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AN OVERVIEW ON HUMAN ETHER-A-GO-GO-RELATED GENE ASSAY

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ABSTRACT: hERG (human ether-à-go-go-related gene) encodes a potassium ion channel which involved in ventricular repolarization at the end of a cardiac action potential. Mutations in hERG channel result in torsade's de pointes. The cardiotoxicity is mainly due to the prolongation of the QT interval caused by the blockage of hERG. Indeed, many therapeutic drugs are withdrawn from the market as well as drug candidates may fail in the late phases of drug discovery because of cardiotoxicity. The ICH S7B and ICH E14 are the safety pharmacology guidelines recommended for evaluating new chemical entities. In the early phase of drug discovery, it is important to screen the compound for the activity on the hERG channels. Many hERG assays are available, including Fluorescent Membrane Potential Sensitive Dye Method, Radioactive Ligand Binding Method, Fluorescence Polarization Ligand Binding Method and Rubidium Flux method, Manual Patch-Clamp Method and automated Patch-Clamp Method, Langendorff's perfused isolated heart method, Non-Invasive Telemetry, and Wireless Telemetry. This review is focused on hERG assay and provides additional information about the long QT syndrome and molecular structure of hERG.

INTRODUCTION: The action potential is considered an electrical basis for the heartbeats, which starts from the specialized pacemaker cells and is transmitted to the atrial, ventricular muscles, and cardiac myocytes. Action potential involves the movement of ions into and out of cardiac myocytes through voltage-gated channels. It includes different phases **Fig. 1**. Phase 1 – Inactivation of Na^+ channels followed by K^+ channels that rapidly open & close, causing a transient outward current.

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Phase 2 – Ca^{2+}channel opens followed by Ca^{2+} ion enter into the cell, resulting in a slow inward current that balances the slow outward (polarizing) leak of K^+ ion. Phase 3 – Ca^{2+} channels close, voltage-gated K^+ channels open, resulting in a K+ ionan outward current efflux that leads to membrane repolarization. In phase 3, the voltage-gated potassium channel categorized as IKur, IKr, IKs delayed rectifier current. The hERG are molecular correlated to IKr(Rapid delayed rectifier potassium current).

Phase 4 – Na^+, Ca^{2+}channel remain closed, and K^+ inward rectifier channels will open and keep the transmembrane potential stable at -90mv [1]. Cardiac K^+ channels are membrane-spanning proteins that allow the passive flow of K^+ ions across the cell membrane along with its electrochemical gradient.

The ion-conducting or pore-forming subunit is typically known as α subunit. Cardiac K^+ channels are often categorized as voltage-gated and ligand-gated channels. In voltage-gated potassium channels, pore opening is coupled to the movement of a voltage sensor within the membrane electric field and they include

- Rapidly activating and inactivating transient outward current (I_{to}).
- Ultrarapid (I_{Kur}), rapid (I_{Kr}), slow (I_{Ks}). delayed rectifier potassium current.
- Inward rectifier potassium current (I_{K1}).

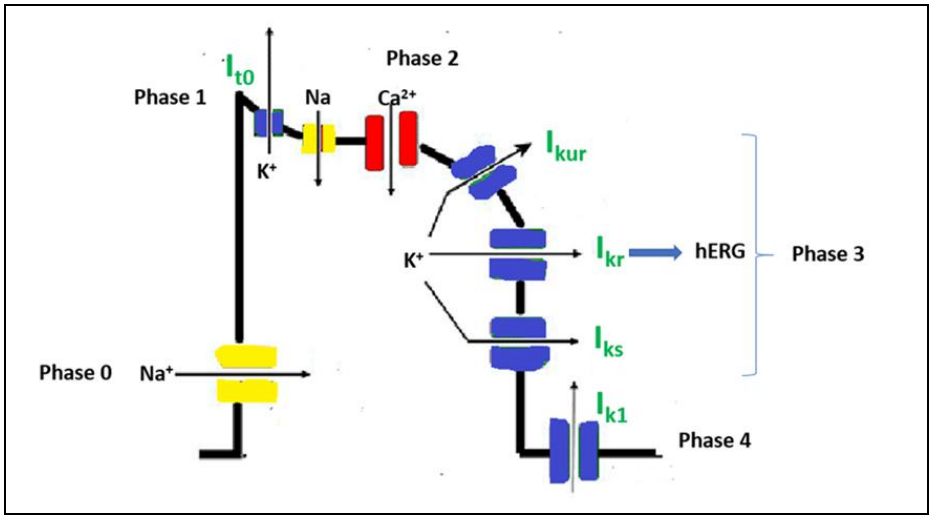

FIG. 1: SCHEMATIC DIAGRAM OF CARDIAC ACTION POTENTIAL

hERG: hERG means human ether-a-go-go-related gene. It is a human gene responsible for channels mediating the 'rapid' delayed rectifier K+ current (IKr) that plays a crucial role in ventricular repolarization in the cardiac action potential, which helps coordinate the heartbeat. The nomenclature hERG is KCNH2. The protein encoded by this gene will be called Kv11.1. It is the alpha subunit of a potassium ion channel. The name of the hERG gene is the homology of the Ether-àgo-go gene, which was discovered from Drosophila when ether-induced leg shaking was observed in flies with mutations in this Ether-àgo-go gene [2, 3]. Ether-àgo-go was named in 1960 by William D. Kaplan and William E. Trout.

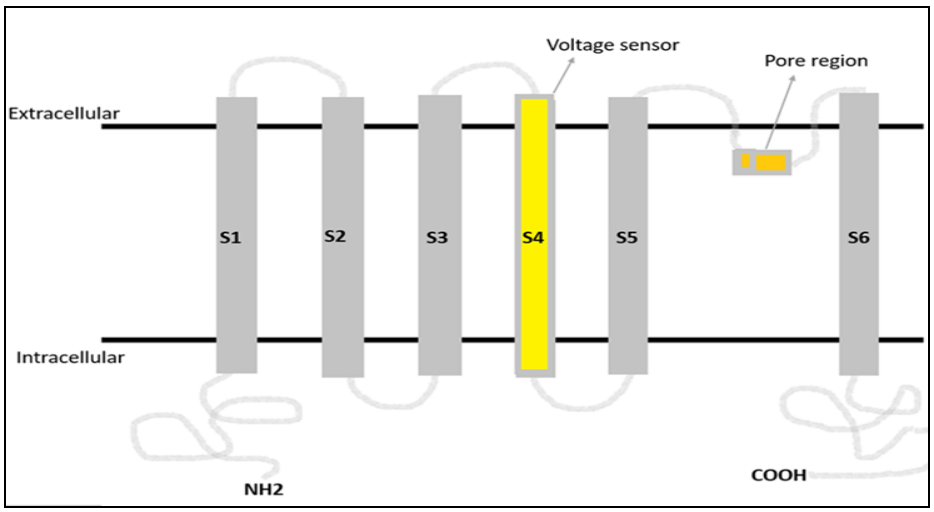

FIG. 2: STRUCTURE OF HERG CHANNELS

Voltage-dependent K^+ channel or hERG channel contains six transmembrane domains and a single pore. It is a tetramer formed of four subunits. Alternating intracellular and extracellular peptide loops connect the six transmembrane domains (S1 to S6). The N-terminus and C- terminus are located on the intracellular side of the membrane. The S4 segment act as the voltage sensor there is link

between S5 and S6 segments which form the ion-conducting or pore region. The S5-S6 linker is responsible for K^+ ion selectivity filter **Fig. 2**. The amino residue such as tyrosine 652 and phenylalanine 656 are considered to be important for drug binding **Fig. 3** [4-7].

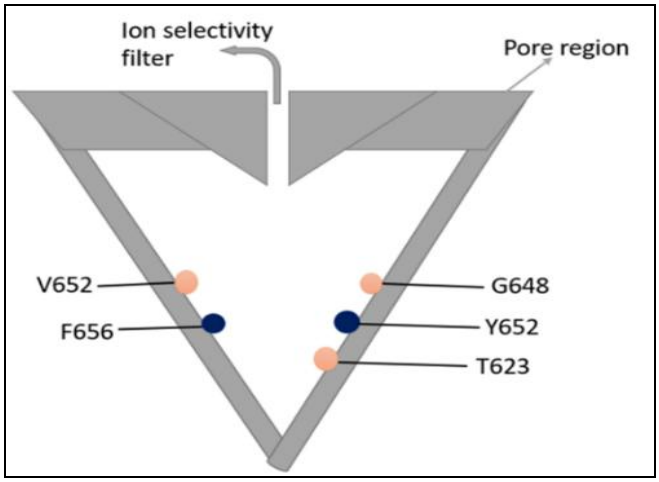

FIG. 3: DIAGRAM REPRESENTS THE AMINO RESIDUES INVOLVED IN DRUG BINDING POCKET IN THE CHANNEL

Long QT Syndrome (LQTS): Action potential is the movement of ions across the cell membrane that contributes to the overall electrical activity of the heart, which is measured by electrocardiogram (ECG). Normally the QT interval (time from the beginning of the QRS complex to the end of the T wave) in the ECG indicates the duration of cardiac ventricular depolarization and repolarization. With the help of ECG the changes in the action potential recording can be monitored.

Long QT syndrome (LQTS) is characterized by QT interval prolongation, which results in ventricular arrhythmias. The QT prolongation occurs with rapid, polymorphic ventricular tachycardia with repeated alteration in the QRS complex is termed as torsade's de points (TdP). The prolongation in the interval can be observed by ECG. TdP leads to worsening of the patient's cardiac condition and cause sudden death.

Long QT syndrome can be congenital (born with a genetic mutation) or acquired (caused by certain medications) [8]. Mutations in the hERG channel are associated with the long QT syndrome and the most commonly used therapeutic medications to block the hERG channel directly, which results in prolongation of the QT interval and leads to increased risk of TdP [9]. In 1989 identified, the overdoses of terfenadine caused prolongation of the QT interval. In 1990, terfenadine was shown to inhibit the delayed rectifier potassium current in isolated myocytes and hERG channel in Xenopus oocytes, leading to Torsades de Pointes.

The International Conference on Harmonisation (ICH) issued two imperative guidance documents called ICH S7B and ICH E14 in 2005 that are still standing today as safety pharmacology guidelines for developing new chemical entities. ICH S7B, titled "Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals describes a non-clinical testing strategy for evaluate the test compound to delay ventricular repolarization. The guideline suggested the *in vivo* QT assay and *in-vitro* I_{kr} assays to evaluate the drugs QT prolongation risk and ICH E14, "titled clinical evaluation of the QT/QTc interval prolongation and proarrhythmic potential for non-antiarrhythmic drugs", requesting that all sponsors should submit the new drug applications for conducting the study to determine whether a drug candidate prolongs the action potential [10-11].

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use has requested the pharmaceutical companies to take serious measures to minimize the risk of drug-induced TdP.

hERG assay is used for assessing cardiotoxicity. Cardiotoxicity occurs when the drug blocks the hERG channels, leading to a prolonging QT interval in the heart. Hence, hERG assay is widely used at an early stage of drug development to predict a drug candidate's ability. Indeed, many medications have been withdrawn from the market due to cardiotoxicity[12, 13]. Drugs with a risk of torsades de pointes include Arsenic trioxide, Astemizole, Droperidol, Erythromycin, Levomethadyl, Quinidine, Sotalol, Mesoridazine, Sparfloxacin, Methadone Opiate, Pentamidine, Bepridil, Chloroquine, Disopyramide, Pimozide, Probucol, Chlorpromazine, Haloperidol, Cisapride, Clarithromycin, Procainamide, Terfenadine, Thioridazine, Halofantrine, Dofetilide, Domperidone, Ibutilide [14, 15, 16]. Scientific studies have reported that many drugs have failed in the

late phase of clinical trials because of cardiotoxic effects such as QT prolongation. Hence, it is important to identify hERG inhibitors in the early phase of drug discovery.

hERG assays were used by 93% of surveyed respondents from 119 pharmaceutical companies for preclinical evaluation of drugs based on the guideline ICH S7B.

hERG Assay: Several methods have been developed to investigate QT prolongation. It is important to screen new chemical entities or drugs for activity on hERG channels. A number of hERG assays are available.

In-vitro methods include Fluorescent Membrane Potential Sensitive Dye Method, Radioactive Ligand Binding Method, Fluorescence Polarization Ligand Binding Method, Rubidium Flux method, Manual Patch-Clamp and Automated Patch-Clamp Method. All the *in-vitro* methods use cell lines such as ashuman embryonic kidney cells (HEK) and Chinese hamster ovary (CHO) cells transfected with the hERG gene.

After transfection, K^+ channel will be expressed on their cell membrane. *Ex-vivo* methods provide more definitive information about arrhythmia compared to *in-vitro* assays. *Ex-vivo* studies include Purkinje fiber, Langendorff perfused isolated heart method and *in-vivo* studies. Nowadays, Non-Invasive Telemetry and Wireless Telemetry systems are widely used in research laboratories. It is automated, which reduces the labor burden and stress on animals. Telemetry studies provide in-depth information in ECG morphology, and the results will be accurate without human error and personal bias [17, 18].

***In-vitro* Methods:**

Fluorescent Membrane Potential Sensitive Dye Method: In this method, the cells were transfected with the hERG gene. The transfected cell has negative membrane potential than wild type cell.

The transfected cells are preincubated with test compound and adye such as bis-(1,3-dibutylbarbituric acid) trimethineoxonol [DiBAC4 (3)], which is lipophilic and negatively-charged that display an increased quantum. The dye is used to monitor membrane potential changes across the cellular membrane using the fluorescence resonance energy transfer (FRET) mechanism. The dye flows into and out of the cell in response to the membrane potential **Fig. 4**.

It interacts with cytoplasmic components to increase the fluorescence, but no such fluorescence is produced in the extracellular solution [19].

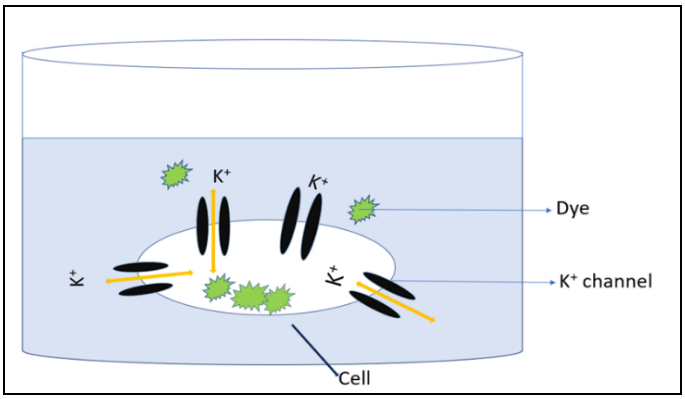

FIG. 4: DYE FLOWS INTO AND OUT OF THE CELL IN RESPONSE TO MEMBRANE POTENTIAL

Suppose a compound blocks the channel **Fig. 5** the membrane potential of the transfected cells increases, which results in increased fluorescence that can be monitored using a fluorometric plate reader for 3-15 min. A newer dye, such as FLIPR membrane potential dye, responds faster than DiBAC4 (3) [20].

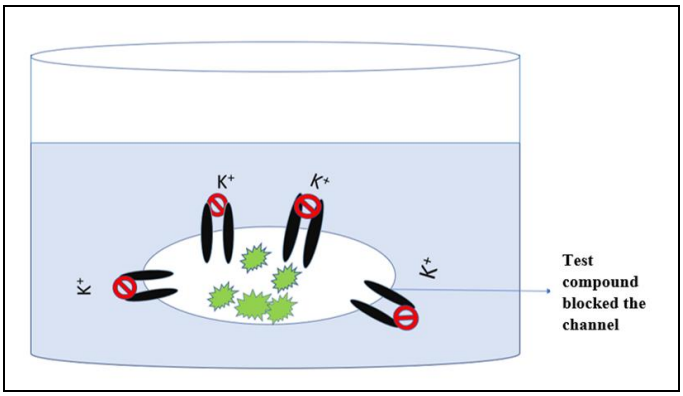

FIG. 5: TEST COMPOUND BLOCKS THE CHANNELS TRANSMEMBRANE POTENTIAL IS INCREASED

Radioactive Ligand Binding Method: HEK 293 cells transfected with the hERG genesis incubated at 37°C for 30-60 min with thetest compound and radiolabelled ligandcompound (*e.g.*,[^{3}H] dofetilide, [^{3}H] astemizole), 34 [^{35}S]-MK-499, 125I]-BeKm-1) [21]. The radiolabelled ligand and test compound compete for binding to the hERG channel **Fig. 6.**

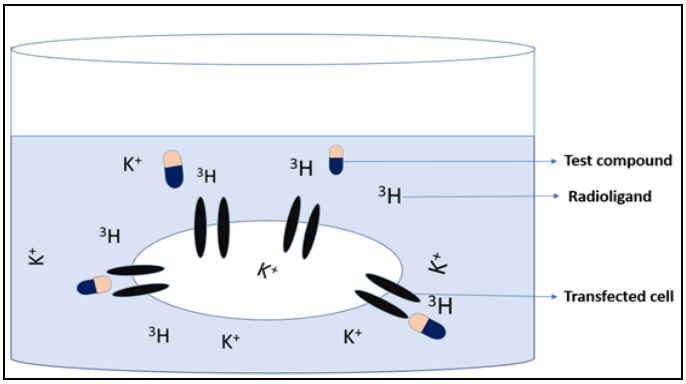

FIG. 6: TEST COMPOUND AND RADIOLIGAND COMPETES FOR HERG CHANNEL

After incubation, the membranes are filtered and washed multiple times with cold buffer [22]. If the test compound has a stronger affinity to the hERG channel, **Fig. 7** the radioligand response will be lower. The radioligand response will be detected by scintillation counter. The activity of the test compound is indicated by the displacement of the labeled compounds. It is an inexpensive method that is widely used in many pharmaceutical companies [23, 24, 25].

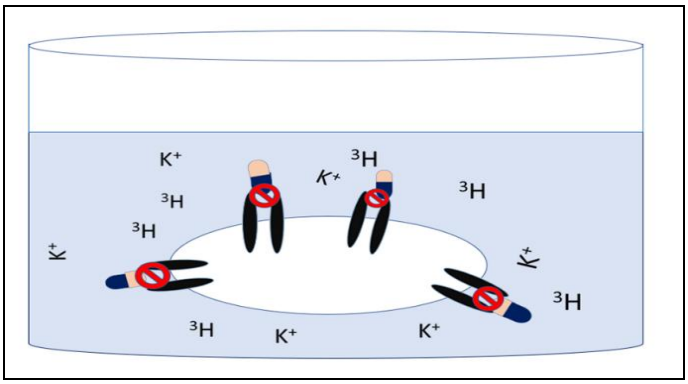

FIG. 7: TEST COMPOUND BLOCKING THE HERG CHANNEL

Rubidium Flux Method: This method is used for screening stronger hERG inhibitors in early drug discovery. The hERG gene is transfected with Chinese hamster ovary (CHO) cells then preincubated in the media which consists of Rb^+ (Rubidium).

After few min, the media is removed, and cells are washed with buffer to remove Rb^+ present in the extracellular solution [26, 27].

Buffer containing test compound and K^+ ion (to manipulate the membrane potential and open the channels) is added to the Rb^+ containing media and incubated **Fig. 8.**

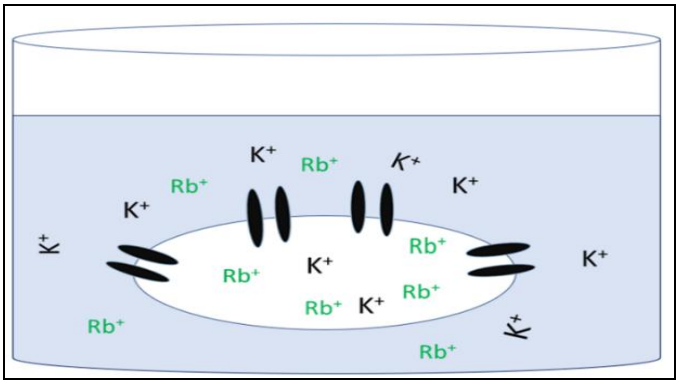

FIG. 8: TEST COMPOUND AND RUBIDIUM CONTAINING MEDIA

If the test compound blocked the channel the Rb^+ will stay inside the cells **Fig. 9**. If the Rb^+ flows into the medium it indicates that the channel is not blocked by the test compound. Rb^+ concentration is measured by either scintillation nor atomic absorption spectroscopy. It is relatively low cost and can provide a high throughput screening [28, 29, 30].

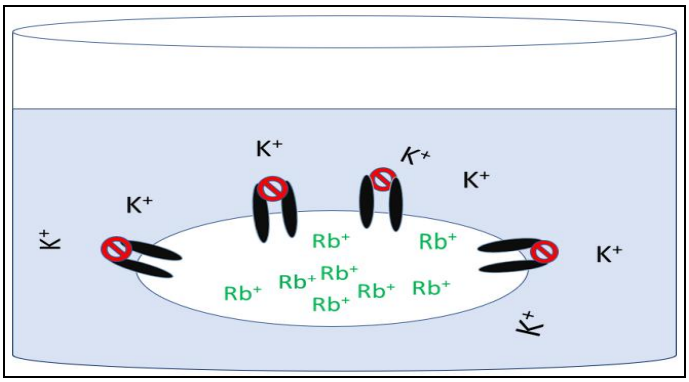

FIG. 9: TEST COMPOUND BLOCKS THE CHANNELS, RUBIDIUM WILL STAY INSIDE THE CELL

Fluorescence Polarization Ligand Binding Method: The membranes prepared from cells (*e.g.*, HEK 293) transfected with hERG. When Fluorescence Polarization (FP) ligand in the solution. It emits plane-polarized light is depolarized owing to rapid rotation.

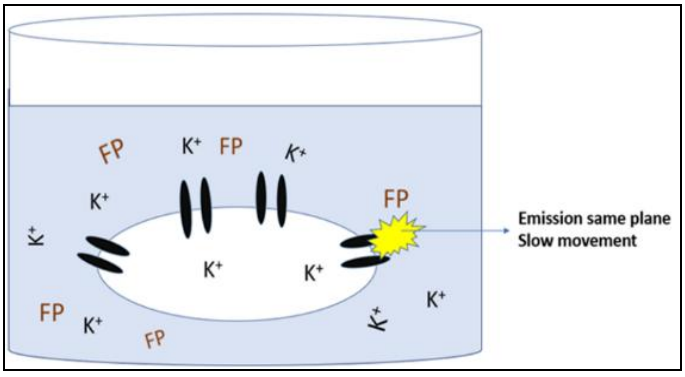

FIG. 10: BOUND FP CAUSE EMISSION IN SAME PLANE

The FPligand and test compound compete for binding to the hERG channel. If FP ligand binds to the channels which as emission in the same plane with slow movement **Fig. 10**. When the test compound inhibits the hERG channel, the unbound FP ligand **Fig. 11** causes the fluorescence emission in the random plane and a fast movement [31].

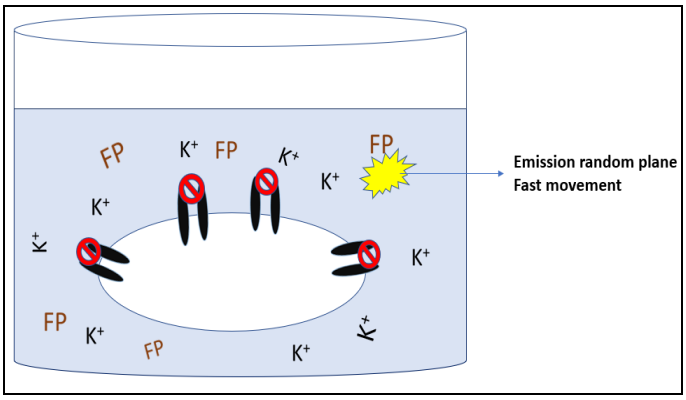

FIG. 11: UNBOUND FP CAUSE EMISSION IN RANDOM PLANE

Manual and Automated Patch-clamp Method: This technique is still considered the gold standard for *in-vitro* assays. The effects of drugs on the hERG channels can be evaluated by the Manual patch-clamp method. The instrument comprises an electrode, glass capillary, microscope, amplifier, computer, recording software, micromanipulator, carbogen camera. An electrode is inserted in the glass capillary filled with buffer. While observing under a microscope, the capillary is attached to a micromanipulator, and of the capillary, the tip is placed in contact with the membrane of transfected cells that expresses the hERG channel. When negative pressure is applied, the capillary is patched tightly to the cell membrane, forming a high resistance seal.

Further application of negative pressure, a small portion of the membrane is pulled into the capillary tip and ruptured, forming a whole-cell patch **Fig. 12**. K^+ ion channel protein in the cell undergoes conformational changes and the channel will be opened in response to the applied positive voltage. The K^+ ions move out of the cell through the ion channel creating an electrical current [32]. If the test compound blocks the hERG channel, the ionic current will be reduced. Patch-clamp can also monitor the ion channel events at millisecond level.

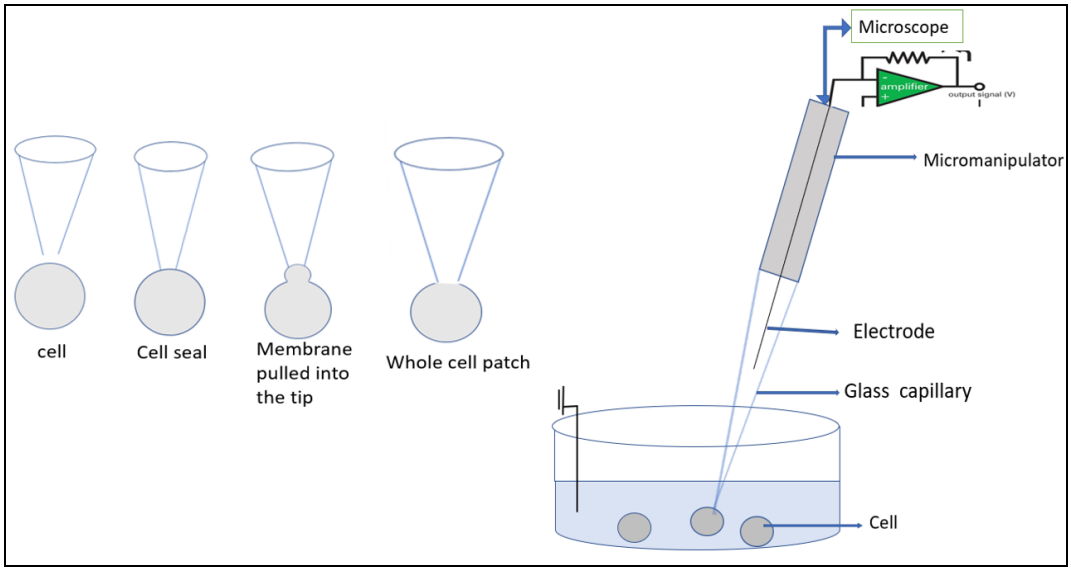

FIG. 12: SCHEMATIC DIAGRAM OF MANUAL PATCH CLAMP

Under Good Laboratory Practice (GLP), a manual patch-clamp is performed for drug candidates in investigational new drug (IND) filings. The advantage of this method is providing reliable data even for the compounds of higher IC_{50}. The patch-clamp method is widely used for studying ion channels activity. Even though time-consuming, this approach is used with computer-controlled robotized platforms, which allows high throughput screening with recombinant ion channels [33, 34]. An automated patch clamp system is performed on a microplate with multiple wells. The cells are placed into a patch plate containing 384 wells with multiple tiny pores or holes on the bottom. When negative pressure is applied to the pores, it draws the cells and holds them there.

A membrane agent called modifier (*e.g.*, Amphotericin B, nystatin) is added below the hole, which produces a small opening in the cell membrane known as a perforated patch. The electrical conductance occurs through the perforated patch. The application of drugs into each well is performed automatically by the liquid handling robot. The response curve and IC_{50} values were calculated automatically using the software. Before and after the addition of the test compound, the current is monitored. This technique is the method of choice for hERG inhibitors screening. The drawback of this method is lipophilic compounds can adhere to the surface of the well, which results in false IC_{50} measurements [35, 36].

***Ex-vivo* Methods:** Purkinje fibers play a crucial role in the conduction and propagation of the electrical impulse located in the inner ventricle walls of the heart. They are also susceptible to development early after depolarizations, believed to be responsible for fatal torsade's de points. Some studies suggested that many ventricular arrhythmias are actually initiated in the Purkinje fiber conduction system [37]. Isolated Purkinje fibers can detect drug-induced effects on the action potential configuration and gives in-depth information about transient outward K^+ion current and the rapid, slow forms of the delayed rectifier current. Purkinje fibers can be isolated from guinea pig, rabbit, or dog [38]. Their polarization and action potential prolongation in these tissues are similar to the human heart.

Purkinje cells were isolated from rabbits by the following procedure. The rabbit was euthanized, and the heart was removed and placed in potassium- and glucose-enriched extracellular solution oxygenated kept at room temperature [39]. Purkinje fibers are dissected along with small pieces of ventricular muscle to prevent damage in fibers. They are divided into 2–3 pieces and pinned through the ventricular muscle to the silicone bottom of the experimental chambers. Transmembrane voltage is recorded with a glass microelectrode having a tip resistance of 10–2 MΩ [40, 41].

Langendorff's Perfused Isolated Heart: In this experiment, the heart is removed from the animal and immersed in a warm water organ bath/chamber followed by cannulation through the aorta and retro perfused. A peristaltic pump is used to deliver the perfusion solution at a constant flow rate. Krebs Henseleit Buffer (KHB) is used as a perfusion solution in a wide range of studies [42, 43]. KHB is composed of $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, glucose 11 mM, NaCl 118.5 mM, $NaHCO_3$ 25.0 mM, KCl 4.7 mM and a range of $CaCl_2$ 1.2-1.8 mM and 95% O_2 +5% CO_2 is supplied to the KHB maintained at 37°C. A sidearm to the perfusion cannula is used for administering the drugs. A ventricular balloon is inserted in the left ventricle to record the left ventricular pressure (LVP). The balloon is inflated through a water-filled syringe attached to one end of the catheter to achieve ventricular pressure. The other end of the catheter is attached to a pressure transducer to monitor the systolic and diastolic pressure. The force of contraction is increased due to the filling of the ventricles. The pressure developed in the left ventricle can be measured with the help of a pressure transducer. The thermocouple is used to monitor the temperature, and it is inserted into the right ventricle. All pressure transducers are connected to the computer devices, which record the ECG continuously throughout the experiment [44, 45, 46].

***In-vivo* QT Assay:** Guineapigs are used for *in-vivo* QT assays. They are anaesthetized with intraperitoneal injection. The animal was placed on a heat pad with circulating water at a temperature of 37–39 °C and was mechanically ventilated by tracheostomy. The pulse oximeter is used to monitor the Oxygen saturation in the animal. For the drug administration and to monitor the blood pressure the jugular vein and the carotid artery were cannulated, respectively. The ECG electrode pins were placed on the limb and chest of the animal, and the ECG signals were recorded [47, 48]. After the drug administration, the animal was observed for 30 min to evaluate the effects of drugs.

Non-Invasive Telemetry: The electrocardiogram instrument is still considered a gold standard for investigating the effects of drug candidates on cardiac electrophysiology. In most cases, electrocardiograms are often used as a component in non-clinical evaluation studies such as safety pharmacology and toxicology assessment.

Non-Invasive Telemetry is widely used in research laboratories for cardiotoxicity studies. The commonly used species is a dog and the majority of evidence confirms that dogs are the most predictive preclinical species with similar human electrocardiography. Guinea pigs and primates were also used. The non-Invasive Telemetry system provides anin-depth analysis of normal and abnormal changes in ECG morphology. It also monitors the beat-by-beat changes in QT intervals and the heart rate [49].

Advantages of telemetry are non-invasive procedure, low stress levels the animal will be conscious and can move freely throughout the experiment, data collected over long periods of time, produces highly informative data. But it is expensive method. It is used for assessing the hERG block by drug candidate followed by lengthening QT interval induced by TdP can be observed directly in the live animals with the help of ECG. Before starting the experiment, animals was acclimatised with electrodes and jackets required for non-invasive telemetry monitoring. After administering the test compound, heart rate and the ECG signals were monitored [50]. Previous studies were carried out in moxifloxacin to evaluate and quantify the changes in ECG parameters by the Non-Invasive Telemetry system on Beagle dogs were treated with moxifloxacin fluoroquinolone antibiotic. Animals were acclimatized with jackets required for non-invasive telemetry monitoring. On the test days, ECG leads and jackets were placed on the animals. Moxifloxacin at a dose of 30 mg/kg was administrated. Heart rate and ECG were monitored for 2 h of predose and 22 h of post-dose. It has been reported that administration of moxifloxacin results in QT prolongation [51].

Wireless Telemetry System: Telemetry methods are now being employed in various fields, and the goal of this system is to provide experimenters or researchers with desired physiological data, and the research animals will be conscious and undisturbed [52]. Wireless Telemetry system is easy to handle for the researcher because of automated physiological measurements, which reduce labor burden and stress on the animal. It is an automated data collection, so there is no need for direct human interaction, which reduces the typical drawbacks in conventional methods. These systems greatly minimized the human effort required for data collection [53].

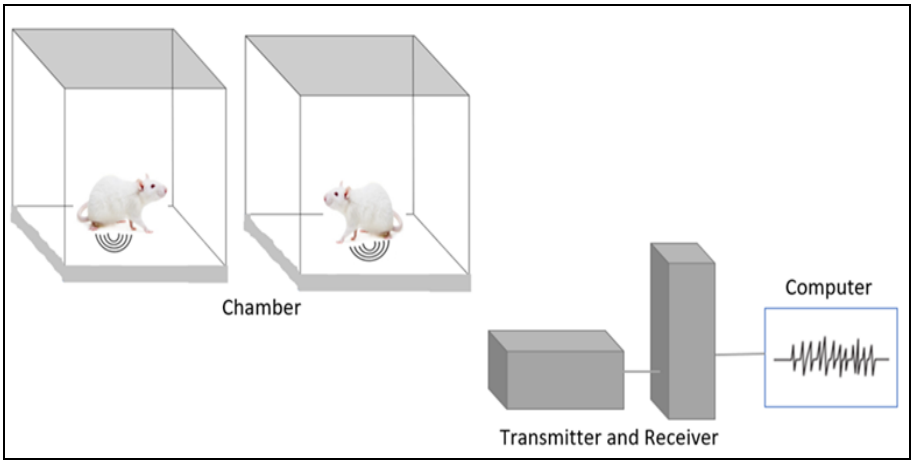

FIG. 13: SCHEMATIC DIAGRAM OF WIRELESS TELEMETRY AND AUTOMATED MEASURING OF PHYSIOLOGICAL PARAMETERS

The telemetry device consists of a chamber, sensors, a battery, transmitter, and Receiver. The chamber, which consists of a disposable footplate electrode (small implanted device) permits automated and wireless measurements from a small animal. When the animal is placed in the chamber, the transmitter sends out physiological data through radio waves detected by a receiver. The ECG signal is recorded automatically in **Fig. 13**, which is considered a better data storage device. The results will be devoid of human errors and personal bias [54].

CONCLUSION: Cardiotoxicity is a major concern in drug discovery because many drugs lead to the risk of Torsade's de Pointes (TdP) arrhythmia, which causes sudden cardiac death.

To reduce the risk, safety pharmacological studies are carried out. Many drug candidates have been failed in the late phase of preclinical safety studies because of QT interval prolongation, so it is essential to screen the drug candidate activity on hERG channel earlier in the drug discovery process. Nowadays, several hERG assays have been developed. *In-vitro* hERG assays are capable of testing hundreds of compounds per day. *Ex-vivo* and *in-vivo* studies provide more definitive information about arrhythmia than *in-vitro* assays. A telemetry system is an advanced technique that provides automated data collection, so there is no need for direct human interaction, which reduces the typical drawbacks of conventional methods. This review emphasis upon the hERG assays which play a prominent role in drug candidate selection and provides brief information about the molecular aspects of the hERG channel.

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