of the heart might contribute to the observed effects of bile acids in humans.

To this end I aimed to develop and characterise a whole-heart model of the fetal heart in ICP, in order to assess the effects of bile acids on impulse propagation in the heart. Two whole-heart models commonly used in cardiovascular research include the Langendorff-perfused heart and the working-heart models. The Langendorff-perfused heart model, involving retrograde perfusion via the aorta, is a well-established model that has long been used to study cardiac function. It has been applied using a number of adult animal and human models (Stewart 1909; Kobayashi et al. 1983; Schuster et al. 2010), but only a few studies were found reported in new born hearts and none in fetal hearts. The model benefits from being simple to set up and inexpensive to perform (Skrzypiec-Spring et al. 2007). It produces highly reproducible results, and experiments are not time-consuming or technically demanding. A number of physiological and pharmacological parameters can be measured including heart rate, ECG and contractile function (Skrzypiec-Spring et al. 2007).

Use of this model will allow better translation of the effects observed *in vitro* to those observed in future using *in vivo* animal models and in human patients clinically. Starting with fetal mouse and newborn rat heart models, preliminary studies were performed to assess the feasibility and reproducibility of drug effects in developing rodent heart models. With expected difficulties in cannulating such small hearts, the benefit of cannulation and perfusion in these models was determined. The response of new born animals of different ages was also compared to determine the stability of the model and how effects might alter with development. Studies were performed using optical mapping and ECG to characterise the effects of the bile acid taurocholic acid (TC) and standard agents on the model.

3.2 Materials and Methods

3.2.1 Animals

All work was carried out according to standards set out in the UK Animals (Scientific Procedures) Act 1986. Day 18 fetal mice (C57 BL/6) were kind gifts from Professor Catherine Williamson's group. Neonatal and adult female rats (Sprague-Dawley) were purchased from either Harlan UK, Charles River UK or were obtained as kind gifts from Pete Rawson of the Central Biomedical Services, Imperial College, London.

3.2.2 Materials

Di-4 ANEPPS and 4-aminopyridine were purchased from Sigma-Aldrich, UK. These were dissolved in 100% DMSO and serially diluted to ensure study concentrations contained no more than 0.01% DMSO. Di-4-ANEPPS was used at final concentrations of ~0.04 mM. Taurocholic acid was dissolved directly in the same solution used for control treatment on study days at required concentrations. Perfusion (Tyrode's) and cardioplegic solutions were prepared as described in Section 2.3.

3.2.3 Evaluation of fetal mouse heart preparation

With the help of my supervisor, Dr Alexey Glukhov, I worked to develop an *ex vivo* model of the fetal heart (fetal heart model, FH) in ICP for this project. Initially hearts from Day 18 fetal mice were studied. To obtain hearts, pregnant female rats were sedated in an anaesthetic chamber with isoflurane. Following cervical dislocation, the whole of the uterus was excised and placed in Tyrode's solution. Hearts from individual pups were then surgically dissected using Dumont #5/45 forceps (WPI, UK) under a microscope and placed in Tyrode's solution containing voltage sensitive dye, Di-4-ANEPPS, in a heated incubator (37°C) for 30 minutes. Following this they were placed on an observation platform for optical mapping studies.

3.2.4 Development of neonatal rat heart whole heart model

Based on the outcome of the fetal mouse studies, hearts from 0 to 3 day old neonatal rats (Sprague-Dawley; ~7 grams) were also evaluated as a model of the human fetal heart in ICP. Due to surgical difficulties in cannulating the much smaller aorta of the fetal and neonatal hearts (see Figure 3-1), initial studies were performed without aortic perfusion and hearts were only superfused with heated solution. Further effort was applied to develop the surgical techniques for the cannulated FH model. Following the excision process described previously, hearts were placed in cardioplegic solution (4°C) in a petri dish under a microscope. The aortic arch was then cut away just below the first bifurcation, to ensure a significant length of aorta was left. Using Dumont #5/45 forceps (WPI, UK), a 21-gauge cannula was inserted into the aorta and tightly held in place with suture thread. Perfusion solutions were heated (37°C) using an in-line heating block and temperature was measured using a thermocouple placed directly in the solution. Perfusion was performed using a peristaltic pump (Watson-Marlow, Cornwall, UK) at various flow rates to determine an ideal rate for perfusion.

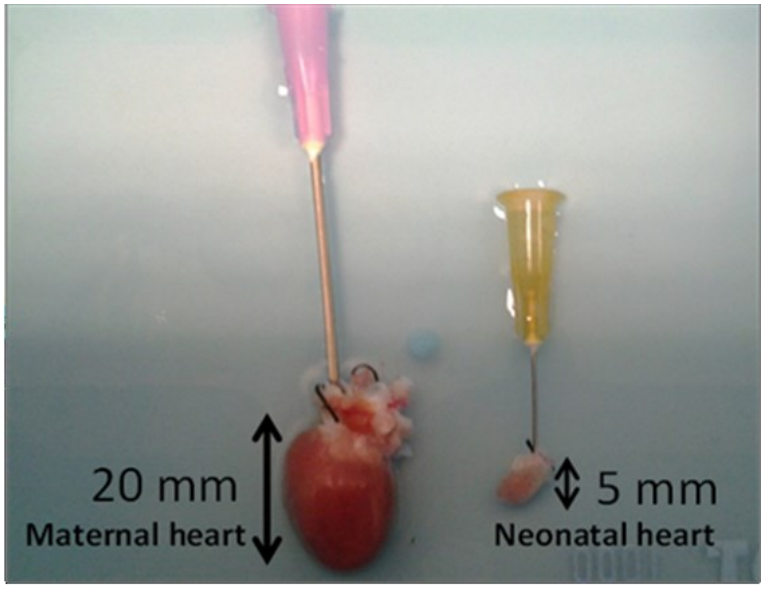


Figure 3-1 Image of cannulated adult female rat heart and neonatal rat heart

Photograph highlights dimensions of maternal heart (MH) and fetal heart (FH) models and sizes of cannulas required.

3.2.5 Experimental protocols

3.2.5.1 Optical mapping procedure

The procedure for optical mapping studies is as described in section 2.4.2.

3.2.5.2 ECG recording procedure

The electrocardiogram was recorded as previously described in section 2.5.2.

3.2.5.3 Drug treatment protocol

Hearts were superfused with either vehicle or taurocholate (400 μM), maintained at approximately 37°C, according to the protocol shown in Figure 3-2 below. The effect of cannulation on conduction velocity using optical mapping, was evaluated in hearts either superfused with solution or superfused and perfused via the aorta with only vehicle treatment. Following vehicle treatment, baseline heart rate, PR interval and conduction velocity values were compared against those from adult hearts. In order to characterise the response of the model to ion channel blockers, we evaluated the effect of a standard agent (4-aminopyridine, 4-AP) that is known to prolong action potential duration (APD). 4-AP is a potassium channel blocker that inhibits the rapidly activating delayed rectifier potassium channel (I_{Kr}) involved in ventricular repolarisation. Effects of 4-AP at concentrations of 0.5 and 5 mM were tested on APD, ECG and heart rate.

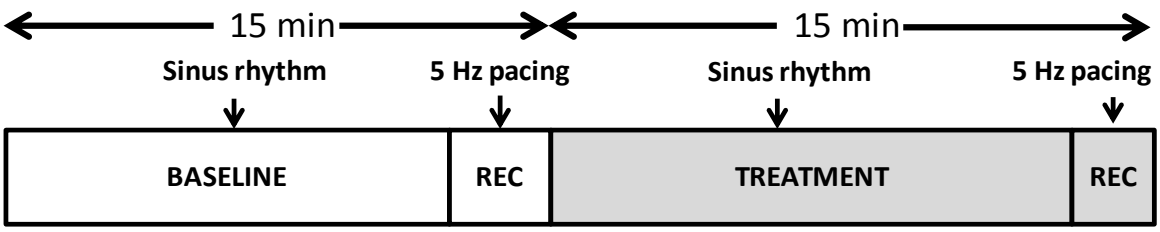


Figure 3-2 Experimental protocol

Hearts are treated with vehicle for a 15 minute baseline period to allow acclimatisation, and then recordings were taken. They were then treated for a further 15 minute period with either vehicle or drug treatment and recordings obtained again. Hearts were paced at 5 Hz during the recording period. REC – recording period.

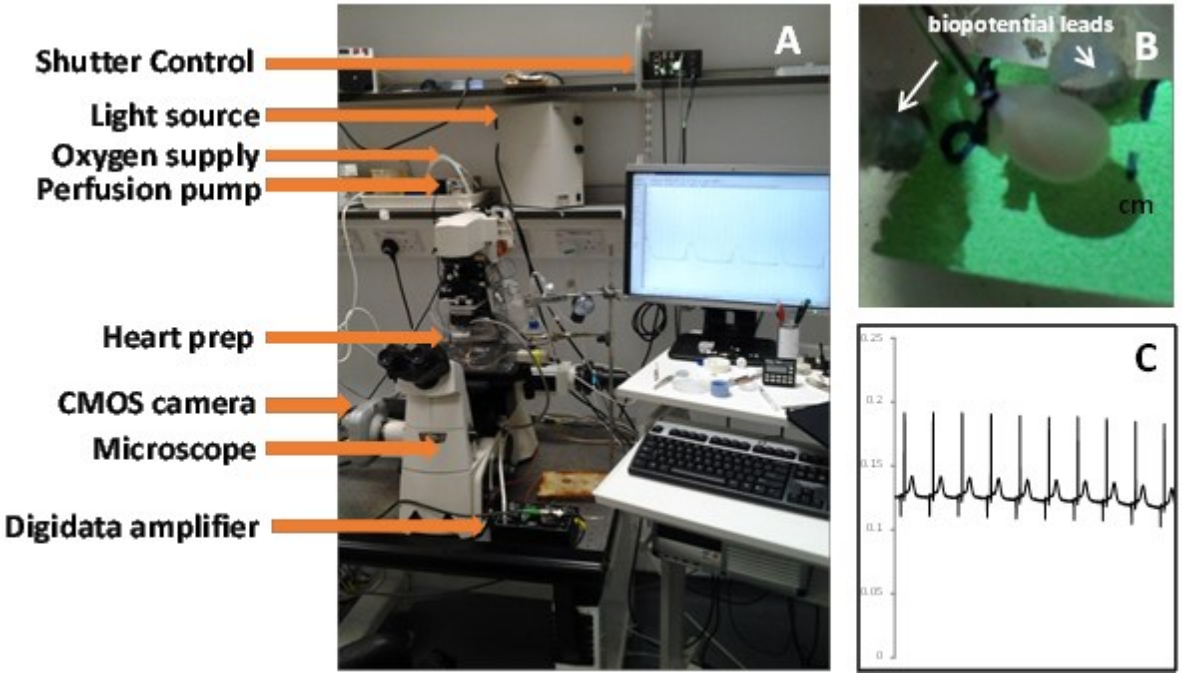


Figure 3-3 Experimental set-up for neonatal heart ECG recording

A. *Photograph of study room showing location of heart preparation, optical mapping CMOS camera and microscope* ***B.*** *placement of biopotential leads and* ***C.*** *Representative traces from day 0 neonatal rat hearts*

3.2.5.4 Comparison of effect of age and duration of perfusion on PR interval and heart rate in developing rat hearts

A comparison was made of the effect of taurocholic acid in neonatal rat hearts of different ages to assess its effects during development and identify an ideal model for studies. The comparison was made on hearts from Day 0, 14 and 21 day old neonatal rats. Hearts were cannulated as described previously and treated according to the protocol shown in Figure 3-2.

3.2.6 Statistical analysis

Conduction velocity, APD_{70} and PR interval recordings were analysed and plotted using GraphPad Prism 5 software. Results are presented as absolute or percentage change values (mean ± SEM). Statistical analysis was carried out comparing treatment to control using 1-way ANOVA and Bonferroni's post hoc test. p values less than 0.05 were considered to be statistically significant.

3.3 Results

3.3.1 Difficulties in preserving day 18 fetal mouse hearts made it a non-viable model

We initially assessed the use of fetal hearts from day 18 mice as a model for our studies. We had difficulty in preserving the hearts in a viable condition. Once the mother was culled, all pups had to be removed from the uterus and hearts excised. Hearts could not be preserved for long enough periods in cardioplegic solution to remain viable in studies. In the few hearts that were used, due to the small size of the hearts a good resolution could not be achieved to allow conduction velocity analysis. Superfusion of neonatal hearts with TC appeared to produce conduction slowing as observed in activation maps (see Figure 3-4A), but it did not alter action potential duration (Figure 3-4B). After culling a number of animals we were only able to obtain more than an N of 2, this was considered wasteful and further studies were discontinued. Data obtained from these 2 hearts is presented below, although this is not a sufficient sample size for accurate analysis.

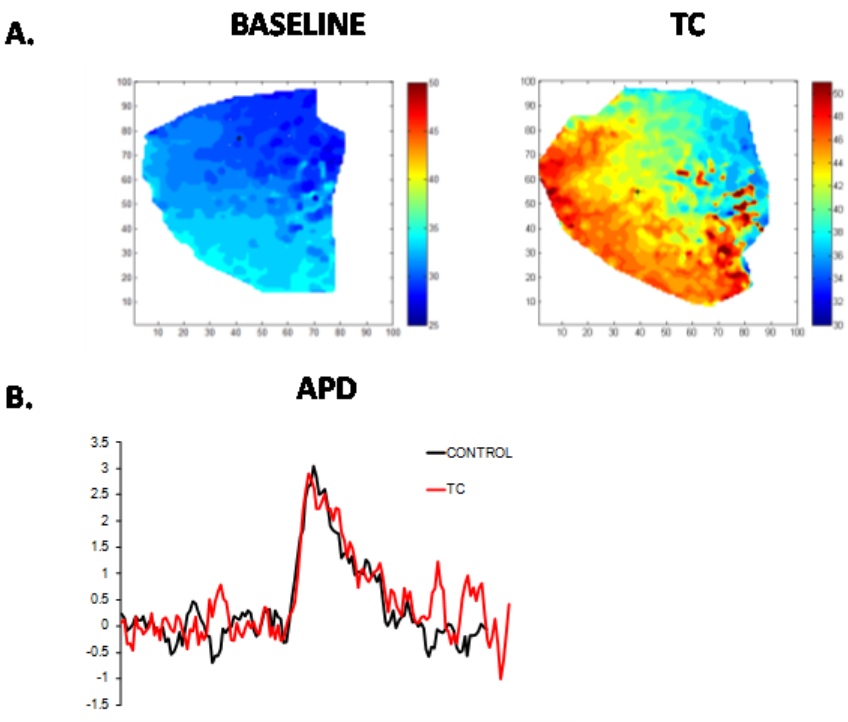


Figure 3-4 Effect of TC on conduction velocity and action potential duration in fetal day 18 mouse hearts

A *Representative activation maps showing a decline in conduction velocity with taurocholate (400 µM) treatment in day 18 mouse hearts (N=2).* ***B.*** *Representative optical action potential traces from baseline and taurocholate treatment showing no significant changes in duration.*

3.3.2 Cannulation prevents ischaemia induced conduction slowing in neonatal rat hearts

Based on the results in fetal hearts, we therefore tried hearts obtained from neonatal rats which are larger. These were initially studied without aortic cannulation but with improvements in technique we were able to cannulate hearts and reduce ischemia caused by lack of oxygenation. Ideal flow rate for aortic perfusion was between 1.5-2 mL/min. Comparison of activation maps from uncannulated and cannulated hearts shows a significant decline in conduction velocity in uncannulated hearts (see Figure 3-5A). This was improved by cannulating the hearts, as shown in Figure 3-5B, plots of mean % change from baseline show a significant increase in conduction velocity with cannulation. Mean % change from baseline values were -15±8.0% and 11±2.0% in uncannulated and cannulated rat hearts respectively. Therefore, further studies were performed with cannulation.

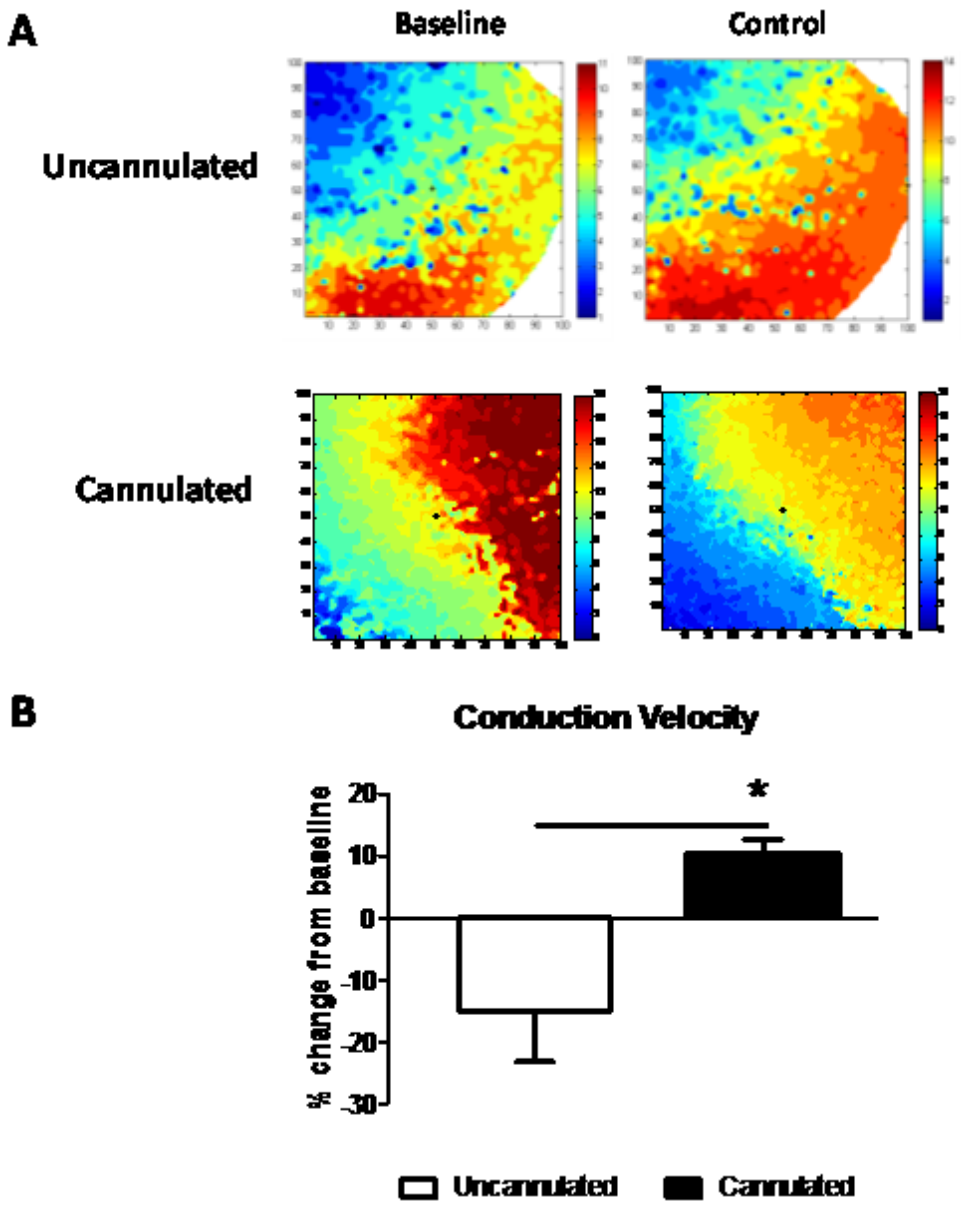


Figure 3-5 Improvement in conduction velocity with cannulation

A *Representative activation maps from uncannulated (top panel) and cannulated (lower panel) hearts.* ***B.*** *Plot of mean % change from baseline values from uncannulated (N=5) and cannulated hearts (N=5). Showing a significant increase in conduction velocity ($p<0.05$).*

3.3.3 4-aminopyridine prolongs APD_{70} in neonatal rat hearts

To our knowledge this is the first time that neonatal rat hearts have been used for electrophysiology studies. Therefore, it was important to characterise the model by assessing the effect of standard agents on cardiovascular parameters. 4-AP, a potassium channel blocker, known to prolong APD was evaluated. Treatment of neonatal day 0 hearts with 5 mM 4-AP resulted in prolongation of APD at 70% (APD_{70}) repolarisation with increasing duration of exposure (see overlay traces in Figure 3-6A). Mean % change from baseline values were -9.4±9.2% and 217.7±144.9% in the vehicle and 4-AP treated groups respectively.

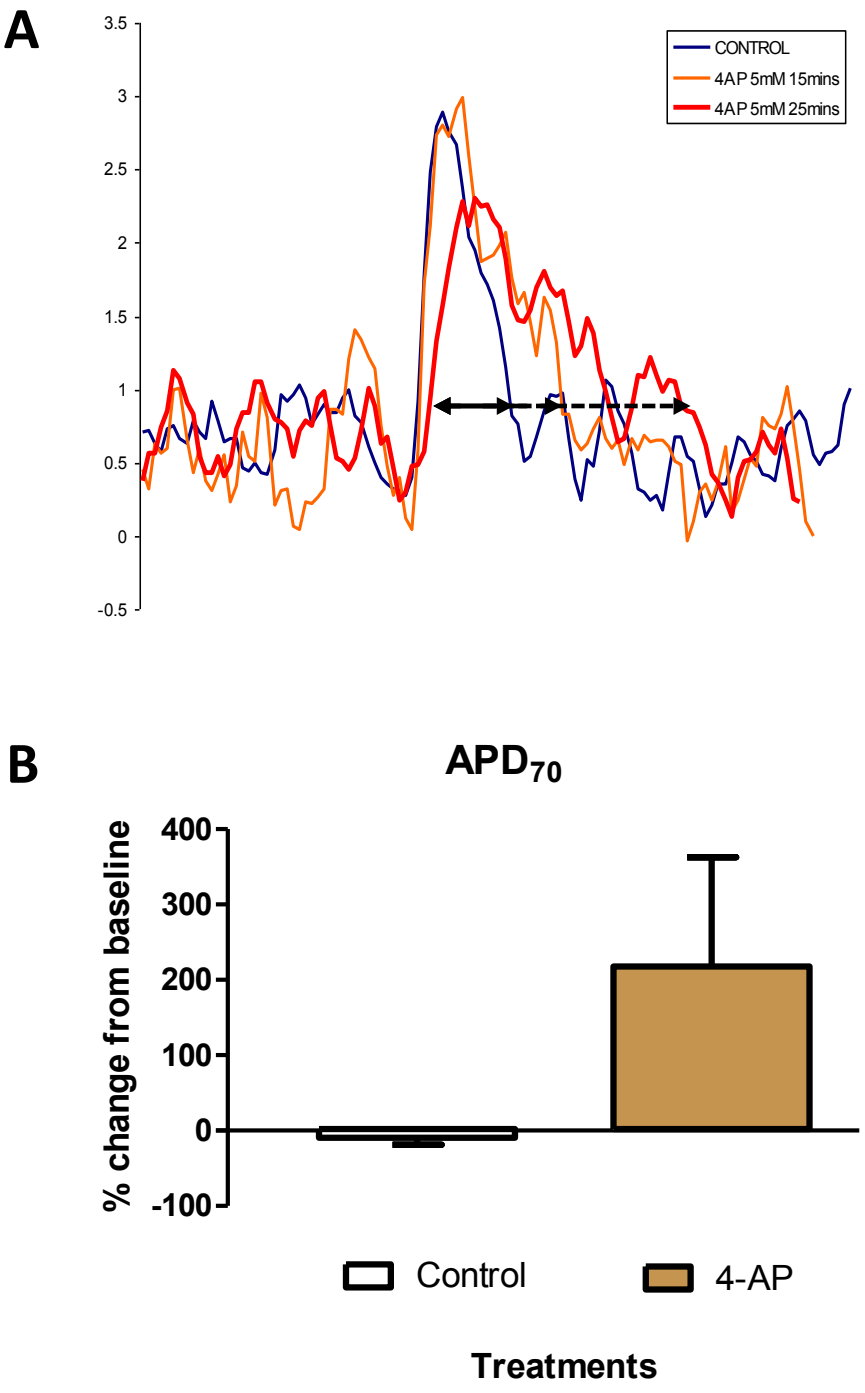


Figure 3-6 Effect of 4-aminopyridine (4-AP) on action potential duration at 70% repolarisation (APD_{70})

***A**. Representative optical action potential traces at baseline, 15 and 25 minutes from the start of perfusion of 5 mM solution showing increase in duration. **B.** Plot of mean % change from baseline in control (N=4) and 4-AP treated neonatal rat hearts (N=2).*

3.3.4 4-AP does not prolong ECG intervals or heart rate in neonatal rat hearts

The effect of 4-AP on ECG and heart rate were also evaluated. As shown in Figure 3-7A, overlay and analysis of ECG traces following 4-aminopyridine (0.5 mM) treatment revealed no changes in ECG PR and QT intervals compared to baseline. Similarly no changes were observed in heart rate compared to hearts in the control and 4-AP groups. Mean % change from baseline values were 8.5±7.3% and -5.1± 4.1% in control and 4-AP treated groups respectively.

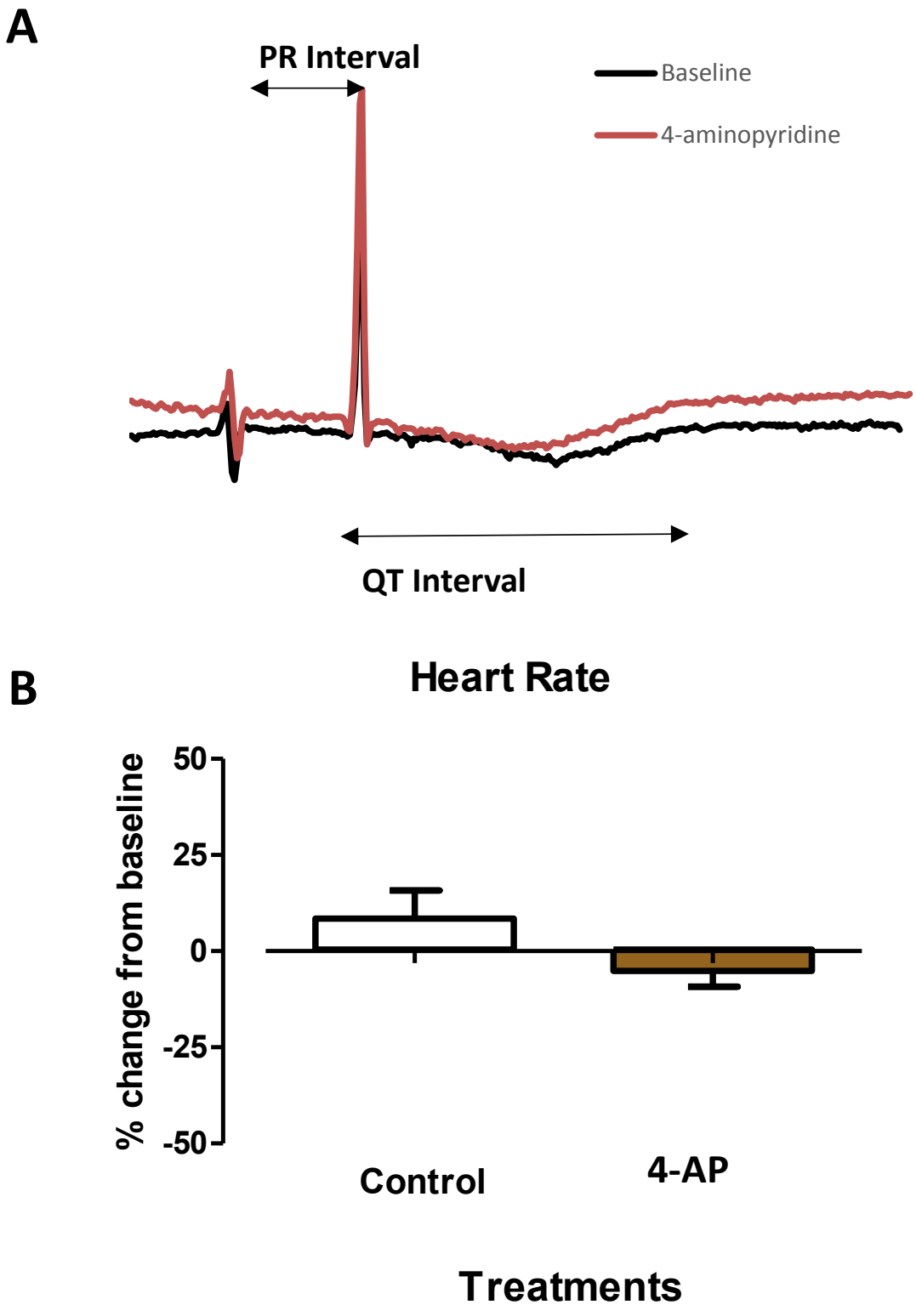


Figure 3-7 Effect of 4-AP on electrocardiogram (ECG) and heart rate

A. Representative ECG trace overlay following baseline and 4-AP (0.5 mM) treatment **B.** Plot of mean % change from baseline in heart rate from control (N=5) and 4-AP treated neonatal rat hearts (N=3).

3.3.5 Evaluation of age and duration of perfusion on PR interval and heart rate in neonatal rat hearts

Based on observations that in ICP PR interval is prolonged in human fetal hearts but not the maternal heart (Strehlow et al. 2010) we performed a pilot study to identify an ideal age for the fetal model and observe how the effects of taurocholic acid (TC; 400 µM) alter with age. TC significantly increased PR interval in Day 0 neonatal rat hearts compared to those in the control group. Values were 11±3.5% (TC) and -1.4± 1.8% (Control) (see Figure 3-8A). Application of a similar concentration to 14 and 21 day old hearts did not produce a significant change in PR interval. Values were 18±7.5% (TC) vs 12±5.4% (Control) in the 14 day old rat hearts and -0.70±7.9% (TC) vs 8.6±8.9% (Control) in the 21 day old rat hearts.

To ensure that hearts would be stable over the study duration, we assessed the effect of drug perfusion for 15 and 30 minutes on heart rate. Mean % change from baseline values were -2.7±2.4% with control (N=6), -1.2±2.2% with TC (N=6) treatments after 15 minutes of perfusion and –3.8±4.7% with control and -7.2±2.4% with TC after 30 minutes of perfusion (see Figure 3-8B).

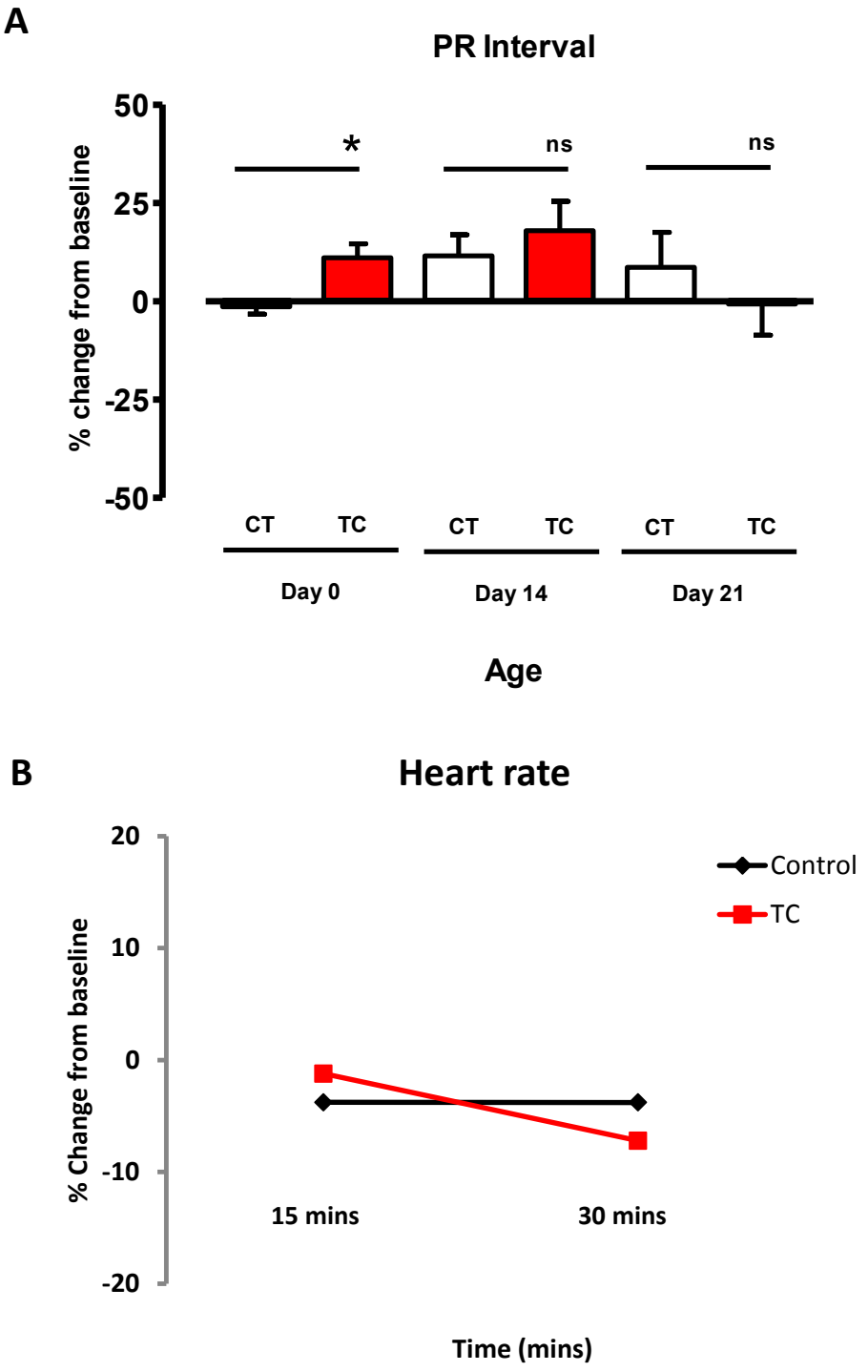


Figure 3-8 Comparison of the effect of age and duration of perfusion with TC on PR interval in developing rat hearts

A*. Plot of mean % change from baseline values in PR interval following 15 minutes perfusion with vehicle and TC from Day 0, Day 14 and Day 21 neonatal rat hearts (N=3-6 per group).* ***B****. Plot of mean % change from baseline values in heart rate following 15 and 30 minutes perfusion with control and TC treatment in Day 0 neonatal rat hearts (N=4 per group). * indicates significant value, P>0.05. ns, not significant.*

3.3.6 Comparison of baseline heart rates, PR interval and conduction velocity in neonatal and adult rat hearts

To further characterise the isolated perfused FH, baseline cardiovascular parameters were measured and compared to those of the well characterised adult heart, used for the MH (see Figure 3-9). Baseline heart rate values were 189±12 and 234±10 b.p.m in the FH and MH models respectively. PR interval values were 46±0.6 ms and 47±0.8 ms in the FH and MH models respectively. Conduction velocity values were 0.5±0.1 m/s and 0.7±0.1 m/s in the FH and MH models respectively. None of the differences were statistically significant ($P>0.05$).

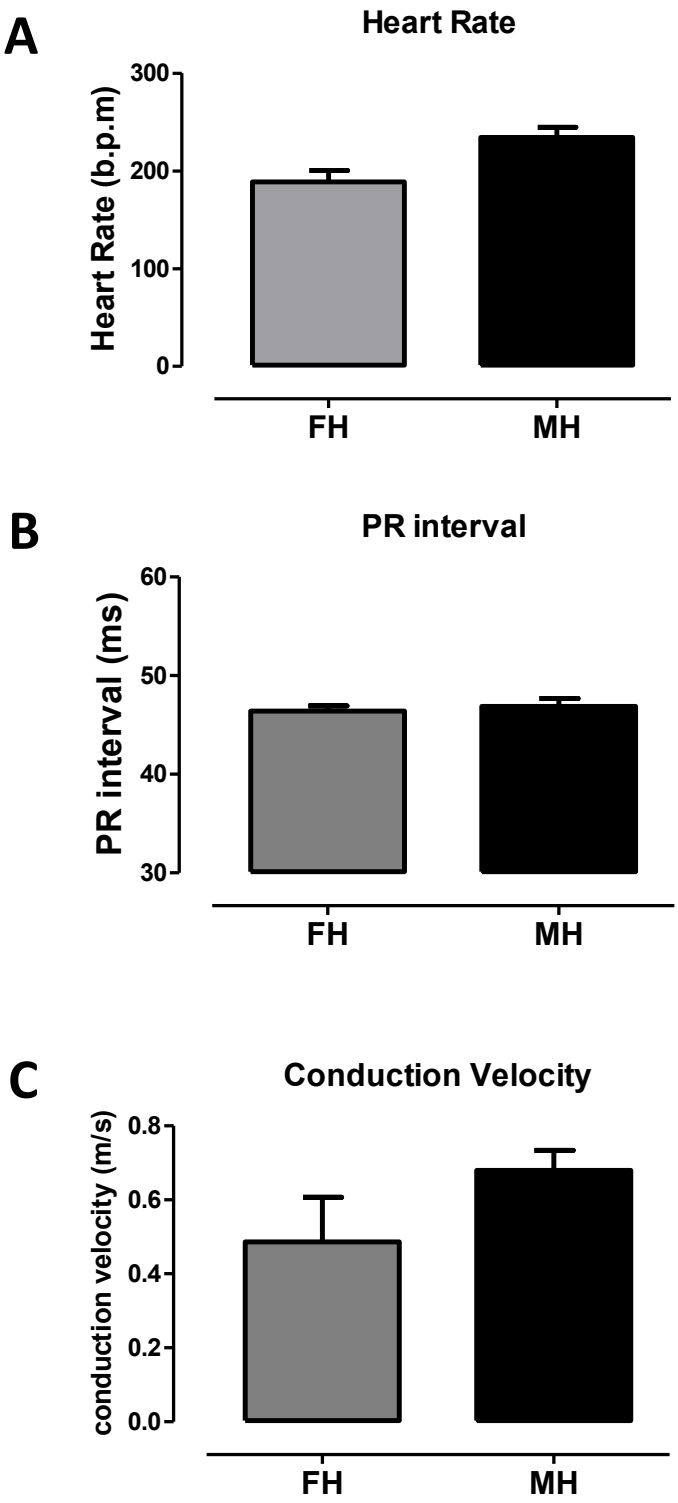


Figure 3-9 Comparison of baseline heart rate, PR interval and Conduction velocity in fetal (FH) and maternal (MH) heart models following vehicle treatment.

A*. Mean plot of heart rates from fetal (N=10) and maternal (N=10) hearts* ***B.*** *Mean plot of PR interval from fetal (N=10) and maternal (N=10) hearts.* ***C.*** *Mean plot of conduction velocity from fetal (N=10) and maternal (N=10) hearts.*

3.4 Discussion

In this section, we aimed to develop a whole-heart fetal model for evaluating the effects of toxic bile acids and to identify any beneficial properties for ursodeoxycholic acid (UDCA) in fetal hearts as is observed in Intrahepatic Cholestasis of Pregnancy (ICP). Previous studies that have been performed in neonatal cardiomyocytes only provide limited information on cardiac physiology(Gorelik et al. 2002; Sheikh Abdul Kadir et al. 2010). It was successfully demonstrated that following treatment with the bile acid, taurocholic acid (TC), PR interval is prolonged in neonatal rat hearts similar to what is observed in human fetal hearts in ICP (Strehlow et al. 2010). We observed that unlike fetal mouse hearts, neonatal rat hearts can be easily cannulated to allow aortic perfusion and produce reproducible results Figure 3-1. In order to better characterise the model, we also investigated the effect of standard agents on cardiovascular parameters and compared baseline values against the established adult heart model. To my knowledge this is the first time that this model has been characterised and used for electrophysiology studies.

3.4.1 Preliminary studies in E18 fetal mouse hearts

As a number of *in vitro* studies of ICP to date have been performed in mouse models (Milona et al. 2010; Papacleovoulou et al. 2011), we initially evaluated the feasibility of fetal mouse hearts as an ICP model. Although use of a fetal heart might have allowed for better translation of observed effects to those of human fetuses, we experienced a number of difficulties in the use of this model which led to it being discontinued. Primarily, we found it to be wasteful in terms of the number of pups available *in utero* and those that could be used on study days. Perhaps this could have been anticipated, considering the duration of the experimental protocol and the limited time for which the pups could be kept alive following the death of the mother. Added to this at about one millimetre in length, cannulation of the aorta was not feasible resulting in hearts only being superfused with solution. As will be pointed out below with neonatal rat hearts, aortic perfusion is important for oxygenation of

the myocardium. A lack of cannulation results in hearts becoming ischemic and can alter cardiovascular parameters.

Literature searches on the use of fetal mouse hearts for optical mapping studies did not identify any studies evaluating electrophysiological changes for prolonged periods which might confirm that difficulties are not restricted to our group. Optical mapping has been used in identifying anatomical features and in much shorter experimental protocols (Benes et al. 2014). The combination of these factors led us to conclude that fetal hearts are not ideal as a whole-heart model for studying acute bile acid effects in ICP.

3.4.2 Development of neonatal rat hearts as a model of the fetal heart in ICP

Since fetal hearts were not a viable model for the purposes of this project, further developmental work focused on the use of neonatal rat hearts. In our group we have performed a number of studies previously using cardiomyocytes isolated from neonatal rat hearts to study the effects of bile acids *in vitro* (Gorelik et al. 2002; Sheikh Abdul Kadir et al. 2010). Therefore, we aimed to develop the use of this whole-heart model for our studies. Due to difficulties in cannulation, preliminary studies were performed without cannulation and perfusion with oxygenated solution. However, we identified a reduction in conduction velocity even in vehicle superfused hearts which was caused by cellular hypoxia. In cardiomyocytes, hypoxia leads to membrane depolarization, inhibition of sodium ion channels and cellular uncoupling via inhibition of gap junctions (Shimoda & Polak 2011; Dhein 2004). This in turn leads to a slowing of the speed of impulse propagation through the heart (Klabunde 2004). An ischemic mechanism for the conduction slowing observed was confirmed by the significant improvement observed with cannulation.

Only a small number (3) of publications were identified where this model had been applied previously. Using supplemented serum free medium for perfusion, Wiechert and colleagues evaluated the use of isolated-perfused neonatal hearts for efficient gene transfer(Wiechert et al. 2003). Hearts were found to be stable despite perfusion for 24 hours. The isolated-perfused neonatal heart has been used to

study the effect of changes in external calcium concentrations on myocardial contractility and functional stability (Riva & Hearse 1991). In the third publication found, isolated-perfused neonatal hearts from 1 to 10 days old were applied to study the effect of ischemic preconditioning during hypoxia (Oštádalová et al. 2002). Although, these publications were useful for setting up the model but only provide limited information on electrophysiological changes or the effect of standard agents.

Therefore, it was important to characterise cardiovascular parameters in the model. Consistent with effects observed by other authors, we observed a prolongation in action potential duration in this model with 4-AP (Baiardi et al. 2002). Similarly, no changes were observed in heart rate as previously reported. And our data is consistent with a number of clinical reports showing that despite 4-AP being a potassium channel blocker it does not prolong the ECG QT interval at concentrations similar to plasma concentrations (Isoda & Segal 2003).

Comparison of basal PR interval, heart rate and conduction velocity values to those in maternal hearts did not identify any statistically significant differences. The ventricular conduction velocity values observed in the neonatal rat are lower than those observed in adult rats and humans (Klabunde 2004). This might be indicative of a greater role of slow sodium and calcium ion channels in impulse propagation in the developing heart. Furthermore, we observed a basal heart rate value of approximately 190 b.p.m. in our neonatal hearts which is higher than values of 135-155 b.p.m. reported by Wiechert and colleagues (Wiechert et al. 2003). However, with limited information on these parameters we were unable to further compare these values or PR interval.

3.4.3 Limitations of model

Despite the greater benefit of using a whole-heart model over cellular models, a number of limitations have to be recognised with the use of this model. Firstly, as with other isolated heart models, this model lacks the influences of humoral and neuronal factors on the heart. Therefore whatever effects observed in the model could be reduced or absent as a result of input from the sympathetic or parasympathetic systems. Changes in blood pressure can have a reflex response on heart rate

(Klabunde 2004). As studies have shown that bile acids can affect cardiomyocyte contractility and blood pressure, any effects observed on heart rate might be different in whole animal models.

Secondly, during gestation and following birth rapid changes occur in the heart (Davies et al. 1996). The impact on cardiac electrophysiology of a transition from fetal to neonatal circulation on blood flow has to be considered. As we showed with neonatal hearts of increasing ages, the responses of bile acids can be reduced during development. By using hearts from new-borns rather than foetuses, there might be significant differences in the effects observed in our model to what might have been observed if fetal hearts was used.

Thirdly, isolated heart models are associated with a deterioration of contractile function with prolonged perfusion (Skrzypiec-Spring et al. 2007). We observed that although the neonatal rat heart was more stable than fetal mouse hearts, it showed a greater deterioration in function compared to the maternal heart. Beyond the 30 minute period evaluated in this study there was a decline in heart rate with longer durations of perfusion (data not shown). As such the model can only be used for acute studies, and is limited for evaluating the chronic effects of bile acids or agents.

Finally, the process of isolation and cannulation of hearts can cause them to become ischemic. This can lead to myocardial injury and preconditioning that can alter baseline cardiovascular parameters (Skrzypiec-Spring et al. 2007). This was seen in this study by an improvement in conduction velocity from baseline in vehicle treated cannulated hearts. Although this limitation is not restricted to the neonatal heart, they appear to be more pronounced.

3.4.4 Further work

Only a limited characterisation of the isolated-perfused neonatal rat as a model of ICP was performed in this project and a number of further studies will be required. As a novel model, a number of conditions applied to this model are based on studies performed in adult hearts. Therefore, different processes applied to this model will need to be reviewed including the constitution of perfusate

solutions, temperature and oxygenation. This also includes analysis of a larger number of standard agents at different concentrations to study changes in the response of the model. By doing this, the effect of changes in the expression of key ion channels during development will be better understood. The model can also benefit from perfusion at perfusion pressures more appropriate for hearts of this age based on its mean arterial pressure(Oštádalová et al. 2002). For this project we used a constant flow rate that was not altered with changes in heart rate, however, this is not reflective of normal physiological conditions *in vivo*.

3.4.5 Conclusions

The isolated-perfused neonatal rat heart is a simple and easily reproducible model that can be applied in cardiac electrophysiological studies. It can be used in investigational studies of electrical activity and cardiac function in developing hearts and their response to pharmacological agents. In this project, through the application of high concentrations of bile acids, we have been able to develop it as a model of the fetal heart in ICP. Preliminary results obtained in this section show that it could provide good translation of cardiac arrhythmias. Further work will be required to better characterise the heart and improve the basic set-up of the model.

4 Identifying a Mechanism of Bile Acid Induced PR interval prolongation in ICP

4.1 Introduction

Intrahepatic Cholestasis of Pregnancy (ICP) is characterised by fetal arrhythmias which can lead to sudden death. Interestingly, although these arrhythmias are commonly reported in the fetus, the maternal heart appears to be affected to a lesser extent or not at all (Reid et al. 1976). Despite extensive studies to investigate the nature and causes of the disease, little is known about the nature of fetal arrhythmias and death *in vivo*. It is unclear how initially benign arrhythmias then degenerate into sudden life threatening conditions. This might be expected considering the obvious difficulties associated with obtaining electrocardiogram (ECG) recordings in foetuses. Added to this, although ICP can be due to a genetic predisposition in some patients, none have been described that are associated with the cardiovascular system (Savander et al. 2003).

A search of PubMed and similar databases reveals a paucity of literature on the subject; however, where available the fetal arrhythmias reported appear to have an atrial origin including cases of atrial fibrillation, atrial flutter, supraventricular tachycardia and bradycardia. A study by Strehlow and colleagues (Strehlow et al. 2010) of foetuses in ICP revealed a significant prolongation in the mechanical PR interval compared to healthy patients, measured by echocardiography. This was despite no significant difference between the heart rates of both subjects. Other authors have reported contrasting observations in heart rate with both cases of bradycardia and tachycardia. Refractory supraventricular tachycardia was observed at 28 weeks' gestation by Shand and colleagues (Shand et al. 2008). In a clinical case report by Al Inizi and colleagues, tachycardia (220-230 bpm) with atrial flutter was observed in a fetus by ultrasonography (Al Inizi et al. 2006). In contrast, Reid and colleagues reported bradycardia in 14% of 56 ICP cases observed (Reid et al. 1976).

However, the majority of non-clinical studies investigating the mechanism of arrhythmias in ICP have primarily focused on ventricular effects of bile acids. These studies have applied cardiomyocytes isolated from the ventricles of rodent hearts to investigate the mechanism of action of toxic bile acids such as cholic acid and its conjugated form taurocholic acid (Williamson et al. 2001; Binah et al. 1987).

In neonatal cardiomyocytes, taurocholic acid (TC) caused a bradycardic effect by reducing cardiomyocyte contraction rate (Williamson et al. 2001). In a cellular model of the fetal heart (containing ventricular cardiomyocytes and myofibroblasts) it induced conduction slowing that was inhibited by UDCA (Miragoli et al. 2011). Only a couple of studies were identified that evaluated the effects of bile acids in the atrial region. In a study in hearts from adult rabbit hearts, TC was observed to inhibit calcium and potassium ion channel currents in sino-atrial node preparations (Kotake et al. 1989). TC has also been found to induce arrhythmias in human atrial myocardium, while UDCA levels were shown to be significantly reduced in patients with atrial fibrillation (Rainer et al. 2013). Added to these, TC also depolarised the resting membrane potential, enhanced Na^{+}/Ca^{2+} exchanger (NCX) tail current density and induced afterdepolarisations.

Beyond ICP, elevated circulating bile acid levels have also been known to induce cardiovascular arrhythmias. An example is seen in jaundice, where there have been reports of severe sinus nodal dysfunction (Bashour et al. 1985). Cholestatic liver diseases have been associated with bradycardia via overproduction of nitric-oxide (Mani et al. 2002) and also QT interval prolongation in some patients (Moezi & Dehpour 2013). The effects of bile acids in atria from fetal or new-born hearts were not investigated, therefore, the aim of this study was to investigate the effects of bile acids on impulse conduction in the atrial region of the fetal heart. Based on previous studies in our group demonstrating a reduction in contraction rate by TC via modulation of muscarinic receptors (Sheikh Abdul Kadir et al. 2010), we hypothesised that the bradycardia and conduction slowing fetal symptoms in ICP are mediated by inhibition of muscarinic receptors. The effect of TC on ECG intervals and heart rate were investigated in Langendorff-perfused whole-heart models of the fetal and maternal heart. Following on from this the effect UDCA and other standard agents were also evaluated to identify a mechanism for the observed effects. Patch clamp studies in isolated atrial cardiomyocytes were performed on calcium ion currents.

4.2 Materials and Methods

4.2.1 Animals

All work was carried out according to standards set out in the UK Animals (Scientific Procedures) Act 1986. Sprague-Dawley neonatal (~7g) and adult female rats (~150-400g) were either purchased from Harlan UK, Charles River UK or were kind gifts from Phil Rawson of the Central Biomedical Services, Imperial College, London.

4.2.2 Materials

Stock solutions of nifedipine and UDCA were dissolved in 100% DMSO. These were serially diluted to ensure study concentrations contained no more than 0.01% DMSO. Acetylcholine, atropine, mibefradil, and verapamil were dissolved in purified water as stock solutions at a concentration of 10 mM and diluted in perfusate solution on study days to achieve required concentrations. When not used immediately, stock solutions were stored at -20°C until required. TC was dissolved directly in perfusate solution on study days at required concentrations. Perfusion (Tyrode's) and cardioplegic solutions were prepared as described in Section 2.3.

4.2.3 Isolation and cannulation of hearts

The process of isolation and cannulation of hearts has been described in full in section 2.3.2.

4.2.4 Treatment protocol

To replicate conditions observed in ICP, individual neonatal rat hearts were divided into groups and treated with TC at concentrations of 0 (vehicle), 40, 100 and 400 μM. This was used as the fetal heart model (FH). Hearts were first perfused with vehicle solution and allowed to acclimatise for 15 minutes before being treated with drug solutions for a further 15 minutes Figure 4-1. Based on results obtained, further studies were performed with TC in combination with UDCA (1 μM) or UDCA alone. Other studies were also performed with standard agents to understand the mechanism of observed

effects. Standard agents used and their concentrations were acetylcholine (1 µM), atropine (1 µM), verapamil (1 µM), mibefradil (1 µM) and nifedipine (10 nM).

Hearts from adult female rats were treated with increasing concentrations of TC at 0, 40, 100 and 400 µM according to Figure 4-2. This was possible because adult hearts did not experience the same degree of deterioration with prolonged perfusion as was seen in the neonatal rat hearts. Follow up studies were also performed with UDCA and standard agents as described in Figure 4-1. For specific sample sizes for each group, see relevant sections below.

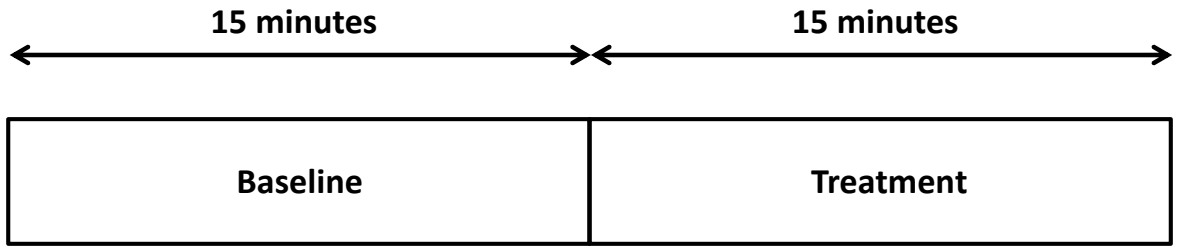


Figure 4-1 Standard treatment protocol

The above treatment protocol was used for FH studies and standard agent studies. Recordings were performed throughout the treatment period.

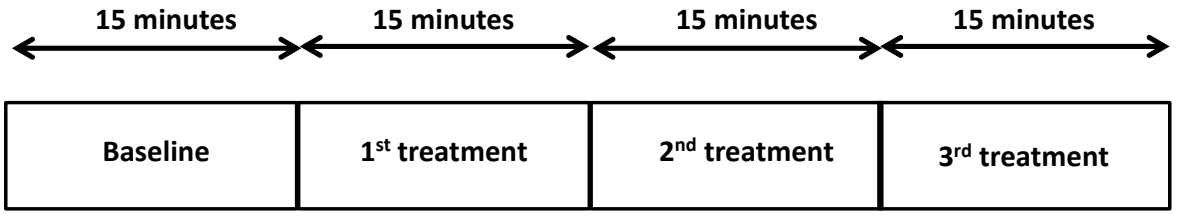


Figure 4-2 Protocol used for MH studies with taurocholic acid

The above treatment protocol was used for MH studies with TC at 0 (baseline), 40 (1st treatment), 100 (2nd treatment) and 400 (3rd treatment). Recordings were performed throughout the treatment period.

4.2.5 Electrocardiogram recording

Electrocardiogram (ECG) recordings were performed throughout the duration of treatment of FH and MH models. The procedure for ECG recording is as described in section 2.5.2. Please note that accurate

PR interval measurement could not be performed in some hearts due to poorer ECG signals, therefore data was excluded and sample sizes were lower than for heart rate measurement.

4.2.6 Statistical analysis

Statistical analysis was performed using GraphPad prism 5 software (GraphPad, La Jolla, USA). Values following treatment for each heart were subtracted from baseline and averaged for each treatment group. Means were obtained and comapred to all groups and control using One-way analysis of variance (ANOVA) and Bonferroni's post-hoc test to determine statistical significance. Values were considered to be statistically significant with a p value less than 0.05 ($p<0.05$).

4.3 Results

4.3.1 UDCA inhibits taurocholic acid induced PR interval prolongation in the fetal heart model

Clinical studies have shown that the mechanical PR interval (measured by echocardiography) is significantly prolonged in fetuses in ICP(Strehlow et al. 2010). This is without any corresponding changes in heart rate. Treatment of the FH model with taurocholic acid at different concentrations initially caused a gradual shortening in ECG PR interval. Percentage change from baseline values were -0.8±1.2, -5.4±1.6 and -7.0±1.2% at 0 (vehicle; N=7), 40 (N=4) and 100 μM (N=3) respectively (see Figure 4-3B). These changes were not statistically significant, P>0.05. However, at 400 μM (N=6) the opposite effect was observed with a statistically significant ($p<0.05$) increase of 8.2±2.4% was observed compared to vehicle treatment (Figure 4-3A and B). Co-administration of UDCA 1 μM (N=6) significantly inhibited this effect (2.1±2.0%, $p<0.05$). As is also observed in clinical cases, no significant changes ($p>0.05$) were observed in heart rate at all concentrations of taurocholic acid tested or with UDCA co-administration (Figure 4-3C). Mean % change from baseline values in heart rate were 6.4±4.8, -3.8±2.4, -8.2±5.5, 6.6±5.4 and 0.9±2.1 with TC 0 (N=9), 40 (N=7), 100 (N=6), 400μM (N=9) and TC 400 μM with UDCA 1μM (N=8) treatments respectively.

The different responses seen with taurocholic acid suggested to us that taurocholic acid has different pharmacological activities at lower and higher concentrations in the FH model. However, before we could evaluate this we needed to understand how the MH model responds to similar concentrations of taurocholic acid.

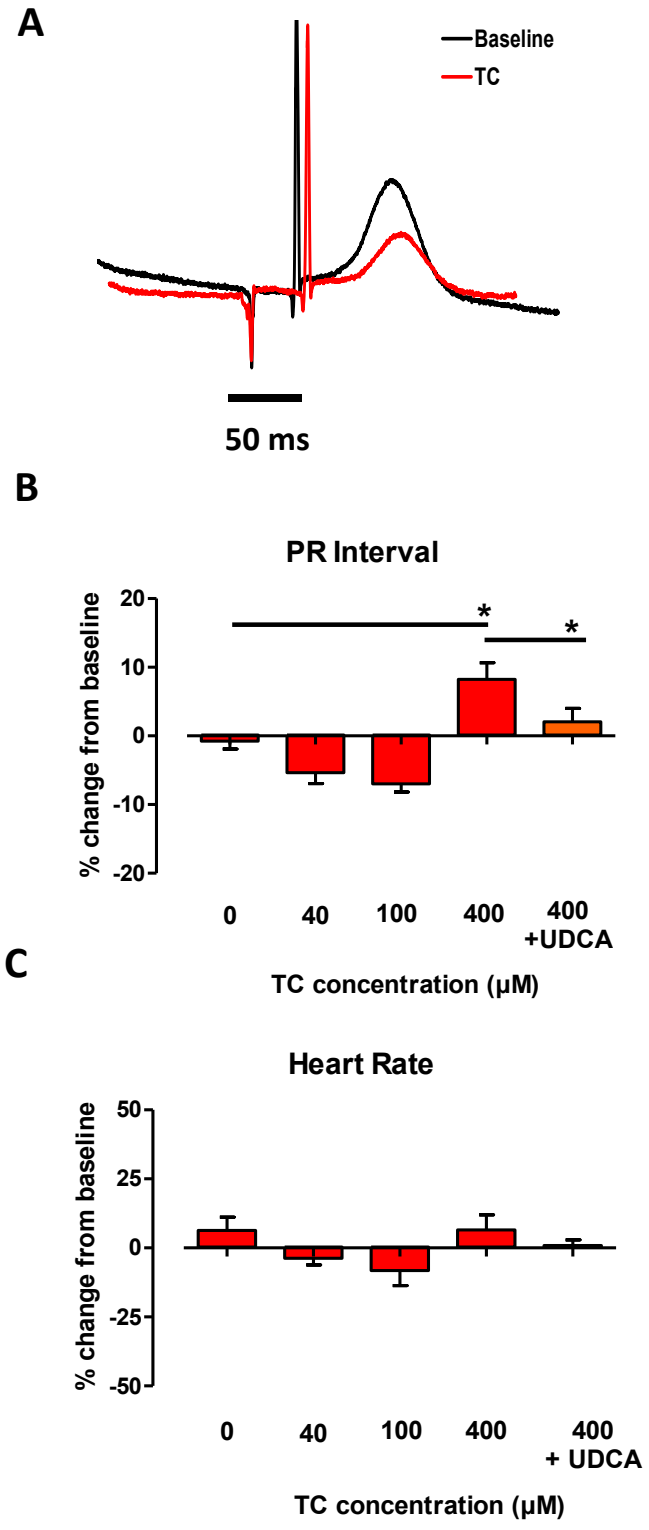


Figure 4-3 Effect of taurocholic acid (TC) on PR interval and heart rate in fetal heart model

A. *Representative ECG trace overlay following baseline and TC 400 μM treatment. Plots of mean % change from baseline values in PR interval* ***(B)*** *and heart rate* ***(C)*** *following treatment with TC 0, 40, 100, 400 μM and TC 400 μM with UDCA 1 μM treatment. * - statistically significant value (P<0.05)*

4.3.2 Taurocholate does not produce significant changes in PR interval or heart rate in the maternal model

Since the maternal heart appears to be protected against the effects of elevated bile acids in ICP, we compared the effect of TC observed in the FH model to the MH model. The results show that although a gradual increase in PR interval was observed with higher concentrations of TC, the effects observed were not statistically significant ($p>0.05$) (see Figure 4-4B). Percentage change from baseline values were 2.0±3.4, 2.7±0.5, 5.2±1.1, 7.3±1.7% at 0 (vehicle; N=3), 40 (N=3), 100 (N=3) and 400 (N=3) µM respectively. Similarly, although a decline was observed in heart rate at the highest concentration of 400 µM (Figure 4-4C), this change was also not statistically significant ($p>0.05$). Percentage change from baseline values were -0.66±4.7, -0.40±1.6, -4.5±2.8 and -19±10% at 0 (N=5), 40 (N=3), 100 (N=3) and 400 µM (N=3) respectively.

These results demonstrate the maternal is less sensitive to the effects of bile aids compared to the fetal hearts. There were small concentration-dependent increases observed in PR interval at 40 and 100 µM in the FH, as opposed to a decrease seen in the FH, which could suggest a difference in the regulation of cardiac conduction in both models. Together these results correlate well with clinical observations in ICP where arrhythmias are generally not reported in the mothers and validate the use of these models. Based on these results, we then sought to understand the mechanism of TC induced PR interval prolongation in fetuses, the inhibitory effects of UDCA and why the maternal hearts respond differently.

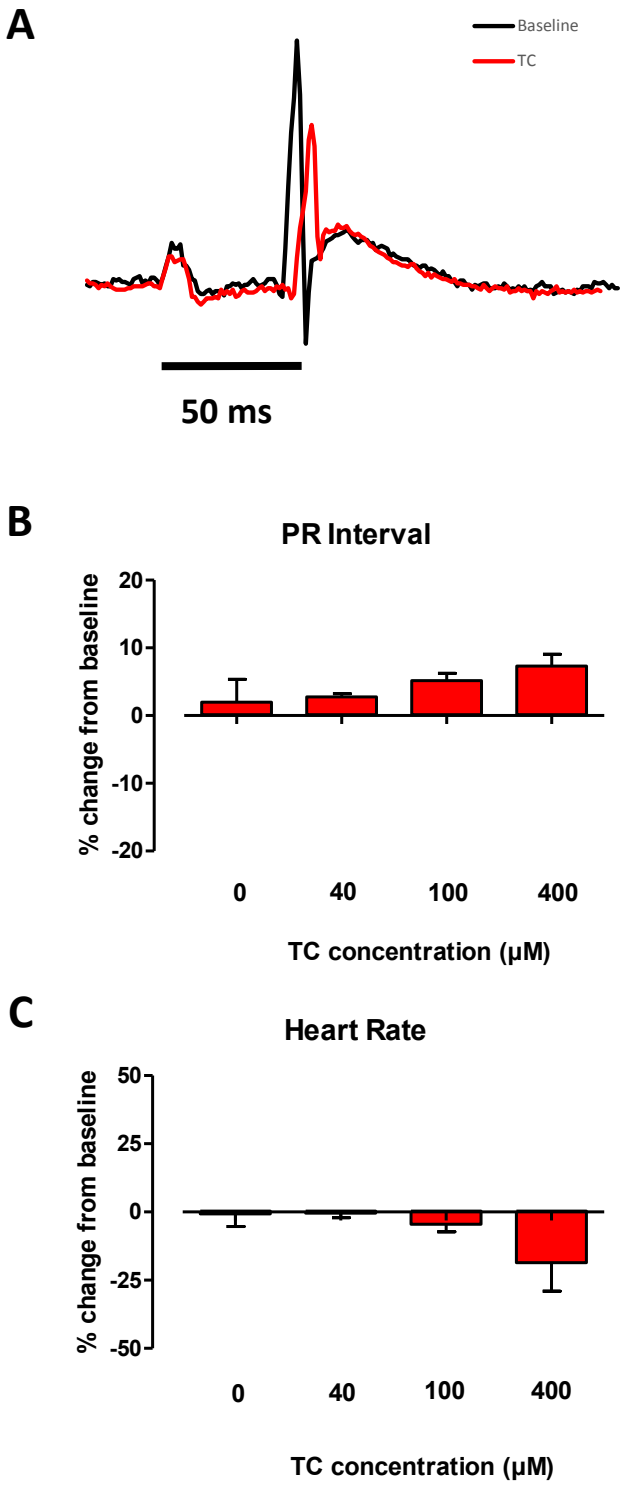


Figure 4-4 Effect of taurocholate (TC) on PR Interval and heart rate in maternal heart model

Representative ECG trace overlay following baseline and TC 400 µM treatment. Plots of mean % change from baseline values in PR interval ***(B)*** *and heart rate* ***(C)*** *following treatment with TC 0, 40, 100, and 400 µM treatment. * - statistically significant value (P<0.05).*

4.3.3 UDCA does not affect cardiovascular parameters in both the fetal and maternal models

Since UDCA had a protective effect compared to taurocholic acid, it was necessary to evaluate the effect of UDCA treatment alone. UDCA is known to be safe clinically with minimal side effects at clinical doses. As can be seen in the ECG overlays (Figure 4-5A and B), in both the FH and MH models, UDCA 1 μM did not produce any significant changes in PR interval. Values in control (N=7) and UDCA (N=4) treated hearts were -0.8±1.2 and -2.8±2.2% respectively in the FH model. In the MH model values were in 2.0±3.4 and -1.1±0.9 in control (N=5) and UDCA (N=3) treated hearts respectively. Similarly, no significant changes were observed in heart rate in both models. Values in control (N=9) and UDCA (N=4) treated hearts were 6.4±4.8 and 5.4±4.9% respectively in the FH model. In the MH model values were 0.7±4.7 and -0.9±2.9% in control and UDCA treated hearts respectively.

The concentration of UDCA applied is similar to plasma concentrations achieved in human patients during treatment. Therefore, our results translate well with clinical findings and further helped to validate our models.

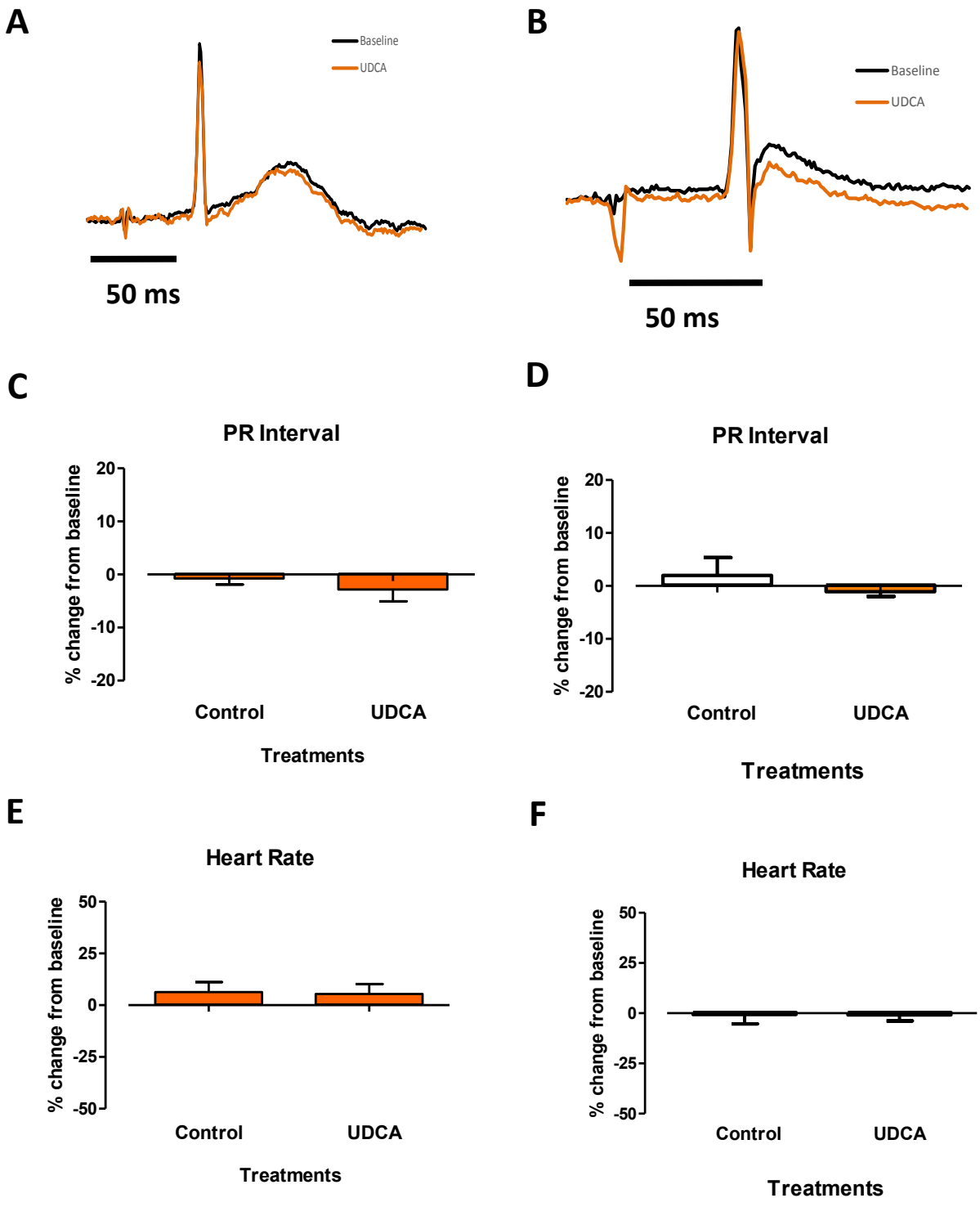


Figure 4-5 Effect of UDCA on PR interval and heart in fetal and maternal heart models

*Representative ECG overlay traces with timescale bars from FH **(A)** and MH **(B)** models following UDCA1 µM treatment. Plots of mean percentage change from baseline values in PR interval following UDCA 1 µM treatment in FH **(C)** and MH **(D)** models. Plots of mean percentage change from baseline values in heart rate interval following UDCA 1 µM treatment in FH **(E)** and MH **(F)** models.*

4.3.4 Activation of the muscarinic receptor by acetylcholine does not prolong PR interval in the fetal model

Based on our initial hypothesis that TC prolongs PR interval by activation of muscarinic receptors, we evaluated the effect of the muscarinic agonist acetylcholine. At a concentration of 1 μM, acetylcholine produced a small decline in PR interval in the FH model (Figure 4-6C), although the effect was not statistically significant. Percentage change from baseline values were -0.8±1.2, -7.1±5.8 and -3.4±1.4% in control (N=7), acetylcholine (N=3) and atropine (N=4) groups respectively. No changes were observed in the MH model at a similar concentration of acetylcholine (Figure 4-6D). Percentage change from baseline values were 2.0±3.4 and 1.4±2.2% in control (N=5) and acetylcholine (N=3) groups respectively. A statistically significant decline was observed in heart rate in the FH model with only a smaller decline in the MH model that was not statistically significant (Figure 4-6E and F). Percentage change from baseline values were 0.8±1.2, -29±11 and 2.2±4.9% in control (N=7), acetylcholine (N=3) and atropine (N=4) groups respectively in the FH model. While in the MH model, percentage change from baseline values were -0.7±4.7 and -8.2±7.4% in control (N=5) and acetylcholine (N=3) groups respectively.

Since a decline was observed in PR interval in the FH model with an agonist, it could only be assumed that an antagonist might reverse this effect. Therefore, we further investigated the role of the muscarinic receptor by evaluating the effect of an antagonist atropine. Application of atropine (1 μM) did not produce any significant changes in PR interval or heart rate in the FH model (Figure 4-6C). These results helped to suggest that the effect of TC in the FH is not mediated by the muscarinic receptor.

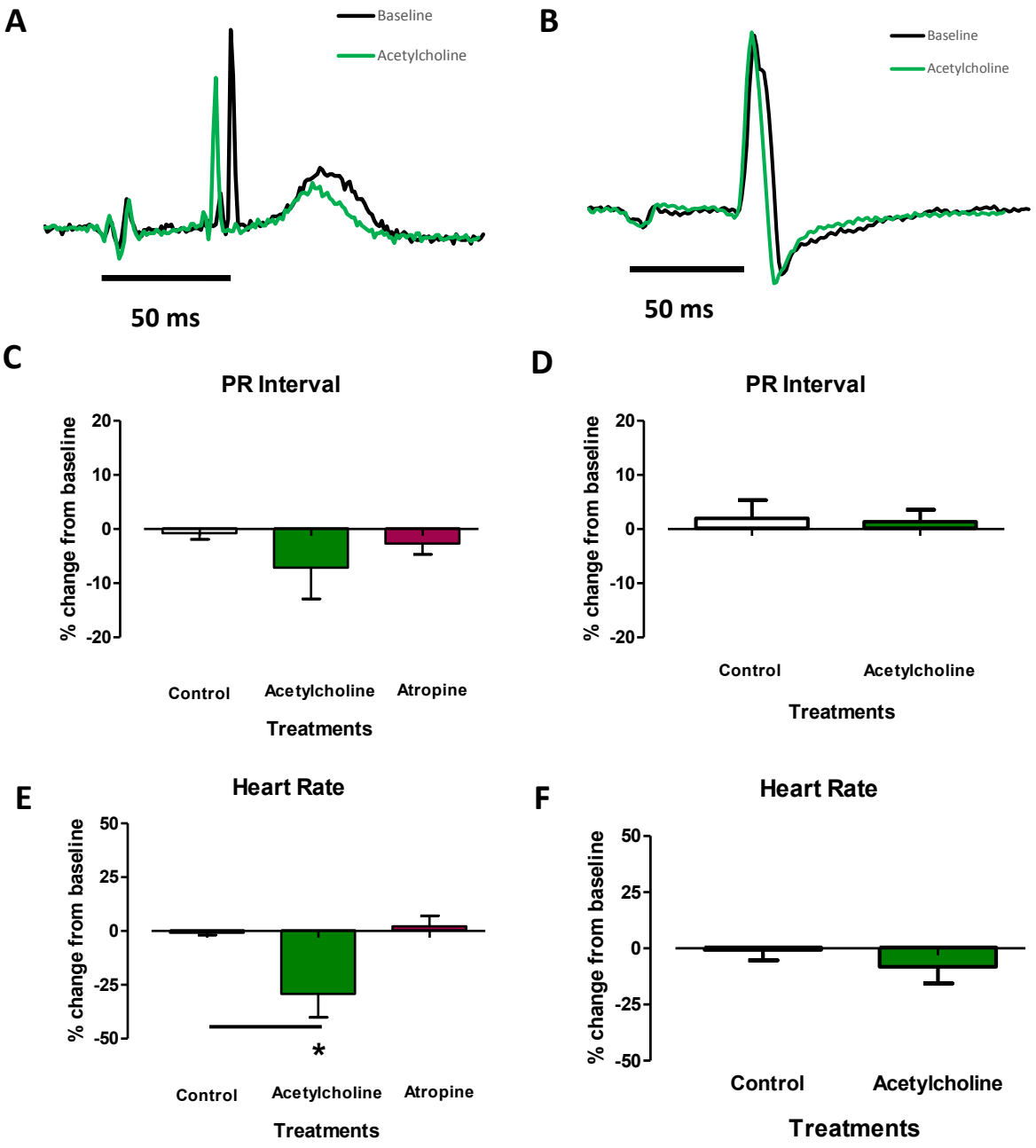


Figure 4-6 Effect of acetylcholine and atropine on PR interval and heart rate in FH and MH models

*Representative ECG overlay traces with timescale bars from FH **(A)** and MH **(B)** models following Acetylcholine (1 µM) and Atropine (1 µM) treatment. Plots of mean percentage change from baseline values in PR interval following Acetylcholine (1 µM) and Atropine (1 µM) treatment in FH **(C)** and MH **(D)** models. Plots of mean percentage change from baseline values in heart rate following Acetylcholine (1 µM) and Atropine (1 µM) treatment in FH **(E)** and MH **(F)** models.*

4.3.5 Inhibition of the muscarinic receptor by atropine, does not inhibit the effect of taurocholic acid on PR interval

To further determine if the effect of TC in the FH model is by activation of muscarinic receptors, we evaluated the effect of co-administration of TC with the muscarinic channel inhibitor atropine. Administration of atropine (1 μM) did not inhibit the reduction in PR interval previously observed with TC 40 μM or the significant increase at the highest concentration of 400 μM. Percentage change from baseline values were -0.8±1.2, -5.4±1.6, -5.3±2.5, 8.2±2.4, 8.0±1.1% in control (N=7), TC 40 μM (N=4), TC 40 μM plus atropine 1 μM (N=6), TC 400 μM (N=7) and TC 400 μM plus atropine 1 μM (N=3) groups respectively (see Figure 4-7). A similar evaluation of its effect on heart rate revealed no significant changes. Percentage change from baseline values were -0.8±1.2, -5.4±1.6, -5.3±2.5, 7.2±6.1, -1.7±1.8% in control (N=7), TC 40 μM (N=4), TC 40 μM plus atropine 1 μM (N=6), TC 400 μM (N=8) and TC 400 μM plus atropine 1 μM (N=4) groups respectively (see Figure 4-7C).

Based on the effects of standard agents alone and in combination with TC, we were able to conclude that the effects of TC were not mediated by the muscarinic receptor and had to alter our initial hypothesis.

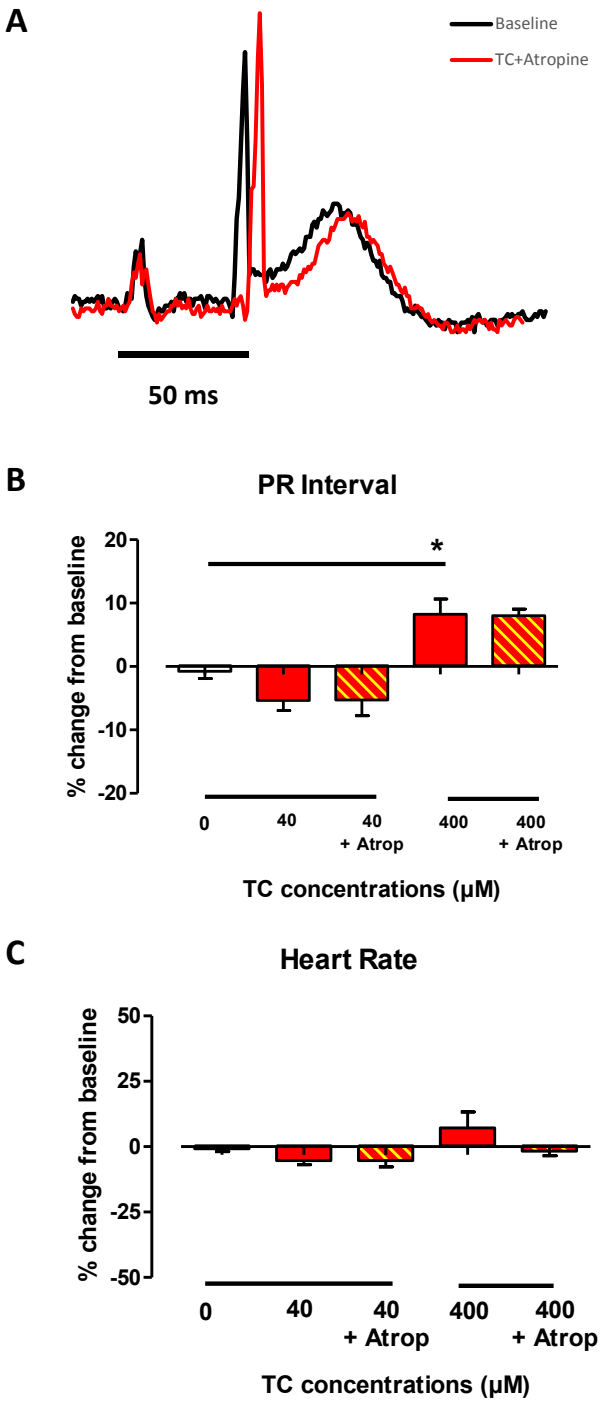


Figure 4-7 Effect of atropine on taurocholate induced changes in PR interval and heart rate in fetal heart model

*Representative ECG overlay traces with timescale bars from FH **(A)** model following TC (400 µM) and atropine (1 µM) treatment. Plot of mean percentage change from baseline values following TC 0 µM, 40 µM, 40 µM with atropine 1 µM, 400 µM and 400 µM with atropine 1 µM in PR interval **(B)** and heart rate **(C)** in FH model.*

4.3.6 The non-selective calcium channel blocker, verapamil, shortens PR interval in the fetal model but prolongs it in the maternal model

Following the revision of our hypothesis, we evaluated an alternative mechanism of PR interval prolongation. It is well established that calcium channel blockers can induce PR prolongation and AV block clinically. This is due to the role of calcium channels within nodal cells of sino-atrial and atrio-ventricular nodes of the heart. Unlike in non-nodal cardiomyocytes, depolarisation in nodal cells is triggered by the entry of calcium and not sodium ions into the cell. Since TC has been shown to block calcium ion channels *in vitro* (Binah et al. 1987), we assessed the effect of a calcium channel blocker verapamil.

At a concentration of 1 µM, verapamil, induced an increase in conduction velocity (shortening of the PR interval), similar to the effect observed in the atrial appendage, in the fetal heart model (see Figure 4-8C). This effect was reduced with UDCA 1 µM co-administration. Percentage change from baseline values were 0.8±1.2, -7.4±2.9 and -1.7±1.4% in control (N=7), verapamil (N=4) and verapamil plus UDCA (N=4) groups respectively. These changes are in contrast to an apparent prolongation observed in the maternal model (Figure 4-8D) which did not appear to be inhibited by UDCA co-treatment. This effect was not statistically significant. Percentage change from baseline values were 2.0±3.4, 94±60 and 94±54% in control (N=3), verapamil (N=3) and verapamil plus UDCA (N=3) groups respectively.

No changes were observed in heart rate with verapamil alone or in combination with UDCA in the FH model (Figure 4-8E). Percentage change from baseline values were 6.4±4.8, 5.2±4.8 and -7.6±7.7% in control (N=9), verapamil (N=4) and verapamil plus UDCA (N=4) groups respectively. A significant decline was observed in the MH model that was only partly inhibited by UDCA co-treatment (Figure 4-8F). Percentage change from baseline values were 0.7±4.7, -50±1.2 and -31±14% in control (N=5), verapamil (N=3) and verapamil plus UDCA (N=3) groups respectively. From these results we were able to identify a difference in the regulation of impulse propagation in developing hearts compared to adult hearts.

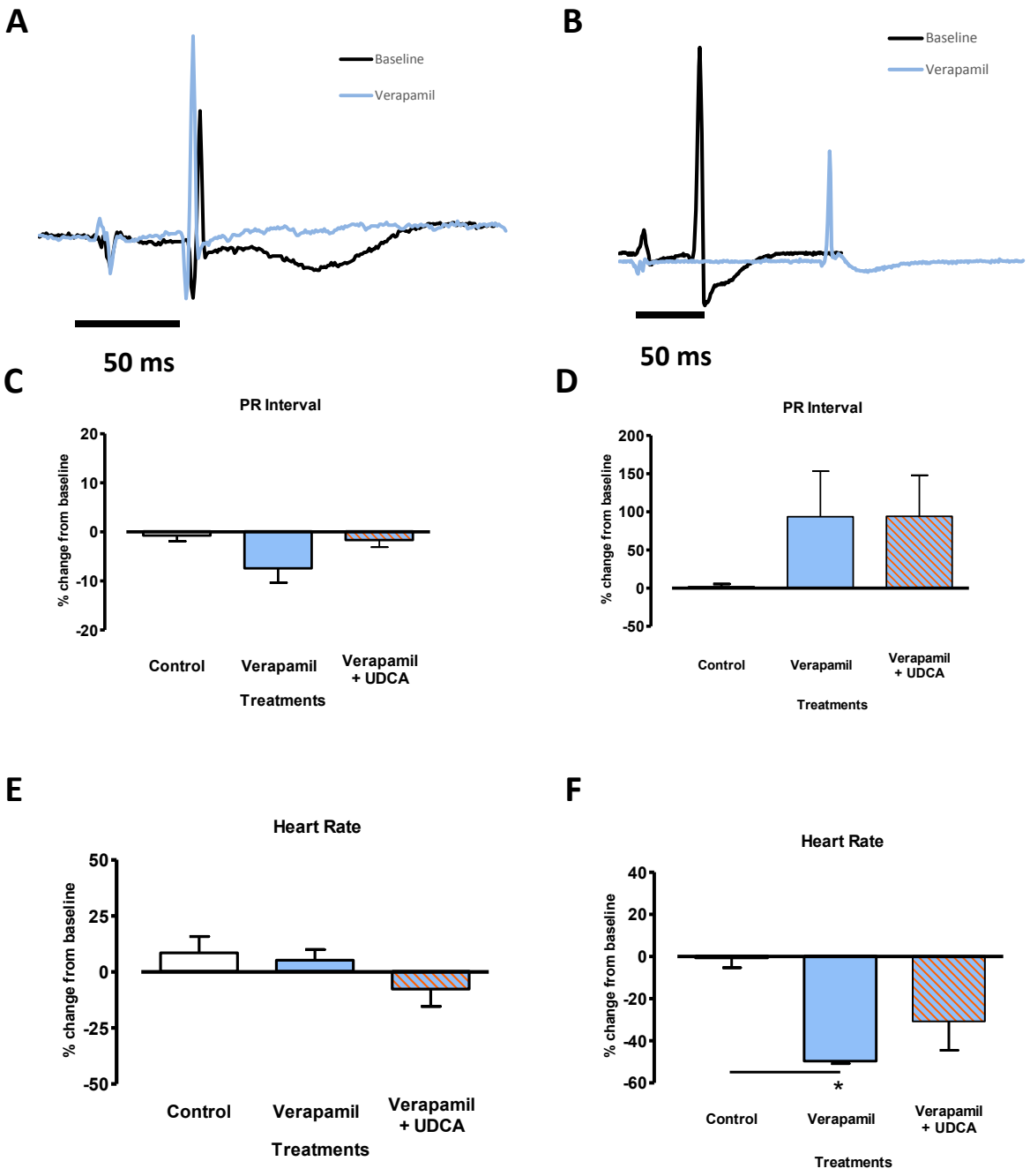


Figure 4-8 Effect of verapamil on PR interval and heart rate in fetal and maternal heart models

*Representative ECG overlay traces with timescale bars from FH **(A)** and MH **(B)** models following verapamil (1 µM) treatment. Plots of PR interval mean percentage change from baseline values following verapamil (1 µM) treatment in the FH **(C)** and MH **(D)** models. Plots of heart rate mean percentage change from baseline values following verapamil (1 µM) treatment in the FH **(C)** and MH **(D)** models.*

4.3.7 The T-type selective channel blocker, mibefradil, prolongs PR interval in the fetal and maternal models

Since, verapamil is known to be a non-selective calcium channel blocker, the effect of more selective agents were evaluated in both models. There are two types of calcium channel sub-types found expressed in the heart – the T- and L-type calcium channels (Ono & Iijima 2010). There is a higher expression of T-type channels in cardiomyocytes of the nodal region compared to other cardiomyocytes of the atrial region (Ono & Iijima 2010).

Mibefradil, is a calcium channel blocker, that is selective for the T-type channel subtype. Following administration of mibefradil 1 μM, only a small prolongation in PR interval was observed (see Figure 4-9C), and this effect was not statistically significant. Percentage change from baseline values were 0.8±1.2, 5.5±1.3 and -2.4±3.5% in control (N=7), mibefradil (N=6) and mibefradil plus UDCA (N=5) groups respectively in the FH model. This effect was much more pronounced in the MH model and did not appear to be inhibited with UDCA (Figure 4-9D). Percentage change from baseline values were 2.0±3.4, 58±18 and 35±17% in control (N=3), mibefradil (N=3) and mibefradil plus UDCA (N=3) groups respectively in the MH model.

No significant changes were observed in heart rate in both models with mibefradil alone or in combination with UDCA (Figure 4-9E and F). Percentage change from baseline values were 6.4±4.8, 2.6±3.8 and 1.1±3.5% in control (N=9), mibefradil (N=7) and mibefradil plus UDCA (N=5) groups respectively in the FH model. While percentage change from baseline values were 0.7±4.7, -14±7.6 and -13±6.8% in control (N=5), mibefradil (N=3) and mibefradil plus UDCA (N=3) groups respectively in the MH model.

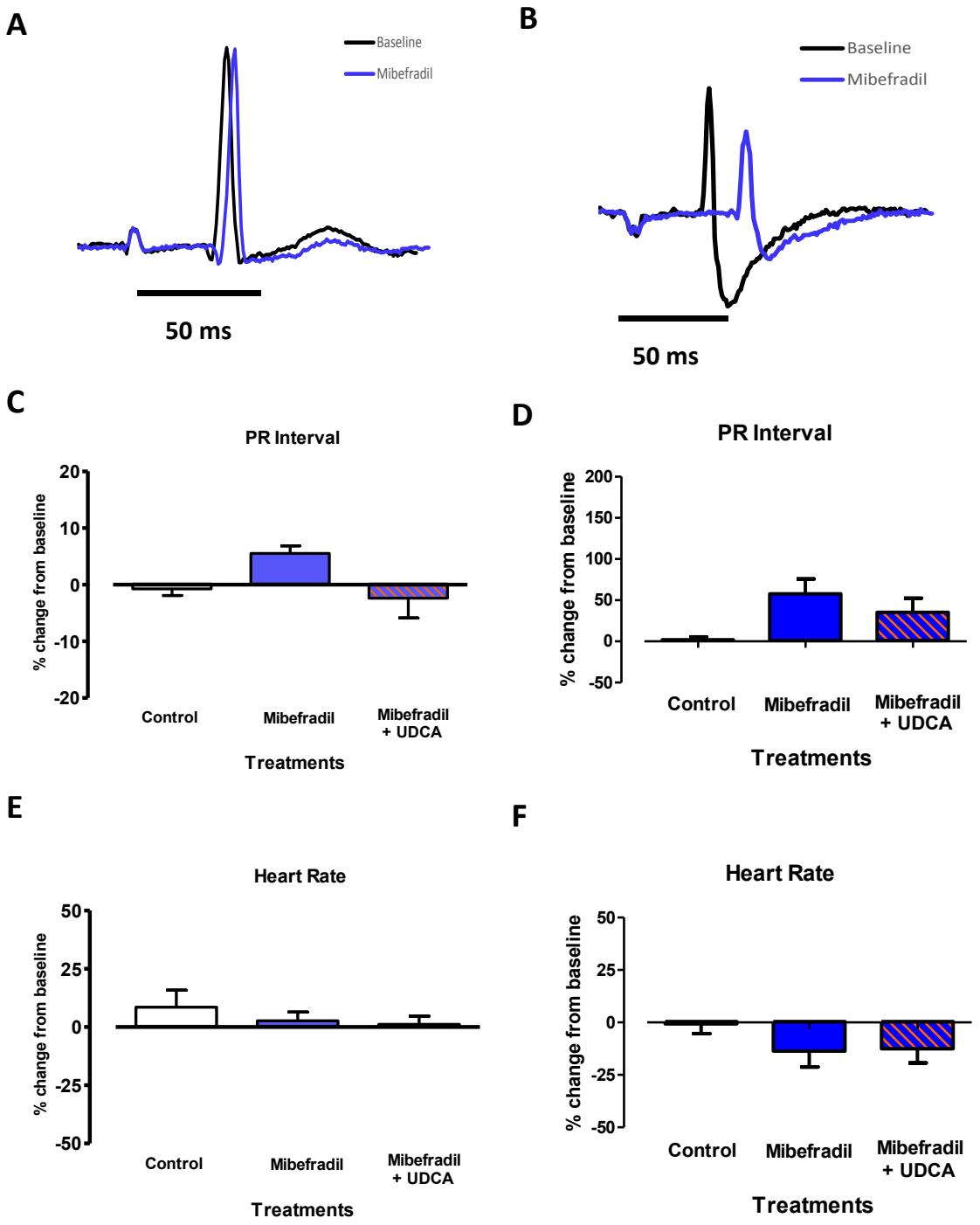


Figure 4-9 Effect of mibefradil on PR interval and heart rate in fetal and maternal heart models

*Representative ECG overlay traces with timescale bars from FH **(A)** and MH **(B)** models following mibefradil (1 µM) and mibefradil (1 µM) with UDCA (1 µM) treatment. Plots of PR interval mean percentage change from baseline values following mibefradil (1 µM) and mibefradil (1 µM) with UDCA (1 µM) treatment in the FH **(C)** and MH **(D)** models. Plots of heart rate mean percentage change from baseline values following mibefradil (1 µM) and mibefradil (1 µM) with UDCA (1 µM) treatment in the FH **(C)** and MH **(D)** models.*

4.3.8 The L-type selective calcium channel blocker, nifedipine, produces a modest shortening of PR interval in the fetal model

In contrast to mibefradil, nifedipine has a greater selectivity for L-type calcium channels over the T-type subtype (Sarsero et al. 1998). Unlike the PR interval prolongation observed with mibefradil, at a concentration of 10 nM nifedipine, a small decline in heart rate was observed although these were not statistically significant Figure 4-10. Percentage change from baseline values in PR interval were -0.8±1.2 and -2.3±1.1% in control (N=7) and nifedipine (N=5) groups respectively. While percentage change from baseline values in heart rate were 6.4±4.8 and 2.2±6.0% in control (N=9) and nifedipine (N=5) groups respectively.

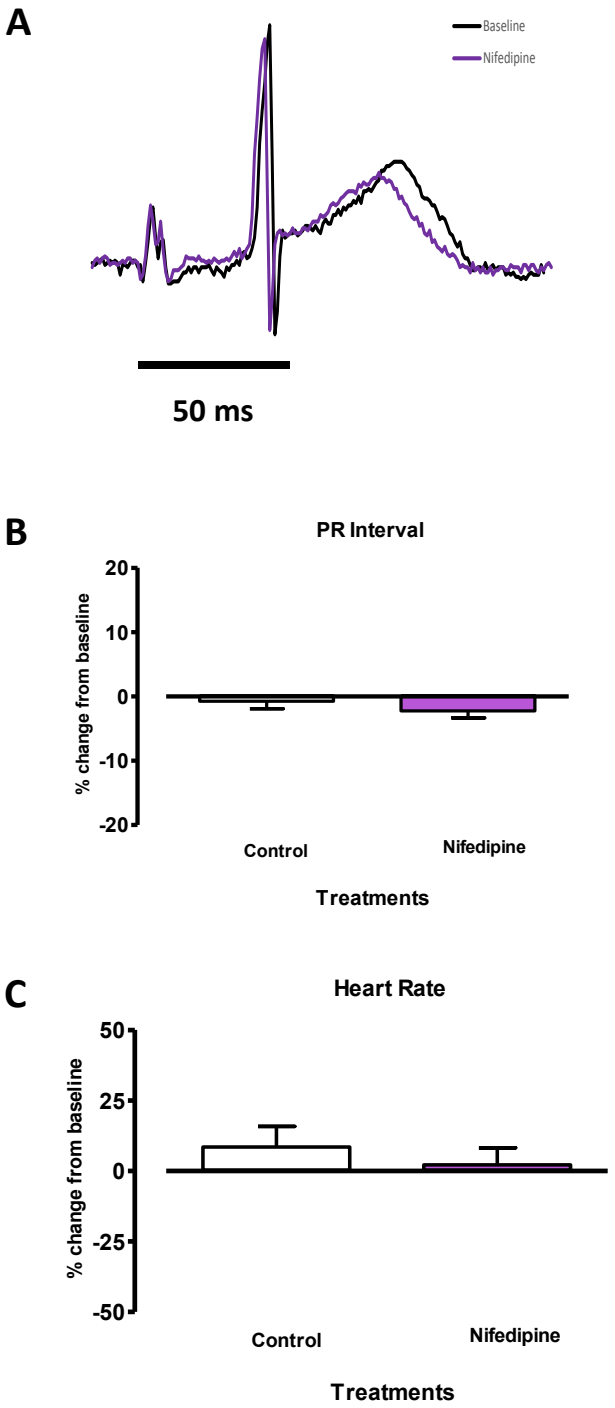


Figure 4-10 Effect of nifedipine on PR interval and heart rate in fetal and maternal heart models

*Representative ECG overlay traces with timescale bars from FH **(A)** model following nifedipine (1 µM) treatment. Plot of mean percentage change from baseline values following nifedipine (1 µM) treatment in PR interval **(B)** and heart rate in FH **(C)**.*

4.4 Discussions

UDCA is widely prescribed as first line therapy for ICP. Administration of UDCA helps to reverse changes in maternal bile acid levels which can reduce the incidence of fetal complications. In this section, we demonstrated that UDCA did not produce any adverse effects. We also show a protective effect for UDCA against TC induced PR interval prolongation using a model of the fetal heart in ICP. No significant changes were observed in a model of the maternal heart at similar concentrations of TC. Unlike in previous studies using ventricular cardiomyocytes, no changes were observed in heart rate in our whole-heart model. This effect did not appear to be linked to activity at the muscarinic receptor as has been previously reported(Sheikh Abdul Kadir et al. 2010), but appears to be partly linked to an inhibition of the cardiac T-type calcium ion channel subtype.

4.4.1 Translation between whole-heart bile acid effects and clinical findings

The findings of our study translate well with clinical observations in ICP patients. UDCA is known to be very safe clinically, with very few and minor side effects reported at therapeutic doses (Hempfling et al. 2003). Of the side effects reported none have been linked to the cardiovascular system and are usually seen with higher doses of the drug, which are not given in ICP. It did not affect any cardiovascular parameters in both of our models, and so our data can be considered to be reflective of clinical cases.

In contrast to the safe and beneficial profile of UDCA, an elevation in other maternal bile acids has been shown to result in prolongation in the mechanical PR interval in fetuses in ICP (Strehlow et al. 2010). The mechanical PR interval is an indirect index of atrio-ventricular conduction duration measured by echocardiography (Glickstein et al. 2004). It can be a useful index of the electrocardiogram (ECG) PR interval considering the difficulties associated with direct ECG measurements in fetuses. Our results with TC are consistent with this finding and have the added advantage of being direct measurements from the ECG. They were also comparable in duration - in the study by Strehlow and colleagues, the mechanical PR interval was found to be approximately 12%

longer in ICP than in healthy patients, while values in our study were approximately 8% longer with TC treatment than controls. To our knowledge this is the first time the effects of bile acids on ECG have been shown in an *ex vivo* model.

The concentrations of TC evaluated in this study were consistent with the defined total maternal bile acid levels in ICP. ICP is diagnosed based on fasting maternal bile acids above 10 μM, although healthy patients with plasma concentrations of 11 μM have been reported (Pascual et al. 2002). However, higher fetal complication rates are observed with fasting maternal bile acid levels above 40 μM, with no increase in fetal complications below this concentration despite it being above the diagnostic level (Glantz et al. 2004). In our studies we explored concentrations of TC from 40 to 400 μM, which are concentrations where fetal complications would be expected. The significant PR interval prolongation we observed was only observed at the highest concentration of 400 μM. This concentration is much higher than normal levels in ICP where PR interval was observed clinically(Glantz et al. 2004; Geenes & Williamson 2009). This could obscure the results from this study as it would suggest that our models are less sensitive to the effects of bile acids than human fetuses.

4.4.2 The effects of bile acids in a whole-heart model were not linked to muscarinic receptor inhibition

The mechanism by which bile acids cause arrhythmias in fetuses is not well understood. In previous studies in our group using isolated cardiomyocytes, it was observed that taurocholic acid treatment caused a reduction in contraction rate, calcium transient amplitude and cellular synchronisation(Sheikh Abdul Kadir et al. 2010). These effects were abolished by siRNA knockdown of muscarinic M_2 receptors indicating that the effect of taurocholic acid was by partial agonism of the receptor. In the heart, parasympathetic activation via the vagus nerve causes a decrease in heart rate by acetylcholine mediated stimulation of muscarinic receptors (Klabunde 2004). We therefore hypothesised that taurocholic acid induces a bradycardic effect via activation of cardiac muscarinic receptors.

Treatment of the fetal heart model with muscarinic agonist, acetylcholine did not induce bradycardia or prolong PR interval. Similarly, co-administration of a muscarinic receptor antagonist, atropine, did not inhibit the effects of TC observed. Although not significant, the gradual decline in heart rate at lower concentrations could suggest partial agonism at the muscarinic receptor by taurocholic acid. This is supported by the significant decline induced by the full agonist, acetylcholine. However, this can only be speculated considering the magnitude of the effect and the lack of inhibition by atropine. These findings led us to reject our hypothesis and to evaluate an alternate mechanism of action.

A number of factors might explain the lack of correlation between effects observed in the cellular and whole-heart models. The cellular models made use of only ventricular cardiomyocytes without atrial or nodal cardiomyocytes. However, heterogeneity is known to exist in muscarinic receptor function and receptor occupancy between atria and ventricles albeit in adult hearts (Brodde & Michel 1999). Furthermore, the cellular models do not take into account potential compensatory effects of basal sympathetic tone to those of any parasympathetic muscarinic inhibition in the heart (Klabunde 2004). As such the cellular model only provided limited information regarding cardiac function and so is limited in its predictive value.

Our finding that bradycardia is not induced in whole-heart models by bile acids is consistent with observations in population studies of ICP. In an analysis of fetuses in 14 ICP patients, bradycardia was not observed compared to fetuses from healthy patients (Strehlow et al. 2010). In a separate study of 56 patients with ICP, bradycardia was only observed in 14% of fetuses (Reid et al. 1976). In perhaps the largest cohort study of ICP with 713 confirmed cases of severe ICP, despite cardiotocographic abnormalities being recorded in 163 cases bradycardia was seen in only 11 of those cases (Williamson & Geenes 2014). Although literature reports identify bradycardia in ICP, these tend to be clinical reports on individual cases and are not a consistent observation. It is also complicated by paradoxical reports of tachycardia in some fetuses (Al Inizi et al. 2006). These contrasting reports of bradycardia

and tachycardia could be indicative of bile acid suppression of the sinus node leading to Sick Sinus Syndrome (Semelka et al. 2013).

4.4.3 Inhibition of T-type calcium channels provides a possible mechanism for PR prolongation by taurocholic acid.

It is still unclear how an elevation in maternal bile acid levels can lead to PR interval prolongation in fetal hearts. ECG traces from our models with taurocholic acid revealed a prolongation of PR interval in the fetal model with a smaller effect in the maternal model which was not statistically significant. Our efforts to identify a mechanism for this conduction slowing effect focused on the role of muscarinic receptors and calcium ion channels. Parasympathetic nerves innervate the heart by the release of acetylcholine which binds to muscarinic receptors found principally on cardiomyocytes of the sinoatrial and atrioventricular node (Klabunde 2004). Apart from the negative chronotropic effect already mentioned this can also lead to a negative dromotropic (conduction slowing) effect through the AV node (PR interval prolongation) via activation of acetylcholine sensitive potassium channels (K_{ACh} channels) and hyperpolarization of cardiomyocytes. The PR prolongation we observed with taurocholic acid was not inhibited by a muscarinic antagonist, atropine, in our models suggesting that this effect was not mediated by agonism of muscarinic receptors.

An alternative mechanism of conduction slowing is inhibition of calcium ion channels. This is because depolarisation in AV nodal cells is regulated by calcium ion entry rather than sodium ion entry as occurs in non-nodal cells (Klabunde 2004). Therefore blockade of calcium channels will reduce the speed of impulse propagation through the AV node. Since taurocholic acid has been shown to block calcium ion channels in cardiomyocytes, we evaluated the effect of other standard calcium channel blockers. It was surprising to observe the opposite effect in the fetal heart model with the non-selective calcium channel blocker verapamil since prevailing knowledge would have suggested otherwise. Although this was initially unexpected, a review of published data showed that most of the

common knowledge on cardiac physiology is based on studies performed in adult hearts. There is little information on cardiac electrophysiology in new-born and developing hearts.

When we compared the effect of the T-type selective blocker, mibefradil, in the fetal and maternal models at similar concentrations, although not significant, a prolongation was observed in the maternal model. This is unlike effects in ICP where the maternal heart appears to be protected. It is possible that this is due to the poor selectivity of mibefradil for T-type channels over L-type channels. Mibefradil is more selective than most other calcium channels for the T-type channel, however, a number of studies have shown that it can cause a voltage dependent block of partially inactivated L-type channels (Leuranguer et al. 2000a; Bezprozvanny & Tsien 1995). Therefore although our results identify a mechanism of prolongation in fetal hearts, it is probably only one of different mechanisms by which taurocholic acid prolongs PR interval.

4.4.4 Potential mechanisms of PR interval prolongation in fetal hearts

For a long time, PR interval prolongation was thought to be benign (Mymin et al. 1986). In the absence of complete atrio-ventricular block, every atrial impulse is transmitted to the ventricles and so the heart rate is normal. However, more recent studies in diverse populations have associated it with an increased risk of atrial fibrillation, pacemaker implantation and death (Cheng et al. 2009; Nielsen et al. 2013). It has also been associated with endothelial dysfunction in high-risk cardiovascular patients (Tse et al. 2011). Therefore even without progression to atrio-ventricular block, PR interval prolongation on its own can have significant implications clinically.

Along with PR interval prolongation, the arrhythmias that have been reported in ICP include atrial flutter, atrial fibrillation and supraventricular tachycardia. These arrhythmias are usually associated with the development of re-entry circuits in the heart. Put simply, a re-entry circuit occurs when a conduction pathway is blocked or slowed down leading to incoming impulses being conducted through an accessory pathway (Gaztanaga et al. 2011). This can be by an anatomical or functional mechanism. The anatomical re-entry mechanism is based on a region of inexcitable tissue surrounded

by a circular pathway through which wavefronts can re-enter. In functional re-entry on the other hand the obstacle is not caused by inexcitable tissue but by heterogeneities in the electrophysiological properties of tissue. They do not have fixed pathways and are dynamic since cells are reexcited as soon as they recover from refractoriness. Functional re-entry can be promoted by a path of slowed conduction that allows sufficient delay for proximal refractory tissue to recover (Gaztanaga et al. 2011). By decreasing conduction velocity within the AV node we propose that bile acids can create re-entry circuits within the atrium. This could lead to the cases of atrial fibrillation that are observed. However, since we did not observe this in our model other triggers must be required along with PR prolongation to induce fibrillation.

In our study we showed that the effect of TC can be reversed with UDCA treatment. Interestingly, in a study of atrial fibrillation a significant increase was found in serum levels of non-UDCA bile acids and a decrease in conjugated UDCA levels compared to healthy subjects (Rainer et al. 2013). Among the intermediate steps by which this could lead to atrial fibrillation is the release of neurohumoral factors (Chan et al. 2011). These factors such as aldosterone and natriuretic peptides are raised in atrial fibrillation and can affect atrial remodelling (Chan et al. 2011; Mayyas et al. 2013). Adequate studies will need to be performed on the heart to evaluate this.

4.4.5 Potential mechanisms of UDCA inhibition of PR interval prolongation in fetal hearts

The aim in my thesis was to understand how UDCA protects against fetal arrhythmias in ICP. However to do this, it was necessary to first understand how toxic bile acids induce arrhythmias. From our results we propose that by inhibiting the T-type calcium channel subtype, TC is able to prolong the PR interval in fetal hearts. Since UDCA reversed the effect of TC, these results would suggest that UDCA might be able to directly modulate T-type calcium ion channels or intracellular calcium handling. However, further studies will need to be performed to establish an exact mechanism for this effect.

No published data was found on the binding affinities of bile acids on calcium channels expressed in the heart. Unconjugated TC, cholic acid, has been shown to reduce sympathetic ganglion neuronal N-

type calcium channel open probability (Lee et al. 2012). We initially speculated that TC binds directly to T-type calcium channels and that UDCA acts as a non-competitive inhibitor of TC at an alternate binding domain. Alternatively, UDCA might have stronger binding affinity but weaker blockade of the channel pore, and therefore not affect calcium entry and prolong PR interval. Although not demonstrated for the T-type subtype, there is evidence to show that interaction between agonists and antagonists of the L-type calcium channel can either be competitive or non-competitive depending on membrane voltage binding site occupancy (Zahradníková et al. 2007). We attempted to test this hypothesis using a radioligand binding assay to determine how both bile acids bind to calcium ion channels; however, we were unable to develop the assay due to difficulties with achieving ligand displacement and establishing a binding curve (results not shown). The duration of this project did not allow us to further test this hypothesis. Further studies will need to be performed to establish an exact mechanism for this effect.

4.4.6 Limitations

We observed good translation of effects in this study to clinical observations however a few limitations have to be highlighted. By using an isolated heart model we have not been able to assess the influence of the external autonomic nervous system (sympathetic and parasympathetic) innervation of the heart. Vagal nerves of the parasympathetic system innervate the sino-atrial and atrio-ventricular node as well as atrial muscle via muscarinic receptors (Klabunde 2004). This can lead to a reduction in conduction velocity and prolong PR interval. Therefore, in our model where this was absent any effects we observed are limited in their predictive value. Added to this, studies have shown that the intrinsic cardiac nervous system undergoes significant changes during postnatal development (Horackova et al. 2000). Since our model uses hearts from new-borns, there might be significant differences in the response observed in fetal hearts. The effects of bile acids might be more pronounced *in vivo*.

Our studies investigated the effects of bile acids over a few minutes however elevations in maternal bile acids in ICP can last for several weeks. Therefore our model only shows the effect of bile acids

under acute conditions. The fragility of neonatal rat hearts limits the duration for which perfusion can be performed before a significant deterioration is observed in cardiovascular function. The short treatment period might help to explain why no arrhythmias were observed beyond PR interval prolongation. The long-term toxicity of bile acids could not be determined in our studies and so it limits our understanding of how arrhythmias develop and progress in ICP.

A key limitation of the present study is that UDCA was only tested at one concentration when ideally it should have been evaluated at a range of concentrations. This is partly due to the limited amount of time that was available for this project, had more time been available a concentration curve would have been performed to determine the response and ideal inhibitory concentration of UDCA.

Finally, the process of isolation and cannulation of hearts can cause hearts to become ischemic. This can have a significant impact on the speed of impulse propagation through the heart even in the absence of drug treatment. Therefore effects on conduction slowing might only be more pronounced in this model. Also the impact of this on neonatal and adult hearts has not been assessed. However, since the neonatal heart has already been shown to be more sensitive to deterioration, it is potentially more sensitive to ischemia as well causing the more significant changes in PR interval with bile acids.

4.4.7 Future work

To date assessments of bile acid effects on the heart, including the present study, have only been performed in *in vitro* studies. To provide better translation to clinical effects and overcome the limitations already mentioned, assessments in an *in vivo* model will have to be performed. Data will especially be required from fetuses as they are likely to be more representative of clinical findings. Echocardiography studies using indirect indices of the ECG might be one way to assess cardiovascular parameters and overcome the difficulties of obtaining fetal ECGs. Better still more data needs to be obtained from ICP patients and fetuses to understand the nature of arrhythmias and their progression throughout the period of ICP and following resolution.

As already mentioned we only evaluated the acute effects of bile acids in this study. Therefore future work will need to focus on their chronic effects. Beyond their effect on ion channels and membrane potential, bile acids can also be cytotoxic resulting in damage to the membrane and cell death. As well blocking AV nodal calcium channels, TC might also cause injury to atrial tissue that could promote arrhythmias. One of the ways by which this could be assessed is in studies of neonatal rat hearts from pregnant rats fed with bile acid diets for prolonged periods during the third trimester. This model is currently used in mice but as we pointed out in the previous section evaluation of cardiovascular function in fetal or new-born mice can be difficult. A similar treatment in rats using our model could be an alternative.

As was pointed out, some of the effects on heart rate might be due to suppression of the sinus node by bile acids. Also PR interval prolongation in some cases is caused by a shift in the origin of the leading pacemaker within the heart. However, we were not able to assess these factors in this project. It will be interesting to determine how the location of the leading pacemaker might be affected with different concentrations and prolonged treatment of bile acids.

The binding affinity of TC and UDCA on calcium ion channels will need to be assessed in future studies. Due to problems with the radiolabelled verapamil (Verapamil Hydrochloride, [N-Methyl-3H]) we used for this study we were unable to perform a radioligand binding assay in this project. This was despite making a lot of effort to optimise the assay. To confirm that both agents act via a calcium channel mechanism, their receptor affinities could be evaluated using a radioligand binding assay or similar assay.

Finally, this study only assessed the effects of one bile acid, taurocholic acid, on cardiovascular parameters. The effects of other bile acids and their conjugated forms known to be elevated in ICP should also be assessed. This can be performed alone or in combination – in order to reflect conditions in ICP. Conjugated forms of UDCA, such as tauro-ursodeoxycholic acid (TUDCA) should also be assessed since UDCA is rapidly conjugated following oral absorption in the liver.

4.4.8 Conclusions

To conclude, we evaluated the effect of the bile acid, TC, on ECG PR interval and heart rate in a model of the fetal heart in ICP. Our results show a similar prolongation in PR interval and no changes in heart rate as are already observed in ICP. This conduction slowing effect was not seen in the maternal heart model at similar concentrations. More importantly we show that UDCA can inhibit the effects of TC. From investigating the mechanism of TC induced conduction slowing, we rejected our original hypothesis that the effects of TC were mediated via muscarinic receptors since this was not confirmed in this study with an agonist. However, we revealed a possible mechanism of conduction slowing by T-type calcium channel inhibition. However, this effect was also observed in the maternal heart and so it is unlikely to be the only pathway for PR interval prolongation. Further work will have to be performed to identify other mechanisms that might contribute to bile acid induced PR interval prolongation in ICP and how this might degenerate into sudden life threatening arrhythmias.

5 A Study of the Effect of UDCA on Bile-Acid Induced Fetal Ventricular Conduction Slowing

5.1 Introduction

Fetuses in Intrahepatic Cholestasis of Pregnancy (ICP) have an increased risk of pre-term delivery and intrauterine death (Kondrackiene et al. 2005). In previous decades, perinatal mortality cases have been as high as 10-15% of cases but these have improved more recently to about 3.5% of cases with active management (Reid et al. 1976; Geenes & Williamson 2009). A UK wide study of ICP patients demonstrated that the risk of preterm delivery and stillbirth increases with increasing maternal bile acid levels (Nelson-Piercy 2015). Preterm delivery was seen in 25% of cases with a 5 fold increased risk, while stillbirth occurred in 1.5% of cases and had a 2.6 fold increased risk compared to healthy patients. The causes of intrauterine death in ICP are poorly understood as they can occur suddenly without prior indications and even in cases where maternal bile acid levels have been lowered (Sentilhes et al. 2006). These also occur despite no significant symptoms in the mother beyond itching.

Therefore, it has become important to understand the toxic role of bile acids in the fetus and how the mother is protected. A number of possible mechanisms by which bile acids can lead to fetal toxicity have been proposed. Bile acids have been known to cause vasoconstriction of placental chorionic veins which can lead to reduced fetal blood flow and asphyxia (Sepulveda et al. 1991). In a rodent model of ICP, maternal cholestasis was shown to induce oxidative stress and apoptosis in the placenta (Perez et al. 2006). Similar non-specific morphological changes have also been reported in human patients (Geenes & Williamson 2009). Specifically in the heart, *in vitro* studies have shown that bile acids can cause calcium handling dysfunction in rat cardiomyocytes which can potentially lead to arrhythmias (Gorelik et al. 2002) and can cause a loss of synchronized beating of cardiomyocytes (Williamson et al. 2001).

In the previous chapter, we assessed the nature of atrial specific conduction slowing in Intrahepatic Cholestasis of Pregnancy (ICP) and the benefits of ursodeoxycholic acid (UDCA) treatment. This was based on a number of reports identifying arrhythmias with an atrial origin in ICP including atrial fibrillation and PR interval prolongation (Al Inizi et al. 2006; Strehlow et al. 2010). However, it was

interesting for us to observe that none of the reported cases of arrhythmia led to sudden death of the fetus. Atrial arrhythmias can cause discomfort and fatigue and lead to significant long-term complications; however, by comparison ventricular arrhythmias can be more life-threatening and can lead to sudden cardiac death (John et al. 2012; Koplan & Stevenson 2009). Similar conditions where bile acid levels are elevated such as in Primary Billiary Cholangitis have been associated with impaired ventricular contractility and electrophysiological changes such as QT interval prolongation (Mozos 2015) which can lead to the sudden life-threatening arrhythmia Torsades de Pointes. Therefore, since ventricular arrhythmias are potentially more life-threatening, this led us to investigate the toxic effects of bile acids and the antiarrhythmic effects of UDCA on ventricular function in our whole-heart models of ICP.

UDCA (Ursodiol®) serves as effective treatment for reducing maternal itching and restoring the bile acid profile. There is also some clinical evidence to suggest that it can reduce the risk of preterm labour, respiratory distress and hospitalization(Bacq et al. 2012). As with the aetiology of ICP, the mechanism of action of UDCA is not well understood in spite of its widespread use and safety. We sought to investigate the effects of the toxic bile acid, taurocholic acid (TC), on ventricular function and how UDCA might work to inhibit these effects. We initially hypothesised that UDCA can protect against bile acid induced arrhythmias by hyperpolarising the membrane potential of myofibrolasts. This section studied the effects of TC and UDCA on the electrocardiogram (ECG) QT interval and ventricular impulse propagation using optical mapping of electrical activity. Furthermore, with the use of standard agents it further attempted to understand the mechanism of UDCA's inhibition of conduction slowing in the fetal heart and identify why fetal and maternal hearts respond differently in ICP. A better understanding of the mechanism of action of UDCA could help to improve its application in ICP and reduce the incidence of sudden fetal death.

5.2 Materials and Methods

5.2.1 Materials

Stock solutions of nifedipine, UDCA and the voltage sensitive dye, Di-4-ANEPPS, were dissolved in 100% DMSO. These were serially diluted to ensure study concentrations contained no more than 0.01% DMSO. Lidocaine, mibefradil, taurocholic acid and verapamil were dissolved in purified water. When not used immediately, stock solutions were stored at -20°C until required. Perfusion (Tyrode's) and cardioplegic solutions were prepared as described in **Section 2.3**. Di-4-ANEPPS was used at final concentrations of 0.04 and 20 μM for optical mapping of adult and neonatal hearts respectively.

5.2.2 Protocol for immunostaining of heart sections

Sections from gestation day 18, one-day old neonatal rats, adult mouse hearts (normal and infarcted) and intestinal tissue (positive control) were stained for the presence of myofibroblasts (MFBs) to validate the hypothesis that UDCA produces its effects through these cells in the heart. Hearts were initially fixed in 15% then 30% Zamboni-glucose fixative solution. Following this, whole neonatal hearts or sections of adult hearts were flash frozen using optimal cutting temperature (OCT) fluid in isopentane. Fixed tissue was then cryosectioned into 5 μm thick slices on Superfrost Plus glass slides (ThermoFisher, UK) and stored at -80°C until they were stained with antibody against the MFB marker, alpha smooth muscle actinin (α-SMA) to detect the presence of MFB. Analysis was performed on a Nikon Eclipse TE200 epifluorescence microscope (Nikon, Japan) at 4 times magnification. The staining protocol was performed with the help of Dr Ivan Diakonov and Caroline Osobowale.

5.2.3 Isolation and cannulation of fetal and maternal models

The process of isolation and cannulation of hearts has been previously described. Please see section 2.3.2.

5.2.4 Electrocardiogram recordings

ICP is associated with sudden fetal death that has been linked to cardiac arrhythmias. In cirrhotic patients, with elevated bile acid levels similar to ICP, it has been observed that the QT interval, duration of impulse propagation through the ventricles, can be prolonged (Mozos 2015). Therefore, the effect of TC on QT interval prolongation was assessed in our models. Electrocardiogram (ECG) recordings were performed throughout the duration of treatment of FH and MH models. Recordings were performed as previously described in section 2.5.2.

5.2.5 Optical mapping recordings

Optimal mapping was performed using a MICAM Ultima camera (SciMedia, USA). The procedure for optical mapping is described in section 2.4.2. Using the same set-up, neonatal hearts were also stained with FLUO-4, a calcium sensitive dye, and changes in time to peak (time for calcium release from the sarcoplasmic reticulum via ryanodine receptors), amplitude (a measure of the peak intracellular calcium concentration during each contraction) and time to decay (time for reuptake of intracellular calcium into the sarcoplasmic reticulum via SERCA receptors) were measured.

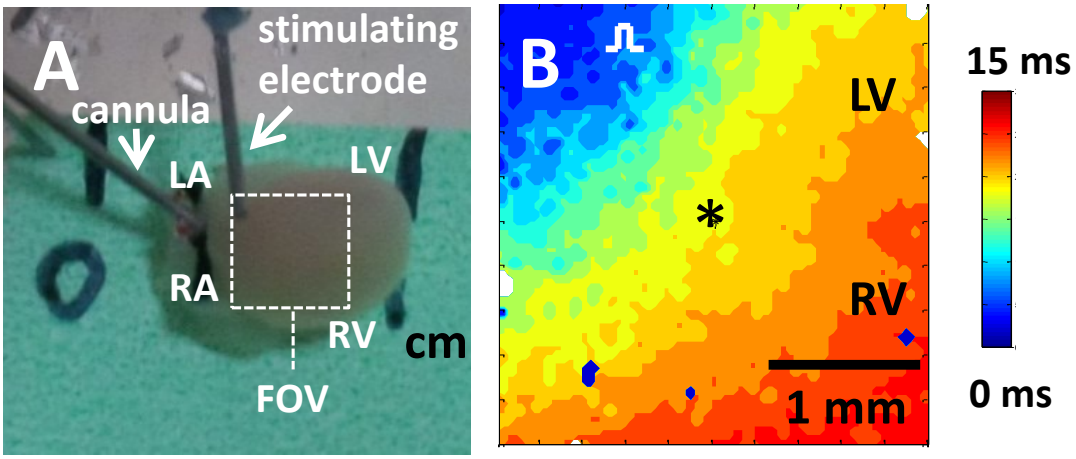


Figure 5-1 Optical mapping of fetal heart model.

A. *Image of cannulated fetal heart showing placement of stimulation electrode and anatomical regions.* ***B.*** *Reconstructed activation map with scale bar timescale. LA – Left Atria, LV – Left Ventricle, RA – Right Atrium, RV – Right Ventricle; FOV – Field of View; ms – milliseconds; π – point of stimulation.*

5.2.6 Experimental protocols for ECG recordings and optical mapping studies

Following isolation, neonatal hearts were divided into 4 groups (n=4-5 per group). The protocol for optical mapping and ECG recordings in the FH model is shown in Figure 5-2. Hearts were initially perfused with the vehicle solution (Tyrode's solution) for a 15 minute period to allow the preparation to stabilise and to obtain baseline recordings. This was followed by a further 15 minute perfusion period with either vehicle, taurocholic acid (TC) at a concentration of 400 µM or TC 400 µM with UDCA 1 µM treatments. Recordings were performed at the end of each 15 minute period.

For the MH model, hearts were divided into 2 groups (n=4 per group) as with the FH model, however, following the 15 minute equilibration period, hearts were treated with TC at a concentration of 400 µM for 15 minutes (Figure 5-3). In a separate group, hearts were treated with UDCA 1 µM for 15 minutes following a 15 minute baseline period. For optical mapping studies, 2 second recordings were performed at the end of each treatment period. While for ECG studies, recordings were performed throughout the treatment periods.



Groups	Baseline	Treatment
1.	Vehicle	Vehicle
2.	Vehicle	TC (400 µM)
3.	Vehicle	TC (400 µM) + UDCA (1 µM)
4.	Vehicle	UDCA (1 µM)

Figure 5-2 Treatment protocol.

The above protocol was used for the fetal heart model optical mapping studies. Hearts were divided into 4 groups and treated with vehicle for an initial 15 minute acclimatisation period followed by a further 15 minute drug treatment period. TC – taurocholic acid, UDCA – ursodeoxycholic acid. N=4-5 per group.

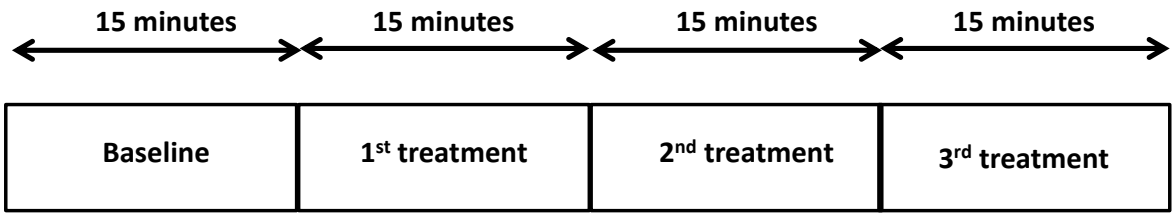


Figure 5-3 Maternal treatment protocol

The above protocol was used for the maternal heart model. Recordings were performed at the end of each treatment period. N=4-5 per group.

5.2.7 Whole-cell Patch-clamp in neonatal cardiomyocytes

The effect of vehicle, TC (100 µM) or TC (100 µM) plus UDCA (100 nM) on calcium channel amplitude were studied using the whole-cell patch-clamp technique. Lower drug concentrations were used in these studies compared to whole-heart studies since this analysis was performed in cardiomyocytes. Calcium currents from single cells were recorded using Axoclamp 200-B amplifier (Axon Instruments) and Clampex data acquisition software. L-type currents were recorded over 200 ms from a holding potential of -40 mV to a test potential of -45 to +60 mV in 5 mV increments. Current amplitude at 0 mV was taken as peak current. Results were analysed offline using Origin and Clampfit. Peak current amplitude for each cell was divided by cell capacitance and this value was termed calcium current density. Mean values were plotted as a current-voltage (I-V) relationship.

5.2.8 Protocol to measure PKA phosphorylation by Western blotting

Neonatal rat ventricular cardiomyocytes were used to evaluate bile acid induced phosphorylation of protein kinase A (PKA). The procedure for the Western blotting technique is as described in section 2.7. Specifically for this study, cells (~500,000 per dish) were either treated with vehicle, UDCA (1 µM) or TC (400 µM) for a period of 15 minutes. Following this, cells were centrifuged at 6000 rpm for 5 minutes to obtain a pellet. The supernatant was discarded and cell pellet stored at -80°C until protein measurement. The primary antibodies used – purified mouse anti-PKA RIIβ and purified mouse anti-PKA RIIβ (pS114) – were obtained from BD Biosciences, UK.

5.2.9 Statistical analysis

Conduction velocity and PR interval recordings were analysed and plotted using GraphPad Prism 5 software. Results are presented as percentage change from baseline values (mean ± SEM). Specific statistical analysis performed for each dataset is shown in the figure legends. p values less than 0.05 were considered to be statistically significant.

5.3 Results

5.3.1 Absence of myofibroblasts in fetal and maternal heart model immunostaining

Based on previous observations in human fetal hearts that showed an increase in MFB during the third trimester of pregnancy, we initially hypothesised that the ability of UDCA to inhibit conduction slowing in cardiomyocytes was mediated by hyperpolarisation of MFB membrane potential. We immunostained our models to confirm the presence of MFBs. Hearts from fetal, new-born and adult mice and rats were stained for the presence of α-SMA, a marker of MFB expression. Staining with α-SMA results in a bright golden stain.

We observed that outside of blood vessels (indicated by arrows), α-SMA was not found present in either fetal or new-born myocardial tissue (Figure 5-4A and B). A similar observation was found in heart sections taken from adult mice and rats (Figure 5-4C and E). To confirm our findings we evaluated MFB expression in infarcted hearts and rat intestines where MFBs are highly expressed. As expected positive controls (8 week myocardial infarction, MI, hearts and adult rat intestine) showed a high expression of α-SMA in both blood vessels and other regions (Figure 5-4D and F).

Based on this we rejected our initial hypothesis that the effects of UDCA in the ventricles were mediated by MFB expression.

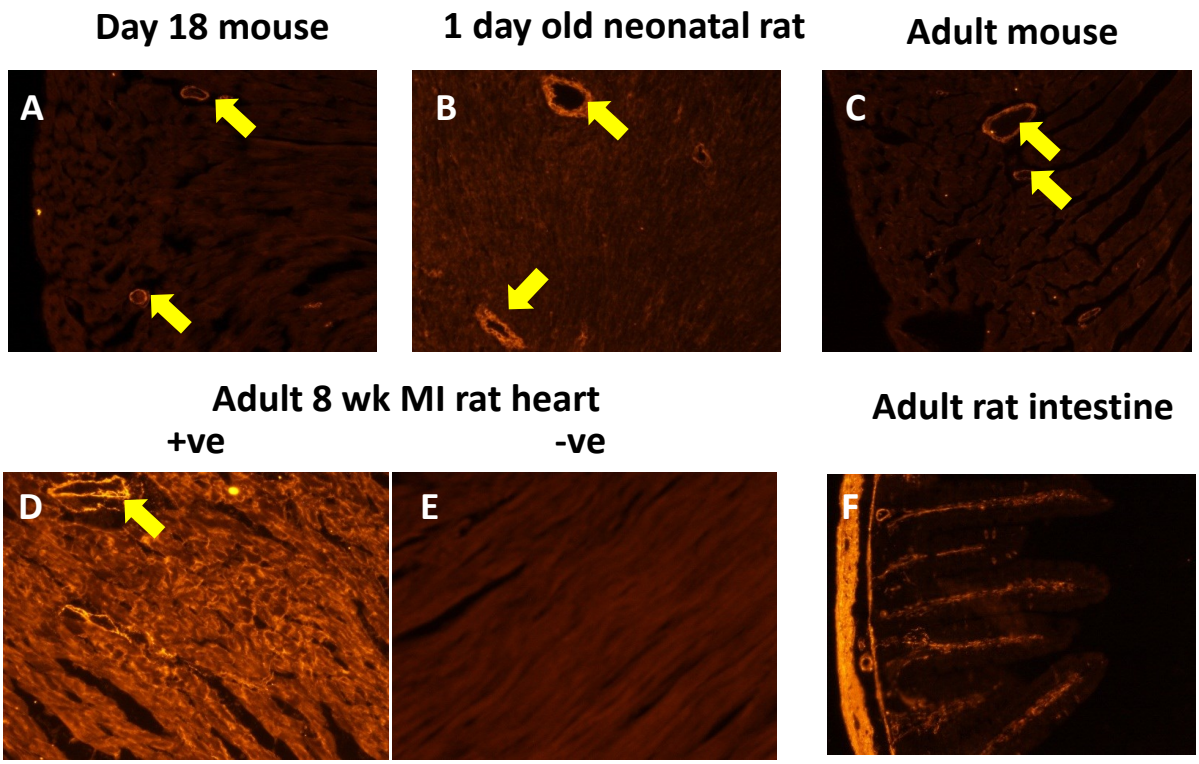


Figure 5-4 Images from immunostaining of heart cross sections.

Representative images of alpha-SMA staining (bright golden brown) from ***A.*** *Day 18 mouse heart* ***B.*** *1-day old neonatal rat heart* ***C.*** *Adult mouse heart* ***D.*** *Adult rat heart following 8 weeks of myocardial infarction* ***E.*** *Adult rat heart without infarction* ***F.*** *Adult rat intestine.*

5.3.2 TC does not prolong QT interval in the fetal heart model

Since sudden cardiac arrhythmias can be linked to prolongation of ventricular repolarisation, we performed ECG recordings of the QT interval in the FH model with TC treatment. TC did not prolong QT interval in the FH model (Figure 5-5). Treatment of the FH model with TC 400 µM did not produce a significant change in QT interval compared to control treated hearts ($p>0.05$). Percentage change from baseline values were -7.4±6.4% and -8.6±5.7% in control (N=4) and TC (N=5) treated groups respectively.

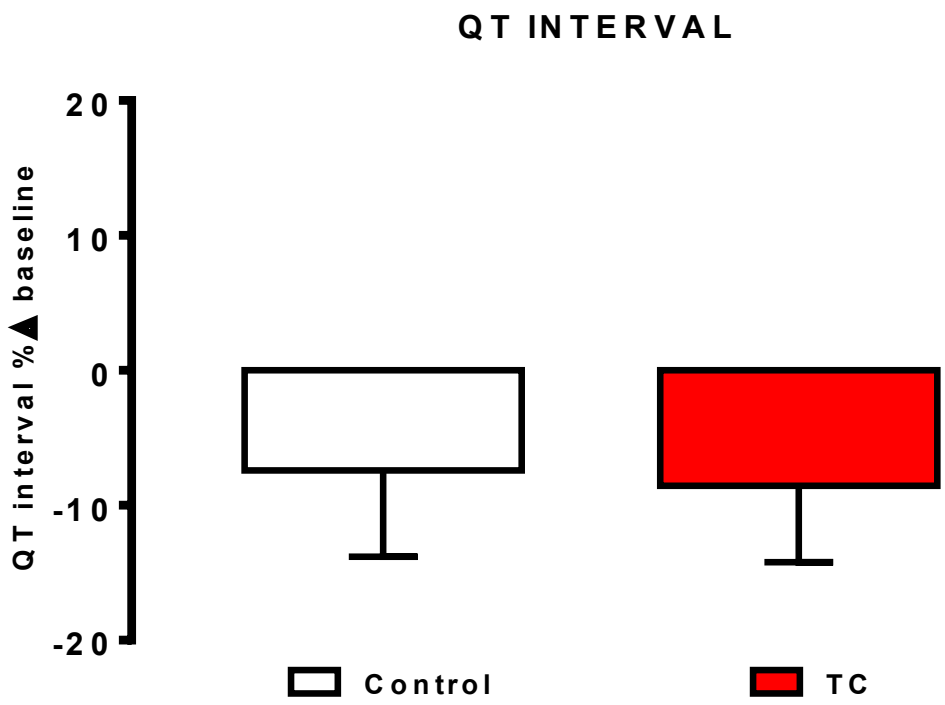


Figure 5-5 Effect of TC on ECG QT interval

Plot of mean % change from baseline values in ECG QT interval in control (N=4) and TC 400 µM (N=5) treated groups.

5.3.3 UDCA protects against ventricular conduction slowing induced by TC

Miragoli and colleagues showed in a cellular model of the fetal heart containing (cardiomyocytes and MFBs) that TC causes a slowing of conduction velocity (Miragoli et al. 2011). Therefore, we evaluated the effect of TC on conduction velocity in our FH model.

In optical mapping studies, significant declines in conduction velocity were observed in the ventricles of the FH model with TC 400 μM (see Figure 5-6). TC treatment caused a significant decline in conduction velocity (-38.9±5.1%; $p<0.05$) compared to hearts in the vehicle treated control group (10.5±2.1%). However, this effect was significantly inhibited when UDCA (1 μM) was added to the TC treatment (0.2±5.8%; $p>0.05$ compared to control group; $p<0.05$ compared to TC group). Consistent with its safe clinical profile, UDCA did not affect CV when administered alone (-0.2±5.8%; $p>0.05$). Representative activation maps are shown in Figure 5-6A. For plots of mean percentage change from baseline values see Figure 5-6B. These results translate well with previous studies in cellular models and confirm that TC can induce conduction slowing in the ventricles of fetal hearts.

Analysis of optical mapping action potential duration revealed no significant changes ($p>0.05$) in APD_{50} with TC (8.9±7.3%), TC plus UDCA (12.0±12.0%) or UDCA alone (-11.0±12.0%) compared to the control group (12.0±6.0%) (see Figure 5-6C). However, this might be due to the noisy nature of signals obtained from optical action potentials. Small but significant changes might be obscured by background noise. Separate studies using better techniques such as microelectrodes will have to be performed to accurately determine effects on APD.

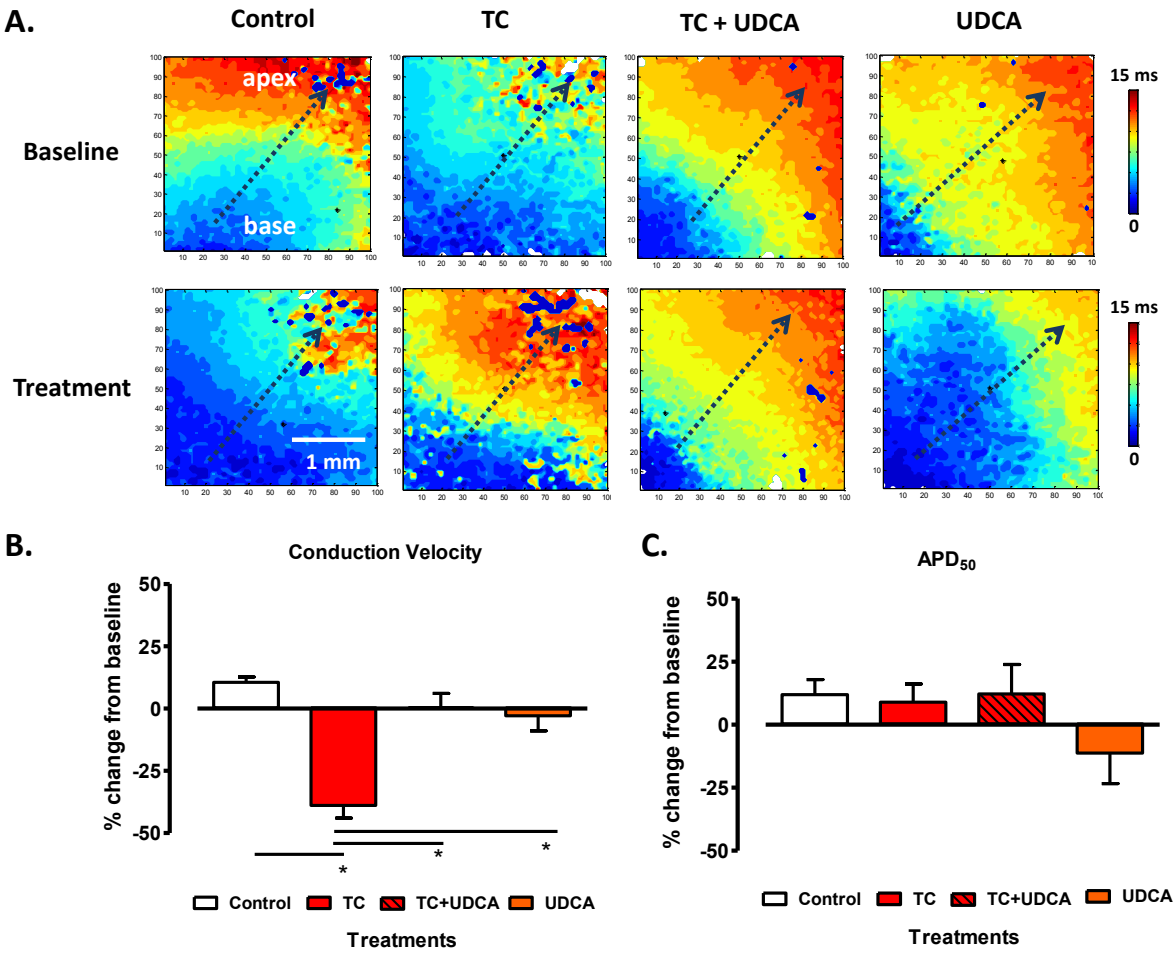


Figure 5-6 TC induced conduction velocity slowing in FH model is inhibited by UDCA

A. *Graph of mean % change from baseline values in conduction velocity following control, TC (400 µM), TC (400 µM) and UDCA (1 µM) and UDCA (1 µM).* ***B.*** *Reconstructed activation maps for each of the 4 treatment group with timescales and scale bar. Dotted arrows indicate direction of action potential propagation from point of stimulation. n=4 per group.1-way ANOVA with Dunnett's multiple comparison post-hoc test. N=4 per group * indicates statistically significant values.*

5.3.4 TC induces a smaller decline in conduction velocity in the MH model

In ICP, arrhythmias and sudden death can occur in fetuses despite no similar effect in the mother. Based on the effects in the FH model, we evaluated the effects of TC in the MH model to determine if it also induces changes in conduction velocity.

When the effect of TC was assessed in the MH model, only a modest decline in conduction velocity was observed (see Figure 5-7). At the same concentration, TC (400 μM) induced a significant decline in conduction velocity (-7.4±1.8%; $p<0.05$) compared to vehicle treated hearts (11±1.7%; $p<0.05$). Again UDCA alone did not significantly alter conduction velocity (6.7±4.4%) compared to the control group.

These results demonstrate that bile acids can induce significant effects in the ventricle. These results with TC in optical mapping studies demonstrate that the adverse effects of bile acids are more pronounced in the FH model compared to the maternal model. Similar observations to these are noted in ICP where cardiac complications only occur in the fetus and not the mother. It therefore suggested different mechanisms of impulse propagation in developing hearts compared to mature hearts which needed to be further investigated.

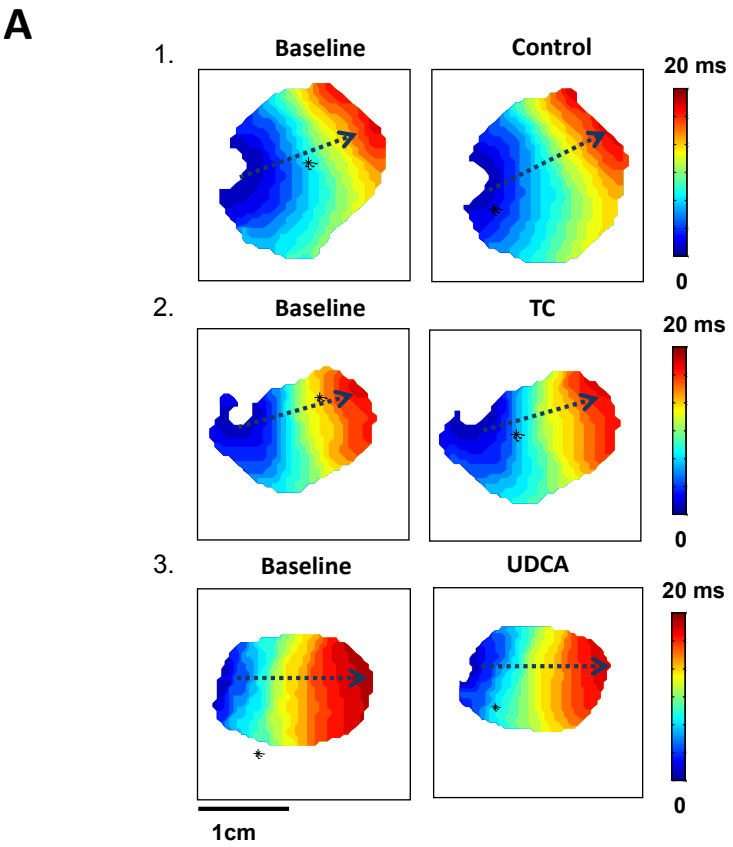


Figure 5-7 TC induces a small decline in conduction velocity in MH model.

A. *Graph of mean % change from baseline values in conduction velocity following vehicle, TC (400 µM) and UDCA (1 µM) treatments.* ***B.*** *Reconstructed activation maps for each of the 3 treatment group with timescales and scale bar. Dotted arrows indicate direction of action potential propagation from point of stimulation. n=4 per group. 1-way ANOVA with Dunnet's multiple comparison post-hoc test. * indicates statistically significant values.*

5.3.5 UDCA inhibits conduction slowing by calcium, but not sodium, channel blockers in the FH model

We then aimed to investigate the mechanism of conduction slowing by TC in the FH model and why this effect is less pronounced in the MH model. Impulse conduction in the heart is primarily mediated by sodium and calcium ion channels; however, other factors including the presence of gap junctions and the size of conducting fibres also play a significant role (Klabunde 2004). As such the speed of impulse conduction varies in different regions of the human heart being fastest in the purkinje fibres (~4m/sec) and slowest through the AV node (~0.05m/sec).

Therefore, to further investigate the mechanism of impulse propagation in the FH model, the effect of a sodium channel blocker, lidocaine 30 µM, was evaluated. Lidocaine treatment resulted in a significant decline in conduction velocity (-60.4±4.5%, $p<0.05$) compared to the control group (FIGURE 5-8). An effect that was not inhibited with UDCA co-treatment (-55.8±3.3%; $p<0.05$). This was then compared to that of a calcium channel blocker, verapamil 1 µM, to determine the contribution of calcium ion channels. When administered, verapamil caused a significant decline in conduction velocity compared to the vehicle treated control group (-20.2±11.0%; $P<0.05$). However, unlike with lidocaine, this effect was inhibited by a similar concentration of UDCA (7.8±7.4%).

These results demonstrate that the protective effects of UDCA are mediated by modulation of calcium and not sodium ion channels.

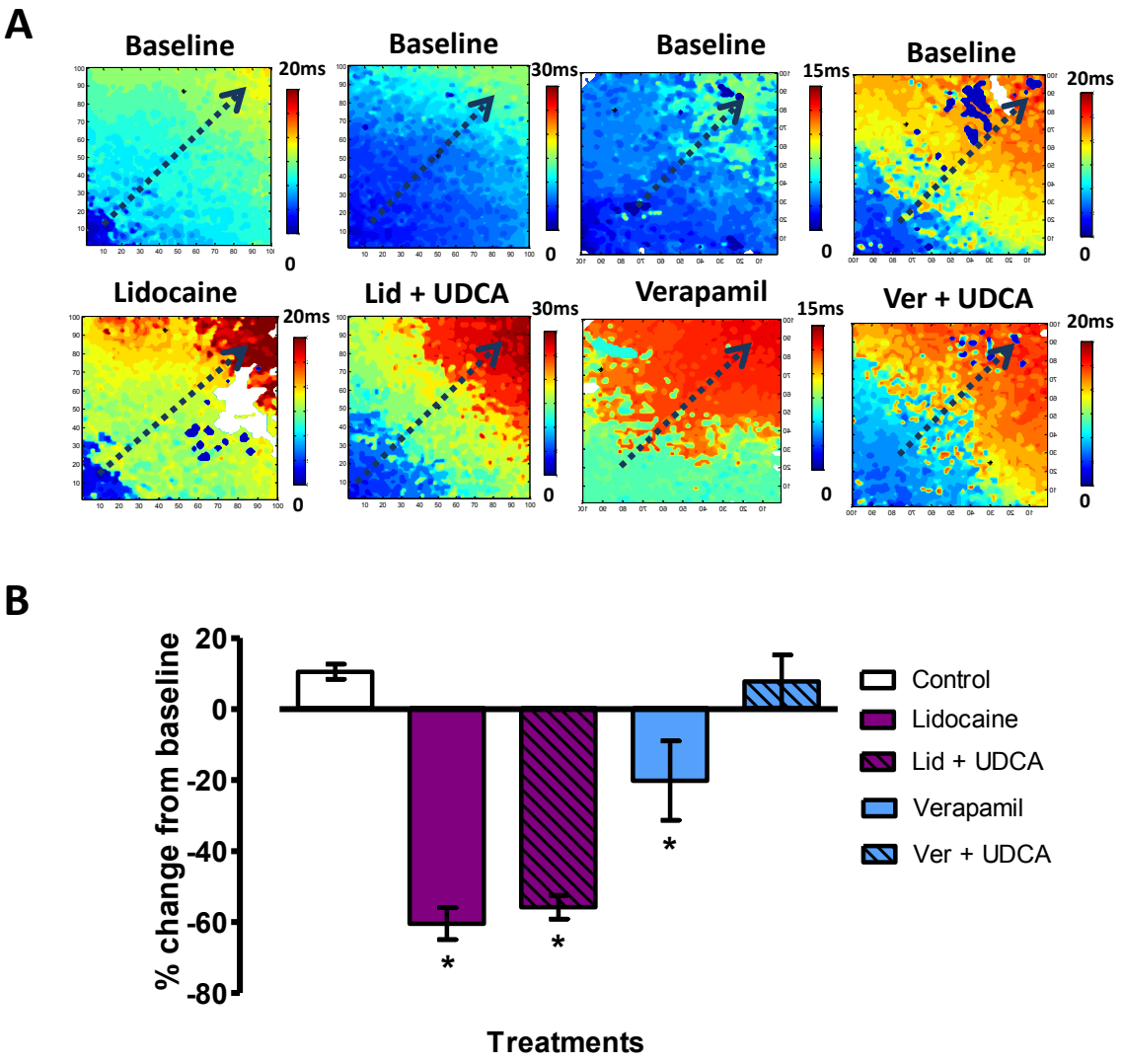


Figure 5-8 UDCA does not inhibit conduction slowing by the sodium channel blocker, lidocaine, but inhibits the effect of the calcium channel blocker, verapamil.

A. *Reconstructed activation maps following baseline (top row) and drug treatments (bottom row), showing timescales. Dotted arrows indicate direction of action potential propagation from point of stimulation.****B.*** *Graph of mean % change from baseline values in conduction velocity following vehicle, lidocaine (30 µM), lidocaine (30 µM)and UDCA (1 µM), verapamil (1 µM) and verapamil (1 µM) and UDCA (1 µM)treatments.n=4-5 per group. 1-way ANOVA with Dunnet's multiple comparison post-hoc test. * indicates statistically significant values. Lid – lidocaine, Ver – verapamil.*

5.3.6 Conduction slowing is not observed in the MH model with verapamil

Based on these results, it was inferred that the conduction slowing effects of TC and its inhibition by UDCA were due to modulation of calcium ion channels in the FH. However, it was still necessary to understand why the fetal heart is more sensitive than the maternal heart to the effects of TC and other bile acids in ICP. Lidocaine and other sodium channel blockers have been well established to reduce conduction velocity in the ventricles of adult hearts (Klabunde 2004), however, the effect of verapamil has not been well studied. Therefore, we compared our results in the FH model to the MH model.

Interestingly, application of verapamil at a similar concentration in the maternal heart model did not induce a significant decline in conduction velocity as was seen in the FH model (2.7±1.4% compared to Control group -1.7±4.1%; $p>0.05$). Representative activation models are shown in Figure 5-9A. Plots of mean percentage change from baseline values are shown in Figure 5-9B.

These results demonstrate that unlike in the FH model, calcium channel blockers do not play a significant role in impulse propagation in the MH model. It also helped to highlight a mechanism by which agents that block these channels can have a pronounced effect on conduction in the fetal heart but not in the maternal heart. From this we hypothesised that the effects of TC are mediated by blockade of calcium ion channels in the fetal heart and that UDCA can be protective by modulating these effects of TC at the channel. However such an effect must be mediated by blockade of a specific ion channel subtype.

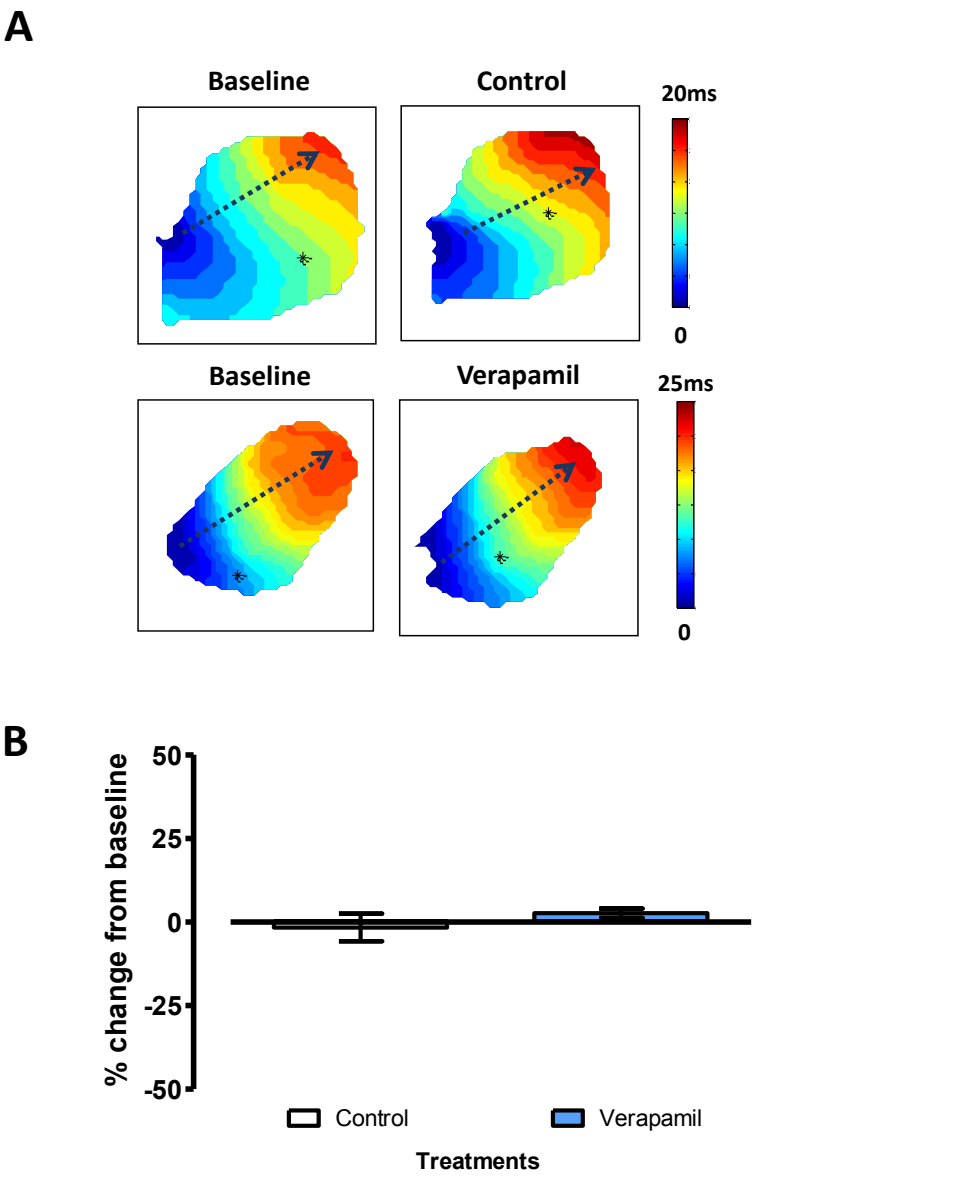


Figure 5-9 Verapamil does not induce conduction velocity slowing in MH model

A. *Graph of mean % change from baseline values in conduction velocity following vehicle and verapamil (1 µM) treatments in the MH model.* ***B.*** *Reconstructed activation maps for the two treatment groups with timescales. Dotted arrows indicate direction of action potential propagation from point of stimulation. n=3-6 per group. 1-way ANOVA with Dunnet's multiple comparison post-hoc test. * indicates statistically significant values.*

5.3.7 UDCA inhibits conduction slowing by a T-type calcium channel blocker, mibefradil

Calcium ion channels are present in both developing and mature hearts. However, in healthy adult hearts only L-type calcium channels are found expressed, but in fetal and developing hearts both L- and T-type calcium channels are present (Ono and Iijima, 2010). Only in disease conditions such as hypertrophy is the T-type channel found re-expressed in adult hearts. We previously evaluated verapamil, but since verapamil has poor selectivity for calcium ion channel subtypes, we evaluated the effect of the more selective agents - Mibefradil (T-type selective) and Nifedipine (L-type selective).

As shown in FIGURE 5-10, application of mibefradil (1 μM) resulted in a significant decline in conduction velocity compared to vehicle treatment (-23.0±6.2%; $P<0.05$). In contrast, with nifedipine (1 μM), there was no significant change in conduction velocity. Similar to TC, the effect of mibefradil could be inhibited by addition of UDCA 1μM (-0.5±4.9%; $P>0.05$). Representative activation models are shown in Figure 5-10A. Plots of mean percentage change from baseline values are shown in Figure 5-10B.

From this we were able to conclude that the impulse propagation through the ventricles of developing hearts is mediated by T-type calcium ion channels which are not found expressed in mature hearts. L-type calcium channels do not play a role in impulse propagation in developing or mature hearts. However, we still needed to confirm that TC and similar effects produce their effects by blockade of calcium ion channels.

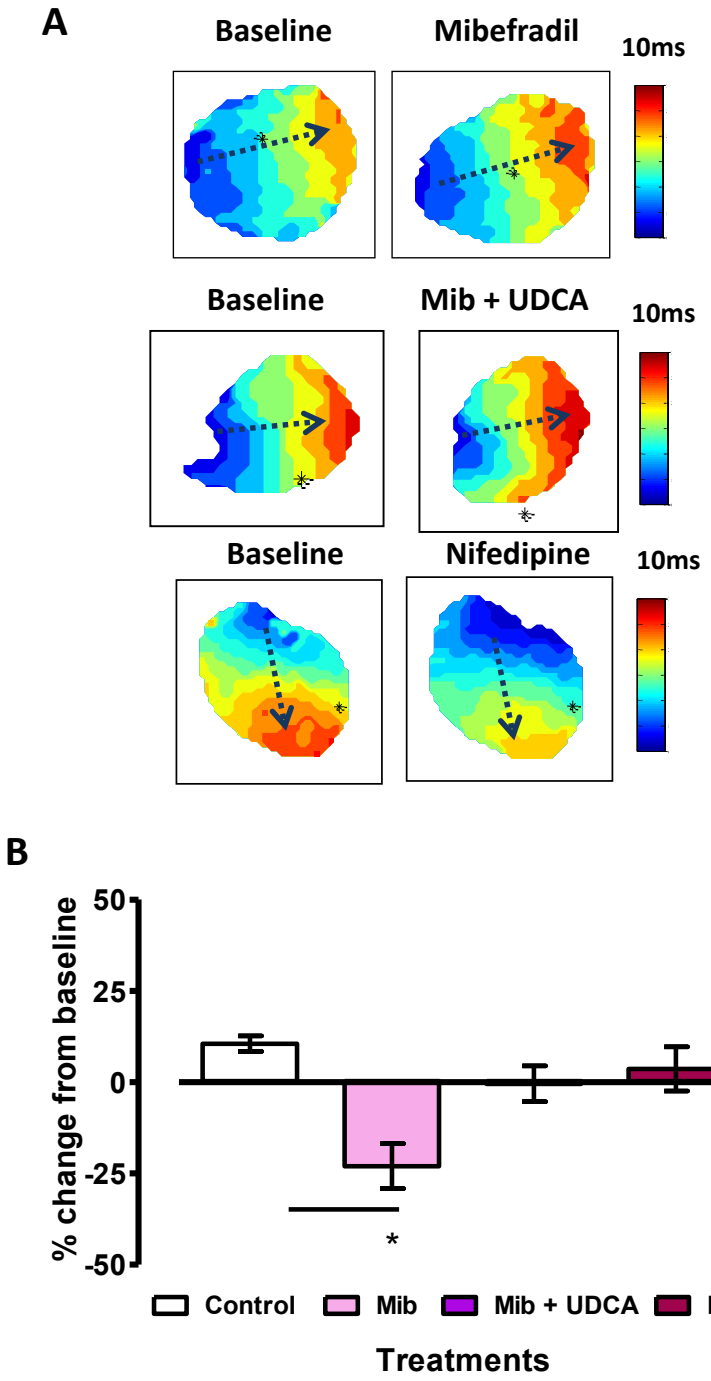


Figure 5-10 Conduction slowing only occurs with a T-type selective calcium channel blocker

Graph of mean % change from baseline values in conduction velocity following vehicle, mibefradil (µM), Mibefradil (1 µM) plus UDCA (1 µM) and nifedipine (1 µM) treatments in the FH model. ***B.*** *Reconstructed activation maps for each of the treatment groups with timescales. Dotted arrows indicate direction of action potential propagation from point of stimulation. n=4-6 per group. 1-way ANOVA with Dunnet's multiple comparison post-hoc test. * indicates statistically significant values.*

5.3.8 Taurocholic acid inhibition of L-type calcium current amplitude in neonatal cardiomyocytes is inhibited by UDCA

Two studies have previously shown that TC can act as an inhibitor of calcium ion currents. In a study in adult rat ventricular cardiomyocytes, TC decreased the slow inward calcium current (Binah et al. 1987). Using a microelectrode voltage clamp technique in a rabbit sinoatrial node preparation, TC produced dose-related decreases in calcium current (Kotake et al. 1989). Therefore we evaluated to see if similar effects are produced in neonatal rat ventricular cardiomyocytes using the whole-cell patch-clamp technique. The effects of TC were initially evaluated on the L-type calcium ion currents.

At a concentration of 100 µM, TC induced a significant decline in peak calcium current amplitude compared to vehicle treatment. TC caused an approximately 40% decline in L-type calcium current amplitude (-238.1 ± 54.69 pA versus Control group -424.8 ± 56.28 pA; $P<0.05$). As with the whole heart preparations, this effect was inhibited by co-administering UDCA100 nM (-319.4 ± 44.62 pA). Interestingly, although the effect was not significant UDCA appeared to produce a small decline in current amplitude alone. Representative current traces are shown in Figure 5-11A. Plots of mean normalised current trace values are shown in Figure 5-11B.

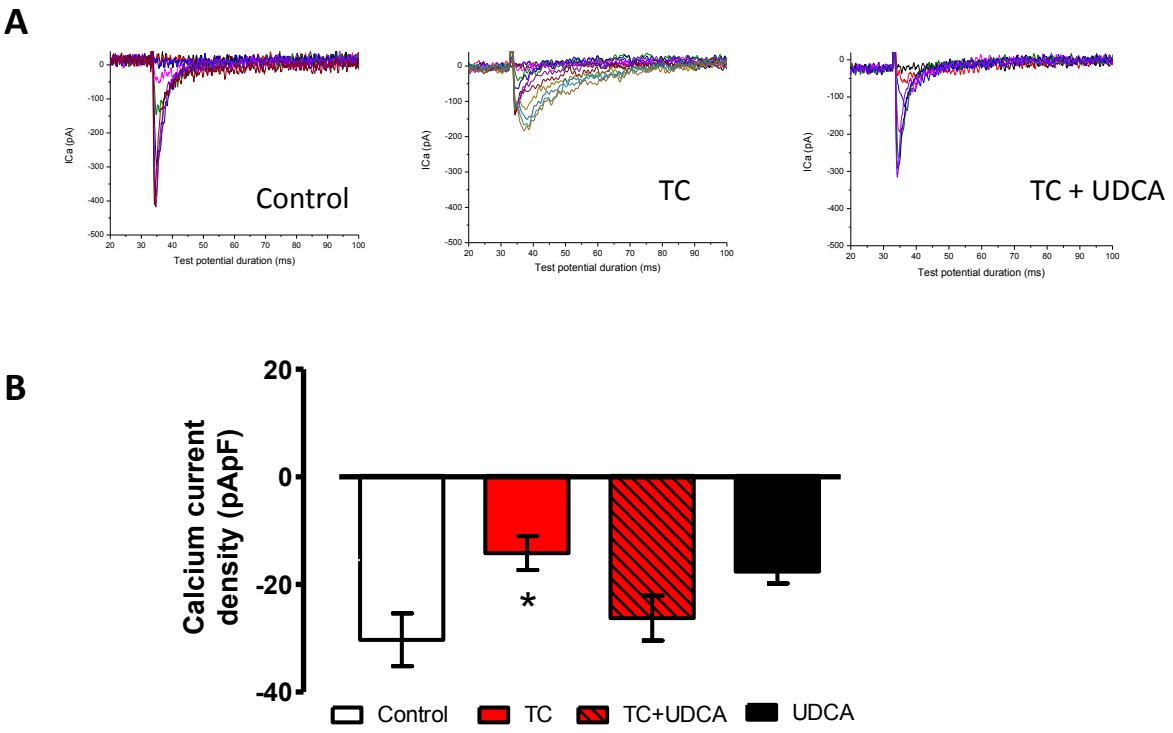


Figure 5-11 UDCA inhibits reduction in L-type calcium current amplitude by TC in neonatal cardiomyocytes.

A. *Reconstructed L-type current traces with control, TC (100 µM) and TC (100 µM) plus UDCA (1 µM) and UDCA alone (1 µM) treatments.* ***B.*** *Graph of mean normalized calcium current amplitude for each of the drug treatments. Currents to normalized to cell capacitance. (n=7-14 cells per group). Student's t-test. * indicates statistically significant value compared to control.*

5.3.9 Co-administration of TC and UDCA leads to an increase in T-type current amplitude in neonatal fetal cardiomyocytes

Although the effects of TC have been demonstrated on L-type currents, no studies have shown its effects on the T-type channel subtype. Therefore to confirm that the effects of TC were mediated specifically through the T-type subtype we also tested the effects of TC at this channel in neonatal rat cardiomyocytes.

TC 100 µM plus UDCA 100 nM co-treatment resulted in a statistically significant increase in current amplitude compared to controls (Figure 5-12A). Mean normalised calcium current density (pA/pF) values were -13.5± 1.8 for control (N=9), -9.9±0.9 for TC (N=11) and -22.3±1.2 for TC plus UDCA (N=10) treated groups. Mean normalised calcium current density (pA/pF) values in human fetal cardiomyocytes were -9.2±2.0 for control (N=9), -5.6±1.1 for TC (N=9) and -10±1.8 for TC plus UDCA (N=8) treated groups.

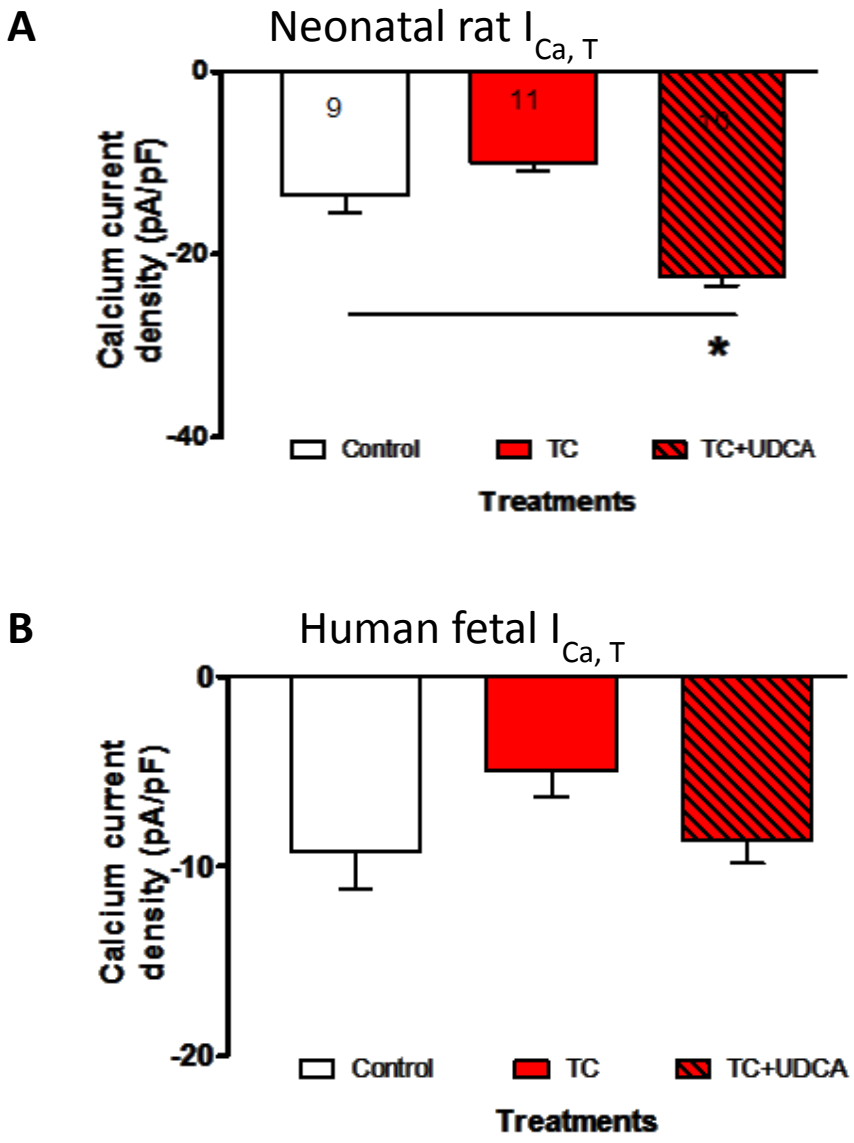


Figure 5-12 Effect of TC on T-type current amplitude in neonatal rat and human fetal cardiomyocytes

A *Normalized current density plots following in control, TC and TC plus UDCA treatments in neonatal rat cardiomyocytes (N=9-11 per group).* ***B*** *Normalized current density plots following in control, TC and TC plus UDCA treatments in human fetal cardiomyocytes (N=9-11 per group).* indicates statistically significant value compared to control group measured using Student's t-test*

5.3.10 Taurocholic acid and UDCA do not alter calcium transient parameters

Along with its effects on calcium channels, we aimed to better understand TC's effect on calcium channels and intracellular calcium handling and how UDCA modulates these effects in the FH model. *In vitro* studies have shown that TC treatment caused a reduction in calcium transient amplitude (Gorelik et al. 2002). Therefore, using optical mapping of hearts in the FH model stained with the fluorescent calcium binding dye, FLUO-4, we measured changes in the time to peak, amplitude and time to decay of calcium transients with TC and UDCA and compared them to the effects of the standard calcium channel blocker, verapamil.

Although TC and UDCA did not produce a significant change in Time to Peak, however a significant reduction ($p<0.05$) was observed with verapamil. Mean percentage change from baseline values were -9.4±3.7, 2.4±6.1, 6.9±7.6 and -39±4.0% for Control, UDCA, TC and verapamil treatments respectively. No significant changes were observed in the Time to Decay with all treatments ($p>0.05$). Mean percentage change from baseline values were -0.7±4.5, -2.0±1.1, -2.7±3.0 and -1.9±6.1% for Control, UDCA, TC and verapamil treatments respectively. Although a large decline was observed with verapamil, no significant changes were observed in the amplitude of calcium transients with any of the treatments ($p>0.05$). Mean percentage change from baseline values were -5.2±12.0, -9.6±12.0, -9.9±3.2 and -40.0±6.1% for Control, UDCA, TC and verapamil treatments respectively. Figure 5-13 shows mean percentage change from baseline plots of calcium transient parameters – Time to Decay (A), Amplitude (B) and Time to Peak (C).

In the absence of data from radioligand binding studies to determine if TC binds directly to calcium ion channels, we compared its effects on calcium handling compared to a standard calcium channel blocker, verapamil. From these results we identified a potential difference in how TC can modulate calcium channel function compared to standard agents.

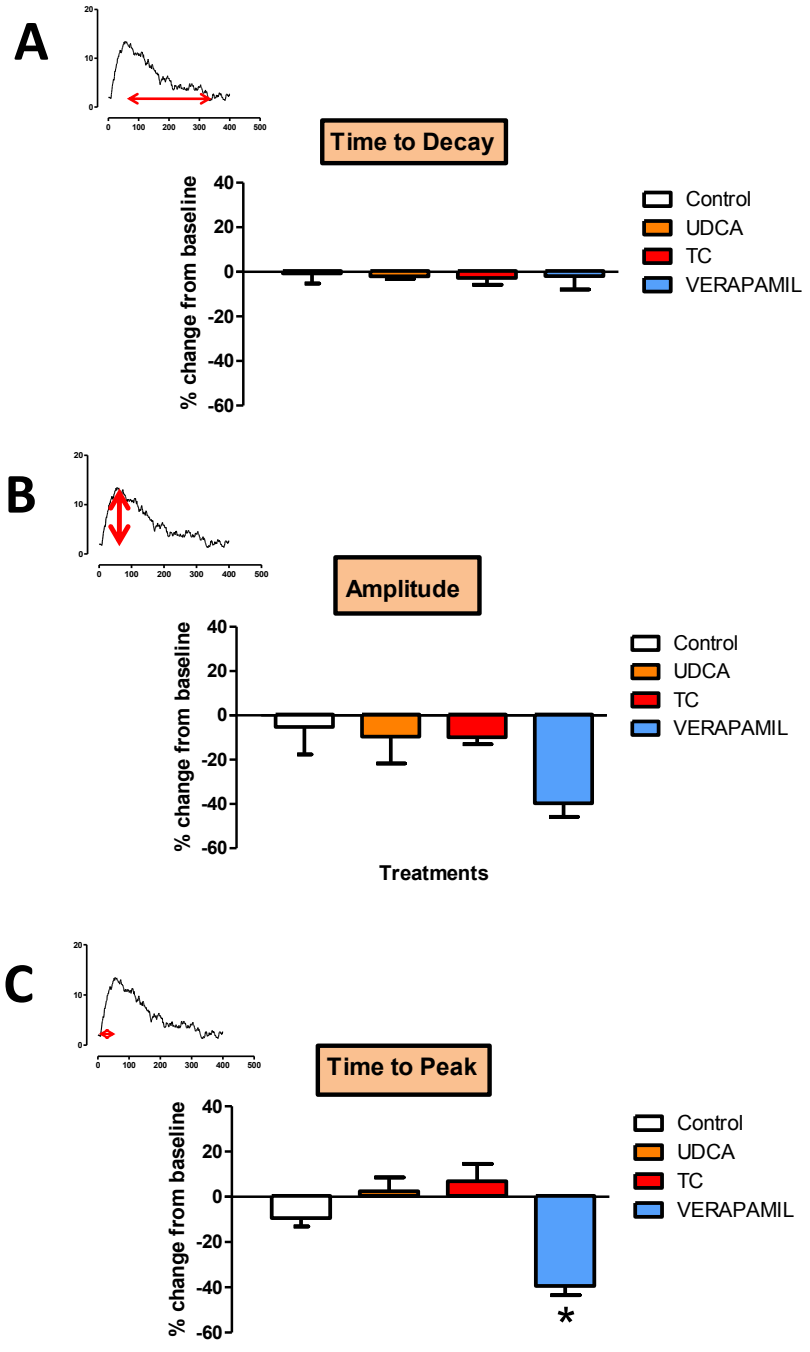


Figure 5-13 Effect on calcium transient parameters of TC, UDCA and verapamil

Effects of control, UDCA, TC and verapamil on ***A.*** *Time to Decay* ***B.*** *Amplitude* ***C.*** *Time to peak in FH model. Values are mean percentage change from baseline values.* indicates statistically significant values compared to the control group using One way ANOVA with Dunnett's multiple comparison test*

N=3-6 per group

5.3.11 Taurocholic acid and UDCA both induce phosphorylation of protein kinase A

We also investigated how UDCA can modulate the effect of TC on calcium ion channels. One of the properties of ion channels is that they can be modulated by intracellular second messengers leading to modifications in channel structure and function (Ismailov & Benos 1995). This can lead to either an increase or a decrease in current entry through the channel. Cardiac calcium ion channel function is particularly known to be modulated G-protein coupled receptors leading to increases or decreases in heart rate, conduction velocity and contractility among other parameters (Kamp & Hell 2000). One of the intermediary pathways by which this can occur is phosphorylation of amino acid residues by protein kinases such as Protein Kinase A (PKA) (Kamp & Hell 2000). PKA phosphorylation can lead to channel opening and increase calcium entry into the cell.

Therefore we evaluated the effect of UDCA on PKA phosphorylation and compared this to the effects of TC and other calcium ion channel blockers. We observed that even though UDCA induces PKA phosphorylation, TC also induces a similar effect. However, vehicle treated cells and selective calcium channel blockers (mibefradil and nifedipine) did not induce PKA phosphorylation (see Figure 5-14).

Although, these results identified a potential molecular mechanism by which UDCA modulates calcium channel function, it did not provide a conclusive pathway that is different from the effects of TC on calcium ion channels. Further studies will be required to determine how these bile acids induce PKA phosphorylation.

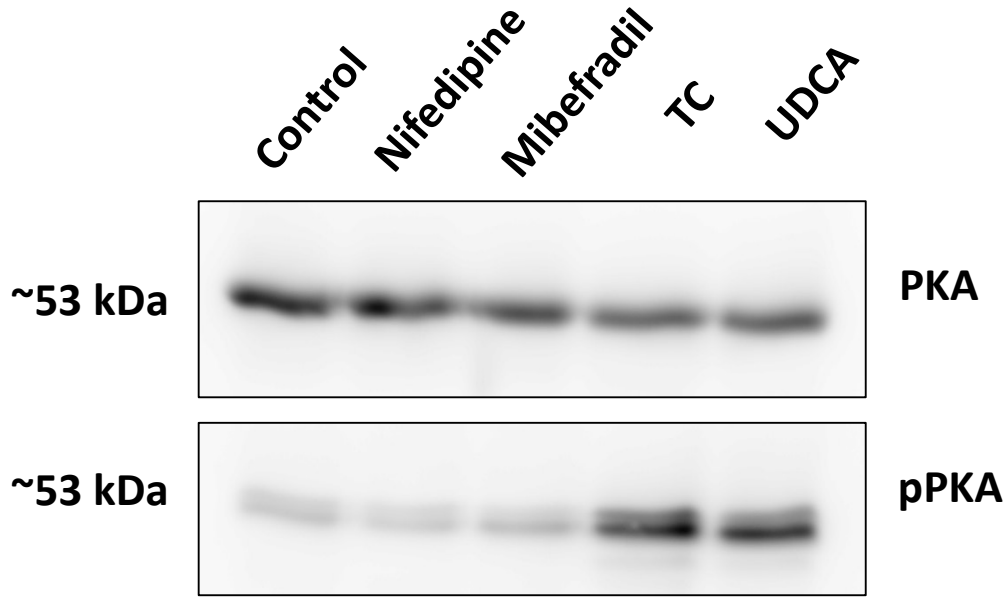


Figure 5-14 TC and UDCA phosphorylate PKA in neonatal cardiomyocytes

Representative Western blot images with total PKA activity (top row) and phosphorylated PKA (pPKA) (bottom row) activity following Control, Nifedipine, Mibefradil, TC and UDCA treatments. Bands were only observed with TC and UDCA treatment.

5.4 Discussion

In this section, potential mechanisms by which bile acids can cause sudden fetal death in ICP triggered by ventricular arrhythmias were evaluated. It also evaluated the effects of the frontline treatment, UDCA, and its mechanism of action. We demonstrated that the bile acid, TC, can cause significant slowing of impulse propagation in the ventricular myocardium which was inhibited by co-treatment with UDCA. Using standard agents, the mechanism of impulse slowing was shown to be by blockade of calcium ion channels and in particular the T-type calcium channel subtype which is highly expressed in fetal hearts but not adult hearts. As with standard agents, TC was shown to reduce the amplitude of calcium currents in cardiomyocytes; however, unlike standard agents it did not affect the amplitude of calcium transients in a whole-heart model. UDCA was shown to potentially modulate effects at calcium channels.

5.4.1 TC induced fetal arrhythmias is not linked to a higher expression of myofibroblasts

In studying heart sections from human fetal hearts during development, Miragoli and colleagues observed that there is a higher expression of MFBs during the third trimester that coincides with the period of ICP (Miragoli et al. 2011). This was shown to decline following birth and to be absent in adult hearts. Based on this finding the authors constructed a cellular model of the fetal heart containing both cardiomyocytes and MFBs and an adult model containing only cardiomyocytes for evaluating the effects of bile acids (Miragoli et al. 2011). Conduction slowing was observed in the fetal model with TC which was attributed to the greater expression of MFBs. Therefore, we initially hypothesised that the protective effects of UDCA will also be mediated through MFBs in our models.

In developing our whole-heart models we compared MFB expression in third trimester fetal hearts, new-born hearts and adult hearts for the expression of MFBs but we did not find any MFBs to be present in any of the models. Therefore, even though conduction slowing was also observed in our models this could not be attributable to effects mediated by MFBs and led us to reject the hypothesis. A possible explanation for the discrepancy between the two findings might be attributable to species

differences. Our staining was performed in mouse fetal hearts while the observations by Miragoli were in human fetal hearts. Another plausible explanation might be the process of isolation and culturing of cardiomyocytes which has been shown to change the phenotype of fibroblasts, leading to a greater proportion of MFBs in cell cultures due to hypoxic conditions (Baum & Duffy 2011). Added to this, following infarction a higher amount of MFBs are found present in the heart (Chen & Frangogiannis 2013). Therefore it can be assumed that the observation in human fetuses was triggered by hypoxia during abortion and is not a normal phenotype of the heart.

5.4.2 TC induced conduction slowing can trigger fatal arrhythmias in fetal hearts

TC treatment caused significant conduction slowing in the ventricles of the FH model as observed with optical mapping. Despite the conduction slowing effects observed in optical mapping studies, we did not observe any significant changes in ECG QT interval. This discrepancy can be explained by the fact that the QT interval comprises both the duration of ventricular depolarisation and repolarisation. Due to limitations in the sampling rate and background noise with our recording equipment, we were not able to accurately measure the QRS duration which defines only the period of ventricular depolarisation. We should point out that we did not correct QT interval for changes in heart rate. Although a formula for QT correction in rats has been proposed, it is not reliable at the lower heart rates observed in this model (Kmecova & Klimas 2010). Also since no changes were observed in heart rate in both groups this would not have been required.

The effects of TC on conduction velocity are similar to the effects observed in the cellular model by Miragoli and colleagues (Miragoli et al. 2011). By demonstrating a conduction slowing effect with TC in the ventricles, we demonstrate a mechanism by which bile acids can trigger fatal arrhythmias in fetuses. This can be facilitated by the development of re-entry circuits in the ventricles. When re-entry circuits are limited to a particular region (local re-entry) i.e. the atria or ventricles it can lead to atrial or ventricular tachycardia (Klabunde 2004). However, when the circuit is between the atria and ventricles (global re-entry) it can result in supraventricular tachycardias such as has been reported in

ICP (Geenes & Williamson 2009). Rather than impulses being terminated in the ventricles they end up being conducted through the AV node or accessory pathways and re-exciting atrial tissue. These types of arrhythmias can have a sudden onset and can also disappear suddenly, which is similar to what is reported in fetuses in ICP.

Re-entry occurs when the electrical wave propagating through the ventricles breaks locally and forms a rotor; the propagating outward waves from a rotor can break up further to develop new rotors and resulting in ventricular fibrillation (Weiss et al. 2005). Although a number of conditions are required for re-entry circuits to develop, by slowing impulse conduction bile acids provide an important substrate for the breakup of electrical waves into smaller wavelets. Ventricular fibrillation has been the most common cause of sudden cardiac death while atrial fibrillation is the most prevalent clinical arrhythmia (Weiss et al. 2005; Boukens et al. 2015) However, unlike atrial fibrillation which might not be readily life-threatening, ventricular fibrillation is particularly life-threatening since it results in cardiac output being reduced to zero causing tissue and organ hypoperfusion (Klabunde 2004). A review of reports on arrhythmias in ICP identified that all the cases with ECG recordings are from fetuses that survived. No case reports were found with ECG recordings from intrauterine death patients. Based on our data and in the absence of ECG recordings from fatal cases, ventricular fibrillation is a very likely cause of the sudden intrauterine deaths that are reported.

5.4.3 A high expression of the T-type calcium channel subtype predisposes fetal hearts to bile acid induced conduction slowing

It is interesting to observe that only the fetus in ICP develops significant arrhythmias while the maternal heart appears to be protected. We observed a similar pattern in our models as TC induced significant conduction slowing in the FH model with a much smaller effect in the MH model. These findings would suggest that there are fundamental differences in the effects of bile acids on fetal hearts compared to maternal hearts. We were able to demonstrate that the conduction slowing effects of bile acids were mediated by blockade of the T-type calcium channel subtype and that the

protective effects of UDCA were via the same receptor. Although bile acids have previously been shown to bind to calcium ions (Gleeson et al. 1990; Baruch et al. 1991) and block calcium ion channels *in vitro* (Kotake et al. 1989; Binah et al. 1987), this was the first time that the specific role of calcium channel subtypes in fetal arrhythmia development in whole hearts has been demonstrated.

There are two calcium channel subtypes present in the cardiomyocytes namely the L- and T-type subtypes (Ono & Iijima 2010). The L-type subtype is found expressed in all regions of both fetal and adult hearts. T-type calcium ion channels are known to be expressed in fetal hearts, but there is a decline in their expression during development (Leuranguer et al. 2000b; Cribbs et al. 2001). They are completely absent in the adult ventricular myocardium where their expression is limited to pacemaker tissue with a critical role in pacemaker function (Hagiwara et al. 1988; Zhang et al. 2013). They occur as two predominant isoforms – Cav3.1 and Cav3.2 – in the heart. T-type channels are found re-expressed in adult hearts only in cardiac conditions such as hypertrophy (Ono & Iijima 2010). It has been suggested that T-type channels contribute to the development of pressure-overload induced hypertrophy in mice (Chiang et al. 2009). However, little is known about the role of T-type channels in the ventricle of developing hearts. Studies have supported a contribution to calcium signalling, proliferation and growth of the heart (Senatore et al. 2012; Vassort et al. 2006). Calcium channels do not affect conduction velocity in adult hearts under normal conditions and might only contribute when there is reduced excitability (sodium channel function) or decreased gap junction coupling (Shaw & Rudy 1997). We show here that calcium channels and in particular the t-type subtype are important for impulse propagation in developing hearts.

5.4.4 UDCA is protective in a whole-heart model of ICP

In the treatment of ICP, UDCA under such brand names as Urso® and Ursodiol®, is widely used as frontline treatment for the restoration of the maternal bile acid profile and can potentially reduce fetal complications including pre-term delivery and intrauterine death (Palma et al. 1997). Although UDCA is safe clinically its mechanism of action is not well known and any potential benefits on

improving fetal arrhythmias have not been assessed. We show that UDCA protects against bile acid induced conduction slowing while not producing any negative effects on its own in a whole-heart model of ICP. This is consistent with findings by Miragoli and colleagues, where UDCA co-treatment protects against conduction slowing induced by TC in a cellular model of the fetal heart (Miragoli et al. 2011). The safety of UDCA has been reported in several clinical studies and the absence of arrhythmic effects has also been reported *in vitro* in human atrial preparations (Rainer et al. 2013).

We demonstrate here that UDCA is able to inhibit TC induced conduction slowing and this was shown to be by modulation of T-type channels. Therefore, it was necessary to identify a mechanism for UDCA's effect. The activity of ion channels can be regulated in a number of ways including changes in membrane voltage, extracellular ligands, and G-protein coupled receptor second messenger phosphorylation (Wickman & Clapham 1995). Among the enzymes involved in phosphorylation are protein kinases including protein kinase A (PKA). PKA has been widely shown to up-regulate L-type channel activity (Kamp & Hell 2000; Bunemann et al. 1999) with a fewer number of studies also showing that it upregulates T-type calcium currents in different cell types including frog atrial cardiomyocytes (Zhang et al. 2013). This project demonstrated PKA phosphorylation by UDCA in neonatal rat ventricular cardiomyocytes. We propose this as one of the mechanisms by which UDCA is able to inhibit the effects of TC; however, it is unlikely to be the only mechanism since similar phosphorylation was also seen with TC. Further studies will be required to determine if PKA phosphorylation is mediated by activation of cardiac G-protein coupled receptors such as the bile acid receptor, TGR5.

An alternate mechanism by which UDCA might be protective is stimulation of calcium release from intracellular stores. In hamster hepatocytes, UDCA induced an increase in intracellular free calcium (Bouscarel et al. 1993). Similarly, UDCA and its conjugate TUDCA have been shown to stimulate calcium entry through store-operated calcium channels and activate calcium entry in rat liver cells (Aromataris et al. 2008). Although, UDCA administered alone did not stimulate an increase in T-type

calcium currents in patch clamp studies, it caused a significant increase with TC co-treatment in neonatal cardiomyocytes. We were not able to demonstrate a significant increase in intracellular calcium (calcium transient amplitude) in our heart models with UDCA treatment alone using optical mapping of calcium transients, however we did not evaluate the effect of both agents combined.

5.4.5 Limitations of the study

Our neonatal whole-heart model was limited in a number of ways for evaluating sudden fetal arrhythmias in ICP. One obvious limitation is the short duration of the study. Maternal bile acid levels in ICP can be elevated over a period of several weeks; however we only evaluated their effects over a much shorter fifteen minute period. Therefore the outcomes observed in this study can only be used to predict acute effects of bile acids.

By using an isolated-perfused heart preparation the study did not assess the effect of bile acids on nervous innervation of the heart which can be significant. There is evidence to support endogenous synthesis and a role for bile acids in the central nervous system (Mano et al. 2004; Ogundare et al. 2010). Bile acids through the G-protein coupled receptor, TGR5, can activate sensory nerves and stimulate the release of neuropeptides in the spinal cord (Alemi et al. 2013). Bile acids acting via autonomic innervation can modulate cellular responses to liver injury (Fava et al. 2007). Little is known about this interaction specifically in the heart, however, an *in vivo* study on rat hearts demonstrated that bile acids reduce the effect of isoprenaline and invert the effect of adrenaline (Kadlubowski et al. 1984).

We were also only able to evaluate the effects of one bile acid, TC, in this study. Conjugated and unconjugated forms of other bile acids might produce greater effects on the heart. For example, a comparison of the effects of deoxycholic acid (DCA), lithocholic acid (LCA) and cholic acid (CA) in rats found that DCA produced greater hepatotoxic effects compared to LCA, with TC producing the least (Delzenne et al. 1992). Other authors have also shown that LCA is more hydrophobic than other bile acids and have proposed that this can cause it to be more cytotoxic than TC (Williamson & Geenes

2014). Furthermore, taurine conjugated LCA is even more cytotoxic than the unconjugated form at lower concentrations in the bile duct (Benedetti et al. 1997).

5.4.6 Future work

Despite demonstrating a protective effect with UDCA, the present study did not clearly define an intracellular mechanism by which it inhibits the conduction slowing effects of TC. Therefore, future work within our group will aim to delineate this mechanism. This will include an assessment of the role of bile acids on PKA and other calcium channel regulatory enzymes such as protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase (CAMK). The effects of bile acids and other inhibitors on enzyme phosphorylation will need to be assessed using western blotting. The physiological effects on cardiomyocytes also need to be assessed in patch clamp studies and other functional assays. In these studies it will also be important to evaluate the role of the G-protein coupled bile acid receptor, TGR5, in *ex vivo* and *in vivo* models of the fetal heart.

Although we initially aimed to gather clinical data from ICP patients we were not able to gather enough samples during the period of this project. This included data from ECGs and Doppler recordings from fetuses and mothers under healthy and diseased conditions. It will be important to continue gathering and evaluating this data from fetuses to allow a better understanding of arrhythmia development and sudden death.

Finally, as has already been mentioned in the study limitations, it will be necessary to compare the results observed in this *ex vivo* model with those of an *in vivo* model. In such a model, the conduction slowing effects can also be assessed with chronic administration of bile acids. Bile acid-induced cardiotoxic effects can also be determined from heart slices in these studies with the use of immunostaining.

5.4.7 Conclusions

In the present study we demonstrated that UDCA is able to protect against bile acid induced ventricular arrhythmias in a fetal whole-heart model of ICP. Although we did not observe any arrhythmias in this study, the results support our alternate hypothesis that conduction slowing by TC and the inhibition of this effect by UDCA were mediated by modulation of calcium ion channels. In particular the results presented here demonstrate that both bile acids produce their effects via the T-type calcium ion channel subtype. However, further studies will be required to determine the exact intracellular and molecular mechanisms by which UDCA is protective.

6 A Pilot Study to Evaluate the Anti-Arrhythmic Properties of UDCA in Ischemia-Reperfusion Injury

6.1 Introduction

So far this thesis has focused on evaluating the antiarrhythmic properties of UDCA for the treatment of fetal arrhythmias caused by elevated maternal serum bile acids, with the aim of improving and expanding its clinical application. Beyond liver disorders, potential applications for UDCA have also been proposed in other cardiac pathologies from studies performed in human and animal models. This includes improvements in vasodilatation in coronary heart disease patients by UDCA treatment (Sinisalo et al. 1999) and improvement in peripheral blood flow in chronic heart failure patients (Von Haehling et al. 2012). UDCA can potentially reduce the incidents of heart transplant rejection. A retrospective study found that cholestasis patients undergoing UDCA treatment during cardiac transplantation had a lower incidence of acute rejection episodes following transplantation compared to controls (Bahrle et al. 1998). This has also been reported in a rat model of heart transplantation (Olausson et al. 1992). Since bile acids form the end products of cholesterol breakdown, a number of studies have evaluated possible benefits for UDCA in atherosclerosis. Although a study reported improvements in cholesterol levels in Primary Biliary Cholangitis (PBC) patients (Poupon et al. 1993), others have not reported such a benefit in non-PBC patients (Woollett et al. 2003).

Of interest to our group were reports highlighting beneficial effects for UDCA in myocardial infarction. Previous studies in liver diseases had shown that UDCA and its taurine conjugate, TUDCA, can protect against cell apoptosis (Benz et al. 2000; Paumgartner & Beuers 2002; Rodrigues et al. 2003). More recently studies of myocardial infarction in rats demonstrated that TUDCA treatment can reduce the size of infarcts compared to controls (Rivard et al. 2007). In studies of ischemia-reperfusion (IR) injury in rat hearts, Lee and colleagues demonstrated that UDCA can improve time to contraction (time for the heart to start contracting again once perfusion was started again) and reduce lactate dehydrogenase (a marker of tissue damage) compared to controls (Lee et al. 1999). In a separate study, intravenous administration of UDCA prior to ligation of the left anterior descending coronary artery can reduce the size of infarcts in rat hearts following ischemia and reduce the release of pro-

apoptotic markers (Rajesh et al. 2005). Based on this evidence we aimed to determine if UDCA can also protect against arrhythmias associated with IR challenge. During the process of reperfusion following ischemia, cardiomyocyte death can occur due to a number of factors. Ischemia can result in accumulation of intracellular sodium and calcium ions, resulting in acidosis (Turer & Hill 2010), while reperfusion can in turn cause rapid alterations in ion flux and mitochondrial membrane depolarization resulting in enhanced cytotoxicity. Although the actual mechanism by which this leads to arrhythmia is still being understood, a number of possible mechanisms have been proposed. One of these is the increased inhomogeneity in action potential duration in and around the ischemic zone (Wit & Janse 2001). Other factors include gap junction uncoupling and membrane depolarisation in the ischemic border zone (Wit & Janse 2001).

This section of the thesis evaluates data obtained from a pilot study designed to determine if UDCA can protect against arrhythmias, such as ventricular fibrillation, which are observed during reperfusion following cardiac ischemia. The need for better therapeutic approaches of IR injury has been highlighted by other authors (Rouschop & Leemans 2008; Hausenloy & Yellon 2013). As a safe drug that has already been clinically approved, we hypothesised that UDCA can prevent arrhythmias. This is based on our previous evidence that UDCA is able to inhibit conduction slowing by hyperpolarising myofibroblast (MFB) membrane potential in a cellular model of the fetal heart (Miragoli et al. 2011). This is further supported by the observations that serum UDCA levels, compared to those of other bile acids, are significantly lower in atrial fibrillation patients (Rainer et al. 2013). We hypothesised that by hyperpolarising MFB, which are re-expressed during ischemia, it can also protect against reperfusion-induced arrhythmias. Due to difficulties throughout the period of the project in developing a chronic ischemia model, only an isolated-perfused global ischemia-reperfusion heart model was assessed. The effects of UDCA were assessed on arrhythmia development using optical mapping and ECG techniques. The results demonstrated no beneficial effects with the use the drug in this model; however, further studies are required to understand the mechanism of this negative finding and to assess the benefits of UDCA in other models of ischemia.

6.2 Materials and Methods

6.2.1 Animals

All work was carried out according to standards set out in the UK Animals (Scientific Procedures) Act 1986. Adult male Sprague-Dawley rats (~150-400g) were either purchased from Harlan UK, Charles River UK or were kind gifts from Phil Rawson of the Central Biomedical Services, Imperial College, London.

6.2.2 Materials

Stock solutions of UDCA and the voltage sensitive dye, Di-4-ANEPPS, were dissolved in 100% DMSO. These were serially diluted to ensure study concentrations contained no more than 0.01% DMSO. When not used immediately, stock solutions were stored at -20°C until required. Perfusion (Tyrode's) and cardioplegic solutions were prepared as described in **Section 2.3**. Di-4-ANEPPS was used at a final concentration 20 µM for optical mapping of hearts.

6.2.3 Experimental protocol

6.2.3.1 Procedure for isolation and cannulation of hearts

The process of isolation and cannulation of hearts has previously been described. Please see section 2.3.2.

6.2.3.2 Optical mapping procedure

The procedure for optical mapping studies is as described. Please see section 2.4.2.

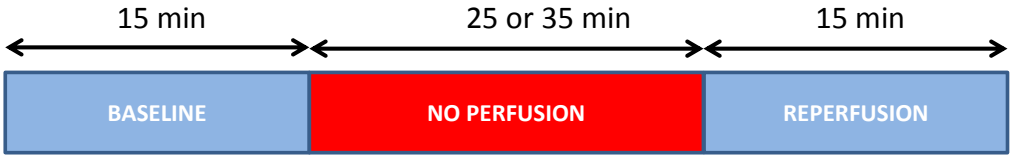
6.2.3.3 ECG recording procedure

The electrocardiogram recording process has previously been described. Please see section 2.5.2.

6.2.3.4 Ischemia-reperfusion protocol

The anti-arrhythmic properties of UDCA were evaluated in an acute model of cardiac global ischemia-reperfusion. Hearts were initially perfused with either vehicle (Control group) or UDCA (1µM) in

Tyrode's solution for a 15 minute period to obtain a baseline recording and allow hearts to acclimatise (see Figure 6-1). Following this, perfusion was stopped for a period of 35 minutes to induce ischemia before being restarted for a further 15 minutes. Based on the outcome of this study a shorter ischemia period of 25 minutes was also evaluated. The durations of ischemia were chosen based on data showing significant induction of infarction in the myocardium with these durations of ischemia (Palmer et al. 2004; Rossello et al. 2015). Hearts were superfused in a chamber to keep the temperature at 37°C throughout the period of ischemia. Optical recording was performed at the end of the baseline period and again at the end of the reperfusion period. ECG recordings were performed throughout the study duration. For specific sample sizes see sections below.



Groups	Treatment	Treatment	Treatment
Control	Vehicle*	None	Vehicle
Treatment	UDCA	None	UDCA

*** Tyrode's solution**

Figure 6-1 Ischemia-Reperfusion protocol

The above protocol was used for acute global ischemia-reperfusion model. Following a baseline perfusion period, perfusion was stopped to induce ischemia and then perfusion of hearts was continued for a further period.

6.2.4 Statistical analysis

Conduction velocity was analysed using BV analyse and MatLab software and plotted using GraphPad Prism 5 software. Results are presented as absolute or percentage change values (mean ± SEM). Statistical analysis was carried out comparing treatment to control using 1-way ANOVA and Bonferroni's post hoc test. p values less than 0.05 were considered to be statistically significant.

6.3 Results

6.3.1 UDCA treatment does not improve conduction velocity following 35 mins ischemia

The effect of UDCA 1 µM was initially assessed in a model of 35 minute global ischemia (perfusion stopped for 35 minute period). A number of the hearts developed ventricular fibrillation (VF) in each group following reperfusion. Representative ECG traces from the Control and UDCA groups are shown in Figure 6-2A. The percentage of hearts that developed fibrillation were 20% in the Control group (1 of 5 hearts) and 57% in the UDCA group (4 of 7) (see Figure 6-2B). No significant changes ($p>0.05$) were observed in CV in the UDCA treated group compared to the vehicle treated group. The total sample size from which CV could be calculated was reduced in both groups due to the induction of VF in some hearts tested. As shown in Figure 6-3B, percentage change from baseline values in CV were -21±12% in the Control group (mean from N=4 of 5 treated hearts) and -2.8±6.3% in the UDCA treated group (N=3 of 7 treated hearts). These results demonstrated no improvement in arrhythmia development with UDCA 1 µM treatment.

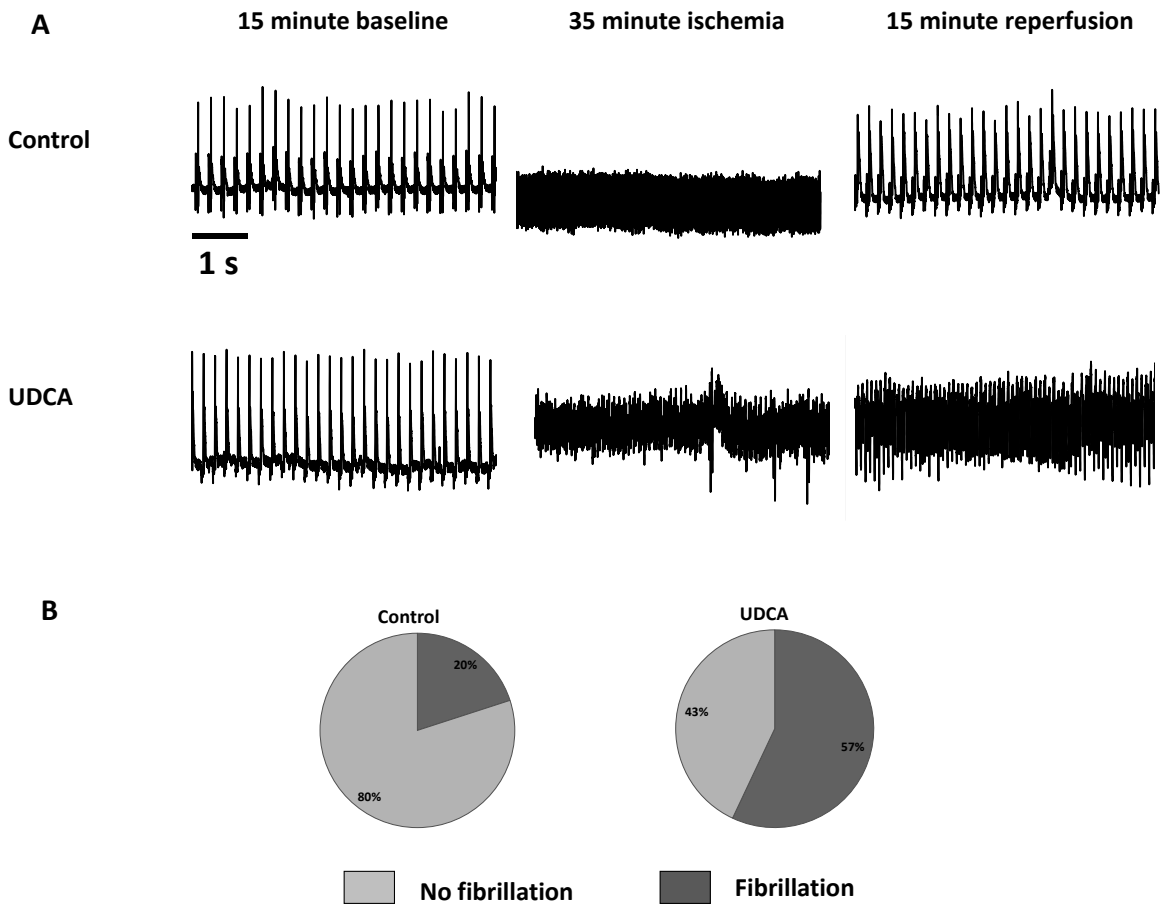


Figure 6-2 Induction of ventricular fibrillation following UDCA treatment

A *Representative ECG traces from control and UDCA treated groups in the 35 minute ischemia protocol demonstrating induction of fibrillation following reperfusion of hearts.* ***B*** *Pie charts showing proportion of hearts that developed VF in Control (N=5) and UDCA (N=7) groups following 35 minutes of ischemia.*

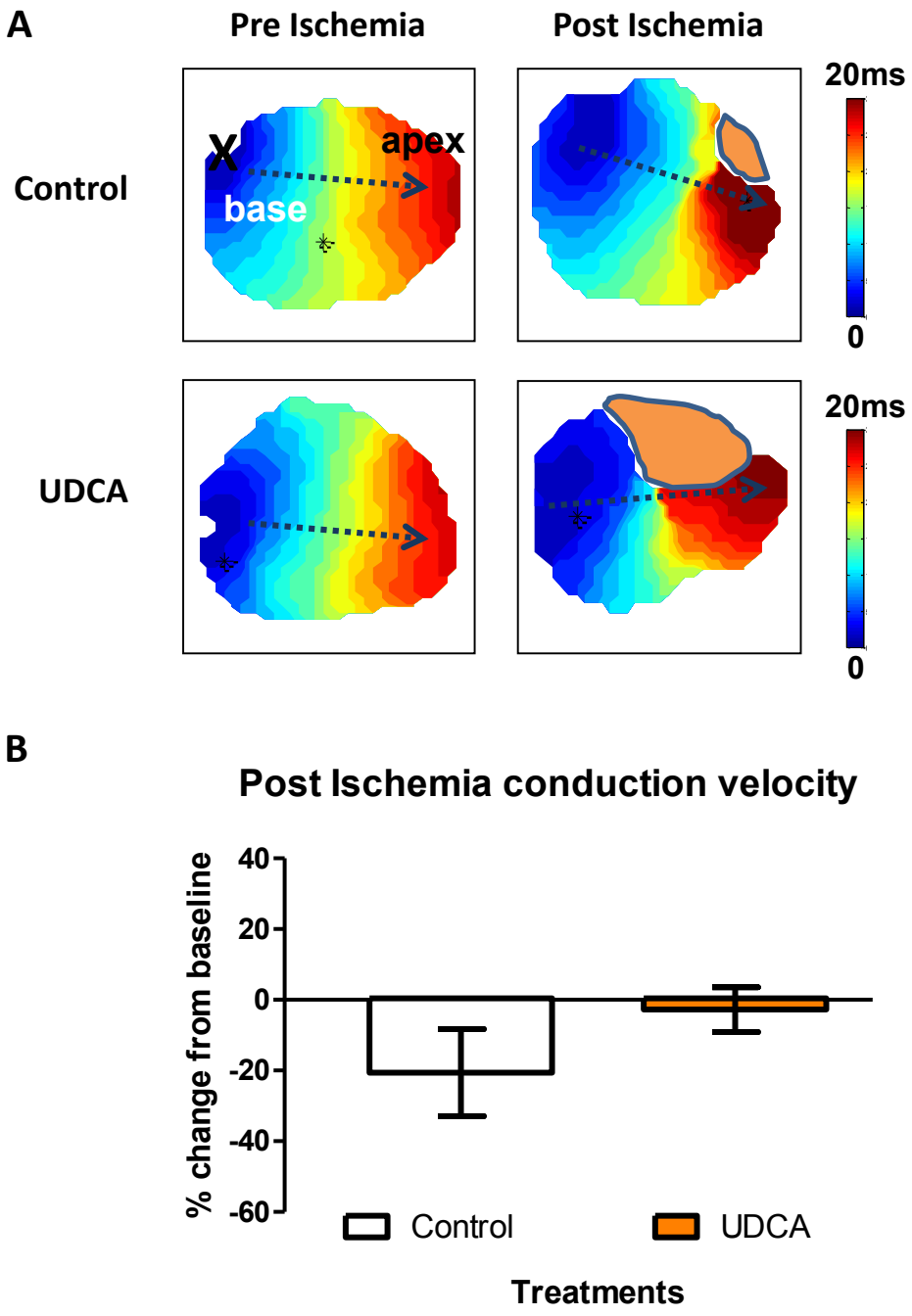


Figure 6-3 Effect of UDCA treatment on CV following 35 minutes of ischemia

A *Representative activation maps (with multi-colour timescale bar) at the end of the baseline period (pre-ischemia) and reperfusion (post-ischemia) periods from Control and UDCA treatment groups. X indicates point of stimulation.* ***B*** *Mean percentage change in CV from baseline plots in Control (N=4) and UDCA (N=3) treatment groups.*

6.3.2 UDCA treatment does not improve conduction velocity following 25 mins ischemia

Since no improvements in CV were observed with 35 minute ischemia, the duration of ischemia was shortened to 25 minutes and the effects of a higher concentration of UDCA evaluated. As with the 35 minute protocol, no significant changes were observed in CV from baseline in the UDCA (5 μM) treated group compared to the vehicle treated group following 25 minutes of ischemia. Similar to the longer protocol, the total sample size from which CV could be calculated was also reduced in both groups due to the induction of ventricular fibrillation (VF). Representative reconstructed activation maps are shown in Figure 6-4A. As shown in Figure 6-4B, percentage change from baseline values in CV were -3.5±13% in the Control group (mean from N=3 of 4 treated hearts) and -14±11% in the UDCA treated group (N=2 of 5 treated hearts). Percentage of hearts that developed VF were 25% in the Control group and 60% in the UDCA group (see Figure 6-4C).

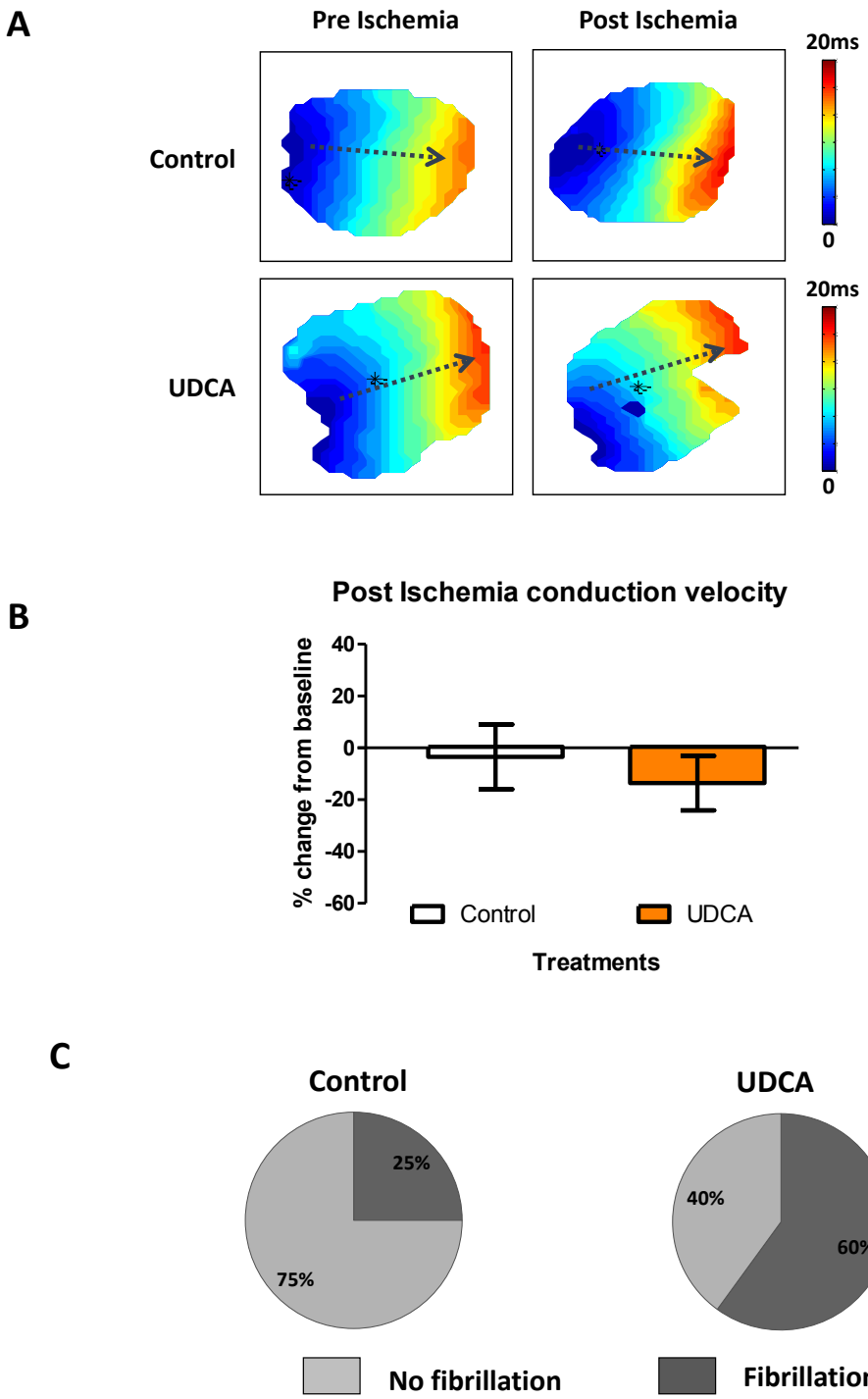


Figure 6-4 Effect of UDCA treatment on conduction velocity following 25 minutes of ischemia

A *Representative activation maps (with multi-colour timescale bar) at the end of the baseline period (pre-ischemia) and reperfusion (post-ischemia) periods from Control (N=3) and UDCA (N=2) treatment groups.* ***B*** *Mean percentage change in CV from baseline plots in Control and UDCA treatment groups.* ***C*** *Pie charts showing proportion of hearts that developed VF in Control and UDCA groups following 25 minutes of ischemia.*

6.4 Discussion

The aim in this section was to identify a possible protective anti-arrhythmic benefit for UDCA against IR induced arrhythmias. Due to the limited duration of this project, this work served as a pilot study for more thorough future investigations by our group to further assess the mechanism of UDCA's action. The results demonstrate that compared to vehicle treatment UDCA did not improve conduction velocity or reduce the incidence of ventricular fibrillation in an acute model of global ischemia. Indeed, a higher percentage of hearts were observed to develop fibrillation when treated with UDCA compared to vehicle treatment. Beneficial effects were not observed even with a higher concentration of UDCA. To our knowledge, this is the first time that the benefits of UDCA on electrophysiological parameters in acute IR injury have been assessed.

6.4.1 Administration of UDCA does not protect against IR arrhythmias

The absence of a protective effect with UDCA treatment was an interesting observation as other authors have proposed that UDCA can protect against myocardial damage following ischemia. It has been shown that UDCA can reduce the size of infarcts following ischemia (Rajesh et al. 2005) and enhance recovery of cardiac contractile function during reperfusion in an isolated-perfused model of IR injury (Lee et al. 1999).

In the absence of mechanistic studies in our models, the mechanism of UDCA's effects can only be speculated. The study by Rajesh and colleagues did not highlight cases of arrhythmia. It was performed in anaesthetised rats with intravenous injection of UDCA; therefore it is difficult to compare this to our model. Similarly, it only assessed the effects of UDCA on ischemia induced in a small region of the heart as opposed to the global ischemia applied in this study. In contrast, Lee and colleagues used a similar model of the isolated-perfused heart for assessing the effects of UDCA in IR injury. Compared to the study by Lee and colleagues, this study applied much lower concentrations of UDCA (1-5 µM versus 20-160 µM). In any case, it is unlikely to explain the higher incidence of arrhythmias compared to vehicle treatment. Another difference between the 2 studies is the difference in the duration of

reperfusion following ischemia. This study reperfused hearts for 15 minutes compared to the 30 minute period used in the study by Lee and colleagues. However, the induction of fibrillation we observed occurred in most cases within the first few minutes of reperfusion. Therefore this is also an unlikely explanation.

A possible explanation for UDCA's effects might come from our previous finding in this study that UDCA can modulate cardiomyocyte calcium ion channels. This is significant as calcium overload is one of the mechanisms of cellular toxicity induced by reperfusion. Ischemia leads to the depletion of cellular ATP inactivating ATPases resulting in reduced active Ca^{2+} efflux. This limits the reuptake of calcium by the sarcoplasmic reticulum and results in calcium overload (Kalogeris et al. 2012). If as demonstrated in hepatocytes, UDCA is also able to increase intracellular calcium levels in cardiomyocytes this can contribute to calcium overload mediated toxicity and arrhythmias. However this can only be speculated in the absence of further studies.

The degree of irreversible ischemic myocardial cell injury that occurs in the heart is dependent on the duration of ischemia (Reimer et al. 1977). Even relatively small differences in the severity of the ischemic condition can markedly affect the degree of tissue injury (Apstein et al. 1977). Similarly, the change in phenotype of fibroblasts to MFBs tends to occur with more prolonged periods of ischemia. Therefore any model to assess drug effects on IR needs to carefully take these factors into account. We hypothesised based on previous studies from our group (Miragoli et al. 2011) that UDCA might be protective by a modulatory effect on MFBs, however, upon review we concluded that the short duration of ischemia applied in this study was perhaps not ideal for assessing UDCA's effects. Although sufficient to induce irreversible myocardial injury in rat hearts (decreased contractile function, myocardial oxygen consumption and significant mitochondrial oedema) (Palmer et al. 2004), the short 25 and 35 minute periods of ischemia can be considered acute and were not sufficient to induce MFB expression. MFB expression might only be identified after 3 days of myocardial infarction (Christia et

al. 2013; Virag & Murry 2003). Beneficial effects for UDCA could well be identified in a chronic model with confirmed MFB expression.

6.4.2 Limitations and future work

The first limitation of this study was the small sample size that was used, which did not allow firm statistical conclusions to be reached on the effects observed. The study was not sufficiently powered to assess the statistical significance of the changes in fibrillation rates observed in control and treatment groups. Therefore future studies will have to be performed with a larger sample size to determine the statistical significance of these changes.

Another obvious limitation of this project is the absence of a mechanistic study to understand the observed effects of UDCA. It is necessary to understand the findings in this study as they will also help to better clarify the role of bile acids in IR injury. Such studies would have to focus on other targets of UDCA that have been proposed in this thesis (i.e. T-type channels) and by other authors such as ion channels located on intracellular calcium stores (Bouscarel et al. 1993).

This study only evaluated the anti-arrhythmic properties of UDCA in an acute model of IR. The 25 and 35 minute periods of ischemia applied in this project were not long enough to induce infarction that leads to the development of MFBs. Future studies in our group will focus on evaluating UDCA's effects in a chronic *in vivo* model of infarction. Although we initially proposed to study a chronic model in this project, difficulties in obtaining surgical training and project approval during the period of this project meant it could not be done.

6.4.3 Conclusions

In this pilot study, administration of UDCA did not significantly improve CV during reperfusion following ischemia. It also appeared to increase the incidence of fibrillation compared to vehicle treated hearts. Based on this, we concluded that UDCA does not protect against IR induced

arrhythmias. However, further studies will be required to better understand the mechanism of this effect and to evaluate the effect of UDCA in chronic ischemia and *in vivo* models.

7 Final Discussion and Conclusion

7.1 Overview

Ursodeoxycholic acid (UDCA) is an endogenous tertiary bile acid formed by the intestinal breakdown of primary bile acids. Like other bile acids it is involved in regulating cholesterol levels and fat absorption. The therapeutic effects of UDCA in the treatment of hepatobiliary diseases have long been known in Chinese traditional medicine, but only more recently in Western modern medicine. UDCA under such brand names as Ursofalk®, Actigall® and Urso® is used in the treatment of gall stones, Primary Biliary Cholangitis (PBC) and Intrahepatic Cholestasis of Pregnancy (ICP). Beyond these benefits in liver diseases, possible beneficial effects have also been shown for UDCA in the cardiovascular system. UDCA is able to reduce infarct size in rats (Rajesh et al. 2005), improve peripheral haemodynamics (Wong et al. 1999; Baruch et al. 1999) and protect against conduction slowing effects in cell cultures (Miragoli et al. 2011). It has been shown that levels of UDCA and its conjugates, compared to those of other bile acids, are significantly reduced in patients with atrial fibrillation (Rainer et al. 2013). Therefore, this thesis aimed to evaluate possible the functional effects of UDCA in disease models of the fetal heart in ICP and the adult heart in ischemia-reperfusion injury. For this, whole-heart models of both conditions were developed and the mechanisms of arrhythmia development were assessed.

7.2 Using the isolated-perfused neonatal rat heart as a model for cardiac electrophysiology studies

Due to the many limitations of cellular models for studying arrhythmias, it was necessary to develop a whole-heart model to better understand the mechanism of bile acid induced arrhythmias in ICP. Cardiac cellular models typically assess effects only on cardiomyocytes, whereas the whole-heart is composed of other cell types including fibroblasts, endothelial cells and vascular smooth muscle cells. Among other factors, it was proposed that a whole-heart model would help to better assess the effects of bile acids on sinus rhythm, impulse propagation in an intact structure and the changes in

electrophysiological properties that occur in a contracting heart. Although the isolated-perfused heart is commonly used in electrophysiological experiments, the vast majority of studies are performed in adult hearts. A limited number of reports were found that applied developing rat hearts (Riva & Hearse 1991; Wiechert et al. 2003) and none with fetal rat hearts. We found just a single publication that used neonatal and fetal pig hearts (Schuster et al. 2010). In this project we developed and characterised the neonatal rat heart as a model of the human fetal heart in ICP. In pilot studies, it successfully reproduced the bile acid-induced conduction slowing effects observed in previous cellular model studies performed in our laboratory. We also went on to further demonstrate a translation of ECG PR interval prolongation observed in human fetuses in ICP in this model (Strehlow et al. 2010). From this we concluded that data from the isolated-perfused whole hearts from new-born rats (between 0-3 days old), correlate well with the observations in human fetal hearts and make it a suitable model for assessing the functional effects of UDCA in ICP.

To our knowledge this is the first time that isolated-perfused neonatal rat hearts have been applied for electrophysiological studies. We propose that it can be a useful model for studying electrocardiogram effects of not just bile acids but also other standard agents in developing hearts. As I found out in this project the majority of literature data on drug effects is from studies performed in adult hearts and it is generally assumed that these effects are similar in new-borns. However as the contrasting effects of verapamil on the PR interval in the neonatal and maternal heart models showed (see Chapter 4), the potential effects of drugs in developing hearts cannot be directly inferred from data obtained in adult hearts. Conversely, the common use of isolated neonatal cardiomyocytes for evaluating pharmacology or physiology changes in adult hearts can lead to inaccurate conclusions and such data should therefore be approached cautiously. Despite a wealth of information on changes in ion channel expression and the electrocardiogram during development (Davies et al. 1996; Wetzel & Klitzner 1996; Grandy et al. 2007) by comparison there was limited data on drug effects in developing heart.

A number of factors need to be considered if the model is to be used for ECG recordings. From a technical side it is recommended that due to the relatively short duration of the QRS interval (approximately 10 ms), ECG recordings need to be performed at higher sampling rates than were used in this project (i.e. >1 kHz) to accurately detect significant changes of less than 1 ms. Also species differences in ion channel expression and function need to be considered in interpreting any findings. For example, the predominant current underlying ventricular repolarization is the 4-AP sensitive transient outward potassium current (I_{to}) in adult rat hearts, whereas the delayed rectifier potassium current (I_k) predominates in hearts of larger species such as in pigs and humans (Gussak et al. 2000). This contributes to a more rapid ventricular repolarization and the absence of a distinct isoelectric interval between the QRS interval and the T wave in rodent ECG recordings (Gussak et al. 2000). As a result, use of this model for evaluating QT interval changes will be limited.

Another important consideration is the choice of perfusion solution used for the model. We observed a deterioration in heart rate with prolonged perfusion (up to one hour), despite the decline in the adult heart being less and allowing it to be used for much longer. This is in contrast to the findings of Wiechert and colleagues who were able to perfuse the neonatal rat heart for up to 24 hours (Wiechert et al. 2003) with minimal change in heart rate. The difference is likely to be in the choice of perfusion solution, Tyrode's solution, used in this project. The 24 hour model was perfused with serum-free medium supplemented with hydrocortisone, amino-acid, ascorbic acid and antibiotic supplements. Also, as was observed by Riva and colleagues, it is also necessary to use higher calcium concentrations (between 1.8 to 2.5 mM) in the perfusate than is normally used for adult hearts (1.2 mM) (Riva & Hearse 1991). Although this does not affect the heart rate, lower concentrations were shown to cause a deterioration in left ventricular developed pressure.

Overall, despite initial difficulties with cannulation, with improved surgical techniques we found this model to be easy to be set up and inexpensive and that it produces readily reproducible results of electrophysiological changes.

7.3 Critical role of T-type calcium ion channels in the developing heart make it more susceptible to bile acid induced arrhythmias

We hypothesised that the ability of UDCA to inhibit conduction slowing was by modulation of intracellular calcium concentrations in cardiomyocytes. Calcium ions and the voltage gated calcium ion channels play a critically important role in cardiac function and cardiomyocyte excitation and contraction (Bers 2002). As a result agents that alter either the intracellular calcium concentration or channel opening can affect cardiac function. In this thesis it was shown that bile acids, which have always been thought of simply as aids of digestion, can also act as signalling molecules in the heart and affect cardiac calcium ion channels. In particular it showed that the bile acid, TC, can inhibit calcium ion currents leading to significant impairment in impulse propagation in the fetal heart, with UDCA treatment being protective. This was proposed as a mechanism for cardiac arrhythmias in fetal hearts in ICP.

It is well documented that the hydrophilic bile acid UDCA can alter intracellular calcium ion concentrations in non-cardiac cells, including hepatocytes (Bouscarel et al. 1993; Aromataris et al. 2008) and pancreatic cells (Voronina et al. 2002) via release from intracellular calcium stores. In contrast, other studies in cardiomyocytes have shown that the bile acid, TC, can inhibit calcium current (Binah et al. 1987; Kotake et al. 1989) and calcium transients (Gorelik et al. 2002). What is less understood is the exact mechanism by which they alter intracellular calcium. A number of studies have shown that bile acids are able to bind directly to calcium ions although with varying affinities (Gleeson et al. 1990; Rajagopalan & Lindenbaum 1982; Moore et al. 1982). One might suggest that similar binding of calcium ions in cardiomyocytes could lead to a reduction in free calcium ions and calcium entry into cardiomyocytes affecting excitation and contraction. Alternatively bile acids might alter calcium ion levels by release from intracellular calcium stores. Choleretic bile acids (that increase bile flow) such as UDCA and TDCA are able to activate store operated calcium channels in liver cells while cholestatic bile acids (decrease bile flow) such as LCA inhibit these channels (Aromataris et al. 2008).

This is supported by a study in hamster hepatocytes that shows that UDCA treatment results in an increase in intracellular free calcium via a release from intracellular stores (Bouscarel et al. 1993). However, optical mapping of the FH model did not show any changes in calcium transient amplitude (peak intracellular calcium concentration) in this study with UDCA alone; therefore this might need to be investigated further.

A more likely mechanism by which UDCA is able to inhibit conduction slowing, as was demonstrated in this thesis, is direct modulation of T-type calcium ion channels. In particular we showed that the T-type Ca^{2+} channel subtype plays a critical role in impulse propagation in developing hearts. T-type calcium ion channels are present in all regions of the developing heart but are only expressed in conductive tissue of healthy adult hearts (Ono & Iijima 2010). Only under disease conditions such as cardiac hypertrophy are they present in ventricular myocardium. In conductive tissues, it is thought to affect intrinsic heart rate by regulating the slope of diastolic depolarisation (Mangoni et al. 2006). Inhibition of these channels with specific blockers such as mibefradil or nickel can result in bradycardia (Hagiwara et al. 1988). A role for T-type channels in conductive tissue has also been proposed. Knock out of genes for the T-type channel subunit, $Ca_v3.1$, in mice has been shown to result in slower AV node conduction (prolonged PQ interval) compared to normal mice, although this was not seen with $Ca_v3.2$ knockout mice (Mangoni et al. 2006). The importance of this calcium channel subtype in developing heart ventricular myocardium is not well understood. Along with contributions to calcium signalling, studies have shown a potential role in proliferation and growth of the heart which is supported by their reappearance in damaged adult hearts during remodelling (Senatore et al. 2012; Vassort et al. 2006). This helps to explain how agents like bile acids that modulate calcium channel functions in developing hearts can have profound effects on heart rhythm such as is seen in ICP. Although the difference in expression of T-type calcium channels in fetal and adult hearts can explain the conduction slowing effects of bile acids in the ventricles, we concluded that it is not likely to be the only mechanism involved in AV nodal impulse slowing in fetal hearts since a similar effect was

observed in the maternal heart. In ICP, PR prolongation has only been reported in fetal but not maternal hearts.

An important question that arises from this work is the potential adverse cardiac effects of T-type selective calcium channel blockers (CCBs) prescribed for pregnant mothers on their fetuses. Available literature reviews have not highlighted any arrhythmia risks at therapeutic doses of calcium channel blockers (Alabdulrazzaq & Koren 2012). However, this is information gathered mainly from L-type selective blockers. There is a limited number of approved T-type CCBs, the popular agent, mibefradil, has been withdrawn from clinical use due to a potential for drug interactions. Future development of T-type selective drugs will need to consider not just the teratogenic risk but also the cardiac side-effects on fetuses.

7.4 Implication of findings for clinical application of UDCA

The findings in this thesis help to identify potential applications for UDCA in cardiovascular conditions linked to liver and bile duct dysfunction. A number of cardiovascular abnormalities have been associated with cholestasis in humans and also in animal models (P J Oliveira et al. 2003; Paulo J. Oliveira et al. 2003; Chayanupatkul & Liangpunsakul 2014). These include morphological changes such as increased atrial volume caused by the increase in toxic bile acids. Other changes include increased heart rate, elevated cardiac output and defective cardiac contractility as a result of impaired β-adrenergic receptor function (Chayanupatkul & Liangpunsakul 2014). In ECG recordings, prolongation of the QT-interval is frequently observed in patients with cirrhosis (Mozos et al. 2011; Bernardi et al. 2012). Even though UDCA is currently used for the treatment of cholestasic diseases there appears to be limited evaluation of its benefits on the accompanying cardiac conditions. By normalising the bile acid profile it might help to reduce the incidents of cardiomyopathies in chronic cirrhosis patients.

Although UDCA is used as frontline in ICP for restoring maternal bile acid profiles and itching, it is not currently approved for the treatment of fetal arrhythmias in ICP. However, analysis of clinical data

from patients has proposed that it might also help to reduce the incidents of fetal complications (Bacq et al. 2012; Grand'Maison et al. 2014). Although none of the publications identified a definite correlation between UDCA treatment and arrhythmia protection, they did not rule out a benefit. Based on the results of this project, we propose that with close monitoring of the fetal electrocardiogram, UDCA can be trialled for the reversal of PR interval prolongation in ICP.

Another point to consider is that in this thesis we demonstrated beneficial effects for UDCA at lower concentrations than peak plasma concentrations achieved at a standard dose in ICP (Dilger et al. 2012). The protective effects of UDCA were achieved at a concentration of 1 μM. This has potential implications for the future doses of UDCA that are applied in ICP treatment and dose selection for clinical trials in fetal arrhythmia treatment. Indeed a couple of recent trials have assessed the benefits of low dose (600 mg/day) (Joutsiniemi et al. 2015) and a very low dose (300-450mg/day) (Grymowicz et al. 2016) of UDCA for the treatment of ICP and identified beneficial effects. This is important especially in the light of reports of side-effects at high doses of UDCA in PSC treatment (Lindor et al. 2009). It is also important as higher doses of UDCA do not always correlate with improved outcomes in diseases, an example of this is seen in NASH (U. F. H. Leuschner et al. 2010; Adams et al. 2010).

Finally as UDCA was demonstrated to be a modulator of T-type channels, its benefits in adult cardiac conditions associated with re-expression of T-type channels, need to be considered clinically. T-type channels are re-expressed in ventricular myocardium during hypertrophy (Ono & Iijima 2010), in post-infarction remodelling (Huang et al. 2000) and are modified in cardiomyopathies (Sen & Smith 1994). Although not evaluated in this study, UDCA's potential benefits could be tested in these diseases to determine if it can reduce hypertrophy and scar formation.

7.5 Future directions

In recent years, an increasing awareness of the role of bile acids as signalling molecules has resulted in a lot of interest in understanding their roles in different organ systems in both health and disease.

As mentioned previously, UDCA levels compared to those of other bile acids have been shown to be reduced in serum from atrial fibrillation (AF) patients (Rainer et al. 2013). The exact role of UDCA in normal cardiac function will need to be explored. Indeed the link between AF initiation and progression and UDCA levels needs to be better understood as it can have major consequences for the management of this common cardiac condition.

It will also be interesting to consider the potential role of G-protein coupled receptors in the cardiac function of bile acids. Identification of the G-protein coupled bile acid receptor, TGR5, in 2002 has helped to identify a role for bile acids in cardiac homeostasis. In recent studies within our group we have shown that siRNA knockdown of TGR5 receptor in neonatal rat ventricular cardiomyocytes can prevent bile acid inhibition of contraction rate and calcium transients (unpublished data). Added to this, as was reported in a previous study, hydrophobic bile acids can alter cardiomyocyte contraction rate via muscarinic (M_2) receptor agonism. We show here that both UDCA and TC phosphorylate protein kinases which can act as signalling molecules for G-protein coupled receptors. Understanding the intracellular pathways by which this occurs and the regulatory effect on other receptors and ion channels will be beneficial.

Finally, the mechanism of the negative effect of UDCA in ischemia-reperfusion injury needs to be investigated further as it might reveal new insights into infarction and have profound consequences for patients and treatment approaches. Other authors have suggested that UDCA can help to reduce infarct size following occlusion (Rajesh et al. 2005).

7.6 Conclusion

This thesis presents work demonstrating beneficial properties for UDCA in the treatment of bile acid induced fetal arrhythmias in ICP. It provided a novel *ex vivo* model of the fetal heart which shows good correlation with clinical data. It highlights a mechanism by which UDCA can be protective through

modulation of cardiac T-type calcium ion channel subtypes. In the absence of a beneficial effect in ischaemia models its use would be disputed in clinical therapy.

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