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Assessment of Drug-Induced Cardiotoxicity during Rat Embryo Development

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Abstract

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The potassium ion channel (hERG/I_{Kr}) is important for normal heart function and drug-induced blockade of the channel in adult humans can lead to irregular heart rhythms (arrhythmia). The ion channel is also essential for early cardiac function in the embryo and therapeutic drugs which block this channel have been shown to cause birth defects in animal studies. A wide range of birth defects have been seen including cleft lip/palate, distal limb defects and heart malformations.

These malformations are associated with periods of hypoxia and altered blood flow in the embryo associated with the drug-induced heart rhythm disorders and bradycardia. It is also well known that other experimental procedures causing periods of hypoxia in the embryo can give rise to similar defects as those seen with drugs that block the hERG/I_{Kr} channel. Paper I on the thesis deals with risk assessment for use in pregnancy of drugs which block hERG/I_{Kr}.

Evaluation of the risk of birth defects is largely based on the results of experimental studies on animals. Guidelines for how such standard tests are to be performed were determined by regulatory authorities several decades ago. However, there are examples where safety studies for drugs blocking hERG/I_{Kr}, although fulfilling regulatory guidelines, have been carried out at a suboptimal dose range and failed to detect teratogenicity. A consequence of this is that the teratogenic potential of hERG/I_{Kr} blocking drugs have been missed in standard safety testing. The results of the paper I show that the teratogenic properties of the drug astemizole (withdrawn from the market several years ago because of fatal cardiac arrhythmias in adults related to the blockade of hERG/I_{Kr}) were missed in the initial safety studies.

Paper II shows that several drugs that block cardiac ion channels other than hERG/I_{Kr} can also disrupt fetal cardiac function during embryonic development. However, the concentrations required to cause these changes are much higher than is likely to occur during normal use of the medicines and based on these results that are not considered a risk when taken during pregnancy.

Paper III deals with the possible teratogenicity of erythromycin. From the Swedish Birth Defects Register there have been signals that use of erythromycin (which has hERG/I_{Kr}-blocking properties) during pregnancy is associated with an increased risk of cardiovascular malformations. Paper III shows that the levels of erythromycin needed to disrupt fetal cardiac function during embryonic development are unlikely to occur after normal oral treatment with erythromycin.

Paper IV shows that the embryonic rat heart undergoes major changes in sensitivity to blockade of specific cardiac ion channels during the organogenic period. This is an important observation from the perspective that there may be periods during embryonic development when the embryo is more or less sensitive to the effect of drugs that affect specific ion channels.

To conclude, papers I-IV show that the study of drug effects on the gestation day 13 rat embryonic heart, together with the use of computational assisted image analysis of the cardiac response, provides an *in vitro* model for hazard identification of compounds with the potential to adversely affect heart function in the developing embryo.

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Till Daniel

“The heart is a mystery. If you’ve seen your own heart on video screen, as million by now have done, convulsing and open rhythmically, you may have wondered why this persistent muscle is so faithful in its function from the uterus to the last breath. This rhythmic gripping and relaxing blindly goes on. Why? How?”

Saul Bellow (1915-2005)

*“I pressed down on the mental accelerator.
The old lemon throbbed fiercely.
I got an idea.”*

P.G. Wodehouse (1881-1975)

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I Nilsson, M.F., Danielsson, C., Sköld, AC., Johansson, G., Blomgren, B., Wilson, J., Khan, K.M., Bengtsson, E., Kultima, K., Webster, W.S., Danielsson, B. (2010) Improved methodology for identifying the teratogenic potential in early drug development of hERG channel blocking drugs. *Reproductive Toxicology*, 29(2):156–163.
- II Abela, D., Ritchie, H., Ababneh, D., Gavin, C., Nilsson, M.F., Khan, M.K., Carlsson, K., Webster, W.S. (2010) The effect of drugs with ion channel-blocking activity on the early embryonic rat heart. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, 89(5):429-440.
- III Nilsson, M.F., Webster, W.S. (2013) Comparative effects of erythromycin and clarithromycin on rat embryonic heart function *in vitro*. *Manuscript*.
- IV Nilsson, M.F., Ritchie, H., Webster, W.S. (2013) Temporal effects on the rat embryonic heart of Na⁺, K⁺, Ca²⁺ channel blockers and the human teratogen phenytoin. *Manuscript*.

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List of additional papers

Danielsson, B.R., Danielsson, C., Nilsson, M.F. (2007) Embryonic cardiac arrhythmia and generation of reactive oxygen species: common mechanism for IKr blocking drugs. *Reproductive Toxicology*, 24(1):42-56. *Review*.

Karlsson, M., Danielsson, B.R., Nilsson M.F., Danielsson C., Webster, W.S. (2007) New proposals for testing drugs with IKr-blocking activity to determine their teratogenic potential. *Current Pharmaceutical Design*, 13(29):2979-2988. *Review*.

Khan, K.M., Nilsson, M.F., Danielsson, B.R., Bengtsson, E. (2008) Fully automated heart beat rate determination in digital video recordings of rat embryos. In: Perner, P., Salivetti, O. (Eds), *Advances in Mass Data Analysis of Images and Signals in Medicine, Biotechnology, Chemistry and Food Industry*. Springer-Verlag, Berlin Heidelberg, p 27-37.

Nilsson, M.F., Dencker, L., (2009) Klassificering av läkemedel i samband med graviditet. In: Bjerner, L., Gottberg, L., Werner, S., editors. *Astma och allergi – Handbok vid graviditet och amning*. Åtta45 Tryckeri AB, Solna. p 79-84

Khan Niazi, K.M., Ibrahim, M.T., Nilsson, M.F., Sköld, AC., Ling, G., Nyström, I., (2011) Robust signal generation and analysis of rat embryonic heart rate *in vitro* using Laplacian eigenmaps and empirical mode composition. In: Real, P., Diaz-Pernil, D., Molian-Abril, H., Berciano, A., Kropatsch, W. (Eds), *Lecture Notes in Computer Science*. Springer, Berlin/Heidelberg, p 523-530.

Nilsson, M.F., Sköld, AC., Ericson, AC., Annas, A., Villar, R.P., Cebers, G., Hellmold, H., Gustafson, AL., Webster, W.S. (2013) Comparative effects of sodium channel blockers in short term rat whole embryo culture. *Toxicology and Applied Pharmacology* doi:pii: S0041-008X(13)00296-2. 10.1016/j.taap.2013.06.023. [Epub ahead of print]

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Abbreviations

AP	Action potential
AV	Atrioventricular
bpm	Beats per minute
cAMP	Cyclic adenosine monophosphate
CICR	Ca ²⁺ -induced Ca ²⁺ -release
DHP	Dihydropyridine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
ECVAM	The European Committee for the Validation of Alternative Methods
FDA	U.S. Food and Drug Administration
GD	Gestational day
HCN	Hyperpolarized-activated cyclic nucleotide-gated
hERG	Human ether-á-go-go-related gene
I _{CaL}	L-type calcium current
I _{CaT}	T-type calcium current
I _{Cl}	Chloride current
I _f	Funny current
I _{Kr}	The rapid component of the delayed rectifying potassium current
I _{Ks}	The slow component of the delayed rectifying potassium current
I _{Kur}	Ultrarapid delayed rectifier current
I _{Na}	Sodium current
IP3	Inositol tris-phosphate
i.v.	intravenous
I _{to}	Transient outward potassium current
LQTS	Long QT syndrome
MW	Molecular weight
NSAID	Non-steroidal anti-inflammatory drug
SA	Sinoatrial node
SD	Sprague-Dawley
SR	Sarcoplasmic reticulum
TdP	Torsade de pointes
WEC	Whole embryo culture

Introduction

The heart is the first organ to function during embryonic development, and until recent times, cessation of heart function constituted death.

Margaret L. Kirby, Cardiac Development

Since the experimental work by the French scientist Geoffrey St. Hilaire in the early 1800s, hypoxia as a teratogen has been studied for almost 200 years. The early studies were primarily done in chick embryos, and it was not until the early 1950s with the production of malformed offspring of mice, rat and rabbits kept under low atmospheric oxygen pressure, that the idea of hypoxia as a human teratogen really took off. Although hypoxia (i.e. in the form of low atmospheric oxygen tension) was not accepted as human teratogen by the scientific community, the experimental work at the time had a great impact on experiments that followed, and still influences experiments done in recent time [1, 2].

In the 1960s to the 1980s, there was an increased interest in studying embryonic hypoxia by restricting the uterine vascular function during pregnancy. Experimental models used to induce embryo-fetal hypoxia in mammals were clamping of the uterine artery (Figure 1) [3-5], or treatment with compounds that induce uterine vasoconstriction [6, 7]. The research showed that the early postimplantation rat embryo is surprisingly resistant to hypoxia with many embryos able to survive 3 hours of hypoxia. However, as the embryo grows and develops it becomes more sensitive to hypoxia [3, 4]. Shorter periods of hypoxia (~45 minutes) produced a range of defects in rat offspring, in particular amputation-type defects of the limbs (Figure 2.A). In agreement with the “edema syndrome” described by Grauwiler in the chick (1970), the malformations observed in rat fetuses were preceded by edema, dilated blood vessels, hemorrhage, and blisters with tissue degeneration (Figure 2.B and 2.C) [4, 5].

In the 1990s a hypoxia-reoxygenation hypothesis was presented, suggesting a common mechanism of teratogenesis for vasodilators (e.g. nifedipine, felodipine), vasoconstrictors, clamping of uterine vessels, or low atmospheric oxygen concentration (Figure 3) [8]. Of particular interest was the inclusion in the theory of the established human teratogen and antiepileptic drug

phenytoin, where hypoplasia of the distal phalanges is a part of the fetal hydantoin syndrome [9, 10].

Since the hypoxia-reoxygenation-hypothesis was presented, numerous papers have been produced describing the association of altered embryonic heart function with malformations, growth retardation, and embryonic death in experimental animals. In the majority of the work, focus has been on drugs that selectively block a specific cardiac potassium channel, the so called human ether-a-go-go related gene (hERG), or drugs that block the channel as a side effect [11, 12]. The precise relationship, however, between cardiovascular responses of the embryo, subsequent hypoxia, and the deleterious developmental effects is not fully understood.

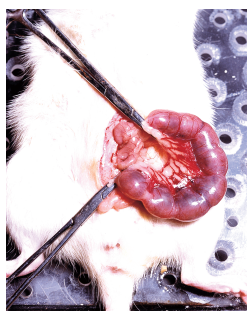


Figure 1. Uterine vascular clamping of one of the uterine horn. Contribution of W.S. Webster.



Figure 2. A. Amputation-type defects of digits of left forelimb after vascular clamping on GD 15. B. Control forelimb (left) and blisters with tissue degeneration of digits after uterine vascular clamping. C. Blisters with tissue degeneration of hind limb after uterine vascular clamping on GD 16. Contribution of W.S. Webster.

The opening lines above from Dr. Margaret L. Kirby's textbook *Cardiac Development* summarize the significance of the heart during our lifetime. The emphasis of this thesis is on pharmacologically induced cardiac malfunction during embryonic or early fetal life. The introduction will give a theoretical background to the biological and technical issues in the thesis. The methods section describes the instrumentation and techniques used in the experiments. The results and discussion section summarizes the biologi-

cal results from the papers and gives future directions for further studies in the field.

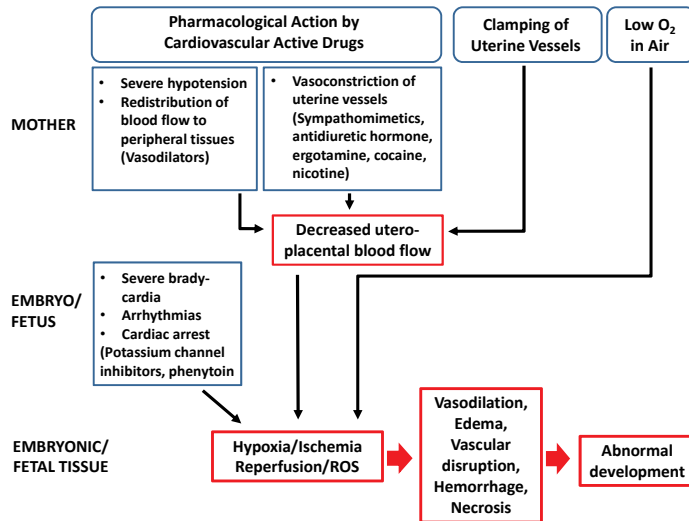


Figure 3. The hypoxia-reoxygenation hypothesis. Adopted from [8].

Physiology of the heart

Physically, the heart may be viewed as an extremely well controlled pump with great endurance and ability to adapt to the surrounding circumstances. The function of the heart is to deliver enough blood at the right pressure, minute-by-minute, 24 hours a day, from year's end to year's end, until it stops. The adult heart is composed of three major types of cardiac muscle: atrial muscle, ventricular muscle, and specialized excitatory and conductive muscle fibers. The atrial and ventricular muscle contract in much the same way as skeletal muscle except that the duration of contraction is much longer. On the other hand, the specialized excitatory and conductive fibers contract only weakly because they contain few contractive fibrils; instead they exhibit rhythmicity and varying rates of conduction, providing an excitatory conduction system for the heart [13].

Ion channels and cardiac action potentials

The heart is dependent on action potentials (APs) to propagate the signal for a contraction and to initiate the contraction of the myocardium. APs are generated through the activity of *ion channels* in the cardiomyocyte surface membrane (sarcolemma). It is important to note that the ion channel nomen-

clature as applied by physiologists and later by molecular biologists has not been fully reconciled and can be somewhat confusing (Table 1).

The APs of the adult atrial and ventricular working myocardium are commonly depicted as in figure 4. A large variety of ion channels contributes to the shape and duration of the AP [14]. Expression of more than 70 ion channel pore-forming α -subunits and auxiliary protein genes have been detected and are differentially expressed in the heart [15]. A few ion transport mechanisms, particularly the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the Na^+ , K^+ -ATPase, also contribute to the membrane potential and AP shape. Despite the variety of ion channels and transporters contributing to membrane potential and the shape of AP they conduct only four ions (Na^+ , Ca^{2+} , K^+ and Cl^-).

The shape of the AP as well as the as the heart rate is modified by autonomic neurotransmitters released by the sympathetic and parasympathetic nervous system that modulates the activity of many of these channels [16].

The specialized excitatory and conductive system of the heart

The specialized excitatory and conductive myocardium that controls cardiac contractions consists of the following basic parts: (a) the *sinoatrial* (SA) *node* in which the normal rhythmical impulse is generated; (b) the *internodal pathways* that conduct the impulse from the SA node to the atrioventricular (AV) node; (c) the AV node, in which the impulse from the atria is delayed before passing into the ventricles; (d) the *A-V bundle*, which conducts the impulse from the atria into ventricles; and (e) the *left and right bundles of Purkinje fibers*, which conduct the cardiac impulse to all parts of the ventricles.

The AP that begins in the SA node spreads immediately into the atria. The SA node controls the beat of the heart because its rate of rhythmical discharge is greater than that of any other part of the heart. The discharge rate of the SA node (70 to 80 times per minute) is considerably faster than the spontaneous rate of either the AV node (40 to 60 times per minute) or the Purkinje fibers (15 to 40 times per minute). Hence, in the mature human heart the SA node determines the rate at which all cardiomyocytes contract and is considered as the pacemaker of the heart [17].

Table 1. Major cardiac ion channels

IUPHR*	Membrane current	Gene & Other names	Physiological activity
CaV1.2, CaV1.3	L-type Ca^{2+} , I_{CaL}	$\alpha 1\text{C}$, $\alpha 1\text{D}$, dihydropyridine sensitive	Ca^{2+} entry, depolarization, pacemaking (?)
CaV3.1, CaV3.2	T-type Ca^{2+} , I_{CaT}	$\alpha 1\text{G}$, $\alpha 1\text{H}$	Ca^{2+} entry, depolarization, pacemaking (?)
NaV1.5	Na^{+} current, I_{Na}	SCN5A	Rapid depolarization, AP upstroke
KV4.3	Transient outward K^{+} , I_{to}	Voltage gated K^{+} channel, A-type	Early repolarization
KV7.1	Delayed rectifier – slow, I_{Ks}	KCNQ1, LQT1	Repolarizing K^{+} current, slow component
KV11.1	Delayed rectifier – rapid, I_{Kr}	KCNH2, HERG, LQT2	Repolarizing K^{+} current, rapid component
K2p3.	Not characterized	TASK-1, KCNK3	Background K^{+} current (?)
HCN1, HCN2, HCN4	Hyperpolarization-activated, I_f , I_h	Cyclic nucleotide gated cation channel pacemaker current, funny current	Pacemaking
Kir2.1	Inward rectifier, I_{K1}	KCNJ2	Background K^{+} current, resting potential
Kir3.1	G-protein gated K^{+} , $\text{I}_{\text{K(Ach)}}$	GIRK1, G $\beta\gamma$ -activated	Receptor activated hyperpolarizing current
Kir6	ATP dependent K^{+} , $\text{I}_{\text{K(ATP)}}$	ATP-dependent channel	Hyperpolarizing during hypoxia (i.e. metabolic stress)
Ncx1**	$\text{Na}^{+}/\text{Ca}^{2+}$ exchange, I_{nxc}	$\text{Na}^{+}/\text{Ca}^{2+}$ exchange	Ca^{2+} extrusion, Na^{+} entry, pacemaking (?)

*International union of pharmacology ion channel compendium

**Technically not an ion channel but transports a single net positive charge for each Ca^{2+} in the opposite direction

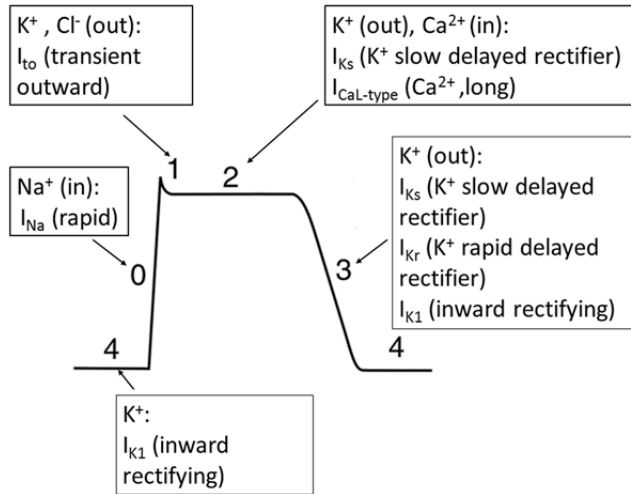


Figure 4. Overview of an idealized action potential (AP) from a mature ventricle with ion currents contributing to the AP.

The embryonic heart

Much of the morphology and physiology of heart development has been studied in avian species, particularly in chick embryos, because like mammals they have four chambered hearts. The human embryonic heart starts to beat during the third week of pregnancy [18-20]. In the chick embryo, electrical activity is first seen at the seven-somite state and contractions begin at the nine-somite stage. In the rat, action potentials and contractions have been measured at GD 9.5 [21].

At the time when the heart starts to beat it is believed that a specialized conduction system does not exist in the heart. Instead the primitive heart tube is composed uniformly of small cardiomyocytes and conduction of the contractile impulse begins at a primary pacemaker region located in the primitive atrium, and presumably in the region of the future SA node. The primitive *pacemaker* contains the fastest beating cardiomyocytes and the conduction impulse spreads sequentially and uniformly from this group of cells towards the primitive ventricular outflow tract [22]. This early basic form of impulse conduction is soon replaced (e.g. GD 12 in rat) by specialized features of the conduction system seen in the adult.

The importance of connexins for the conduction system

All cardiomyocytes are electrically coupled to form a *syncytium* so that when one of the cells becomes excited, the AP spreads to all myocardial

cells. During early embryonic life, the contraction of the heart tube proceeds from inflow to outflow, thus no paths of excitation exists. However, as myocardial cells in the heart tube proliferate and develop contractile properties and chamber-specific characteristics, they begin to differentiate to become a working myocardium, and a specialized myocardium of the conduction system. A major feature of the heart is to enable rapid and coordinated electrical excitation, which is a prerequisite for normal rhythmic cardiac function. *Connexins*, intercellular proteins join cardiac cells and thus establish the electrical syncytium of the heart. There are three types of connexins expressed in the heart, *connexin43* (*Cx43*), *Cx40*, and *Cx45*. The three types of connexins have different conductive properties. *Cx43* is abundant in atrial and ventricular working myocardium [23-26]. *Cx40* has the largest *conductance* and is expressed in atrial tissue and the fast-conduction His-Purkinje system [27, 28]. *Cx45* is expressed in both primary myocardium and in the SA and slow-conducting AV-node [29-31]. In the rat, at GD 12 the expression of *Cx40* and *Cx43* mRNA and proteins support the presence of a conduction system of the rat embryonic heart at mid-organogenesis [32, 33].

Action potentials and autorhythmicity of the embryonic heart

For the most part, APs in the developing heart after around mid-gestation are similar to those in adults. However, the earlier in development one looks the more the electrical activity of myocytes resembles that in conducting cardiomyocytes of the adult SA node (Figure 5) [34].

The APs of early embryonic cardiomyocytes and SA nodal cells have a much slower rate of rise (small dV/dT) and the diastolic potential is more depolarized compared with a mature atrial or ventricular cardiomyocytes. The small dV/dT is due to the fact that voltage-activated Na^+ channels are either absent or are in an inactive state [35]. Therefore, in the earliest embryonic myocytes, the rising phase of the action potential is depending on the voltage-dependent activity of L-type and T-type Ca^{2+} channels. These APs are not affected by Na^+ channel blockers but can be completely inhibited by blocking Ca^{2+} channel activity. The more depolarized diastolic membrane is due to poor K^+ permeability resulting from the presence of few background *inward rectifier* K^+ channels (*Kir*) and a background inward Na^+ conductance [36-38].

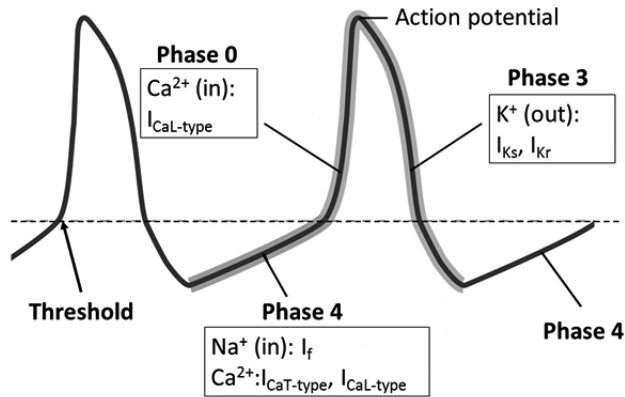


Figure 5. Schematic action potential of SA cardiomyocytes (“diastolic action potential”) and early embryonic cardiomyocytes.

The pacemaker current I_f

In the early 1980s, DiFrancesco first reported and characterized I_f (i.e. “funny current”) because of its slow activation upon repolarization of the AP [39, 40]. This current, often termed the “pacemaker” current, is thought to be a major contributor to the depolarization in spontaneously excitatory cells. Four I_f channel genes have been cloned and all are expressed in SA and AV nodes, and in Purkinje fibers [41]. These channels are gated directly by cyclic adenosine monophosphate (cAMP) and the four isoforms are referred to as HCN1-4 for *hyperpolarization-activated cyclic nucleotide-gated*. The I_f (HCN) channels conduct Na^+ and K^+ roughly equally, but conduct mainly Na^+ at hyperpolarized potential which produces an inward current.

Knockout of HCN4, which constitutes 80% of the I_f channels in the mouse heart, is embryonic lethal, with death occurring between GD 9.5 and 11.5 [42]. HCN4 knockout slows the heart rate about 40% but does not stop it altogether. I_f is likely important for autonomic regulation of heart rate as adrenergic stimulated increase in cAMP shifts the activation to a more positive potentials, leading to greater activation of the current on AP repolarization in pacing cardiomyocytes and an increase in the heart rate [43, 44]. It should be noted that adrenergic innervation does not become functional until very late in development, long after loss of autorhythmicity in ventricular cardiomyocytes [45, 46]. However, levels of hormonally secreted catecholamines can be quite high in the mouse embryo, particularly in response to periodic hypoxic stress which is normal for mammalian and avian embryos [47-50]. An interesting possibility is that the HCN4-null is embryonic lethal because of an inability of the heart to respond to hypoxic stress, which leads to a decrease in heart rate in the embryo (bradycardia). Regardless, I_f chan-

nels appear to have an important role in regulating heart rate during development.

Cardiac Na-Ca exchanger (NCX1)

NCX1 is the principal Ca^{2+} efflux mechanism across the sarcolemma in myocardial cells. NCX1 moves three Na^+ ions across the plasma membrane in exchange for a single Ca^{2+} ion moving in the opposite direction [51, 52]. It has been shown that I_{NCX} likely contributes significantly to the latter third of the depolarization in SA nodal cells, and that I_{NCX} is essential for autorhythmicity in SA nodal cells [53, 54]. In embryonic pace making the rising phase of the AP is due to L- and T-type Ca^{2+} currents, I_{CaL} , and I_{CaT} , both of which are presented in embryonic cardiomyocytes. However, removal of extracellular Na^+ abolishes the spontaneous activity in chick embryonic cardiomyocytes [55]. Hence, the Na^+ -dependence of the embryonic SA node may at least in part be due to the inward current generated by the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX1) [56, 57].

EC coupling in the developing heart

Contraction and subsequent relaxation are elicited by a transient rise followed by a decline in cytosolic Ca^{2+} with each heartbeat. This process involves a number of steps that are collectively known as *excitation-contraction (EC) coupling*. EC coupling may be defined as all steps between depolarization of the sarcolemma and the subsequent delivery of Ca^{2+} to the contractile proteins. Although the general mechanisms involved in EC coupling in developing cardiac muscle are qualitatively similar to EC in adult myocardium, there are important structural and functional differences. Embryonic cardiomyocytes are small, mononucleated, lack T-tubules, and have poor myofibrillar organization compared with adult cardiomyocytes. This relatively poor cytoplasmic organization is to be expected considering that cardiomyocytes have to contract to keep the embryo alive as well as proliferate as the embryo grows.

The steps in the EC coupling process of adult cardiac muscle are well described (Figure 6) [16]. Briefly stated, sudden depolarization of the sarcolemma leads to activation of voltage-gated Ca^{2+} channels to produce a Ca^{2+} current (I_{Ca}). This current provides net Ca^{2+} entry into the cardiomyocyte after which net Ca^{2+} extrusion occurs due to $\text{Na}^+/\text{Ca}^{2+}$ exchange. Contraction does not occur in the absence of I_{Ca} , indication that this current is essential for the EC coupling process. I_{Ca} increases the concentration of Ca^{2+} in vicinity of the *terminal cisternae* of the *sarcoplasmic reticulum* (SR) which causes release of Ca^{2+} from large stores in the SR. This process is referred to as Ca^{2+} -induced Ca^{2+} -release (CICR) [58, 59]. Ca^{2+} release from the SR occurs through ryanodine receptor/ Ca^{2+} release channels which appear to be identi-

cal to the feet structures that span the space between the sarcolemma and the junctional SR [60]. These channels are in close association with the dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels of the sarcolemma. The DHP receptor in adult cardiac muscle appears to function as a Ca^{2+} channel and not a voltage sensor that directly controls release of Ca^{2+} from the SR as it does in skeletal muscle [61]. Essentially, the amount of Ca^{2+} entering the cytoplasm via the SR during systole (about 80% in larger mammals including humans) is taken back up by the SR and that entering across the plasma membrane mostly via Ca^{2+} channels is extruded by the NCX1 [16].

Thus, a major difference in cardiac EC coupling between the embryonic and adult heart is the primary dependence on extracellular Ca^{2+} for contraction during development. In embryonic and fetal hearts, most of the Ca^{2+} needed for contraction comes from the extracellular space via sarcolemmal Ca^{2+} channels, and perhaps NCX1 operating in reverse mode [16, 34]. Therefore, a major difference between embryonic and adult EC coupling is that Ca^{2+} removal during relaxation is dependent largely on the NCX1 operating in what is considered its normal forward mode extruding Ca^{2+} over the sarcolemma.

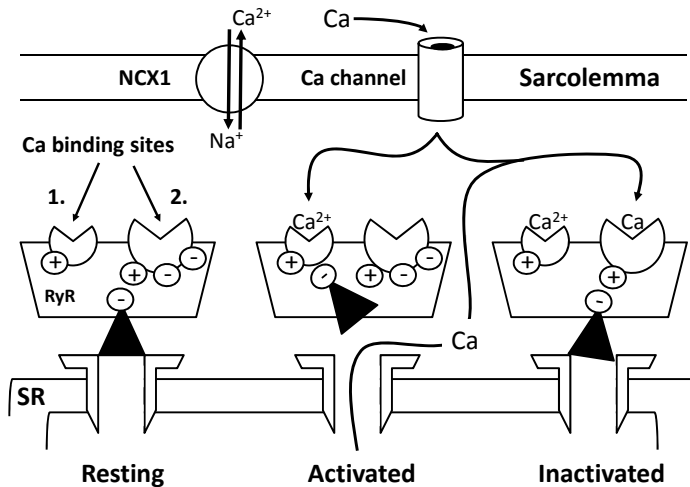


Figure 6. Overview of calcium-induced calcium-release (CICR). Adopted from [16].

Innervation of the heart

The mature heart is supplied with *sympathetic* and *parasympathetic* nerves. The parasympathetic nerves are distributed mainly to the SA and the AV nodes, and to a lesser extent to the muscle of the two atria, and even less to the ventricular muscle. The sympathetic nerves are distributed to all parts of the heart with a strong representation to the SA node [13].

The effects of cholinergic and adrenergic stimulation in the adult heart are achieved by modulation of ion channel activity and the function of the SR in cardiomyocytes. Sympathetic stimulation of β -adrenergic receptors by catecholamines acts on several effector mechanisms via G-protein coupling with adenylate cyclase and subsequent increase of cAMP. The increase in heart rate after adrenergic stimulation is due to an increase in the magnitude of the repolarizing K^+ delayed rectifier current (I_K) combined with an increase in I_f (see above). The increase in I_K increases the rate of repolarization and thereby shortens the AP, while an increase in I_f increases the rate of depolarization in the SA node (Figure 5). In addition, there is an increase in the activity of L-type Ca^{2+} channels. The increase in permeability to Ca^{2+} is partially responsible for the increase in contractile strength of the cardiac muscle. The antagonizing effect on sympathetic stimulation by parasympathetic nerves is also through G protein-dependent mechanism that inhibits adenylate cyclase. The result is reduced elevation of cytosolic Ca^{2+} and subsequent decrease in contractility. In addition, cholinergic stimulation again through a G protein-dependent mechanism activates a background K^+ channel that produces a hyperpolarizing membrane current. The effect of this current is to reduce the beat frequency by slowing the rate of diastolic depolarization. The acetylcholine activated background K^+ channel does not appear to be preset in ventricular myocytes [62-65].

Neural control of the embryonic heart

The onset of a sympathetic and parasympathetic control of the developing heart is largely unknown. In the human heart, muscarinic receptor-mediated cholinergic and β -adrenergic receptor-mediated responses are elicited soon after the initiation of the heartbeat, during the fourth and fifth weeks of gestation, respectively (Figure 7). However, because the cardiac nerves have not developed at this time, this does not represent the onset of neural control of the heart. Formation of morphological and functional control by the autonomic innervation occurs in the human heart well after the appearance of the reactivity to autonomic transmitters. Muscarinic-cholinergic innervation is established at 10-12 weeks. By 15-17 weeks, tachycardia can be elicited by atropine, indicating that there is tonic control of heart rate by the parasympathetic nerves. Bradycardia can be elicited by β -blockers in weeks 23-28, suggesting that sympathetic innervation is functional. This seems to follow

the general pattern that the parasympathetic-cholinergic control of the developing heart is functional earlier than sympathetic-adrenergic neural control [66].

Adrenergic and cholinergic receptors and their associated effector mechanism are present in cardiomyocytes well before autonomic nerve fibers can be found innervating the heart. It is not known why these autonomic receptor-mediated effect mechanisms are present before there is functional innervation. The levels of circulation catecholamines in the embryo may be high from endocrine-like secretion of catecholamines from the developing adrenal medulla and sympathetic chain ganglia [46-48]. Levels of catecholamines increase during periods of embryo hypoxia and it has been proposed that they mitigate the bradycardia and other effects of hypoxia on cardiac function [47, 48]. The oxygen level in the mammalian uterine environment in the early rodent organogenic period (or almost entire first trimester in humans) is low and the embryo may be subjected to episodes of hypoxia due to contractions of the uterus during pregnancy [67].

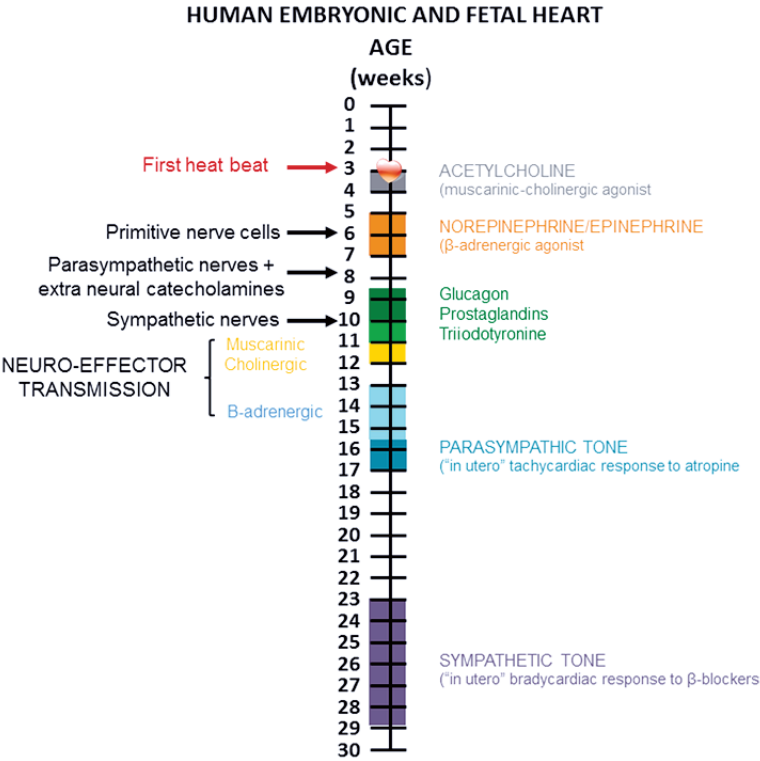


Figure 7. Overview of the innervation and onset of neural control during human heart development. Adopted from [66].

Onset of neurotransmitters

Acetylcholine

In rat, despite the absence of a functional nerve supply both GD 11 [68] and GD 13 mouse [69, 70] embryos in culture respond with an increase in heart rate to pharmacological adrenergic stimulation. Similarly, acetylcholine can reduce the heart rate of human embryos at 3-7 weeks, the magnitude of response increasing with gestational age [66]. Knockout mice for the cardiac receptor subtype (M2) show no prenatal abnormalities [71] suggesting that the receptor is not essential for prenatal survival, or there may be compensatory mechanisms involved in the development of the M2-deficient mice.

Catecholamines

Looking at tyrosine hydroxylase activity in rat embryos, adrenergic biosynthetic enzymes are expressed early by myocardial cells interspersed throughout the myocardium. By GD11.5, they are localized to regions of the sinoatrial and atrioventricular nodes. Gradually expression decreases in these regions but increases along the crest of the interventricular septum, where the bundle of His is located [72].

In GD 9.5 mouse hearts, β -adrenergic agonists increase the spontaneous beating rate, slope of the pacemaker potential and action potential duration with decreased maximum upstroke velocity. L-type Ca^{2+} channel currents are modulated by β -adrenergic receptors probably via a cAMP-dependent pathway [73].

Adenosine

Adenosine is produced by all cells by enzymatic degradation of ATP [74-76]. In mature tissue, adenosine inhibits norepinephrine release from sympathetic nerve endings and causes vasodilation of smooth muscle. Adenosine also has important antiarrhythmic properties and prevents some of the deleterious sequelae of ischemia. In humans, adenosine evokes a sympathetic excitatory reflex mediated by chemically sensitive receptors and afferent nerves in the mature heart. This reflex may be active during exercise and ischemia [77].

Adenosine A1 receptors (A_1R) are expressed in mouse embryonic hearts from the onset of cardiac contractions (GD8), making the adenosinergic system the earliest functionally responsive system in the heart [70]. Between GD 9 and 12, adenosine agonists down-regulate heart rate, possibly by a G protein-mediated mechanism that alters cAMP, ATP-dependent kinase, L-type calcium, sodium or chloride channels, and consequently the pacemaker current [78].

Developmental toxicity of ion channel blocking drugs

A considerable number of studies on the developmental effects of cardiovascular active drugs have been performed (Table 2, and reviewed in [11, 12]. It is beyond the scope of this thesis to discuss them all in detail. However, some aspects will be brought to attention here. Animal studies with drugs that selectively block the rapid component of the cardiac repolarization potassium current (I_{Kr}), encoded by the *human ether-a-go-go related gene* (hERG) have been performed primarily in rats [79-84]. A major effect of selective hERG/ I_{Kr} on development in rats is embryonic death [79, 80, 83, 84]. In a follow-up to the safety evaluation studies of the type III-antiarrhythmic agent L-691,121, Ban et al. (1994) investigated the cause to the high incidence in embryonic death after repeated oral dosing (Figure 8A). Single oral dosing (1 mg/kg, p.o.) on specific days of gestation identified a sensitive period in rats for embryonic death (GD 10-13) (Figure 8B). The observed embryonic death was not instant but occurred within 48 hours after dosage. Malformations of surviving embryos were enlargement of the cardiac tube and pericardium generalized edema, hematoma and deformed brain and forelimbs [84]. *In vitro* studies showed that L-691,121 slowed the rat embryonic heart rate, suggesting that the embryonic death in rats is due to interference with embryonic heart function [84]. The basic mechanism of type-III antiarrhythmic agents (according to the Vaughn-Williams classification) is potassium channel blockade, delaying cardiomyocyte repolarization, thus increasing the action potential duration and slowing the rate of contractions [85].

Table 2. *In vivo* and *in vitro* studies of cardiovascular active drugs.

Drugs	Exposure regimen	Species*	References
Antiarrhythmics:			
Almokalant	<i>In vivo, in vitro</i>	Rn	[79, 80]
	<i>In vivo, in vitro</i>	Mm	[86]
	<i>In vivo</i>	Rn	[81, 82]
Dofetilide	<i>In vivo, in vitro</i>	Rn	[80]
	<i>In vitro</i>	Rn	[87]
Ibutilide	<i>In vivo</i>	Rn	[83]
L-691,121	<i>In vivo</i>	Rn	[84]
d-Sotalol	<i>In vivo, in vitro</i>	Rn	[80]
	<i>In vivo</i>	Oc	[88]
Antiepileptics:			
Carbamazepine	<i>In vitro</i>	Mm	[89, 90]
Dimethadione	<i>In vivo, in vitro</i>	Mm	[91]
	<i>In vitro</i>	Mm	[90]
	<i>In vivo</i>	Rn	[92, 93]
Phenobarbital	<i>In vitro</i>	Mm	[89, 90]
Phenytoin	<i>In vivo, in vitro</i>	Mm	[94-96]
	<i>In vitro</i>	Mm	[89, 90]
	<i>In vitro</i>	Mm, Rn	[97]
	<i>In vitro</i>	Rn	[98]
	<i>In vivo, in vitro</i>	Rn	[99]
	<i>In vivo</i>	Rn	[100]
	<i>In vivo</i>	Oc	[101-103]
Trimethadione	<i>In vitro</i>	Mm	[89]
Valproic acid	<i>In vitro</i>	Mm	[89]
Vasodepressants:			
Felodipin	<i>In vivo</i>	Rn	[104]
	<i>In vivo</i>	Oc	[105, 106]
Nifedipine	<i>In vivo</i>	Oc	[101, 105, 106]
Nitradepine	<i>In vivo</i>	Oc	[105]
Others:			
Cisapride	<i>In vivo, in vitro</i>	Rn	[107]

*Mm – *Mus musculus*, Rn – *Rattus norvegicus*, Oc – *Oryctolagus cuniculus*

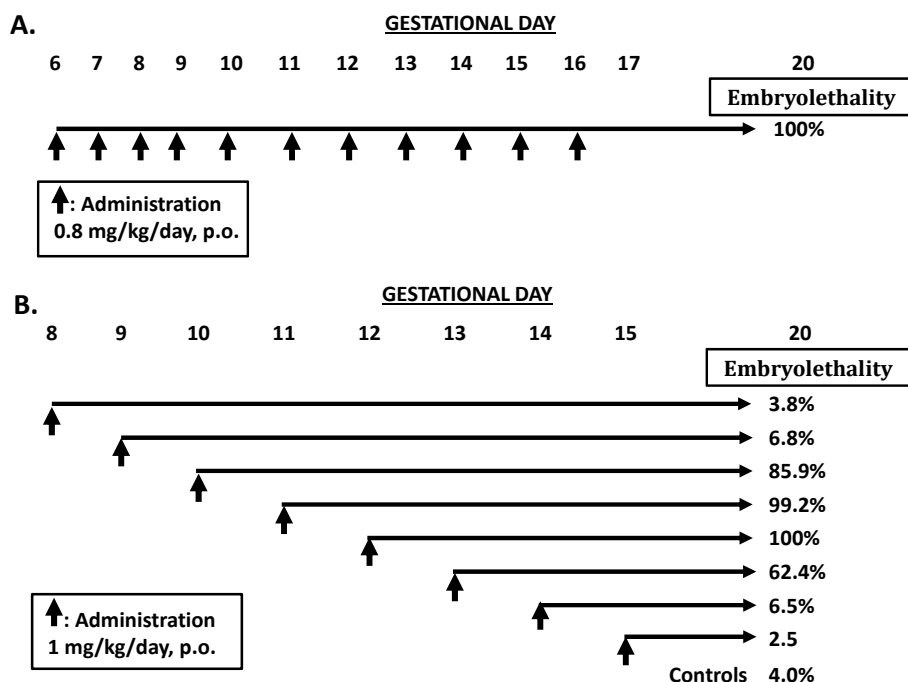
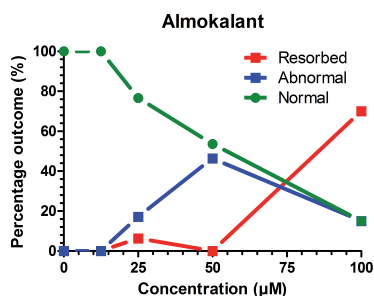


Figure 8. (A) Incidence of embryo lethality of GD 20 following treatment with 1 mg/kg L-691,121 on GD 6-16. (B) Incidence of embryo lethality on GD 20 following treatment with 1 mg/kg L-691,121 on a single day between GD 8 and 15. Adopted from [84].

In *in vivo* studies there is an apparent close relationship between embryonic death and malformations in surviving rat embryos. A major consequence of exposure to selective hERG/I_{Kr} blocking drugs in pregnant rats is embryo lethality (figure 9). This has led to the concern, that a high incidence in embryonic death may mask the teratogenic potential ([83, 107], and reviewed in [12]).

Another point to be made is regarding GD of exposure in *in vivo* and *in vitro* studies. Selective hERG/I_{Kr} blocking drugs cause an array of malformations on specific days of gestation [81, 82]. Various single doses on GD 8 to 16 of almokalant, dofetilide or d-sotalol to pregnant rats, identified a sensitivity period for malformations GD 10-14, with GD 13 as the most sensitive day of teratogenicity with a high incidence of forelimb defects (Figure 10). A high incidence of external and visceral malformations is also observed on GD 11, such as right-sided oblique cleft lip, short tails (Figure 11), and cardiovascular defects [82].

A.



B.

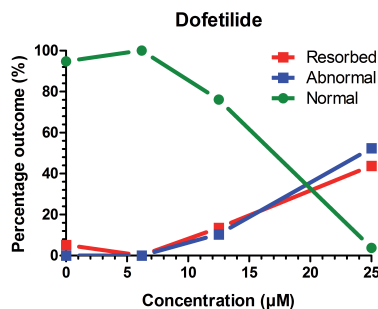


Figure 9. The outcome (resorptions, abnormal embryos, normal embryos after external visual inspection) in percentage, after single oral dosing of the type III-antiarrhythmic drugs almokalant or dofetilide to pregnant rats on GD 13 [80].

As a consequence, the majority of *in vitro* studies investigating the effect on embryonic heart function hERG/ I_{Kr} blocking drugs has been performed on GD 11 or 13 rat embryos [79, 80, 98, 99], or corresponding developmental stages in the mouse [86, 89, 90]. In this thesis, we have used the GD 13 rat embryo as a biological model in our *in vivo* and *in vitro* studies.

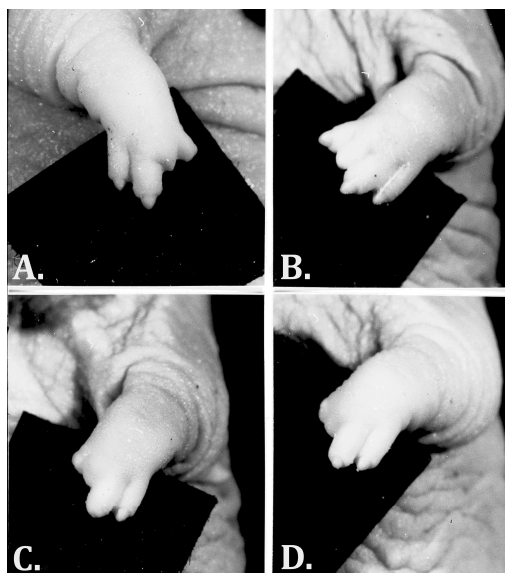


Figure 10. Distal digital defects after almokalant doses on GD 13. Contribution of W.S. Webster.

Drug-induced embryonic hypoxia

A consequence of a malfunctioning heart is inadequate oxygenation of tissues and organs that are normally supplied with oxygenated blood, circulated by the heart. In the literature the words anoxia and hypoxia may often be used synonymously, however, anoxia meaning total lack of oxygen is probably rarely achieved biologically outside of frankly lethal situations. Therefore, hypoxia, generally meaning relative lack of oxygen, is a better term in this context. Studies with hypoxia markers have shown that a teratogenic dose of hERG/I_{Kr} blocking drugs given to pregnant animals on sensitive days of gestation subsequently results in increased hypoxia of embryonic tissues [95, 108, 109]. The increase in hypoxia is a generalized increase in the whole embryo, and not a specific increase in tissues later found to be malformed at birth [95, 108]. Nevertheless, the results support the association between pharmacologically drug-induced hypoxia and birth defects.

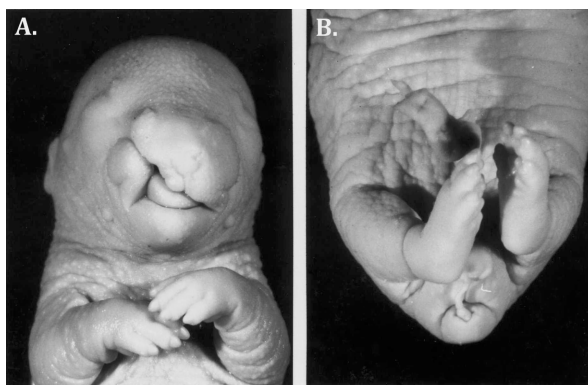


Figure 11. Right cleft lip and short tail after almokalant exposure on GD 13. Contribution of W.S. Webster.

Aims of the thesis

At the time when the aims of the thesis were formulated, there was an extensive literature on hypoxia-related developmental effects of hERG/I_{Kr} blocking drugs in pregnant experimental animals. There was also a suggested association between the pro-arrhythmic potential of a drug in the adult and its potential teratogenicity. However, little was known about the effect on the embryonic heart function of drugs active on ion channels other than hERG/I_{Kr}, and even less was known about the effect on embryonic heart function of drugs with concomitant action on more than one cardiac ion channel. The, overall aim of this study was to explore the teratogenic potential of ion channel blockade of the mammalian embryonic heart.

The specific objectives of the thesis were:

- To investigate if the teratogenic potential of hERG/I_{Kr} blocking drugs may be masked due to the embryo lethality in conventional safety evaluation studies.
- To develop the methodology to assess embryonic heart function of rat embryos in whole embryo culture.
- To examine the effect on rat embryonic cardiac function of pharmaceutical drugs with various ion channel-blocking properties.
- To examine the likelihood of suggested human teratogens to induce hypoxia related malformations via drug-induced heart malfunction in the developing embryo.
- To explore the effect of drugs interfering with cardiac ion currents *in vitro* during the ‘sensitivity period’ for adverse developmental effects in the rat.

Methods

Without deviation from the norm there can be no progress.

Frank Zappa (1940-1993)

In vitro whole embryo culture (WEC) of rat embryos is the main method used in this thesis (paper I, II, III and IV). However, the WEC methodology used in this thesis is somewhat modified from the traditional technique of culturing rat embryos (GD 9-11). In addition to the WEC, an *in vivo* teratology study and a hypoxia probe study were performed in paper I. Methodological aspects and details on selection of animals, justification of drug selection and concentrations, experimental design, and conduction of the experiments etc., are included in this section. For complete details on the experiments, not discussed in this section, the reader is referred to the Materials & Methods sections in specific papers.

Animals

All animal work in this thesis was approved by the animal ethics committee in Uppsala, Sweden, (paper I, II, and III) and the University of Sydney animal ethics committee (paper I and IV). In all experiments Sprague-Dawley (SR) rats were used.

Time-mated Sprague-Dawley (SD) rats (B&K Universal (Sollentuna, Sweden) were used in the *in vitro* WEC studies in paper I. SD rats (Animal Research Centre, Western Australia) were used in the *in vivo* teratology studies in paper I, and in the *in vitro* WEC studies in paper II and IV. SD rats (Møllegaard breeding centre Ltd, Denmark) were used in the hypoxia probe studies in paper I. SD rats (Taconic M&B, Denmark) were used in the *in vitro* WEC studies in paper III. The rats in paper I, II and IV were kept under a 12 hours light/dark cycle and the mating was done over-night. The rats in paper III were kept under a reverse light/dark cycle (6 a.m. dark/6 p.m. light), and mated between 8.00 a.m. and 2.00 p.m. In all experiments the day of vaginal plug or a sperm-positive smear was considered gestational day zero (GD 0).

Drugs

In total, twenty pharmaceutical drugs were investigated in this thesis. Two drugs (astemizole and cetirizine) were investigated *in vivo* and in *in vitro* WEC, and the remaining eighteen drugs were tested in *in vitro* WEC only. An overview of the compounds and the concentrations used in WEC, and reported affinity towards some ion channels is presented in table 3.

In paper I, stock solutions of drugs to be used in WEC were prepared with dimethylsulfoxide (DMSO) and stored in -20°C until used. In paper II and III, stock solutions of the tested drugs were prepared freshly each day. In paper IV, stock solutions of dofetilide, lidocaine and phenytoin were prepared with water and kept cold (4°C) until used. Stock solution nifedipine used in paper IV was prepared freshly each day with DMSO.

Rationale for dose selections *in vivo*, concentrations used *in vitro*, and duration of exposure.

In the teratology study in paper I, pregnant rats were administered a single oral dose of 80 mg/kg astemizole on GD 13. This dose was selected since regulatory studies, in which rats were dosed with 160 mg/kg astemizole in the diet resulted in a 100% resorption rate [110]. In the *in vivo* hypoxia probe study in paper I, pregnant rats were administered astemizole (160 mg/kg) or cetirizine (225 mg/kg) by gavage followed by a hypoxia marker (HypoxiprobeTM, NPI Inc., Belmont, MA), pimonidazole hydrochloride (60 mg/kg, i.v. in the tail vein) 30 minutes later on GD 13 or 16. The doses of astemizole (160 mg/kg) and cetirizine (225 mg/kg) were the same as the top doses in reported teratology studies (Segment I for astemizole; 14 days prior to mating and further during pregnancy, Segment II study for cetirizine; from GD 6-15) in rats [110, 111]. GD 13 and 16 as days of treatment were selected based on previous work showing that single dosing of a hERG/I_{Kr} blocker on GD 13 resulted in a high incidence of forelimb defects, while dosing after GD 15 no external malformations were observed [80].

The concentrations used in the WEC studies were based on preliminary concentration range studies in the individual papers (paper I, II and III), with the exception of the concentrations used in paper IV which are based on the previous findings in paper II. Thus, the concentrations used in the *in vitro* studies were not primarily based on exposure data from the literature, although the final concentrations used in the WECs are discussed in relation to possible human exposure. Generally, by establishing a concentration-response curve based on several concentrations (rather than testing a single or two) a better relationship can be defined. Therefore, concentrations up to 1 mM (dependent of solubility) were initially tested for each drug, and the effect on the rat embryonic heart was recorded. The concentration of each

drug was then progressively reduced (e.g. with a factor 2) until a no observed effect concentration was reached. In a majority of drugs, 4 to 8 concentrations were tested.

Table 3. Overview of investigated pharmaceutical drugs

Drug	Pharmacological action	Conc. tested (μM)	IC ₅₀ I _{Kr} /hERG (μM)	IC ₅₀ I _{Na} /NaV1.5 (μM)	IC ₅₀ I _{CaL} /CaV1.2 (μM)
Paper I:					
Astemizole	Antihistamine	0.02-0.4	0.048	-	-
Cetirizine	Antihistamine	6	>30*	-	-
Paper II:					
Amiodarone	Antiarrhythmic	2.75-88	0.95 [112]	3.0 [113]	~1.0 [114]
Disopyramide	Antiarrhythmic	15-250	84 [112]	28 [115]	100 [116]
Dofetilide	Antiarrhythmic	0.025-3.2	0.021 [112]	>100 [117]	>100 [117]
Flecainide	Antiarrhythmic	2.8-22	2.3 [112]	6.5 [118]	63.4 [119]
Lidocaine	Antiarrhythmic	31-500	-	102 [118]	>100 [120]
Verapamil	Antiarrhythmic	0.15-4	0.38 [112]	5.7 [118]	27.4 [121]
Quinidine	Antiarrhythmic	2.5-40	1.4 [112]	5.2 [118]	14.9 [114]
Mibefradil	Vasodilator	0.6-10	1.0 [112]	0.3 [122]	0.1 [123]
Nifedipine	Vasodilator	0.9-28.8	275 [124]	>100 [125]	0.07 [121]
Amitriptyline	Tricyclic anti-depressant	5-20	4.7 [112]	1.0 [118]	23 [126]
Indapamide	Diuretic	275-2,200	~75 [127]	79 [128]	>1000 [128]
Niflumic acid	NSAID	12.5-200	-	73 [129]	~100 [130]
Phenytoin	Antiepileptic	50-800	100 [131]	23.5 [132]	~30 [133]
Nickel chloride	n/a	1,000	-	-	-
Atenolol	β1-adrenegic antagonist	1,000	-	-	-
Paper III:					
Azithromycin	Antibiotics	100-1.000	>300 [134]	-	-
Clarithromycin	Antibiotics	25-500	45.7 [135]	-	-
Erythromycin	Antibiotics	25-500	38.9 [135]	-	-
Paper IV:					
Dofetilide	Antiarrhythmic	0.4	0.021 [112]	>100 [117]	>100 [117]
Lidocaine	Antiarrhythmic	125-250	-	102 [118]	>100 [120]
Nifedipine	Vasodilator	0.9-1.8	275 [124]	>100 [125]	0.07 [121]
Phenytoin	Antiepileptic	100-200	100 [131]	23.5 [132]	~30 [133]

*No effect on hERG current expressed in *Xenopus laevis* oocytes in concentrations tested up to 30 μM.

In paper I, II and IV the exposure time in WEC was 1 hour for comparison reasons, whereas in paper III the rat embryos were expose over a three hour period with the heart rate examined every hour. The reason for the prolonged exposure time in paper III was macrolide antibiotics are relatively

large drug molecules (MW ~600-700) and may need longer time to be transported from the culture media, and distribute into the embryonic tissue.

Whole embryo culture

Culture methods of rodent embryos are based on the pioneer work by Nicholas [136], however, it was with the further development, reliability and adaptability of the method by New and co-workers, that the mammalian whole embryo culture (WEC) method was widely established in the scientific community [137].

It has been generally accepted that whole embryo culture methods greatly facilitate studies on normal and abnormal embryonic development under controlled experimental conditions. However, the period of investigation is limited to the main phase of organogenesis. Most likely due to the failure of the allantoic placenta to develop in culture, growth of embryos (measured by total protein synthesis) is often found to be retarded. Therefore, normal rates of embryonic/fetal growth beyond the time (~GD 11 in rats) at which the allantoic circulation becomes functional is unlikely to be maintained in WEC [138].

One of the main purposes of using the WEC method has been to observe and interpret deviations from normal *in vitro* development, often with the intention of analyzing the effects of toxic substances. Endpoints measured in rodent WEC are morphologic abnormalities (i.e. deviation from “normal” development *in vitro*) of the cultured embryos [139, 140]. The rat WEC method is one of the four *in vitro* assays validated by the European Centre for the Validation of Alternative Methods (ECVAM) [141]. In the study the accuracy of rat WEC to predict developmental toxicity was estimated to ~80%. Recently, however, the method was challenged with a novel set of pharmaceutical compounds showing the method to be less accurate than previously reported [142].

Short term WEC

The *in vitro* WEC methods of rat embryos used in this thesis are different from the “conventional WEC”. However, the differences are mainly due to the developmental stage used in the particular experiments. Since the embryos in this thesis are cultured for a considerably shorter time than normally in WEC, to avoid any misunderstandings regarding what method is used, the culture method in this thesis is designated *short term* WEC. Although the culture system used at Uppsala university (paper I and III) differed from the

culture system used at the University of Sydney (paper II and IV) (Figure 12), the procedure of the short term WEC; from killing the pregnant animal, explanting the embryos, assessing heart function etc., were basically performed uniformly in all *in vitro* experiments. Technical details on instrumentation are found in table 4. A presentation of the short term WEC method is presented below.

Table 4. Overview of culture systems used for short term WEC

	Uppsala University (paper I and III)	The University of Sydney (paper II and IV)
Gas	Intermittent	Continuously
Incubator system	Roller rotator inside a Binder incubator	Rotating drum culture system (BTC Engineering, Cambridge, UK)
Rotation speed (rounds per minute, rpm)	40	11
Microscope	Olympus SZ40	Leica DP70
Camera system	Ikegami ICD-700P (paper I) Ueye, UI-2210 (IDS Imaging Development Systems GmbH, Obersulm Germany) (paper III)	Olympus DP70 (Olympus Australia Pty Ltd, Melbourne, Australia)
Heating stage	Ref N° 12055/0003 (MTG Medical Technology, Altdorf, Germany)	Linkham MS 100

Explanting of embryos

Pregnant rats (SD) were killed on GD 13 by CO₂ inhalation. The abdomen was opened and the uterus placed in warm (37°C) phosphate buffered saline (PBS). The decidua and Reichert's membrane were removed from each embryo and a small incision was made in the yolk sac, taking care not to damage the vasculature. The embryos were then pulled through the incision by grasping the amnion with forceps, and the amnion was then opened [87, 143].

Assessment of exposure

Each embryo with open yolk sac was then placed in a culture bottle containing pre-gassed Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co, St Louis, MO, USA) (38°C) and cultured for one hour.



Figure 12. Culture systems at Uppsala University (left and middle) and at the University of Sydney (right)

After the recovery period in culture the heart rate was determined for each embryo using a light microscope equipped with a camera. A video was taken off the embryo and saved on a computer hard drive. Because the embryonic heart rate is temperature sensitive [144], during the examination of the embryos the bottles were placed on a heating stage keeping the culture media at 38°C. Damaged or dead embryos, and embryos not fulfilling the minimum beats per minute (bpm)-criteria were discarded. After the embryonic heart rate was recorded, the test substances were added to the culture medium. Controls were treated with vehicles. All treatments were randomized. One hour after addition of the test substances the embryonic heart rate was examined and recorded a second time, after with the experiment was then terminated (paper I, II and IV). In paper III, the culture of embryos continued for another 2 hours before it was terminated. We have previously cultured embryos up to 4 hours successfully [145].

Image analysis of embryonic heart rate

Prior to the work in this thesis, monitoring of rat embryonic heart function was routinely done by visually observing the heart under a light microscope and calculating the number of beats for a period of time. This method is tedious, potentially subjective, and error prone due to the fast rat embryonic heart rate. Methods to automate the assessment of embryonic heart rates have been done over years [107, 144]. As the existing techniques were unavailable to us or shortcoming for the purpose, in parallel to the biological work in the thesis we collaborated with the Centre for Image Analysis at Uppsala University, to develop an efficient and reliable method to assess rat embryonic heart rate *in vitro* from digital recordings [146, 147]. The video recordings in paper I-IV are analyzed with the software developed for the purpose.

The embryonic heart can be detected automatically in the video recordings by the software, if necessary, or different regions of interest (ROI) can be marked manually. In the automated method the heart is localized with the

help of motion information instead of color information (shades of red) because there are instances when the color of the blood is not easily visible due to poor imaging conditions. There are also situations when regions of the embryo have the same color as the blood.

The motion of the heart is represented as a 1D heart signal by taking the cross-correlation between the template frame and the rest of the frames of within the heart region. The template represents either of the extreme states in the heart cycle (i.e. either end-systole or end-diastole). The software used in paper I and II, used a template chosen from the two frames which produced the minimum cross-correlation in the first two seconds of the video. In paper III and IV, further development of the software had been done, and the template used was chosen from the four frames (two from atria and ventricles, respectively). The identification of embryonic hearts in the video recordings in this thesis was manually marked.

Results and discussion

Believe half of what you see and none of what you hear.

Lou Reed

The use of excessive doses in developmental toxicity testing of selective hERG/I_{Kr} blocking drugs may mask teratogenicity

Paper I

A number of proof of principal experiments were performed, testing if the teratogenic potential of the antihistaminic drug astemizole may have been missed in the original teratology studies due to a high rate of embryonic death [110].

The results of paper I show that a single oral dose (80 mg/kg) of astemizole on GD 13 causes distal digital defects of the forelimbs in rat offspring examined near term (Figure 13).

In vitro exposure of GD 13 rat embryos to astemizole (20-400 nM) caused a concentration-dependent bradycardia and arrhythmias at higher concentrations (200-400 nM). The antihistaminic drug cetirizine (6 μ M) served as a negative control in the WEC experiment. Cetirizine, without hERG/I_{Kr} blocking properties, did not affect rat embryonic heart rate *in vitro*. The hypoxia marker pimonidazole showed that astemizole cause hypoxia in the embryo *in vivo*, while cetirizine did not (Figure 14 and table 5).



Figure 13. Forelimbs from GD 20 rat fetuses. **(A)** Forelimb from a control fetus. **(B and C)** Forelimbs from fetuses of dams treated with 80 mg/kg astemizole on GD 13. The limb defects shown are oligodactyly **(B)** and brachydactyly **(C)**.

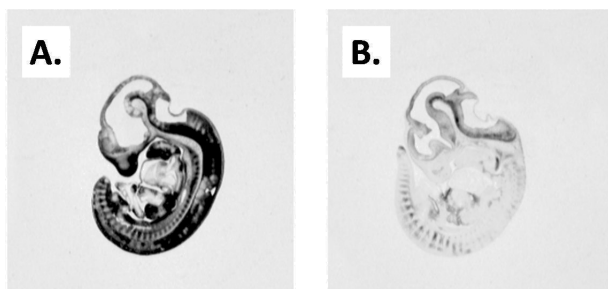


Figure 14. (A) Astemizole-treated embryo stained to show hypoxic areas using pimonidazole. (B) Pimonidazole stained control embryo.

Prior to paper I, similar work has been performed comparing the gastropromkinetic drugs cisapride (hERG/I_{Kr} blocker) and mosapride (non- hERG/I_{Kr} blocker) [107]. In concordance with the results in paper I, the experimental design in the work by Sköld et al. (2001) showed that hERG/I_{Kr} blocking properties may constitute the teratogenic difference of drugs belonging to the same pharmacological class [107]. The high rate of embryonic death (i.e. embryotoxicity) of highly selective hERG/I_{Kr} blocking drugs (i.e. type-III antiarrhythmic drugs) may be a problem when evaluating the safety issues of these drugs [80, 83, 84], however, this may be a problem for selective hERG/I_{Kr} blocking drugs *per se*, which is derived from the old headache in developmental toxicity testing regarding maternal toxicity and dose selection.

Table 5. Immunostaining results from treatment with astemizole, cetirizine or controls (vehicle/tap water) followed by the administration the hypoxia marker pimonidazole. Figures are presented as mean \pm SEM.

Day of treatment	Gestation day 13			Gestation day 16		
	Contr.	Astemizole	Cetirizine	Contr.	Astemizole	Cetirizine
No. of litters	7	4	3	6	4	3
No. of embryos	19	12	8	14	9	8
Average hypoxia staining ($\times 10^{-2}$)	4.9 \pm 0.5	14.4 \pm 1.1*	6.3 \pm 0.7	7.2 \pm 0.7	7.2 \pm 0.5	6.9 \pm 0.6
Ratio drug/control	-	2.9	1.3	-	1.0	0.96

Contr. – controls

*p \leq 0.05

A strategy in toxicological evaluations has been to compensate for small numbers of experimental animals by using excessive doses. Although pragmatic and easy to perform, this strategy may be basically wrong and unsuitable for risk assessment relevant to man, and that the results obtained from such studies may easily give rise to misinterpretation. This strategy is used to a lesser extent for medical substances today, because the dose range used in experimental studies should not deviate that much from the anticipated hu-

man exposure, especially when plasma concentrations are compared rather than doses. Generally in developmental toxicity studies of pharmaceutical drugs, three doses levels are used: a low dose level near the expected therapeutic concentration/a high dose where minimal toxicity is observed (e.g. a decrease in maternal body weight gain), and an intermediate dose. However, a majority of the selective hERG/I_{Kr} blocking drugs may be inevitably linked with the generation of data obtained after administration of excessive doses. As a consequence, conventional or regulatory teratology testing may fail to identify the teratogenic potential of drugs that slow the embryonic heart rate with the observation of increased embryonic death being dismissed as maternal toxicity (reviewed in [12]).

The special feature of selective hERG/I_{Kr} blocking drugs is that they do not affect the heart of the rat dam so large doses can be given to the pregnant animal without toxicity to the dam. The heart of the adult mouse and rat does not need hERG/I_{Kr} for repolarization [79, 148]. In the human the situation is different, the embryonic heart is dependent on hERG/I_{Kr} [98] but so is the adult human heart. Hence it is uncertain if a pregnant woman could be exposed to a high enough dose of almokalant or dofetilide to affect the embryo or if it would affect the mother's heart first.

Consequently, dosing on single GDs and monitoring rat embryonic heart function *in vitro* may be a complement prior to conventional studies to better characterize, and screen the teratogenic potential of hERG/I_{Kr} blocking drugs.

Cardiovascular active drugs affecting the embryonic heart could potentially be harmful during pregnancy

Paper II

Characterization of the developmental toxicity of hERG/I_{Kr} blocking drugs is primarily based on studies with highly selective blockers of the cardiac potassium channel (e.g. type III-antiarrhythmic drugs almokalant or dofetilide) [80-82, 149]. Hence, little is known on the effects on the teratogenic properties of simultaneous blockade of other cardiac ion channels. The study in paper II was undertaken to investigate the effect *in vitro* of a range of pharmaceutical drugs with various combinations of ion-channel blocking activity on the functional activity of the GD 13 rat heart. The hypothesis was that blocking of hERG/I_{Kr} is only one factor that determines the overall effect on the embryonic heart.

An overall finding in paper II was that the effect of drugs blocking multiple ion channels is difficult to predict just by looking at the affinity for individual ion channels. Basically all drugs tested caused a concentration de-

pendent bradycardia, with the exception of the vasodilating agent and Ca^{2+} channel blocker nifedipine (Figure 15).

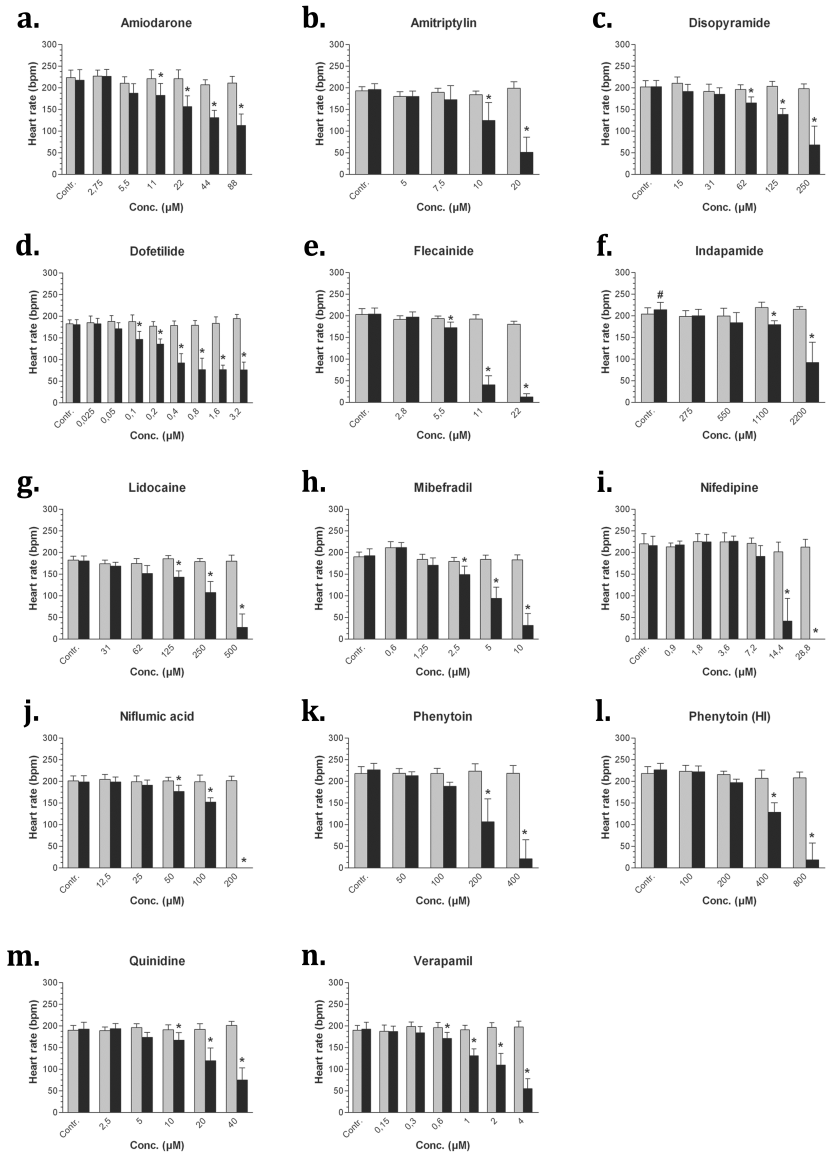


Figure 15. Pre- (gray bars) and post-exposure (black bars) heart rates (mean \pm SD) at various tested concentrations for drugs in [150].

In addition to the overall effect of slowing the rat embryonic heart rate *in vitro*, a number of drugs induced arrhythmia that appeared to be related to either Na^+ channel blockade, or hERG/ I_{Kr} channel blockade (Figure 16).

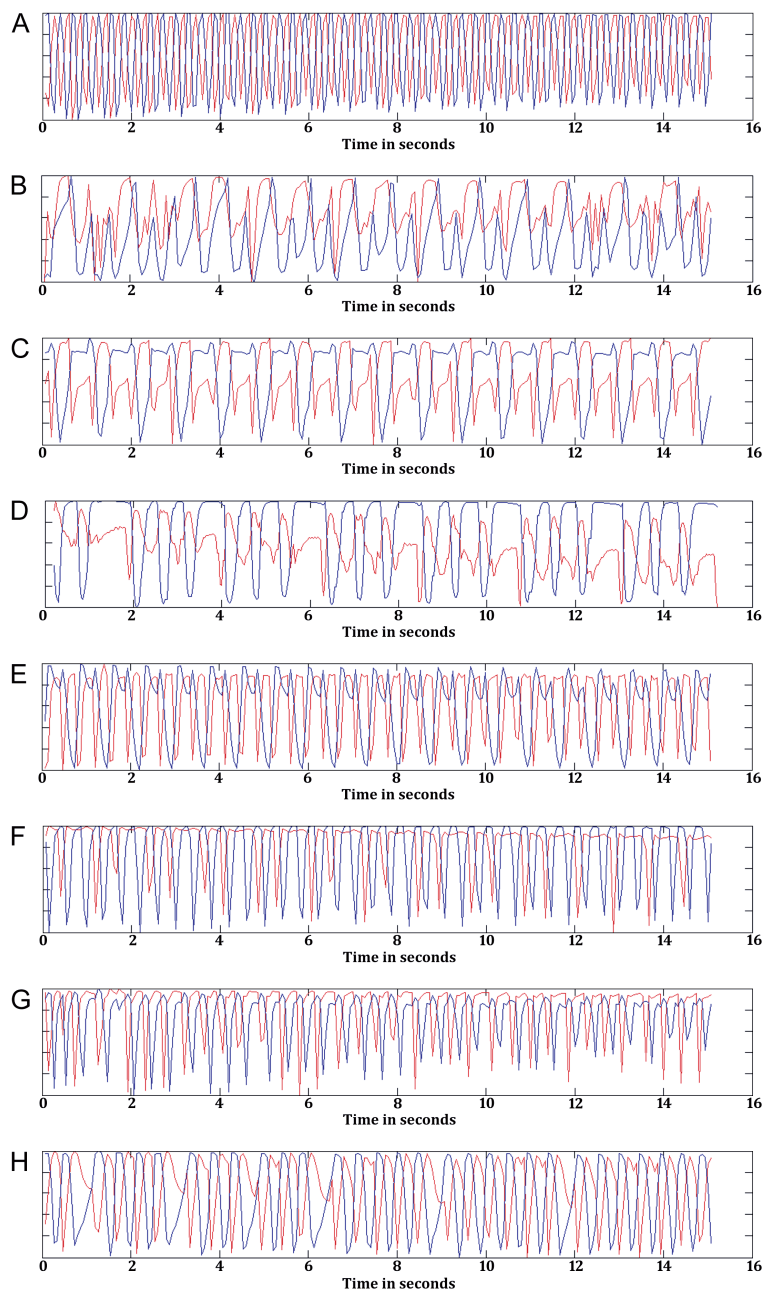


Figure 16. Traces from [150] of embryonic hearts lasting 15 sec. The trace from the atria is read and for the ventricles blue. (A) Control; (B) Dofetilide 400 nM; (C) Lidocaine 250 μ M; (D) Quinidine 40 μ M; (E) Amitriptyline 10 μ M; (F) Amiodarone 88 μ M; (G) Mibefradil; and (H) Niflumic acid 100 μ M)

The arrhythmias induced by the selective hERG/I_{Kr} channel blocking drug dofetilide resulted in an irregular heart rate with the atria and ventricles beating at a different rhythm and with varying inter-beat time (Figure 16B). Blockade of sodium channels also caused arrhythmias; however, the appearance of the arrhythmias was different from those seen with hERG/I_{Kr} blockade. Typical traces showed additional contractions of the atria for every ventricular beat (similar to 2:1 AV block) (Figure 16C).

From a teratological perspective, any drug that can cause bradycardia or arrhythmia in the embryo may be potentially harmful in pregnancy [8]. In practice, however, human exposure levels are much lower than the concentrations associated with the adverse effects observed in this paper.

WEC of macrolide antibiotics do not support blockade of hERG/I_{Kr} as teratogenic mechanism of erythromycin

Paper III

As mentioned previously, characterization of developmental toxicity of hERG/I_{Kr} blocking drugs is primarily based on studies with highly selective blockers of the cardiac potassium channel [80-82, 149]. Paper III was done in response to a publication based on information from the Swedish Medical Birth Register [151], suggesting that the macrolide antibiotic erythromycin may be a human teratogen due to its ability to block hERG/I_{Kr} [135, 152-154]. The hypothesis tested in paper III was that macrolides would have the same effect on embryonic heart function *in vitro* as selective and highly teratogenic hERG/I_{Kr} blockers previously investigated [80, 149, 150]. Three macrolides with different pro-arrhythmic potential were tested [153].

As seen in figure 17 the rat embryos tolerated the extended WEC to 4 hours in culture, as well as the washout procedure. The results show that erythromycin and clarithromycin cause a concentration-dependent slowing of the heart rate (Figure 18). Arrhythmias were only observed at high concentrations and at long exposure time (3 h). In comparison with previous arrhythmias induced by the selective hERG/I_{Kr} blocker dofetilide, the arrhythmias of erythromycin or clarithromycin were of a different sort. No arrhythmias were observed with azithromycin.

Since erythromycin is primarily used to treat infections it is possible that elevated body temperature is an additional risk factor for the embryo. We investigated whether a 2°C elevation would increase the degree of bradycardia caused by erythromycin in the rat embryos. Hyperthermia (40°C), simulating feverish condition in connection to antibiotic therapy did not augment the effect of erythromycin or clarithromycin on rat embryonic heart rate (Figure 19).

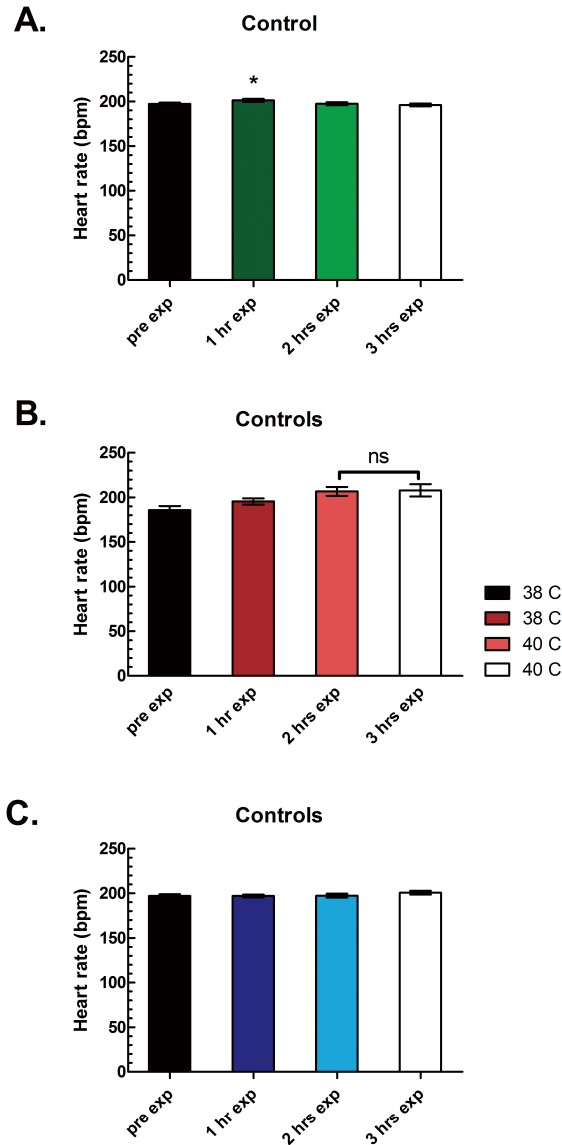


Figure 17. Heart rate (mean±SEM) of control embryos in paper III. (A) Main study, (B) hyperthermia experiments, and (C) washout experiment. The black bar represents pre exposure heart rate in all experiments. Dark green, dark red and dark blue bars represents 1 hour of exposure, respectively. Light green, light red and blue bars represent 2 hours of exposure, respectively. White bars represent 3 hour of exposure in all experiments. In (B) there was a significant difference ($P<0.05$) between all groups except 2 and 3 hours exposure. * $p\leq 0.05$

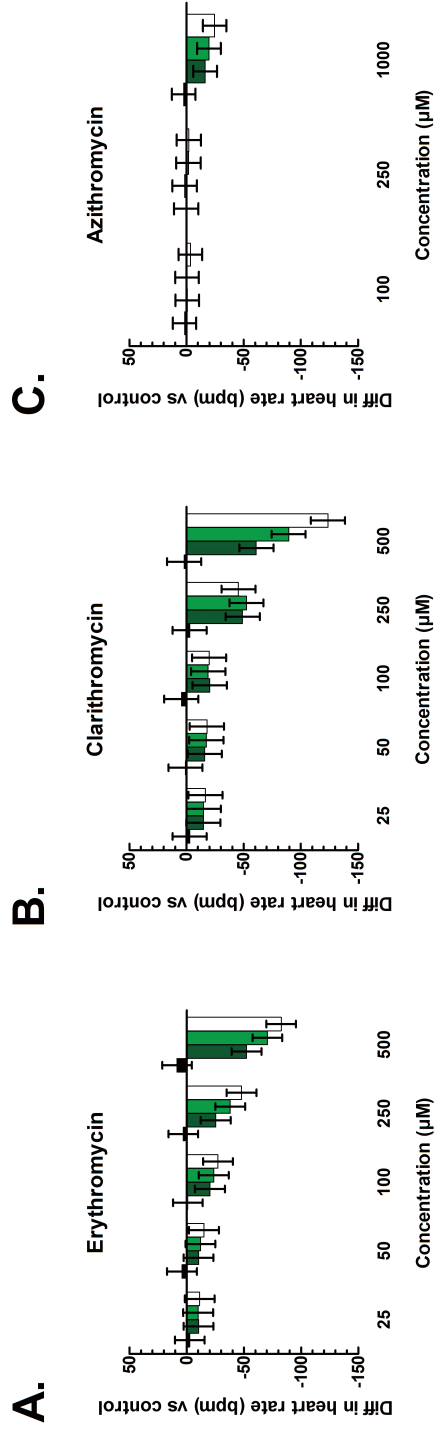


Figure 18. Difference in heart rate (mean \pm 95%CI) compared with controls of (A) erythromycin (25-500 μM), (B) clarithromycin (25-500 μM , or azithromycin (100-1,000 μM). The color representations of the bars are the same as in Figure 17.

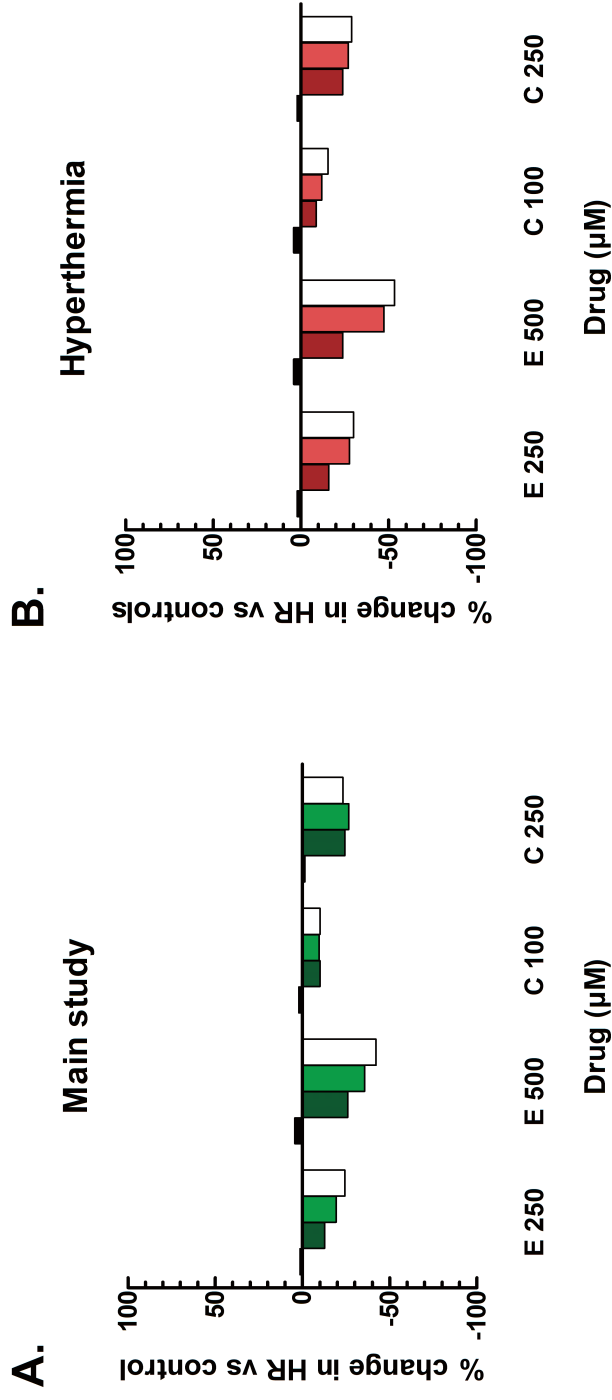


Figure 19. Percentage change in rat embryonic HR compared with controls in, (A) main study at 38°C, and (B) hyperthermia experiment with increased temperature (40°C) during the last two hours of exposure. The color representations of bars are the same as in Figure 17.

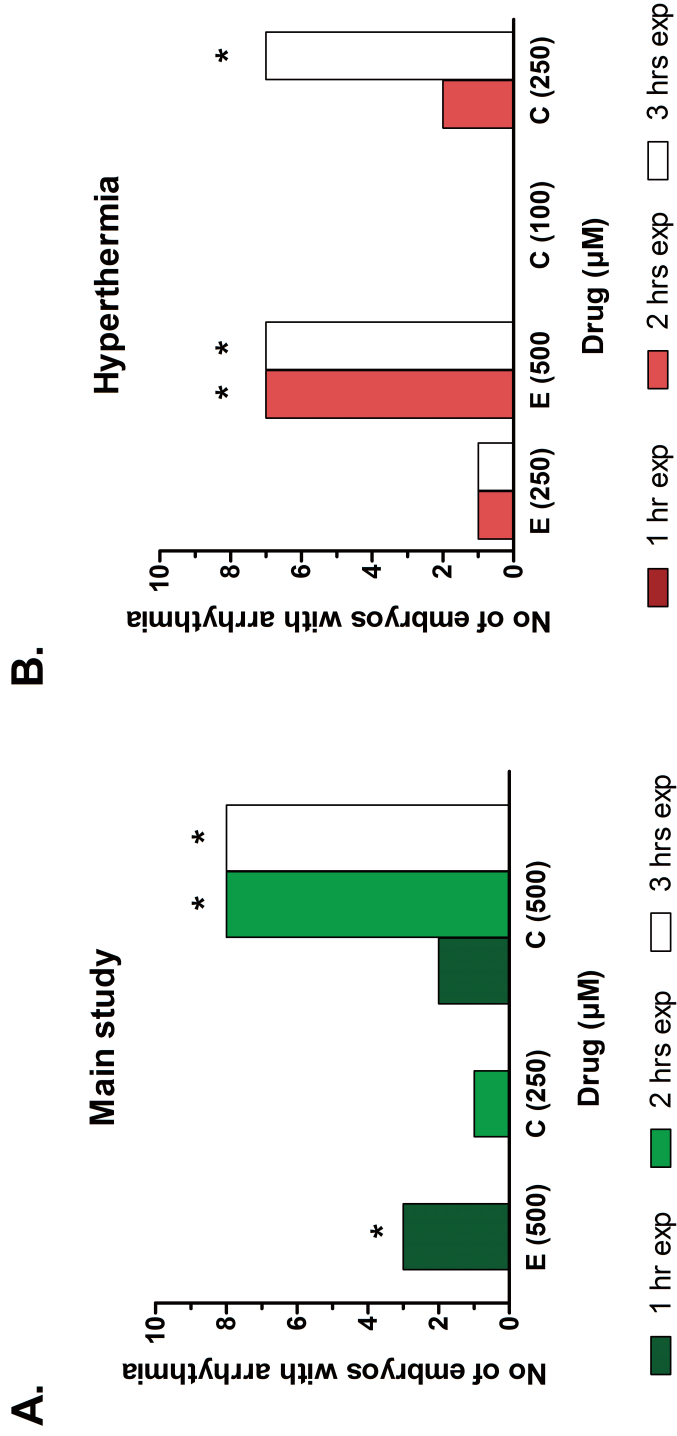


Figure 20. Number of embryos with arrhythmias in, (A) main study at 38°C, and (B) hyperthermia experiment with increased temperature (40°C) during the last two hours of exposure. The color representations of bars are the same as in Figure 17. There was ten embryos in each group (N=10). *P<0.05.

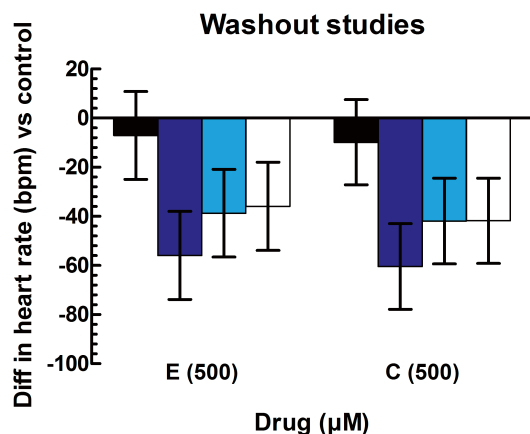


Figure 21 Difference in heart rate (mean \pm 95%CI) compared with controls in the washout experiment of erythromycin (500 μ M) and clarithromycin (500 μ M). The color representations of bars are the same as in Figure 17

The number of arrhythmias increased with increased temperature, and the onset occurred at shorter exposure time compared with 38°C (Figure 20). A positive temperature dependent effect of erythromycin on hERG/I_{Kr} is reported in neonatal (day 1) mouse ventricular myocytes. Increased potency with temperature is also shown in human HEK293 cell. In the latter study the increase in effect with temperature was explained by facilitated transport over the cell membrane and not an effect on the ion channel *per se*. A facilitated distribution of the large drug molecules from the extracellular compartment into the cell would explain the early onset of arrhythmias observed at a higher temperature (40°C).

Washout studies did not markedly improve heart function indicating that erythromycin or clarithromycin do not washout well which may be expected since they are large molecules (Figure 21). We have successfully washed out the effect of sodium channel blockers (MW \sim 400) according to the same protocol as we used in this study [145].

In a study of women awaiting therapeutic abortions (12-22 weeks), placental transfer of erythromycin was studied after a single oral dose of 500 mg erythromycin. Mean maternal blood levels were 27 nM with a range of 0-150 nM. There was some evidence of erythromycin in fetal liver (0.6 μ M). Multiple dosing (4-20) resulted in mean maternal blood levels at the time of operation of 4.7 μ M and mean fetal blood levels were 81 nM, ranging from 0-163 nM. Accumulation in the fetal liver was also reported with repeated dosing (0-1.1 μ M) [155]. The extent to which these measurements are relevant to the immature placenta at 5 weeks gestation (corresponding to \sim GD 13 in rats) is not known but the tissue barrier between the maternal and embryonic circulation is thicker at this stage. Whether this translates into a

more substantial barrier to erythromycin is not known. An additional factor when comparing effects *in vitro* with *in vivo* is protein binding. It is generally considered that it is the “free-concentration” of a drug in plasma that can be transported over cell membranes and be “pharmacologically active”. In humans 75-95% of erythromycin is bound to plasma. The erythromycin in the rat WEC was in a protein-free media. Thus, although erythromycin slowed the heart rate at the concentrations tested (25-500 μM), compared with reported concentrations in plasma after therapeutic doses, taking placental passage in consideration, there is a large margin of safety between the concentration of erythromycin that causes bradycardia in the GD 13 rat embryo and the levels to which a human embryo may be exposed.

The arrhythmias induced by erythromycin were different from those induced by selective hERG/I_{Kr} blockers indicating a different mechanism of action. Hence, based on the observations on rat embryonic hearts in WEC it appears unlikely that macrolides would be teratogenic to human embryos based on their hERG/I_{Kr} blocking properties.

Although, in response to the paper by Källén et al (2005), the Swedish Regulatory Medicines Agencies have issued warnings against the use of macrolides during the first trimester [156]. Other authorities have been less restrictive. According to the classification system for prescribing drugs in pregnancy, erythromycin is assigned to category B in the United States [157] and category A in Australia [158]. The UK Teratology Information Service summarizes as follows: ‘A recent study has suggested a possible increased risk of cardiovascular malformations and pylori stenosis; however, causality has not been established and the individual risk, if any, thought to be low. If a macrolide antibiotic is required in pregnancy then erythromycin would be considered the agent of choice [159]. Somewhat confusing, the warning issued by the Swedish Medical Product Agency states that the same level of caution should be taken regarding other macrolides where animal data indicate a teratogenic risk. However, according to the Swedish classification system for medicinal product use in pregnancy, clarithromycin is assigned to category B:3 (‘Data from pregnant woman is insufficient; classification based on animal data. Reproductive toxicity studies in animals have revealed an increased incidence of fetal damage or other deleterious effects on the reproductive process, the significance of which is considered uncertain to in man’), and azithromycin is assigned to category A (‘Reliable clinical data indicate no evidence of disturbance of the reproductive process’). It should also be noted that if the risk increase of malformation after maternal drug use is less than 2, there is a larger probability that no causality exists in the individual case, than that it exists [160]. The latest estimated risk for congenital cardiovascular malformations reported from the Swedish Medical Birth Register was 1.59 (95%CI: 1.17-2.17) [161].

Temporal effects on rat embryonic heart rate *in vitro* of ion channel blockers

Paper IV

The embryo culture in this study was done at the University of Sydney. Selective hERG/I_{Kr} blockers cause malformations in rats between GD 10-14. The rat embryonic heart start to beat at GD 9-10 and it is believed that the importance of the hERG/I_{Kr} potassium current declines with gestational age. Simultaneous effects on multiple ion channels may also influence the effect of hERG/I_{Kr} blockade. To characterize the ion channels changes in the developing rat heart we used three relatively selective ion channels blockers dofetilide (hERG/I_{Kr}), lidocaine (Na⁺), nifedipine (Ca²⁺), and phenytoin (multi-channel blocker and known human teratogen) at concentrations previously tested *in vitro* [150]. Rat embryos were exposed *in vitro* on GD 10-15.

The rat heart rate *in vitro* showed a gradual increase with gestational age, from GD 10 to 13, but then a decrease for GDs 14 and 15 (Figure 22). The effect of lidocaine on rat embryonic heart function was more severe with increasing gestational age (GD 14-15) (Figure 23). Arrhythmias (either variable inter-beat time or heart block) were only seen in GD 14 embryos. Dofetilide caused a reduction in heart rate for each day tested with GDs 11, 12, and 13 showing the largest decreases. Arrhythmias were also seen on each day (Figure 24).

Nifedipine did not show a clear effect on the rat embryonic heart rate with gestation age as seen with lidocaine or dofetilide. However, on GD 10 nifedipine stopped the hearts in almost all embryos (Figure 25). Phenytoin stopped the hearts in a majority of embryos on GD 10-12. After GD 12 the effect of phenytoin on the rat embryonic heart became less severe with increasing gestational age (Figure 26). Arrhythmias were not seen on any day of exposure with phenytoin.

Paper IV shows that the rat embryonic heart undergoes marked changes in cardiac ion channel dependence with increasing gestational age. The changes in potassium channels are in agreement with *in vivo* teratology studies with dofetilide. The importance of calcium- and sodium channels for rodent embryonic cardiac function is reflected by the high incidences of lethality of nifedipine on GD 10, and the more severe effect of lidocaine with increasing gestational age. The severe effect of the multi-active ion channel blocker phenytoin with high rates of embryonic death early in gestation suggest a predominate effect on cardiac calcium channels.

The results underline the importance of determining the effect on the “function” of the embryonic heart of drugs with multiple ion channel activity. The *in vitro* rat embryonic heart with use of computation assisted image analysis offers a model for hazard identification of compounds that due to

their potential effects on the embryonic hear may pose a risk to the developing fetus.

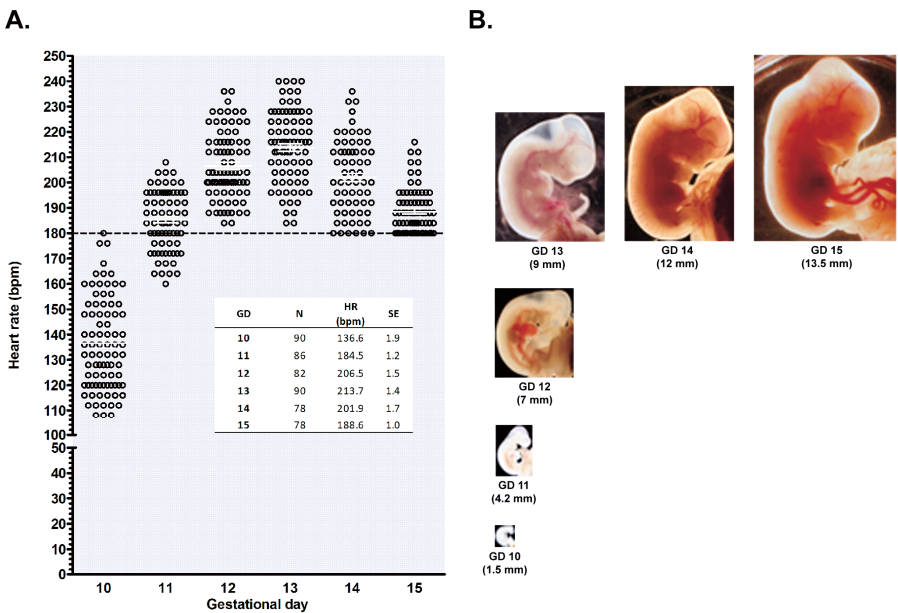


Figure 22. (A) Scatter-plot of the pre exposure heart rates (beats per minute) of each embryo in the study. Rat embryonic heart rate *in vitro* showed a gradual increase with increasing days of gestation form GD 10 to 13 but then declined for GD 14 and 15. The mean heart rate \pm standard error of the mean is depicted for each gestational day and the mean values, standard error of the mean, and the number of cultured embryos (N) are listed in the table. (B) Gross morphology and size of rat embryos GD 10-15.

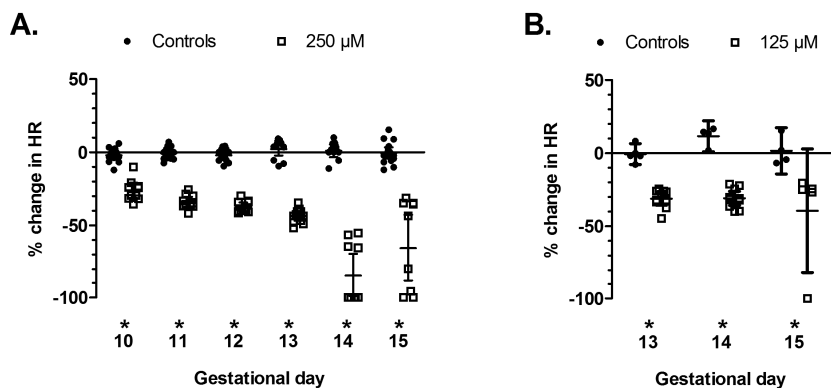


Figure 23. The effect of 250 μ M (A) or 125 μ M (B) lidocaine on rat embryonic heart rate *in vitro*. The change in heart rate (pre-exposure vs. post exposure) for each control (solid circle) and lidocaine-treated (open square) embryos cultured on GD 10-15 is depicted as scatter-plots. The effect of lidocaine on rat embryonic heart was initially tested with 250 μ M on GDs 10-15 (A). Due to severe effects on GDs 14 and 15 the concentration of lidocaine was reduced and 125 μ M was then tested on GDs 13-15 (B). The mean change in heart rate is depicted for each group on each GD. The error bars represent 95%CI. Asterisk denotes a statistically significant difference of the change in embryonic heart rate between control (water) and lidocaine treatment (two-way repeated measures ANOVA and Bonferroni post hoc test, $P < 0.05$).

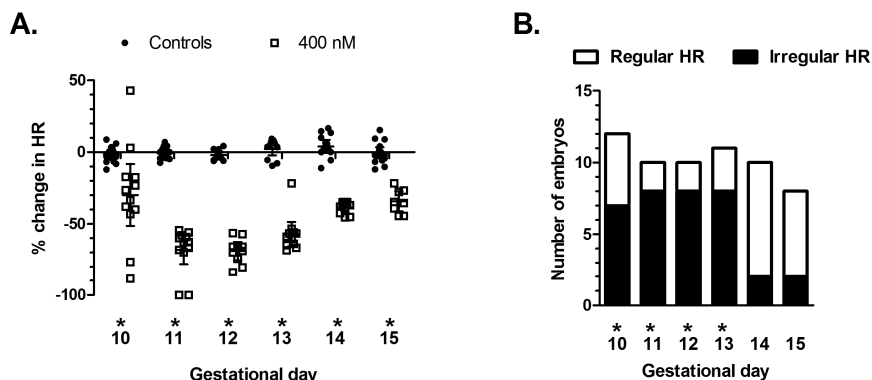


Figure 24. (A) The effect of dofetilide (nM) on rat embryonic heart rate *in vitro*. The change in heart rate (pre-exposure vs. post exposure) for each control (solid circle) and dofetilide-treated (open square) embryos cultured on GD 10-15 is depicted as a scatter-plot. The effect of dofetilide on rat embryonic heart rate peaks at GDs 11-13 with a reduction in heart rate of 60-70% and two embryos out of twelve (2/12) embryos with heart stop on GD 11. The mean change in heart rate is depicted for each group on each gestational day. Error bars represent 95%CI. Asterisk denotes a statistically significant difference in the change in embryonic heart rate between control (water) and dofetilide treatment (two-way repeated measures ANOVA and Bonferroni post hoc test, $P < 0.05$). (B) Dofetilide (400 nM) induced arrhythmias at all GDs tested (although the effects on GDs 14 and 15 did not reach statistical significance). Asterisk denotes a statistical significance between control and dofetilide treatment (Fischer's exact test, $P < 0.05$). No arrhythmias were observed in the control groups. The number of controls (in parenthesis) on each gestational day was; GD 10 (14), GD 11 (16), GD 12 (11), GD 13 (11), GD 14 (14), GD 15 (16).

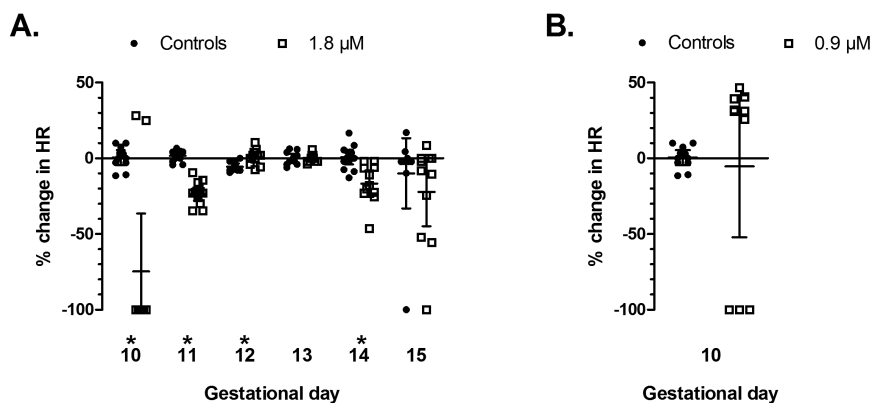


Figure 25. The effect of 1.8 μM (A) or 0.9 μM (B) nifedipine on rat embryonic heart rate *in vitro*. The change in heart rate (pre-exposure vs. post exposure) for each control (solid circle) and lidocaine-treated (open square) embryos cultured on GD 10-15 is depicted as scatter-plots. The effect of nifedipine on rat embryonic heart was initially tested with 1.8 μM on GDs 10-15. Due to severe effects on GD 10, where 8/10 embryonic hearts stopped, the concentration of nifedipine was reduced and 0.9 μM was then tested at GD 10 (B). At 0.9 μM nifedipine, three out of ten (3/10) embryonic hearts stopped (not significant compared with controls, Fisher's exact test) on GD 10. Interestingly the surviving embryos exposed to 0.9 μM or 1.8 μM on GD 10 all showed an increase in embryonic heart rate. The mean change in heart rate is depicted for each group on each GD. The error bars represent 95%CI. Asterisk denotes a statistically significant difference of the change in embryonic heart rate between control (water) and lidocaine treatment (two-way repeated measures ANOVA and Bonferroni post hoc test, $P < 0.05$).

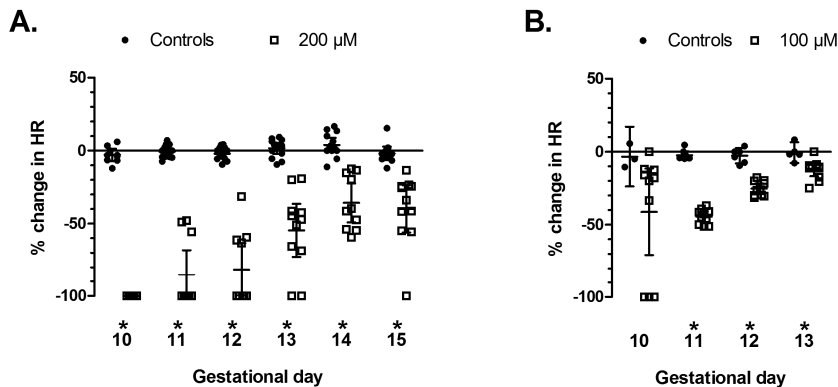


Figure 26. The effect of 200 μM (A) or 100 μM (B) phenytoin on rat embryonic heart rate *in vitro*. The change in heart rate (pre-exposure vs. post exposure) for each control (solid circle) and lidocaine-treated (open square) embryos cultured on GD 10-15 is depicted as scatter-plots. (A) The effect of phenytoin on the rat embryonic heart was initially tested with 200 μM on GDs 10-15. Due to the severe effect on GDs 10-12, where a majority of the embryonic hearts stopped, the concentration of phenytoin was reduced and 100 μM was then tested on GDs 10-13. The mean change in heart rate is depicted for each group on each GD. The error bars represent 95%CI. Asterisk denotes a statistically significant difference of the change in embryonic heart rate between control (water) and lidocaine treatment (two-way repeated measures ANOVA and Bonferroni post hoc test, $P < 0.05$).

Concluding remarks

*There must be some kind of a way out of here, said the joker to the thief,
there is too much confusion, I can't get no relief.*

Excerpt from All along the watchtower, written by Bob Dylan

The work in this thesis has generated the hypothesis that, although all drugs that affect the embryonic cardiac function may be considered potential teratogens, there are basic biological, physiological and pharmacological aspects that needs to be taken in consideration when extrapolating a risk to the human embryo.

The *in vivo* and *in vitro* studies in paper I confirmed the hypothesis and drew attention to the fact that conventional teratology testing of the antihistaminic drug, and selective hERG/I_{Kr} blocking drug astemizole had not demonstrated teratogenicity. Because of species differences between rats and humans, regarding the dependence of the ion channel during embryonic development and in adult life, this may limit the exposure levels that may occur in the human embryo to hERG/I_{Kr} blocking drugs.

The *in vitro* study in paper II was the first of its kind to show that drugs which blocked ion channels other than the hERG/I_{Kr} channel (i.e. sodium and calcium channels) could also have a negative effect on the functioning of the embryonic heart. Paper II illustrates that from a teratological perspective, any drug that can cause bradycardia or arrhythmia in the embryo may be potentially harmful in pregnancy [8], but in practice human exposure levels are much lower than the concentrations associated with the adverse effects observed in this paper.

To further explore the use of WEC combined with image analysis for hazard identification, and possibly risk assessment, we took on the work of exploring the effects of the macrolide antibiotics erythromycin, clarithromycin and azithromycin on the GD 13 rat heart. The *in vitro* studies of the macrolides in paper III clearly show that hERG/I_{Kr} related effects on the embryonic heart would only occur at exposure levels greatly in excess of those likely to occur during normal therapeutic oral dosage. Paper III demonstrates that data on toxicity should be related to possible exposure to man, and the faint idea that something might happen at extreme exposures is of little help for everyday discussions and risk assessment. A point to be made regarding the warning issued by the Swedish Medical Products Agency [156], leading

to the classification of erythromycin as a possible human teratogen, is that several of the *in vitro* studies referred to as models differ from the situation of human embryos (and rat embryos for that matter) [153, 154]. Milberg et al. (2002) for instance, tested the three antibiotics erythromycin, clarithromycin and azithromycin in a sensitized rabbit model where; "...concentrations for all three macrolides were several multiples higher than the expected free plasma concentration in patients to create a maximal proarrhythmic milieu and to better study the underlying mechanisms of proarrhythmia. The experimental setup was designed to reproduce conditions and circumstances that are clinically known to be associated with and increased propensity to develop TdP" [153].

Another point to make concerns the concentration used in this thesis and in other *in vitro* models. A limitation of pharmacological studies is that drug specificity is rare. Most drugs show activity toward a variety of targets and are more often considered selective rather than specific. And with increasing concentration the selectivity of drugs is diminished, and activity on several ion channels can either result in mutual synergies or cancellations of the effect on hERG/I_{Kr} [162, 163]. At the concentrations used in this study it is expected that the selectivity of the tested drugs is wide. However, in terms of using WEC to predict potential teratogenic/developmental hazard, it is our opinion that the net functional effect on the embryonic heart is of primary interest.

The *in vitro* studies in paper IV demonstrate that the rate embryonic heart undergoes dramatic changes in dependence on specific ion channels during development. It underlines the fact that the effect on the embryonic heart is properly investigated and assessed. The GD 13 rat embryonic hearts, together with the use of computational assisted image analysis of the cardiac response, provide a model for hazard identification of compounds with the potential to adversely affect the heart in the developing embryo.

The overall finding of this thesis is that WEC in combination with image analysis could mean another use for *in vitro* culture of rodent embryos. Combining WEC/image analysis-system with electrophysiological recording in rat embryo would be the next step to take, developing an *in vitro* system for safety pharmacology screening purpose, and hazard identification prior to reproductive toxicity studies in animals [79, 144, 146, 147].

Svensk sammanfattning

Kaliumkanalen hERG/I_{Kr} är viktig för normal hjärtfunktion. Läkemedelsinducerad blockering av jonkanalen hos vuxna personer kan leda till oregelbunden hjärtrytm (arytmi). Denna jonkanal är också viktig för hjärtfunktion i embryot och läkemedel som blockerar jonkanalen har visat sig orsaka fosterskador i djurförsök. Exempel är läpp-/gomspalt, förkorning eller avsaknad av tår, samt hjärtmissbildningar.

Dessa missbildningar är kopplade till perioder av syrebrist och förändrat blodflöde i embryot, bland annat i samband med läkemedelsinducerad förändring av hjärtrytmen och sänkt frekvens hos embryots hjärta. Det är också känt att andra experimentella metoder, som orsakar perioder av syrebrist i embryot, kan ge upphov till liknande skador som de som orsakas av läkemedel som blockerar hERG/I_{Kr}.

Arbete I i avhandlingen rör riskbedömning av läkemedel som blockerar hERG/I_{Kr} med avseende på användning under graviditetet. Utvärdering av risken för fosterskador i allmänhet är till stor del baserad på resultaten från experimentella studier på djur. Riktlinjer för hur sådana standardiserade tester skall utföras är bestämt av tillsynsmyndigheter sedan flera årtionden tillbaka. Det finns emellertid exempel där säkerhetsstudier enligt regulatoriska riktlinjer inte lyckats upptäcka fosterskadande egenskaper hos läkemedel som blockerar hERG/I_{Kr} bland annat, på grund av suboptimala dosintervall i säkerhetsstudierna. En följd av detta är att fosterskadande egenskaper av hERG/I_{Kr}-blockerande läkemedel kan ha missats i standardiserade säkerhetstester. Resultaten av Arbete I visar att de fosterskadande egenskaperna hos läkemedlet astemizol missades i de tidiga säkerhetsstudierna på djur. Astemizol är tillbakadragen från marknaden sedan flera år på grund av livshotande hjärtarytmier hos vuxna som resultat av hERG/I_{Kr}-blockad)

Arbete II visar att flera läkemedel som blockerar jonkanaler andra än hERG/I_{Kr} också kan störa fostrets hjärtfunktion under fosterutvecklingen. De koncentrationer som krävs för att orsaka dessa förändringar är emellertid mycket högre än vad som är vanligt förekommande vid normal användning av läkemedlen, och utifrån dessa resultat anses läkemedlen inte utgöra en risk när de tas under graviditet.

Arbete III behandlar eventuella fosterskadande egenskaper hos erytromycin. Data från det Svenska Medicinska Födelseregistret visar på att användning av erytromycin (som har hERG/I_{Kr}-blockerande egenskaper) under graviditeten är förknippat med en ökad risk för hjärt-kärlmissbildningar. Resultat

taten av Arbeta III gör troligt att de nivåer av erytromycin som krävs för att störa fostrets hjärtfunktion under fosterutvecklingen högst sannolikt inte uppkommer hos gravida kvinnor (vid rekommenderad tablettbehandling med läkemedlet).

Arbete IV visar att embryohjärtat hos råtta genomgår stora förändringar gällande känslighet för blockad av specifika jonkanaler under organbildningsperioden. Detta är en viktig iakttagelse utifrån perspektivet att det kan finnas perioder under fosterutvecklingen när embryot är mer eller mindre känslig för effekten av läkemedel som påverkar speciella jonkanaler. I övrigt visar avhandlingen att studier av läkemedelseffekter på embryohjärtat under tidig utveckling med fördel kan studeras i det isolerade embryot *in vitro*. Hos råtta sker det optimalt om embryot tas ut under dräktighetsdag tretton. *In vitro*-modellen i kombination med databaserad bildanalys av embryohjärtats funktion är en användbar metod för riskidentifiering av föreningar med potential att påverka hjärtfunktionen hos det utvecklande embryot.

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