Project Title

Differential Effect of Cholesterol Depletion on Contractile Response of the Porcine Isolated Splenic Artery

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Abstract:

The contraction of vascular smooth muscle cells in porcine isolated splenic artery is regulated by signaling pathways on the membrane before generation of any functional response. The vascular smooth muscle cells consist of regions known as caveolae enriched with cholesterol. Cholesterol-rich membrane rafts serve as attachment site on the plasma membrane to different agonist for regulation of responses. In this study, the extraction of cholesterol from membrane by the treatment of different concentrations of methyl-β cyclodextrin (MβCD) i.e. 0.75mM and 3mM. The extraction of cholesterol dissembles the caveolae of vascular smooth muscle membrane. Considering the depletion of cholesterol from vascular smooth muscle cell membrane, the reduced level of cholesterol in the plasma membrane results in change response of phasic contractions of vascular smooth muscle cells of splenic artery to different vasoconstrictor agonist. However, restoration of depleted membrane cholesterol by ingesting cholesterol externally can maintain the normal contractile response of vascular smooth muscle cells. It is now known that signaling pathways for phasic contractions mainly originates from cholesterol enriched membrane domain known as rafts. Our study aims to test the hypothesis that membrane cholesterol depletion results in differing contractile response of different agonist. Methyl-β-cyclodextrin was used as cholesterol acceptor to deplete cholesterol from membrane of vascular smooth muscle cells of splenic artery. The samples of porcine splenic artery were contracted in Krebs-Henseleit solution using organ bath. Transmission electron microscopy was used to examine the disruption of caveolae in the membrane due to treatment of methyl-β-cyclodextrin. The phasic contractions varied as a result of application of different agonists. These agonists act on different receptors present in cholesterol-enriched membrane rafts on the vascular smooth muscle cells of porcine splenic artery. The Phenylephrine and noradrenaline proved to have the same effect both in the absence and presence of membrane cholesterol. The contractile response to endoperoxide analogue U46619 was reduced. The agonist KCl and 5-HT showed decreased vasoconstrictor effect by the treatment of 3mM MβCD. Conversely, the serological data demonstrated that the caveolae were disrupted due to cholesterol depletion in membrane of vascular smooth muscle cells. The results were demonstrated in terms of PD2±SEM of vasoconstrictor effects of different agonist after cholesterol depletion. The ANOVA with post hoc Dunnett's test was used as statistical interpretation of comparison of control group and treatment group results.

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Abbreviations

cardiovascular diseases (CVD)

coronary artery calcium (CAC)

coronary heart disease (CAD)

Cyclopiazonic acid (CA)

Detergent insoluble glycosphingolipids: (DIGs)

G-Protein-Coupled Receptors (GPCRs) high-

density lipoprotein (HDL) Intermediate

density lipoproteins (IDL), lecithin- cholesterol

acyltransferase (LCAT) low density lipoprotein

(LDL)

Methyl-β-Cyclodextrin (MβCD)

N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA)

Potassium Chloride (KCl)

Sarcoplasmic reticulum (SR) smooth

muscle cells (SMCs) Vascular smooth

muscle cells: VSMCs very low-density

lipoprotein (VLDL)

L type calcium channels (LTCC)

INTRODUCTION

Atherosclerosis is found to be one of the leading causes of cardiovascular diseases (CVD). The building of plaques occurs over time as individual ages and symptoms are not noticeable until it progresses to CVD. Atherosclerosis is believed to be the leading cause of CVD deaths around the world. There are no effective treatments that can reverse atherosclerosis, however, change in lifestyle such as dietary changes, smoking and therapeutic intervention such as satins may lower the risk of the disease progression to CVD. Atherosclerosis occurs due to cumulation of plaques also known as cholesterol (lipid) within the artery walls, as a result reducing blood flow. Cholesterol is known to be a major component of cell membrane and in perspective of CVD it is highly regulated to ensure optimal cellular function is achieved. Regulation of cellular cholesterol can be achieved through synthetic pathways, but also in terms of both deposition and removal of plasma-based proteins. Plasma-based proteins called low density lipoprotein (LDL) and high-density lipoprotein (HDL) regulate plasma cholesterol, however, dysfunction of these process can lead to the aggregation of cholesterol in the cell and cell membrane subsequent alteration of vascular responses, withier through direction effect on smooth muscle or modification of endothelial dependent process. In the case of atherosclerosis, the cumulation of plaques within the blood vessels may lead to ischemic downstream or it may even lead to death.

The therapeutic significance of these processes is the development of drugs that either slowly modify synthetic pathways or the uptake of cholesterol in vascular smooth muscle, e.g., statins or fibrates. In this project I will examine the effect of a novel cholesterol-depleting agent, methyl-β-cyclodextrin, that is capable of rapidly and selectively depleting cholesterol from cell. Of particular interest and examine the effect of this agent on the number of functions of caveolae, a cholesterol-rich part of the cell membrane known to be strongly associated with G protein-coupled receptors and it has been known to influence cell activity. I will cover the role and importance of cholesterol, caveolae, association of G protein coupled receptors (GPCRs) and function of vascular smooth muscle. Secondly, I will discuss some basic importance of methyl-β-cyclodextrin. Lastly, I will discuss the technique used in quantifying caveolae, followed by a summary of the studies undertaken to date using methyl-β-cyclodextrin on vascular smooth muscle.

1.1 Aims and Objectives:

1. To investigate the extent of cholesterol depletion in plasma membrane of vascular smooth muscle cells in the control group, 0.7mM M-β-cyclodextrin and 3mM M-β-cyclodextrin.

2. The contractile response of porcine splenic artery by application of different vasoconstrictor agonists in control group; 0.7mM and 3mM Methyl-β-cyclodextrin.

3. The effect of cholesterol depletion on caveolae.

4. The receptors responsible for contractile response present in cholesterol rich membrane domains.

Table 1. Agonist used and their Respective Receptors

Agonist	**Receptors**
KCl	L-type ion gated Ca2+ channel
U46619	Thromboxane Receptor
Noradrenaline	α1-adrenorecptor
Phenylephrine	α1-adrenorecptor
Carbochol	Muscarinic Receptor
5-HT	Serotonin
Isoprenaline	β1And β2-adrenergic Receptor

1.1 What is Atherosclerosis, Pathogenesis and Mobility

Atherosclerosis also known as ischemic heart disease (IHD), it is a chromonic inflammatory condition of the arterial wall and it is known to be the largest contributor of CVDs. The disease is characterised by atherosclerotic plaques formed in the arterial wall of the arteries, comprising of necrotic cores, calcified regions, accumulated modified lipids and inflamed smooth muscle cells (SMCs), endothelial cells, leukocytes and foam cells. Risk factors associated with atherosclerosis is divided into two main groups: non-modifiable risk factors and modifiable risk factors. Non-modifiable risk factors are factors that cannot be prevented, changed or controlled such as age, which is known to be one of the risk factors associated with coronary heart disease (CAD) and males clinically manifesting the condition at 50-60 years and female about 10 years later. It has been investigated, the leading cause of death those over the age of 65 is CVD. Epidemiological study suggests that family and parental history of myocardial infraction is a contributory factor of coronary disease. The association of a reported family history of earlyonset (before age 55 years) CHD with presence and burden of coronary artery calcium (CAC) in electron beam computed tomography in 8549 asymptomatic men and women has been established(Nasir *et al.* 2004). Modifiable risk factors such as: smoking dietary imbalance diabetes mellitus, obesity are factors that can in theory be changed, prevented or controlled. Established study suggest, an increase in blood pressure to 20/10mm Hg doubles the risk of ischemic heart disease and stroke over the range of 115/75 to 185/115mm Hg in individuals from 40 to 90 years of age (Lewington *et al.*, 2002). Obesity has been associated with CVDs as it increases oxidative stress and the pro-inflammatory effects of certain cytokines.

The mortality rate of CDVs in the United Kingdom remains high. It has been established, there are around 7.6 million people living with heart and cardiovascular disease in the United Kingdom, approximately 4 million which are males and 36 million are females. Approximately 43,000 people under the age of 75 in the United Kingdom die from the disease and more than a quarter of all deaths in the united kingdom are associated with heart and cardiovascular disease (BHF, 2019). Atherosclerosis plaques is known as the buildup of lipids called cholesterol. Cholesterol has been implicated the progression of this disease, thus limiting blood flow the body. Regulation of cellular proteins by lipoproteins remains important integral part of the atherosclerosis formation and clinical manifestation (Figure 1).

1.2 Plasma Membrane

Considering the plasma membrane of eukaryotes, some more domains are described other than rafts i.e., caveolae and tetraspanin rich domains. Tetraspanins have four domains that control the tubule formation of membrane. Cellular processes regulations require proteinprotein interaction. The most effective site for protein-protein interaction is plasma membrane so it serves as place for protein network and complexes. According to evidence the regulation of genes of protein networking at specific time is done by membrane lipids i.e., spatiotemporal regulations by direct interacting with signaling protein. Mammalian cells outer leaflet contains phosphatidyl choline, glucolipids and sphingomyelin while the inner folding consists of phosphatidyl ethanolamine, phosphatidyl inositol bisphosphate and phosphatidyl serine. This inner fold serves as specific site for membrane signal to induce cellular protein and modulate mutual interactions. Temporarily lipids are produced such as triacylglycerol and phosphatidyl3, 4, 5-biphosphate by inner folding of plasma membrane that plays a significant role in cell signaling.

According to Nicolson fluid mosaic model cell membrane is like lipid bilayer in which proteins are embedded. For many years it influenced the view on structure and dynamics of the biological membrane. However, studies investigated the new concepts by changing domains of the membrane. Though, it is claimed recently that the fluid mosaic model is still relevant to understand the structure, function and dynamics of the biological membrane even after 40 years.

1.3 Cholesterol in the smooth muscle cells plasma membrane

Cholesterol is a vital component of mammalian cells. It is the major constituent of plasma membrane and is abundant in membranes of the organelles. It is synthesised in endoplasmic reticulum and is transported to plasma membrane through Golgi apparatus. Concentration of cholesterol is much higher in membrane than any other component of the cell. In plasma membrane cholesterol plays a major role in maintaining the fluidity and permeability of membrane. It is also necessary for membrane trafficking, protein and lipids grouping. Cholesterol in the form of lipoproteins is stored in the liver hepatocytes cells and transported in the blood. These lipoproteins are taken into the cell by endocytosis. Liquid ordered domains are formed by cholesterol which are known as lipid rafts or nanodomains. Cholesterol is major constituent of lipid in plasma membrane while its cellular concentration is regulated tightly (Lange, et al 1999).

There are different types of lipoproteins and are named according to their mass. There are several types of lipoproteins found in the body and serve distinct functions: chylomicron, very low-density lipoprotein (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins

(LDL) and High-density lipoproteins (HDL). Dietary fats are absorbed and delivered to the tissue via VLDL particles in the blood stream. The two main lipoproteins involved in cholesterol metabolism are low density cholesterol and high-density cholesterol. The two main lipoproteins involved in the metabolism of cholesterol have very district role, structure, density and protein composition. LDL is mainly composed of cholesterol whereas HDL consist of low amount of cholesterol. Low density lipoprotein contains a single copy of Apolipoprotein B-100 which covers a huge part of LD particles. LDLs are approximately 18-25nM in size and they mainly store cholesterol esters in their core. structurally, LDL consist of 10% triglycerides, 45% cholesterol, 22% phospholipids and 25% proteins. In the monolayer of LDLs, high amount of cholesterol can be found as compared to other lipoproteins, thus increases cholesterol uptake throughout the blood plasma. Low-density lipoproteins transport cholesterol to the liver though blood plasma into the cells of various tissues, this process is important in the process of cell wall formation. High levels of intracellular cholesterol level activate an enzyme called Acyl-CoA: cholesterol acyltransferase (ACAT): an enzyme that converts cholesterol to cholesterol ester which can be stored in cells when it is not required. high level of cholesterol also decrease the production of HMG-CoA reductase (rate limiting step) and it also decreases LDL receptors. LDL particles can be oxide as they flow within the blood stream and accumulate within the walls of the blood vessels. Scavenger receptors type A are receptors located on the surface of macrophages which enables them to locate oxidised LDL particles and endocytose the oxidised particles. Continuous removal of LDL particles by macrophages overtime can cause inflammation in the wall of the blood vessels and the development of plaque leading to atherosclerosis. For this reason, LDL is known as 'bad cholesterol'.

High density lipoproteins consist of structural protein called Apolipoprotein A-1 produced in the liver and released into blood plasma. HDL collects free cholesterol across the cell membrane from cells via apolipoprotein A-1.HDL main function in the regulation of cholesterol involves: the removal of free cholesterol embedded within its core from other lipoproteins such as chylomicron or LDL, the free cholesterol is then esterified into cholesterol esters by an enzyme called lecithin- cholesterol acyltransferase (LCAT) which enables the movement of esterified cholesterol into the core of HDL. In the regulation of cholesterol homeostasis, HDL uptake cholesterol from cells into the liver cells for synthesis or it can absorb free cholesterol and dispose cholesterol from the remnant of HDL particle via the activity of scavenger receptor B1. This is an important process in the regulation of intracellular cholesterol pool and in the process of atherosclerosis formation.

Change in transmembrane currents fluctuates intracellular free calcium ion concentration that regulates the contraction of smooth muscle cells. Ca^{2+} ions determine the membrane potential and membrane conduction. Plasma membrane of smooth muscle cells contains ion channels and receptors that are involved in regulating currents and these are modulated by agonists,

Ca^{2+} and protein-protein interaction. Sphingomyelin, high cholesterol rafts and non-raft regions containing glycerol lipids are the subdivisions of plasma membrane. Lipid rafts are cholesterol and sphingolipid enriched domains that compartmentalize cellular processes. Cholesterol is essential for the stability of rafts. The more ordered and less fluid region is possible by cholesterol in contrast to surrounding membrane. Lipid rafts are more closely related to range of signaling components including ion channels, receptors and enzymes. Cholesterol made lipid rafts in the cell membrane and these cholesterol-rich membrane regions including caveolae is involved in the regulation of contraction of arteries. Cholesterol and caveolae plays a role in cell signaling. These are also the modulators of contractility. Caveolae is a flask like invagination of cell membrane and is regarded as lipid raft and have rich concentration of ion channels and receptors. Caveolae is abundant in vascular smooth muscles. Depletion of cholesterol in the cell membrane disrupts caveolae and alter the signaling processes. Functions of ion channel is modified by cholesterol depletion like outward K+ current is increased with unaltered cell inward Ca^{2+} current. Caveolae is involved in receptor localisation, Ca^{2+} entry pathways and intracellular calcium release through sarcoplasmic reticulum and has role in agonistic stimulated signaling.

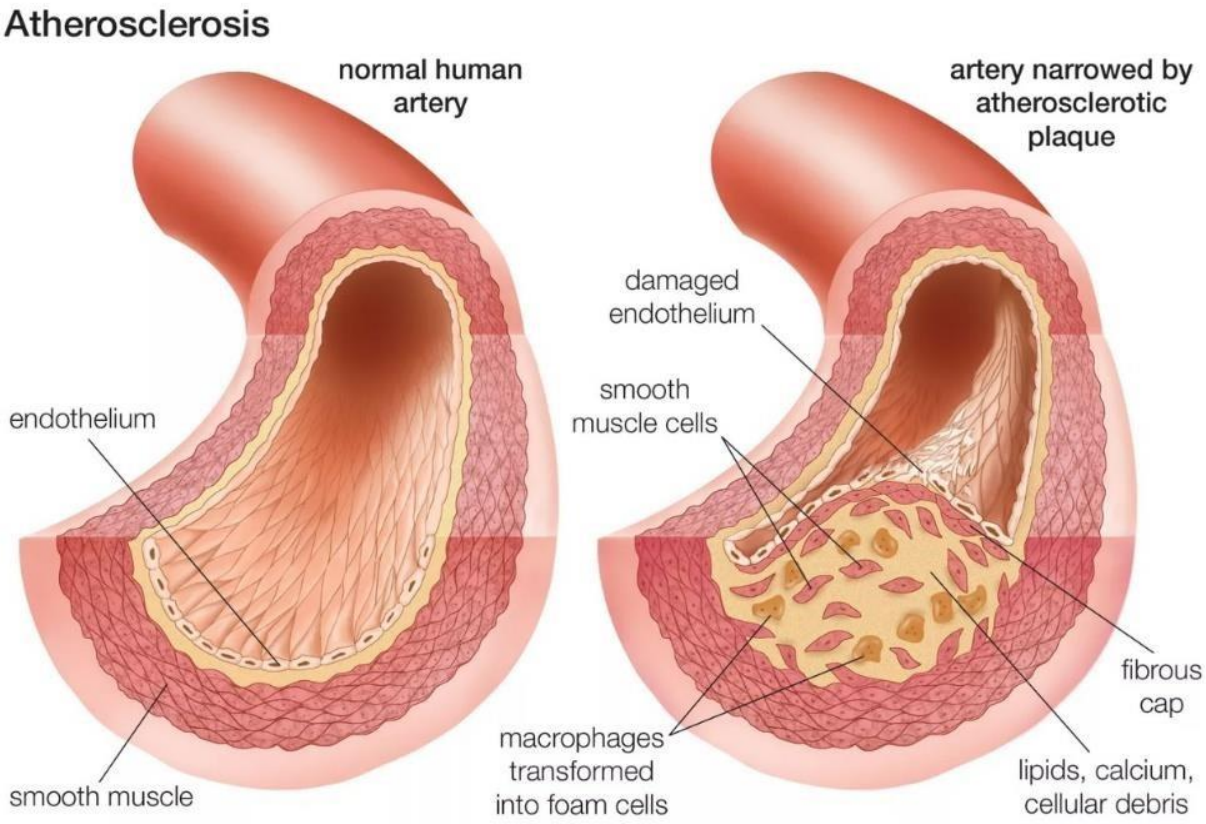


Figure 1. Diagrammatic representation of the normal human artery and narrowing of the artery by plaque formation (adapted from Richard, 2020)

1.4 Cholesterol Depletion

Cholesterol depletion is widely used to study the role of cholesterol in cellular processes. Methylbeta-cyclodextrin is the cholesterol acceptor and results in cholesterol depletion effecting force of contraction and Ca^{+} handling. It impairs pressure-induced vasoconstriction and myogenic constriction. The intracellular calcium stores are depleted resulting in loss of connectivity of signaling between plasma membrane and sarcoplasmic reticulum. By SEM, cholesterol depletion is found to disrupt caveolae. The viability of β-cyclodextrin is further confirmed by agonistic sensitivity. In the cholesterol depleted preparations, the application of adrenergic agonists, 5-hydroxytryptamine, vasopressin, KCl and endothelin affects the membrane depolarization and activation of G-proteins. However, exogenous cholesterol can restore the effect of cholesterol depletion (Nishimura, Vrljic, Klein, McConnell, & Moerner, 2006).

Lipid microenvironment of the membrane receptors affect their conformational structure and function. G-protein coupled receptors are transmembrane receptors and are known as heterodimers involved in cell signaling events. The membrane cholesterol has interaction sites on the receptors that are important in cholesterol sensitivity in receptor function. The receptors form heterodimers and their confirmation are regulated by the membrane cholesterol. The receptor-cholesterol interaction and indirect membrane effects both regulates the heterodimer confirmations. Better therapeutic strategies can be possible if membrane cholesterol regulates G-protein coupled receptor hetero-dimerization.

1.5 Lipid rafts and caveolae

In early 1970s the idea of membrane micro domain was introduced and in 1982, it was demonstrated experimentally by Karnovsky. Karnovsky, demonstrated that multiple phases exist in the lipid environment of a membrane. Sphingolipids and cholesterol form one type of micro domain and it was shown that this type of micro domains was already present in plasma membranes.

After conducting several experiments Van Meer and Siemons named these microdomains as lipid rafts in 1988. Lipid rafts are also defined as heterogeneous, highly dynamics and small microdomains that consist of excess glycosphingolipids, cholesterol and proteins. These are highly organised as compared to their surrounding lipid bilayer. These microdomains regulate neurotransmission and membrane trafficking. Lipid rafts and the plasma membrane can be distinguished based on their lipid composition. The experiment shows that much more cholesterol is present in raft as compared to their surrounding bilayer. Cholesterol serves as dynamic anchor that maintain the rafts together.

Invaginations in plasma membrane are called caveolae. Their diameter ranges from 60 to 80 nanometer and they were identified in 1950 by electron microscope in smooth muscle cells, adipocytes, endothelial cells and fibroblast, these invaginations revealed the presence of caveolae. Their function includes calcium signaling, regulation of various cell signaling pathways and endocytosis (Parton & Richards, 2003). Caveolae-1 is the major constituent of caveolae. The other two isoforms are caveolae-2 and caveolae-3. Caveolae-3 is muscles specific (Song et al 1996). Topology of all these three caveolin proteins are same as they have in C and N terminal domains that are present in the cytoplasm and they have a long hair being transmembrane domain. All these caveolin proteins are formed in the cells more accurately in Golgi apparatus. They are formed as monomers then they begin to form as oligomers when they enter the secretary pathway. When cholesterol is added the exit of caveolin from Golgi apparatus becomes quick for the formation of caveolae. The ability of caveolin to oligomerise is essential. Caveolin 2 is also essential for formation of caveolae. In muscle cells in caveolin 3 is needed for the formation of caveolae if caveolin 3 is lost or it mutated then it results in the dystrophic phenotypes. The presence of caveolin expression is not the only inducer for the formation of Caveolae, although extraction of cholesterol leads to the disruption of caveolae ay the plasma membrane. The extraction of cholesterol in membrane disrupts formation process, this is because cholesterol is required for the incorporation of caveolin into raft domains and it is considered as a critical event for the formation of caveolae. Initially it was thought that cholesterol and caveolin are essential for the formation of caveolae, however several studies show some additional proteins that are called Cavin. This family of protein have four members Cavin 1, Cavin 2, Cavin 3, and Cavin 4. Cavin one is called PTRF Cavin 2 is called STPR Cavin 3 is called SRBC and Cavin 4 which is specific to muscles and it is called MURC. These four proteins are also very essential for the functioning and formation of caveolae. The formation of caveolae is complex and it is a regulated cellular process. To form a single caveolae, 50 to 60 Cavin are necessary to associate with 150 to 200 caveolin monomers.

As it is mentioned already that sub-domain of lipid rafts is represented by Cavalli. The distribution of GMI is shown by confocal microscopy and it is considered as a well-known raft maker and it does not merge with caveolin. Flotillin is considered as another raft marker and it is defined as non-caveolar raft and its merges with GMI. A non-heterogeneous distribution of non-caveolar regions and caveolar regions are exhibited on plasma membrane.

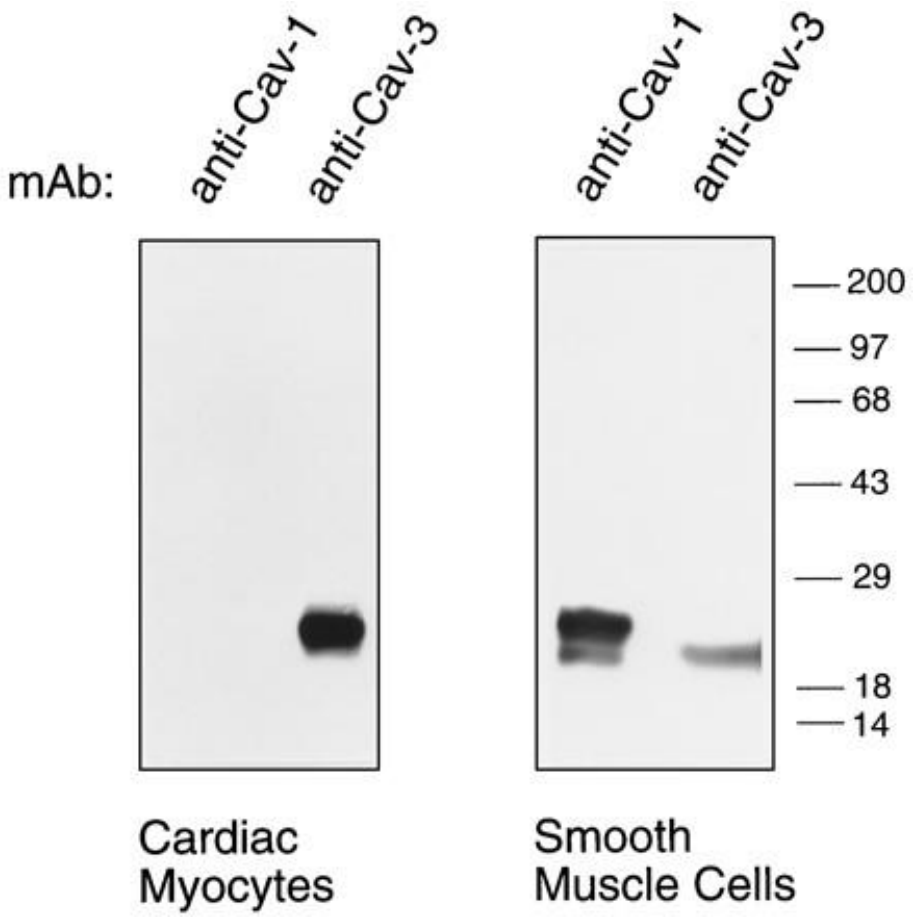


Figure 2. Expression of caveolin-1 and caveolin-3 using primary cell culture. Left panel shows no expression of Cav-1 and the expression of Caveolin-3 in cardiac myocytes. Right panel shows the coexpression of caveolin-1 and caveolin-3 (adapted from Song, et al 1996).

1.6 Lipid Rafts: Role in Signal Transduction

Simon and Van Meer in 1986 presents that in epithelial cell's tight junction difference exists in the lipid composition of exoplasmic leaflet and cytoplasmic leaflet. It showed that heterogeneity exists in the lipid composition. Furthermore, lipid raft theory was proposed by Simon, in which detergent resistant membranes are made of glycosphingolipids. These detergent resistant membranes are enriched with cholesterol and glycophosphatidylinositol (GPI) protein. These rafts operate as site for protein attachment when membranes are moved during signal transduction. This theory is based on grouping of sphingolipids and cholesterol (Simons & Toomre, 2000).

There is still a debate on the definition of lipid rafts; there is variation in the isolation procedures and incubation time span of the lipid rafts, hence there is complexity in comparing the results and different isolation procedures and incubation time has been proposed. Membrane raft study comes in the domain of biophysics and cell biology. In order to do intensely study the lipid rafts, a collaboration is needed between physical and biological aspects. Scientists in the discipline of physics are involved in the composition and mathematical aspects involved in composition whereas the biological scientists are interested in the biological pathway's role and function of lipid rafts.

1.7 Anatomy of Splenic Artery

Splenic artery or lienal artery is a vessel that is responsible for supplying blood to the spleen, and major parts of stomach and pancreas. It is a single artery that originates from the celiac trunk, being one of its longest branches. Through the lienorenal ligament and upper pancreatic border, splenic artery travels anterior to the adrenal gland and left kidney, and posterior to the stomach. Twisting and coiling of this artery is responsible for producing distention in stomach and spleen without disturbing their blood flow (Jauregui, 1999). The splenic artery also splits off into pancreatic branches that include dorsal pancreatic artery, inferior pancreatic artery, and great pancreatic artery. These pancreatic branches basically originate from the proximal part of the splenic artery (Gil-Vernet, Caralps, & Ruano, 1978). The basic and the main function of splenic artery is to supply blood to the spleen, and major portions of stomach and pancreas (Keramidas & Soutis, 2003).

1.8 Layers of Artery

The cardiovascular system is composed of five main classes of blood vessels: arteries, arterioles, capillaries, venules and veins. Arteries are found to be very strong and elasticated in nature, they carry blood away from the heart under high pressure and partitioned into smaller arteries. The wall of artery is further divided into three distinctive layers:

- **Tunica Interna (Inner Most Layer):** it is made up of simple squamous epithelium layers (endothelium) which lays on a connective tissue's membrane with many elastic, collagenous fibers. The purpose of the endothelial layer is to prevent blood cloth, regulate blood flow release nitric oxide to relax the smooth muscle.

- **Tunica Media (Middle Layer):** this layer is the most abundant layer of the arterial wall; it consists of smooth muscle fibers and a thick elastic connective tissue layer.

- **Tunica Externa (Outer Layer):** this layer is the thinnest layer, primarily made up of connective tissue with irregular fibers which is affixed to the surrounding tissues.

1.9 Smooth Muscle Membrane Potential

Difference in electrical potential across the membrane of GIT smooth muscle cells is maintained like other cells that are excitable. Smooth muscle cell resting membrane potential is between 50 to -60 mV. In contrast to other muscle cells and nerves, fluctuation occurs spontaneously in smooth muscle cells membrane potential. These partial depolarisation are equal to changes in the membrane potential between 5 – 15 mV (Ulyanova, 2019).

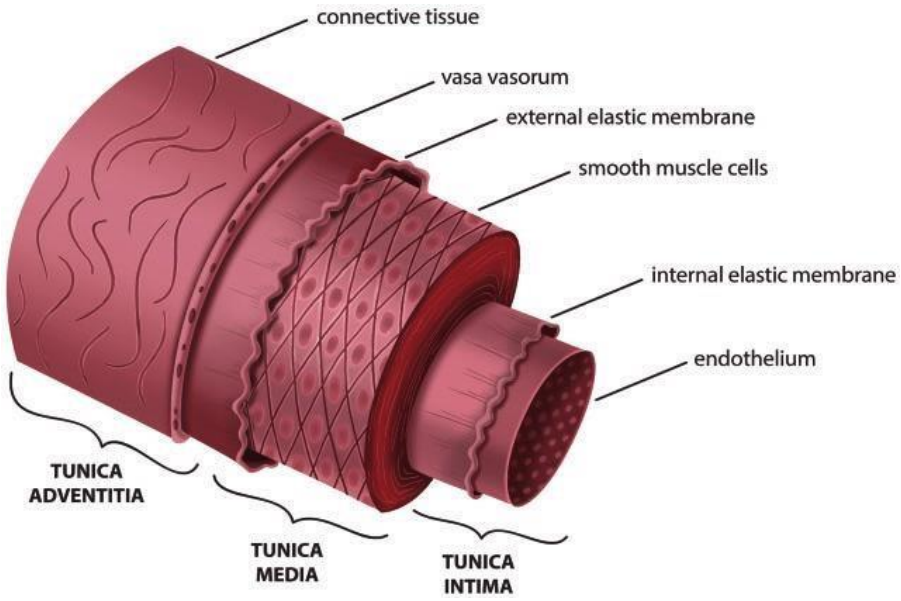


Figure 3. The three major layers of the artery, the tunica intima, tunica media and tunica adventitia (adapted from Enis and Sadikoglu, 2016)

1.10 G-Protein Coupled Receptor

GPCRs are phosphorylated upon agonist binding which initiates receptor internalisation and receptor down-regulation. The downstream signaling processes by G-protein coupled receptors is regulated by mechanism of endocytosis. However, membrane cholesterol has major role in the structure, function and dynamics of GPCRs, although role in endocytosis is unaware. The structure and organisation of the cell membrane is maintained by the cholesterol and is involved in various other cellular processes. GPCRs undergo endocytosis upon agonist stimulations and cholesterol depletion due to cholesterol acceptor such as methyl-β-cyclodextrin which switches the lipid raft independent endocytosis to lipid raft dependent endocytosis (Chini & Parenti, 2004).

In regulating the fluidity and integrity of plasma membrane cholesterol place a vital role. Compartmentalization of intracellular signaling is regulated by cholesterol. Re-organisation of actin skeleton composition is regulated by cholesterol depletion in non-muscle cells such as lymphoblasts and fibroblasts. In phosphatidylinositol, proteins that are bound to membrane their lateral mobility is reduced due to cholesterol depletion. In fibroblasts and endothelial cells if the cholesterol is depleted, it results in increase in the stiffness of cell because of remodeling in actin and it is due to increase in adhesion energy of membranes cytoskeleton. In skeletal muscle cells depletion of cholesterol impairs contractions that results in morphological abnormalities and vacuolization. Furthermore, transmembrane glycoprotein and beta dystroglycan through which extracellular matrix is connected to cytoskeleton is uncoupled due to cholesterol depletion.

Cholesterol enriched domains such as caveolae are necessary for the functioning of cells. In muscle cells these proteins are necessary for regulation of membrane tension and mechanosensing. Despite from cellular mechanics modulation calcium dynamics in several cells are also regulated by caveolae through which activity of ion channels i.e., L type Ca^{2+} channel is regulated. signaling of β1 and β2 adrenergic receptors is guaranteed by the proper localization of caveolae in cardiomyocytes and it provides a role in initiating calcium cycle in cardio myocytes.

1.11 Contraction in Smooth Muscle Cells

Contraction in smooth muscle cells is regulated by changing in the transmembrane currents that results in free calcium fluctuation in intracellular concentration. Over the last decade progress is being made in understanding the impact of calcium and mechanism of calcium in regulating the contractile machinery. The major determinant or schedule is recognized by membrane potential and control of membrane conductance and as results in contractile

response. Smooth muscle cells plasma membrane contains receptors and ion channels that contribute in the modulation of transfer of currents and these are regulated by membrane potential, calcium, agonist and protein-protein interaction (Dreja et al., 2002).

In cells with lower level of cholesterol defects in relaxation and variation in the contraction rates are seen that are revealed through traction force microscopy TFM experiments. Despite some different dynamics and organisation of different cell the contractile machinery of ectomio scene remains conserved.

1.12 Methyl-β-Cyclodextrin

In plasma membrane nano sized micro domains are present that are enriched with cholesterol, gangliocytes and sphingolipids. These rafts are planner in structure and are associated with the lipids of membrane and act as platforms that separate phase for receptors and are components of signaling cascades. These micro domains regulate the efflux and influx of drugs. MβCD being equally strong extracting agent is used to access the role of raft in plasma membrane and its effect on contraction in smooth muscle is evaluated for vascular smooth muscles. Permeability of small non-electrolytes and ions are enhanced when the integrity of lipid rafts is disrupted by the depletion of cholesterol present in the membrane. The most effective agent among several agents used for the depletion of cholesterol is methyl-β-cyclodextrin which is extremely water soluble hepato-saccharide that consists of beta-glucopyranose unit. It is observed that cytotoxic effects of several chemo therapeutic agents are enhanced by MβCD (Zidovetzki & Levitan, 2007).

Segregation of flattered caveolae and membrane lipids is abolished when MβCD extract cholesterol from plasma membrane. In contractile responses of muscles, the modulation occurs because of these structural rearrangements. This modulation is associated with the activity of raft that is associated with potassium channel.

1.13 Stereology

The use of electron microcopy in the quantification of caveolae has become particularly important in lipid raft investigation. Quantitative stereology is a specialised area of microscopic image analysis which can be used to measure biological objects accurately by the means of rationalised steps of sampling combined with mathematical reconstruction. Analysis of solid bodies are based upon statistical derivations of genomics probabilities which requires basic but justifiable assumptions, therefore, complex structures can be characterised (Iglesia et al, 1981). Biological cellular compartments are known to be 3D, hence, interpretation into 2D image sections is required. Estimation of parameters can be performed fast, effective and unbiased. Serology can be conducted using live microscopy images saving time and space or it can be performed using pre-saved 2D images (Howard and Reed, 1998).

Stereology technique enables the quantification of the following parameters: total volume of a tissue, surface area or length of structure population of cells within a tissue, cell size or number. visualisation of 3D morphology which involves fixing and sectioning of samples depending on the specimen(s). Molecular, organic and or organismal samples can be examined from various slices depending on the thickness, this can be achieved using microtome. Microtome is used in cutting ultrathin slices, it utilises a diamond or glass microtomes blades (Figure 5)Thickness of slices may vary from 2cm slices for organs, MRI slices thickness from 3 to 30□m LM or TEM slices thickness from 30 to 100nm and from 2 to 20nm-thick for electron tomographic slices. Microscopic slices using appropriate thickness enables the viewing of specific organelles in different part of the tissues. TEM samples may be placed on a copper mesh which provides support, however depending on the sample there are four different types of copper grids that are available for sample to be place on and analysed (Figure 6). Depending on the viewing area, selection of mesh is especially important, it must be clean and easy for samples to adhere during preparation, as a result sample can be easily analysed.

Stereology slices obtained can be employed to visualise objects using two distinct methods. Serial slices (depending on thickness) can be used for instruments such as MRI, CT scan. The most used stereology method in the area of biological research is simple random sampling also known as systematic uniform random sampling (SURS), slices can used for instruments such as light microscopy and transmission electron microscopy. SURS has become an attractive area of interest as it allows scientists to quantify biological object using probes (Figure 4). A probe is a set of computer-generated shapes that may be used to study tissues. Probe works according to different sets of rules that allow fair and unbiased quantification, it also allows the tissue been studied to be examined without leaving out any space hence, achieving unbiased results (Mayhew and Lucocq, 2015). Volume estimation can be achieved using SURS method, this method uses the Cavalieri principle or point counting estimator. Point counting stereology utilises point grid. Points are evenly spaced from across and down on a grid which is called a lattice. A lattice can be place over an image and points that have landed on area of interest may be counted, point on the outside of area of interest and outside exclusion line will not be considered, results obtained from point counting can be further used for mathematical calculation to obtain accurate volume of objects from various samples (controlled to treated groups) (MBF Bioscience, 2019).

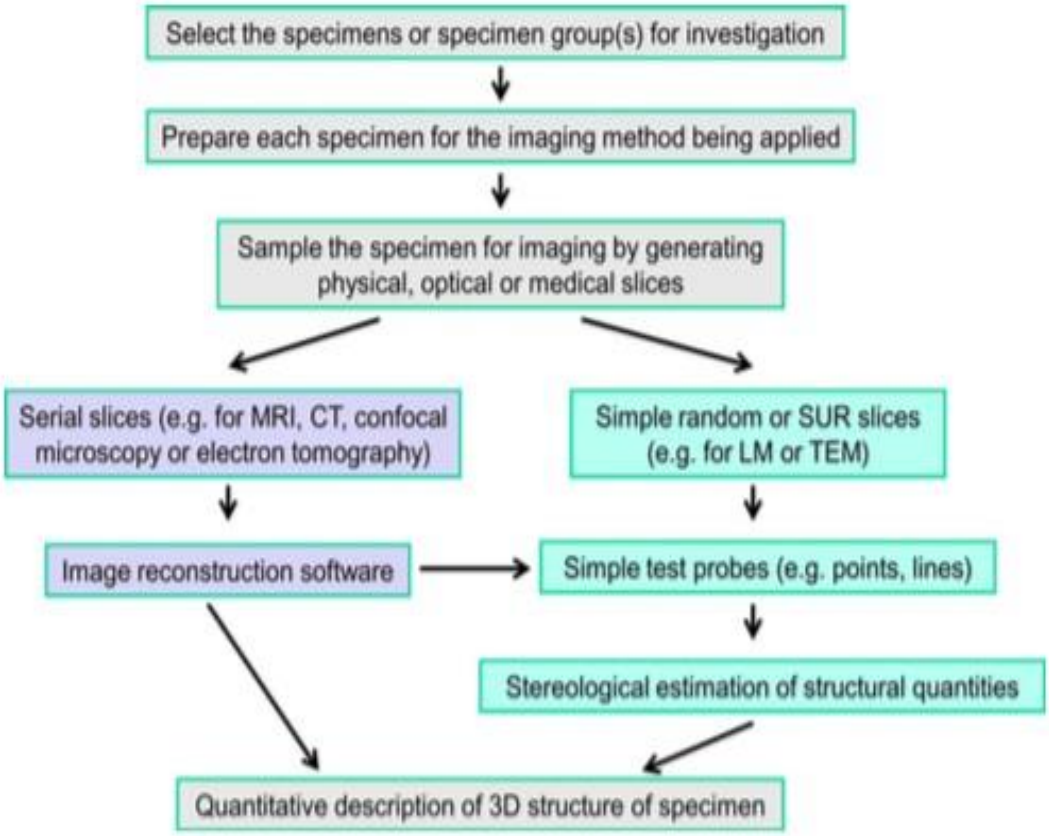


Figure 4. Stereological process used in quantitative analysis of 3D structure of specimen (Mayhew and Lucocq, 2015).

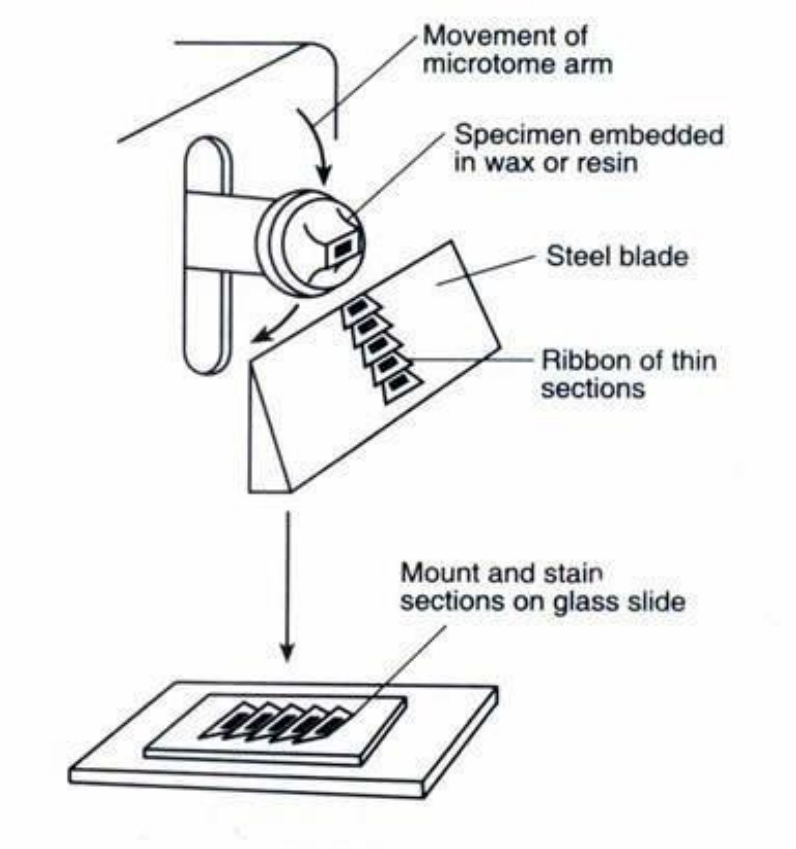


Figure 5. Schematic representation of microtome for sample slicing (Kumar, 2019)

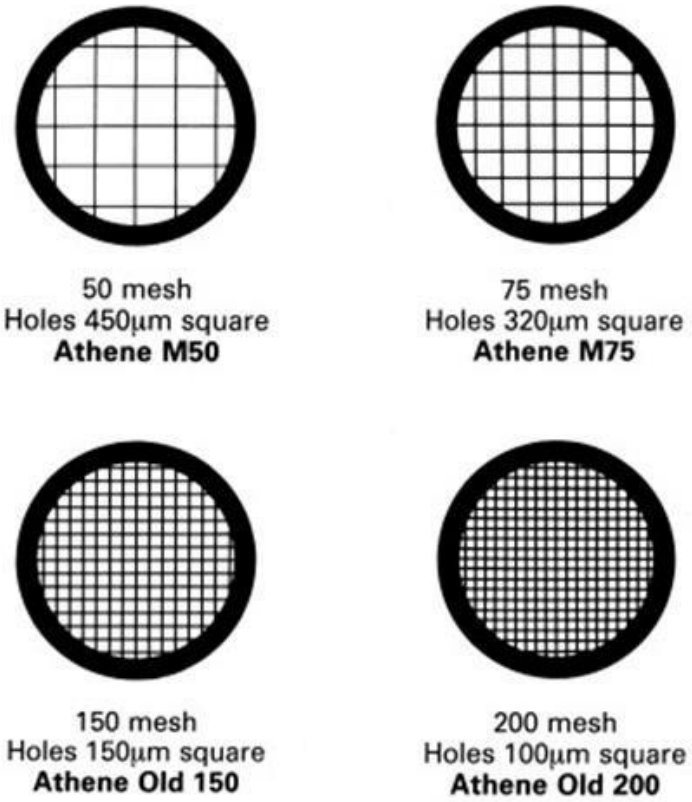


Figure 6. Four main types of grids used in sample preparation (adapted from Agarsceintific, 2019)

LITERATURE REVIEW

A study was conducted that shows the impairment results in contraction due to cholesterol depletion in neonatal rat cardiomyocytes. For the depletion of cholesterol of plasma membrane cyclodextrin called methyl beta cyclodextrin was used. Time resolved Traction Force Microscope (TFM) was used to measure how contractility was affected by cholesterol depletion in neonatal cardiomyocytes. The contractile forces that are generated within myofibrils via actomyosin interactions are called cell contractility. Cells that were used attached to flat substrate their contractile behavior was measured using TFM. For forty seconds traction stress maps were obtained so that spontaneous beating of cardiomyocytes can be obtained and the maximum contractions and relaxations were captured. To measure the total mechanical work that is done by the cell to deform a substrate is called strain energy. The characteristic contractility measurements that are independent of the shape and size of cell was defined by strain energy and cell area ratio. The contraction relaxation patterns of cardiomyocytes were demonstrated by traction stress maps from strain energy per area of the cell quantification. Frequency of 0.3 Hz was applied. It was observed that the contraction in cholesterol depleted cells became irregular. Energy of the troughs and the peaks were increased when the cells were treated with7.5 mmol/L methyl beta cyclodextrin. The strain energy per cell area was evaluated by checking the difference between trough energy and peak energy per cell area and it was observed that contractility decreases overall. Furthermore, the power spectra showed that the depletion of cholesterol yielded spectra that was noisier and the frequency of beating was increased. Thus, contractility was highly affected by cholesterol depletion.

Furthermore, depletion of cholesterol disorganises the myofibers in neonatal cardiomyocytes. It was investigated that whether changed contraction due to cholesterol depletion puts change on the morphology of myofibril. For this purpose, staining was done for sarcomeric alphaactinin, actin and cells were made fixed. One panel was treated with 5 mmol/L and the second panel was treated with 7.5 mmol/L methyl-β- cyclodextrin. In the myofibers organisation of the thin filaments were highly affected due to cholesterol depletion. Lack of cholesterol distorted the z-bands in alpha actinin cells while in control cells z-bands remained parallel and were equally spaced.

To measure the angle between z-bands and their separation distance image analysis was performed to check changes in alpha actinin myofibril. In control cells, the z-band spacing remained regular with value of 1.75 ± 0.09 micrometer whereas z-band spacing in cholesterol depleted cells reduced to 1.49 ± 0.29 μm in cells that were treated with 5 mmol/L cyclodextrin and 1.47 ± 0.31 μm reduction was observed in 7.5 mmol/L treated cells. In control cells the

angle and spacing of z-bands remained the same as was expected. The alignment of z bands was less defined in cholesterol depleted cells (Vial & Evans, 2005).

To adapt sudden changes in membrane tension, caveolae proved beneficial in adult cardiomyocytes. Caveolin was modulated by actin cytoskeleton. Membrane tension and architecture of actin cytoskeleton both are changed by depletion of cholesterol. Caveolin-3 distribution was localised along with z bands in controlled cells while the cells that were treated with 5 and 7.5 mmol/L methyl-β-cyclodextrin homogenous distribution of caveolin-3 disappeared as it happened in controlled cells. The caveolin-3 distribution was distorted and disorganised in cholesterol depleted cells.

In contractile machinery of muscle cells Calcium plays a fundamental role so the change on sarcoplasmic Calcium in cardiomyocytes was observed. For this purpose, comparison of control cells and cholesterol depleted cells was done. Control cells and cholesterol depleted cells were incubated with Fluo-4 AM and their intracellular calcium changings were tracked by imaging. On heat maps fluorescence of Ca was shown for cyclodextrin treated and control cells in the states of relaxation and contraction. The comparison of contracted states in both types of cells showed that the intensity of Ca fluorescence increases in the cholesterol depleted cells. The cells in which cholesterol was depleted cannot buffer cytoplasmic Ca when they were in relaxed state. It was revealed in quantification of intracellular Ca fluorescence that in control cells alteration in Ca spikes was periodic and fluorescence signals moves to zero in the relaxed position of these cells. On the other hand, baseline for calcium intensity was high in cells that were treated with 7.5 mmol/L cyclodextrin was indicating lower cholesterol and was in relaxed position. Spectra for calcium spikes was noisier in cells with low amount of cholesterol. Increase in contraction rate was observed. As cholesterol depleted from cardiomyocytes an increase in the peak frequency of calcium was observed. In cholesterol depleted cells more concentrated calcium events were presented as compared to control cells. TFM results showed that calcium concentration and frequency of calcium spikes both are enhanced and when cell undergoes relaxation impairment was observed in cytoplasmic Ca buffering.

Extracellular Ca also plays a critical role in the contraction of cardiomyocyte cells. Ca channels are present in caveolar domains such as L type calcium channels (LTCC) and plays a role in regulating their activity. Cells were labelled for LTCC to check whether the localisation of LTCC was changed by the change in the concentration of cholesterol in the plasma membrane. Homogeneous distribution of caveolae in control cardiomyocytes. The distribution of Ca 1.2 subunit was less uniform in areas that were devoid of labeling in cholesterol depleted cells. To control LTCC pore’s opening, the PKA phosphorylates Ca 1.2. for measuring cAMP dependent PKA activity enzymatic assay was performed for the correlation of changes in the localisation of LTCC both in treated and control cells. increase in cAMP dependent activity was observed in treated cells that leads to more pore opening events that results in more entry of Ca. so the

deregulation in contraction was observed in neonatal cardiomyocytes. Furthermore, one of the main proteases of muscle tissue is calpain, it is activated by Calcium that is present intracellular. Treatment with methyl beta cyclodextrin increased calpain activity in treated cardiomyocyte cells as compared to control cells. It was observed that due to reduction of cytoplasmic calcium in cholesterol depleted cells, calcium dependent protease degrades the myofibers.

Cholesterol deficiency and reduced substrate stiffness leads to the lowering of VSMCs elasticity and adherence to fibronectin (FN). An assay was performed for measuring the overall levels of cholesterol in the cell. It demonstrated that when 5mM MβCD was utilised for an hour, a decrease in the level of cholesterol (50%) was observed. But contrary to this, administration of 5mM MβCD-CHOL complex for half an hour augmented the overall cholesterol levels. When a comparison study was performed for control VSMCs and those treated with MβCD & MβCDCHOL, it was clearly indicated that 25% of the cells were damaged and destroyed during their treatment with MβCD & MβCD-CHOL. Atomic force microscopy (AFM) was used to generate force curve analysis where specifically used for visualization of the modulus of elasticity of cholesterol including control, MβCD (depleted) and MβCD -CHOL (enriched) VSMCs when cultured on different substrates i.e. 103 kPa, 28kPa and 3.5kPa. Comparison between the elastic modulus was found to be the highest for the Control VSMCs while lowest for the other two when cultured on the substrate 103 kPa. Conversely, culturing on 103 kPa and 28kPa substrates showed a significantly higher modulus of elasticity for MβCD-CHOL VSMCs as compared to the MβCD treated cells. Loading rate (that was figured out from AFM force curves) and cell adhesion force were basically used to evaluate the effect of cholesterol and stiffness of a substrate on the integrin α5β1-mediated VSMCs adherence and attachment to FN. A remarkable decline was observed in the adherence force of cell mediated by integrin on α5β1 substrates 103kPa and 28kPa when both substrate stiffness and cholesterol levels were kept lowered. However, impact of cholesterol on cell adherence was neglected in case of substrate 3.5 kPa. Evaluation of cholesterol enriched therapy i.e. MβCD -CHOL on 3.5kPa substrate alone showed a notable rise in the adhesion force of VSMCs mediated by integrin α5β1-FN as compared to control and MβCD VSMCs. When comparison was done for control, cholesterol deficit and cholesterol augmented VSMCs. Cholesterol-deficit VSMCs was found to be lowering the loading rate of these cells, cultured on 103kPa and 28kPa substrate. It is clearly shown through these findings that cholesterol influence on VSMCs biomechanics is somehow based on the substrate stiffness.

2.1 Cholesterol deficiency: responsible for the reduction in vasoconstrictive action of splenic artery

In order to examine the impact of cholesterol, an ex vivo technique called myograph was adopted. In this method, the effect of cholesterol changes on vascular activity of a native rat

aorta was evaluated. According to the quantitative analysis, cholesterol level in the tissue was reduced by ~50% when MCD therapy was used. But contrary to this, an increase of ~25% was observed in the cholesterol level when MCD-CHOL therapy was applied. To examine the contractility of cholesterol- deficit aorta, 100 μM of phenylephrine was applied to the aortic rings. This resulted in the production of maximum contractile force. i.e., 50% when compared with the control aorta. However, a small increase was also observed in the contractility of aorta enriched with cholesterol when compared with the control group ($P > 0.05$). This result shows that when Methyl-cyclodextrin (MCD) is used, a remarkable decline in the vasoconstrictive activity of the native rat aorta occurred in case of deficit cholesterol while no notable increase was seen in enriched cholesterol.

In 2014 a research was conducted by Naoshad Muhammad and his co-workers that tamoxifen induced cell death is augmented due to cholesterol depletion by methyl-β-cyclodextrin in malenoma cells as its uptake is enhanced in melanoma. A_{375} and B_{16F10} cells were exposed both to methyl-cyclodextrin and tamoxifen in the presence of cholesterol and absence of cholesterol to check the correlation of cytotoxic effects of tamoxifen and less amount of cholesterol. The survival of the cell was checked by colorimetric assay (MTT). It was seen that the survival of cells that were treated with both tamoxifen and methyl-cyclodextrin was reduced to more than 50%. Cells that were cultured in cholesterol containing medium their tamoxifen induced death was abrogated. Cells that were treated alone with tamoxifen and those treated with both methyl cyclodextrin and tamoxifen was compared. It was observed that cells that were treated with both tamoxifen and MCD their total cellular cholesterol was reduced by 25 percent as compared to those that were treated alone with tamoxifen. As A Result, it was concluded that supplementing cells with cholesterol rescues the cells from tamoxifen as their susceptibility towards tamoxifen is enhanced due to methyl-cyclodextrin (Mohammad et al., 2014).

Risak Onodera in 2013 published a study in which it was revealed that in Ihara, KB and M_{213} cells methyl-cyclodextrin causes apoptosis. Due to depletion of cholesterol in lipid rafts. In mice it was observed that after injecting intra-tumoral injection to colon-26-cells, methyl beta cyclodextrin inhibit the growth of these tumor cells. The molecular weight of cyclodextrin is high and it bears aqueous properties the membrane permeability of MCD is poor. For this reason, when methyl-cyclodextrin is uptake into the cells it becomes difficult for MCD to induce apoptosis. Lysosomal storage organelles that are enriched in cholesterol are present in cells, when cyclodextrin conjugates with dextran it enters the cell proved effective in declining the accumulation of cholesterol. In HeLa cells fluorescein tagged methyl-β-cyclodextrin was internalised by clathrin dependent endocytosis. Tetra methyl rhodamine iso thiocyanate (TRITC) association with methyl-β-cyclodextrin was examined in various cells. A slight association was seen among KB cells, M_{213} cells and Ihara cells with TRITC MCD. It was hypothesized that apoptosis interacts with components of plasma membrane and this induction

of apoptosis is due to cyclodextrin. In NR 8383 apoptosis was induced by Non-ionic surfactants such as poly-oxy-ethylene hydrogenate castor oil, tween 20 i.e., glycol sorbitan monolaurate and triton X 100 because the components of membrane such as phospholipids and cholesterol have solubilising effects. Induction of apoptosis by MCD is due to the solubilising effects of MβCD on the components of membrane and MCD's solubilising effect on cholesterol makes its hemolytic effect high. It was reported that lipid rafts i.e., lipid microdomains which are composed of sphingolipids and cholesterol, they contribute to apoptosis. It was revealed by scientists, using the red blood cells of rabbits, cholesterol from lipid microdomains was extracted using MCD to notice the contribution of cholesterol from lipid microdomains in the induction of apoptosis. From lipid rafts cholesterol was released into culture medium in KB cells. The addition of cholesterol in the culture medium inhibits the cytotoxic activity of MβCD. It was strongly suggested that the depletion of cholesterol in rafts of lipids plays a critical role in the apoptosis induction that is due to MCD (Onodera et al., 2013)

Burdyga and co-workers in 2004 conducted a study to check the manipulation of cholesterol in smooth muscle cell plasma membrane. For this purpose, a variety of smooth muscle tissues were treated and it was observed that the cholesterol was solubilised by MCD while it does not affect the majority of lipid components in plasma membrane remained unaffected. The contents of the cholesterol that were extracted was incubated in water soluble cholesterol. In the cells of smooth muscles, the effective manipulation of cholesterol contents the replenishment of MCD with water soluble cholesterol was done.

It was observed that the membrane rafts destabilised by the extraction of cholesterol. When lipid rafts were stabilised by cholesterol, they become solubilising resistant due to non-ionic detergents. This is the reason that it was isolated as detergent insoluble domain known as sphingolipid enriched membrane. Detergent enriched glycosphingolipid is enriched with cholesterol and sphingomyelin in cells of smooth muscles. Raft destabilised due to cholesterol extraction becomes the reason in loss of resistance of raft associated proteins and sphingomyelin both towards nonionic detergents. Their extraction pattern became similar to the extraction pattern of non-rafted glycerol phospholipids.

It was examined that cholesterol extraction disassembled caveolae. Due to extraction of cholesterol, structural reorganisation of plasma membrane in smooth muscle cells occurred followed by caveolae disassembly. It can be reversed by supplementation cholesterol. It was seen that cholesterol extraction did not induce any changes in the morphology of mitochondria and sarcoplasmic reticulum.

Work was done on the inhibition of electrically evoked contraction in the smooth muscles of ureter of rat due to lack of cholesterol. It was demonstrated by MβCD solution that increase of

both the force and Ca concentration in ureter of rat that was perfused by solution containing methyl cyclodextrin (Pucadyil & Chattopadhyay, 2007).

Caveolae is enriched with cholesterol and fatty acids and it contains saturated change that make its structure rigid and highly organised. it exists in smooth muscle cells and adipocytes, but it is highly abundant in endothelial cells. For the formation and stabilisation of caveolae the biochemical marker is cav-1. Cav 1 interacts with various proteins that helps in the regulation of signal transduction in cav-1. Endothelial nitric oxide synthase (eNOS) associates with cav-1. nitric oxide is generated by endothelial nitric oxide synthase by the conversion of L-arginine to L-citrulline. Phosphorylation by kinases and intro cellular calcium concentration regulates this process. The activity of endothelial nitric oxide synthase, calcium signaling endothelium dependent relaxation and nitric oxide production is impaired by the absence of caveolae. Hence, it was concluded that the disruptions in the caveolae may be the reason for many diseases such as essential hypertension. A study conducted by Marcella in 2019 showed that endothelium dependent relaxation that is induced by acetylcholine is reduced due to caveolae destructions. It was shown that in alpha androgen agonists that were used to treat spontaneously hypertensive rats caveolae like structures are decreased as compared to normotensive rats. Decrease in the density of caveolae in vessels is related to impairment in endothelium dependent relaxation that is induced by acetylcholine. The most important finding of this study was that the depletion of cholesterol due to endothelial nitric oxide synthase phosphorylation in the presence of tetrain greatly increases reactive oxygen species production in the vessels and normotensive rats that suggest endothelial nitric oxide synthase uncoupling. endothelial nitric oxide synthase mono reservation is inhibited in the presence of bh4 and L-arginine. This result shows that decrease in the number of Kerala is associated with several diseases and its disruption promotes endothelial nitric oxide synthase uncoupling. the presence of cav-1 is essential for the formation of caveolae structure. after the disruptions of caveolae the contractor contractility effect increases due to phenylephrine stimulation in the smooth muscles of aortas and in mesenteric arteries. A study used an aortic ring of male rat with intact endothelium suggest that contraction in smooth muscles are not modified by the presence of alpha adrenergic agonist, it suggests that in cholesterol rich domains alpha adrenergic agonist are not required for the presence of signaling molecules to cause contraction. In aorta and mesenteric arteries both the release of lower nitric oxide after treating it with dextrin is related to increased phenylephrine contractile effect that is promoted by dextrin. In smooth muscles the maximum relaxant effect that is induced by acetylcholine is related to the disruptions in the structure of caveolae. Text in reduced relax and effects of acetylcholine in vessels. Different studies reported that due to disruptions in the caveolae nitric oxide release decreased and relaxation induced by acetylcholine become impaired. So, it suggests that is structure and

integrity of caveolae is essential for the relaxation that is dependent on endothelium (Babiychuk et al., 2004).

Most of the signaling activities depend on the amount of cholesterol present in the plasma membrane. The cholesterol then combines with other proteins, receptors and ion channels and leads to the formation of lipid raft. The lipid raft formed is responsible for the coordination of signaling within the cells. The β_1 and β_2 adrenergic receptors are mostly present in the heart and they are responsible for producing a positive inotropic effect via increasing Ca^{2+} transient. β_1- adrenergic receptors are in both raft and non-raft portions of the plasma membrane, while most of the β_2-adrenergic receptors (having different components like G_s, G_i, Akt, protein kinase A, nitric oxide synthase, and adenylyl cyclase 5/6) of the heart are located in lipid rafts, primarily in caveolae. Furthermore, analysis of the structure of β_2-adrenergic receptor showed the presence of various binding sites for cholesterol that are responsible for regulating receptor dimerization. When MβCD or methyl-β-cyclodextrin treatment was given in order to make plasma membrane deficient of cholesterol, it disturbed and dislocated the lipid raft and signaling of β_2-receptor in heart muscle cells too. Following MβCD treatment for 1 hour, selective $\beta_2$adrenergic receptor had more substantial positive inotropic effect in the ventricular muscle cells in rat. This occurs as a result of up-regulation caused by signaling of cAMP-protein kinase A. Activation of β_2-adrenergic receptors in heart muscle cells treated with MβCD resulted in much longer cAMP signaling than the normal cells. Treatment with a lipid lowering agent decreased the cholesterol that then amplified the inotropic effect produced by the activation of β_2-receptor in rat myocytes, although the lipid lowering agents induce negative inotropic effect associated with increased nitric oxide output.

2.2 MβCD increases both Ca^{2+} transient and DAF-FM fluorescence but has little effect on the contractility.

Cholesterol deficiency had no discernible effect on the magnitude of atrial contractions. However, a substantial rise in Ca^{2+} transient and DAF-FM fluorescence was found using nitric oxide markers and Ca^{2+} fluorescent in response to cholesterol deficiency. Higher Ca^{2+} transient levels are responsible for amplification of atrial contractions, while nitric oxide generation can decrease it by reducing the sensitivity of Ca^{2+} of troponin complex. In order to improve the magnitude of Ca^{2+} transient and contraction to steady state levels and reduce DAF-FM fluorescence, pre-treatment with an inhibitor of nitric oxide synthase is required. It depicts that basal nitric oxide production is involved in the negative regulation of contraction and Ca^{2+} transient in the cytosol. Application of MβCD, after inhibition of nitric oxide synthase did not result in any improvement in DAF-FM fluorescence but did cause a rise in magnitude of contraction. In atria that were pre-exposed to L-NAME, the deficit cholesterol mediated amplification of Ca^{2+} transient was found to be identical to that of the control preparation. Stimulation of Gi-protein-Akt pathway may be responsible for the rise in nitric oxide production

following cholesterol deficiency. Pre-exposure with pertussis toxin and Akt ½ kinase inhibitor were used in order to investigate this possibility. In atria that were pre-exposed to pertussis toxin, the initial magnitude of contraction and Ca^{2+} transient did not change. Pre-treatment for 40 minutes with Akt inhibitor boosted contraction and Ca2+ transient to steady state values whereas the basal DAF-FM fluorescence reduced after exposure to Akt inhibitor. MβCD treatment when given during Gi-protein or Akt blockade did not rise the magnitude of contraction but it did increase the magnitude of Ca^{2+} transient and DAF-FM fluorescence to same extent as observed in the control sample. These results suggest that MβCD exposure increased the nitric oxide output in Gi-protein-Akt kinase independently, and that nitric oxide inhibited the positive inotropic effect of elevated Ca^{2+} transient (Odnoshivkina, Sytchev, & Petrov, 2017).

2.3 MβCD effects on the Ca^{2+} transient and DAF-FM fluorescence eliminates upon membranes' restoration with normal levels of cholesterol

Filipin III, a fluorescent antibiotic binds to the cholesterol that is present in the membrane. This antibiotic was used for measuring the amount of cholesterol in membrane. During control atria, the measured mean fluorescence of this antibiotic was 123 a.u. (representing 100 percent normal cholesterol amount). When comparison was done between control and treated preparations with MβCD, the filipin fluorescence was found to be decreasing by 12 % and 31 % in case of treatment with 1 mM and 5mM MβCD. The 5 mM MβCD-cholesterol can be used to preserve the original cholesterol levels in membrane. Filipin fluorescence was found to be identical to the control one, when atria were incubated with MβCD-cholesterol complex but after being exposed to 5 mM MβCD. However, in this case no notable effect was found on magnitude of contraction. The pre-exposure time with MβCD plays an important role in determining the magnitude of Ca^{2+} transient and DAF-FM fluorescence. Application of MβCD-cholesterol complex within 10 minutes of MβCD treatment reduced the magnitude of Ca^{2+} transient and DAF-FM fluorescence more rapidly to the pre-treatment baseline with MβCD i.e. within 15 minutes. This shows that cholesterol deficiency is associated with MβCD effects on both the Ca^{2+} transient and DAF-FM fluorescence.

2.4 Cholesterol deficiency reduces the positive inotropic effect of β2 adrenergic receptor agonists

β_2 adrenergic receptors are in the lipid rafts present in heart muscle cells. These receptors get disturbed and delocalised due to cholesterol depletion. After 15 minutes of application of 1mM MβCD, the double labeled immunofluorescent technique showed that localisation between β_2 adrenergic receptors and caveolin 3 was disturbed. The inotropic effect of β_2 adrenergic receptor agonist was evaluated in both control and MβCD treated cells in order to check the role of cholesterol. Use of β_2 receptor agonists at a concentration of 50μM greatly improved i.e magnitude of contractions. Blockade of β_2 receptor by an antagonist eliminated this effect, but

it was fully articulated when β1 receptor antagonists was present. The positive inotropic effect of β_2 receptor agonist was greatly lowered upon reduction in cholesterol levels. The observed rise in the magnitude of contraction was only 13-16% upon pre-treatment with MβCD. Experiments were done on MβCD-cholesterol in order to confirm the role of cholesterol. After application of 5mM MβCD, the inotropic effect of β_2 receptor agonist was considered to be partly recovered if the cholesterol in membrane was preserved by MβCD-cholesterol. The positive inotropic action of β2 receptor agonists was inhibited by Pre-treatment with MβCD.

2.5 Changes in the effect of β_2 receptor agonists on Ca^{2+} transient and DAF-FM fluorescence due to cholesterol depletion

Selective β_2 receptor agonists enhanced the Ca^{2+} transient by 57% ($p < 0.001$ and $n= 7$). Only cholesterol depletion by treatment with 5mM MβCD decreased the selective β_2 receptor agonists' induced amplification of Ca^{2+} transient (only 13% enhancement, $p < 0.01$ and $n= 8$). In this way, when 1 mM MβCD was used for cholesterol depletion, no change was observed in the activity of β_2 receptor agonists on Ca^{2+} transient. These findings show that slit cholesterol depletion inhibit the β_2 receptor agonists induced inotropic effect but independent of Ca^{2+}, while mild to moderate cholesterol depletions could produce the effect partially by decreasing the effect of β_2 receptor agonists on Ca^{2+} transient. Moreover, β_2 receptor agonists are found to be increasing the production of nitric oxide in the atria of mice at lower concentrations of 5 μM. During control conditions and administration of 50 μM, no remarkable change was seen in DAFFM fluorescence. Different effects produced at these two concentrations on nitric oxide may be associated with concentration dependent signaling of β_2 receptors. DAF-FM fluorescence is mainly used for the detection and measurement of nitric oxide therefore, when 50 μM β_2 receptor agonists were applied after cholesterol depletion it leads to the elevation of DAF-FM fluorescence. This is actually indicative of increased nitric oxide production. This study shows that deficit cholesterol decreases the inotropic effect of the β_2 receptor agonists via up regulation of β_2 receptor or nitric oxide signaling. Cholesterol depletion, rather than down regulating changes the β_2 adrenergic signaling. Atrial caveolae plays a significant role in tuning of β_2 adrenergic signaling in order to produce greatest positive inotropic effect. Cholesterol deficiency also disturbs and dislocates these caveolae in the atrial membrane.

2.6 Role of cholesterol depletion in suppressing inotropic effect induced by adrenergic receptor agonist via decreasing the β_2 receptor mediated components

Binding of sympathomimetic drugs or adrenoceptors agonist to the adrenergic receptors resulted in a positive inotropic effect. The magnitude of force of contraction potentiated to 257± 8% in case of the agonist binding while binding of an antagonist to the β_2- adrenergic receptor produced a partial inhibitory response. In case of beta blockers or selective β_2-adrenegric receptors antagonist, the magnitude of force of contraction potentiated to 191±7 % only. This was also investigated by making a comparative study between the positive inotropic effect

produced by adrenoceptors agonists in control and β_2 receptor antagonists used for treating atria. Use of 5 mM MβCD to make cells cholesterol deficit also remarkably reduced the inotropic effect of adrenergic receptor agonists. However, in atria treated with β_2 receptor antagonists' the cholesterol changes had no significant effect on the inotropic effect driven by adrenergic receptor agonists. This data suggests that cholesterol depletion plays a significant role in suppressing sympathomimetics induced enhancement of force of contraction by reducing the inotropic effect of activated β_2 receptor.

Jorgen Vinten in year 2002 investigated how cholesterol supplemented fractions of the plasma membrane are responsible in controlling the contraction of arteries. For this purpose, tail of the rat was used in order to observe the contractions in that specific part of the rat. The study made use of serotonin receptors, α_{1A}-selective agonists, anti-diuretic hormones, and higher K^+ concentrations in order to produce contractions in the denuded rings of vascular endothelium in artery of the rat's tail. The resultant effects were close to the agonist's maximum effects produced at higher concentration. Moreover, the time duration of effects produced was also 3 hours. Considering these two parameters, the effects produced were taken as control. In order to decrease the concentration of cholesterol, the preparations after half an hour were treated with MβCD for a time period of 60 minutes. Following this treatment, the contractions were again recorded and data of 8 experiments was analysed. The analysis showed that MβCD treatment was primarily responsible for decreasing the effect of serotonin receptors, ADH, and Endothelin-1.

A relationship was developed between the control and MβCD treated preparations in the form of cumulative dose-response curve. After decreasing the cholesterol concentration, the doseresponse curve for serotonin receptors was moved tenfold to the right. However, at saturated concentrations of serotonin receptors the force was decreased by half. All these effects were seen in case of serotonin receptors only; none of these responses were noted with α_{1A}-receptor agonist or ALF^{4-}. It was found that after the re-introduction of cholesterol into the MβCD- complex, serotonin receptors effect was gained again. The respective effect of the receptor was not regained promptly, as the preparations continued to impair due to cholesterol deficiency. After regaining of effects, it was observed that the force sustained for one more contraction without supplementing cholesterol continuously. Fura 2 technique was utilised in order to quantify calcium present in the cells. In case of cholesterol deficient preparations, the basal intracellular calcium was found to be substantially high. This was accompanied by an increased basal vascular tone that depends upon calcium. In this case, L-type calcium channel blockers, non-selective cation channels blockers, and store operated calcium channel blockers were found to be unaffected. As the rate of reduction of intracellular calcium ion (due to increased K+ concentration leading to repolarization) in calcium-free solution was found to be

similar to that of the MβCD-treated arteries and control arteries, this shows that calcium extrusion processes were not influenced by the respective treatment.

It was found that during cholesterol-depletion, treatment with α_{1A}-adrenergic receptor agonist and higher K^+ concentrations did not affect the mean intracellular Ca^+ levels; however, response to serotonin receptors was significantly reduced. Contraction due to serotonin receptor depends only on the calcium residing outside the cell and voltage gated L-type calcium channels. Moreover, in order to investigate deficient-cholesterol effect on the release of calcium from intracellular stores experiments were performed extracellular solution that were calcium free. It was found that release of calcium potentiated in response to α_1-receptor activation, which was represented as baseline increase. Moreover, it was found that increase in intracellular calcium due to caffeine was substantially greater in cholesterol-deficient preparations. These findings show that cholesterol-deficient preparations have more calcium loading of sarcoplasmic reticulum. Following cholesterol extraction, calcium release due to serotonin receptor remains unaffected. In order to rule out the possibility that higher intracellular Ca^+ stores would mask the action of MβCD on effects produced by α_1-adrenergic, tissues were treated with caffeine and non-competitive inhibitor of sarcoplasmic reticulum calcium ATPase to reduce calcium from sarcoplasmic reticulum. However, in the presence of calcium residing outside the cells, no difference between control and MβCD-treated preparations was found in response to the effect of selective α_1-receptor. When an agonist is activated, every smooth muscle cell in the walls of arteries exhibit asynchronous calcium oscillations. These oscillations enable spatial and temporal coding of calcium present inside the cells and help in force regulation. The intracellular calcium signal that is estimated from the vessel wall depends upon the frequency, amplitude and portion of the cells oscillating and interweave calcium present inside the cells. A laser confocal scanning microscopic technique was used to measure calcium wave response. In this technique, fluo-4-MβCD-treated and control arteries were used. Fluo-4 is a fluorescent indicator for calcium; it is often used to calculate the calcium present inside the cells. Cells displaying waves and activated by α_{1A}-receptor agonist, serotonin receptor were least affected by the MβCD treatment. However, with serotonin receptor stimulation, the average frequency of the wave was lowered by 37% in treated vessels and with α_{1A}-receptor agonist, it was found to be slightly lowered.by 14%. When the amplitude of the wave was compared, it reduced by 23% with serotonin whereas no effect was seen with α_{1A}-receptor agonist. It is indicated from this that following MβCD therapy, the potential for production of calcium wave is highly retained. And the reduction in response during serotonin receptor activation is mainly due to upstream fault (Dreja et al., 2002).

When the tail artery was viewed under light microscope, the images were found normal. Same was found following cholesterol exclusion and force registration. Caveolin-2, Caveolin-1, cavp60 are the integral protein components of the caveolae membrane. They produced

immunofluorescence signals along the surface of smooth muscle cells. Although, caveolin-2 was weakly marked but similar distribution was found for it. Caveolin-3 was not found. So it was found that cholesterol extraction ad no discernible impact on the allocation and distribution of the following proteins.

Electron microscopic technique was also used to assure that no harm to the tissues occurred during force application. In this, luminal surface was excluded as its endothelium was removed. It was found that in preparations that were not depleted of cholesterol, the smooth muscles of the plasma membrane consisted of several caveolae in aggregated form. However, cholesterol depletion over 60 minutes led to overall modification in the appearance of caveolae. The caveolae either faded (flat) or appeared shallow having large openings. Other than caveolae, Golgi apparatus was also affected having disordered arrangement. Cholesterol depletion for an hour did not cause any conventional signs of tissue damage. When cholesterol was reintroduced in the cholesterol-depleted preparations, caveolae structure and quantity were found to be similar to muscle cells of the control samples. It was found in western blot experimentation, that the cholesterol depletion had no effect on the serotonin receptor-2A and caveolin-1 levels. The protein arrangement of both the depleted and control were found to be identical in silver-stained gels. When sub-cellular fractionation via sucrose density gradient was done, serotonin receptor-2A co-fractionated with caveolin-1 and cav-p60 was found in fractions 5 and 6. While selective α_{1A}- receptors were found in fractions 9 and 10.

Chapter 3

Materials and Methods

All the experimental procedures were performed according to the guidelines of laboratory of the university of Nottingham.

3.1 Tissue Preparation

Porcine spleens were kept in ice and delivered to the laboratory after two hours of slaughter. Porcine spleen was divided into three groups. One group was treated as control, one group treated with 0.7mM MβCD and the third group treated with 3mM MβCD. MβCD dose was determined by the cholesterol lowering action study of MβCD in guinea pigs. The porcine isolated splenic arteries were kept in temperature-controlled environment during the whole study (in the preparation Lab). Tissues were placed on a clean dissection tray containing KrebsHenseleit solution (K-H solution) using dissection tools rough dissection was carried out by dissecting the dorsal branch of the porcine splenic artery. Segments were dissected into 4 to 5mm ring portions. Krebs-Henseleit buffer containing 2% ficoll and pen/strep was transferred into two sperate 20ml vials and gassed with carbogen 95% O_2/5% CO_2. The solution was stored overnight at 4°C and 37°C. This process was repeated for control and treated (0.7mM and 3mM MβCD) segments.

3.2 Preparation of Krebs-Henseleit Buffer Solution:

Hens Krebs and Kurt Henseleit first introduced the buffer solution called Krebs-Henseleit buffer solution. The ingredients of this solution are sodium, potassium, bicarbonate, calcium, chloride, glucose, albumin and phosphate. It is now used in a variety of ways for example in experimental purposes like ex vivo techniques (to study arteries). Glucose energy is useful for continuity of stored cells. Krebs solution is used only for researching and development purpose. For sterilisation of this solution, supplements are added before filtration or instigate aseptically. The powder contents are added during stirring of solution. Continue stirring the solution until whole powder content dissolved. Add this solution in above solution. Now add 0.373 g of calcium chloride di-hydrate in 1 liter solution and stir it properly. After this add 2.1 g sodium bicarbonate. Instead of sodium bicarbonate 28 ml of sodium bicarbonate solution (7.5% w/v) can be added. PH adjustment needed during stirring of the solution in the range of 0.1-0.3 pH as it increases during filtration. The addition of 1N HCL or 1N NaOH is useful. Make up the final volume with water. Sterilization is achieved by sterilization with membrane technique and this membrane has a porosity of 0.22 microns. With proper aseptic technique add the solution into aseptic container. In glass vial with solution add measured 2g ficoll. The function of ficoll is to maintain freshness of splenic artery. Addition of 200ul of modified pen/strep being made for prevention of growth of bacteria. The solution required to be gassed for 20 minutes. The 95%

oxygen and 5% carbon dioxide are ingredients of the gas. As the dry powder salts are hygroscopic in nature store them under dry conditions. Store liquid solution in dark with a temperature range of 2-8 °C. Any disturbance in the powder medium can be checked by

1. Visual appearance as colour change may occur.
2. Granulation/Clumping
3. Insolubility

Any disturbance in the liquid medium can be checked by

1. pH change
2. Precipitate or Particulates
3. Cloudy Appearance
4. Colour Change.
5. Supplements have impact on storage condition and shelf life of medium (Krebs & Henseleit, 1932).

3.3 Isometric tension recordings:

After an overnight exposure and incubation of control and treated segments, contractile examination of was carried out using isometric tension recording (organ bath) These ring segments were hooked to a thread, mounting hook and in 20ml organ water-jacket containing Krebs-Henseleit solution. The gassing process is continued side by side. The temperature was maintained at 37°C. By using amplifier, the upper part attached to a force transducer and lower part is made secured. Wait for 20 min called equilibration period. The tissues were subjected to stress with follow up of resting stress in a range of $2–3\ g\ wt^{-1}$ for (splenic artery). In order to achieve ultimate two responses, diverge below 10% the tissues were subjected to contraction after 2-3 times exposure with 60 mM KCl before initiation of procedure. Tissues were washed at least 3 times after each drug application with Krebs-Henseleit buffer. Tissue was left for 20 min subjected to recovery period. Sometimes fine pair of forceps used to remove endothelium from blood vessels parts. This is achieved by rubbing luminal surface with forceps. After removal of endothelium the parts were placed in organ bath. In order to check either endothelium is removed or not the tissues were presented to 0.1 μM substance P and inability of the tissues to relax is observed. Segments with endothelium and without endothelium were compared (Robert et al., 1999).

3.4 Organ bath:

For physiological and pharmacological investigation of in vitro tissue organ bath is used widely. It is proved to be beneficial for maintenance of perfused tissues for long duration. The procedure will start right after equilibration period by researcher. Researchers perform experiment in two ways either by adding drugs to the organ bath or direct stimulation of the tissue. The tissues react in two ways. They may contract or relax. Isometric transducer is used for measurement of force. Isotonic transducer is used for measurement of displacement. Dose response curves were plotted (tissue response against drug dosage or stimulus potency) after completion of experiment. Then petri-dish were taken along with Krebs's solution and tissues were made in it. The edges of tissues fixed to mounting hook and transducer with the help of silk. For proper mounting of tissue, silk or fine wire is used. A pin may be used to pin the tissue for preparation of the arterial rings. determination of results in form of graph Kaleidagraph Synergy Software is widely used. Firstly, the data obtained in mechanical form then this software is used for conversion of this data into graphs. Isometric transducer is used to measure change in force due to muscle relaxation/contraction. Muscle length remains the same (*Organ Bath Systems*, n.d.).

3.5 Isotonic Transducer:

For proper investigation of isotonic contractions of separated organs and tissues low inertia, high-performance isotonic transducers are fit for use. Diminution (contraction) and Propagation (elongation) is used for the formation of mechanical activity. The transducer would be isometric or isotonic it all depends upon the ration between elongation and contraction. Some tissues exhibit large displacement and a short contraction force. Some tissues exhibit short displacement and large contraction force (Figure 7 and Figure 8).

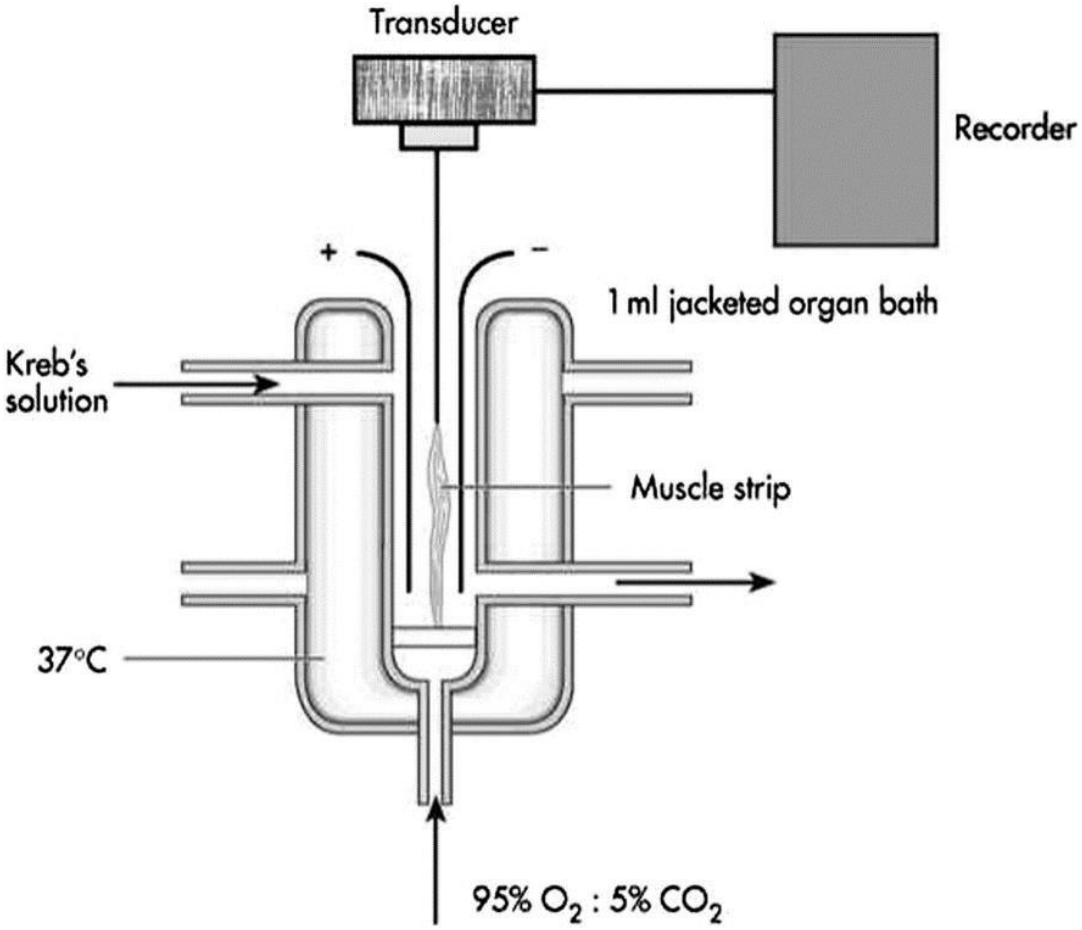


Figure 7. Diagrammatic representation of organ bath in which integrity of the tissue is maintained using a control environment to keep tissue viable through the procedure (Meiss, 1974)

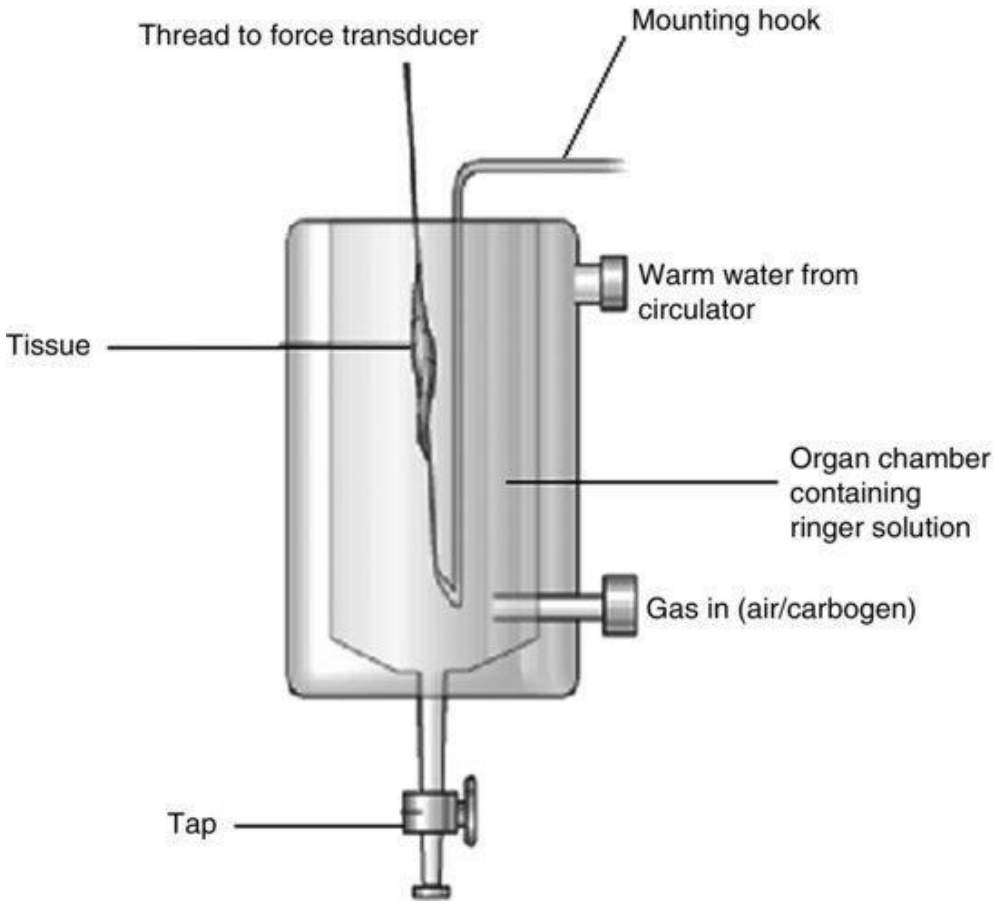


Figure 8. Diagrammatic representation of isotonic transducer in organ bath, it is used to investigate isotonic contractions in isolated organs particularly smooth muscle (Organbath pdf)

3.6Transmission Electron Microscopy

Transmission electron microscopy (TEM) has a vital role in biological and medical fields. TEM utilises electrons as elimination source, whereas light microscopy (LM) uses light as elimination source. The principle of TEM is remarkably like light microscopy; however, specimens are prepared into ultrathin sections which enables the elimination of electron to highly magnified images. Specimens can be magnified up to 2 million times, this allows researchers to visualise structures of interest. TEM utilises high voltage electron beam, an electron gun which emits electrons that travel through a vacuum tube, an electromagnetic lens and fluorescent screen found at the bottom of the microscope.

Specimens must be stained, dehydrated, embedded in raisin, and sliced using an ultramicrotome (diamond knife). Sections must be ultrathin which enables electron to emit through the specimens. A circular grid mesh may be used to secure the specimen, which is inserted into the TEM specimen holder. An electron beam is generated using high voltage and shot out of the electron gun. The electron beam is transmitted down the anode, the beam passes through the specimen and objective lens magnifies the image. Electrons are projected onto fluorescent screen which allows a camera attached to capture images which are used to visualised using computer screen. Images can be analysed directly from the computer or saved for later use. TEM has the capability to visualise small, detailed structures such as proteins, it creates flat images which can be used in stereological analysis and it has better resolution as compared to Scanning electron microscopy (SEM) (CCBER, 2007-11).

Scanning electron microscopy utilises focused gallium ion beam combined with electron microscope used to visualise sample surface. For image of milled surfaces Backscattered electron detector is used to generate an immense series of images. The images produced by Backscattered electron Detector were compiled into a 3D rendered image of sustained and embedded biological tissue. SEM generates 3D images, poorer resolution and only used in visualising the surface of specimen (Bushby et al., 2011).

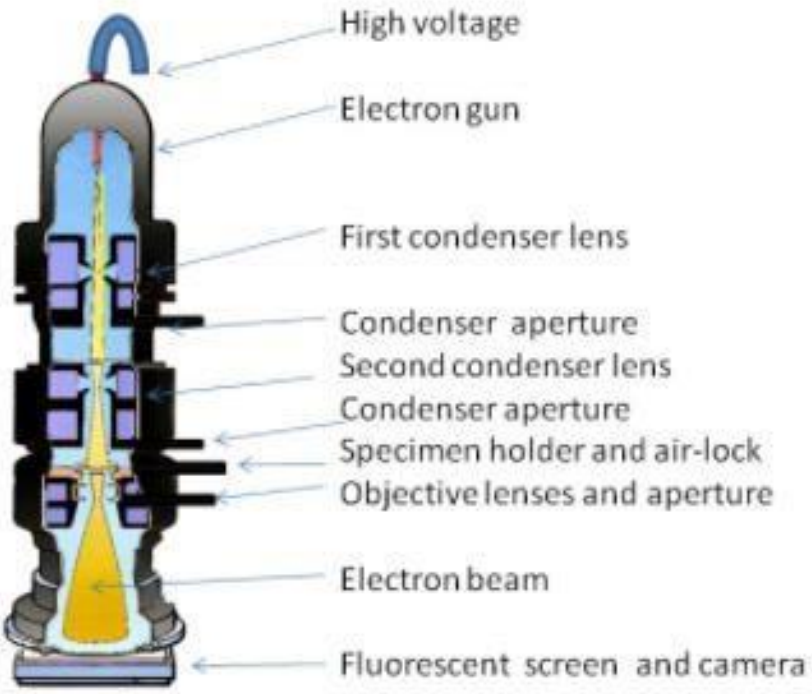


Figure 9. Diagrammatic representation of transmission electron microscopy (CCBER, 2007-11)

3.6.1 Solutions:

The buffer solution is a medium for fixative and has certain properties:

1. In the time of fixation buffer solution maintain the pH and make it constant. In EM the pH of fixative is maintained in the range of 6.5-8.0 by the use of buffer system.

2. The ionic constituent in buffer system prevents the extraction of cellular components or it is also helpful for the prevention of deposition of precipitates of fixative chemicals.(*Chemicals for Microscopy*, 2021).

0.2M Cacodylate buffer, Distilled water, 25% EM Glutaraldehyde, 3% Glutaraldehyde in 0.1M Cacodylate buffer (Electron microscopy fixative), 1% Osmium tetroxide, 0.1M Phosphate/Cacodylate wash buffer, 3% Agarose (Low gelling|), TEM Resin, Grades of ethanol and Propylene oxide.

Sodium cacodylate tri-hydrate is used for the preparation of 0.2 % Cacodylate buffer. Sodium cacodylate trihydrate is the most used buffer solution. For electron microscopy sodium cacodylate tri-hydrate is used with fixatives for specimen preparation. Sodium cacodylate trihydrate is also called cacodylate or cacodylic acid. On thin sections cacodylic acid is used to avoid the microprecipitation. If the specimen is not well rinsed between pre- and post-fixation micro precipitation happened with phosphate buffers. For Electron Microscopy 25% EM Glutaraldehyde is the Universal Fixative. When glutaraldehyde is purified to EM grade care must be needed if EM grade not handled properly, it has a high risk of polymerizing. In order to obtain polymer-free, pure glutaraldehyde special precautions are taken by Electron Microscopy Sciences. Consistent purity and the highest attainable quality is assured when EM grade aqueous glutaraldehyde is assayed. On proper storage glutaraldehyde exhibit a long shelf life. Osmium is referred by osmium tetra oxide. It is mainly used as a secondary fixative of tissues. The molecules interact with lipid molecules. During interaction when lipid reacts with molecules it results in the oxidation of unsaturated bonds of fatty acids and this reaction build on density and contrast to biological samples. Black staining observes due to presence of osmium in normal tissues. Black staining can be visualised by normal eye. In Electron microscope it is also used as mordant and increase lead staining. Mordant means (a molecule which mingle with stain or dye at later on stages) .When equal volumes of 0.2 M Cacodylate stock solution and distilled water (1:1 dilution) is mixed well it results in the preparation of 0.1M Phosphate/ Cacodylate Wash Buffer.(Morris, 1965).

3.6.2 Primary Fixation (3% Glutaraldehyde in 0.1M Cacodylate Buffer):

For the preparation of EM fixative 25 ml 0.2M Cacodylate buffer was transferred into a tube which has already (600ul) 25% EM Glutaraldehyde and stored at 4^0C. 0.2M Cacodylate available as pre-made (2.5ml) containing 1.9ml of distilled water. Samples that were treated overnight

transferred into epindoff tube. After this 1ml of the fixative solution was transferred into epindoff tube. These tubes were stored at 4^0C.These tubes were stored in fridge in order to fix the tissues. In order to obtained reduced physiological change to sample the temperature (room temperature) was maintained the same for sample as well as for fixative (when fixative was used). Preservation of fine structures of the cells is achieved by the fixation of the tissues.

3.6.3 Post Fixing of Samples:

Samples were moved into new glass vials for 1 hour. These glass vials already contain 1% osmium tetroxide. When sample transferred the colour of it turned black. After 1 hour the samples were washed two times with the help of distilled water.

Dehydration: Different concentrations of ethanol were made and then samples emerged in these concentrations respectively:

1. 50% ethanol for 10 minutes twice
2. 70% ethanol for 10 minutes twice
3. 90% ethanol 10 minutes twice
4. 100% ethanol for 15 minutes twice thrice (Vari-Speed Rotator is used for agitation of samples on each ethanol dose throughout the time)
5. 100% propylene oxide (volatile) is used for washing of samples for round about 15 minutes twice.

3.6.4 Infiltration of Samples:

After dehydration, samples were placed with resin-propylene oxide mixture for 1 hour. Reinfiltration of samples were achieved with the use of 1:1 resin-propylene oxide mixture for 24hrs but with the lids of the bottle of sample open in a fume hood. After 24 hours with the use of fresh bottles samples were invade with 100% resin for round about 1 hour.

3.6.5 Embedding of Samples:

A mold was used to shape the samples. Sample labels were printed and placed in the well of the mold, 100% resin was transferred into wells of. In order to correct the orientation of sample, the samples were placed out in the center of the mold under the microscope. For polymerization rubber mold was placed in an incubator at 60^0C for approximately 48 hours.

3.6.6 Sample Cutting for TEM Sectioning:

With the use of sharp glass knife blocks were prepared. The knife blocks were prepared for TEM processing. For trimming of samples, the glass knife was used. Reichert Jung ultra-microtome (LEICA EMUC6) was used for the insertion of resin block and tightened securely. For trimming of sample block razor was used. The sharp edge of the knife was centered in order to align the

knife and sample block. They both aligned so that they were equidistant and parallel. The samples were cut into sections of 0.5 um (500nm). The sections were collected in a boat filled with water for injection. The portions having endothelium and smooth muscle only was shaped down into trapezoidal area. Samples collected in the boat stretched with the help of heating pen. Stretching of the sample was done to strengthen the sample in order to avoid folding.

3.6.7 Preparation of Semi-Thin Slides for Electron Microscopy:

Three samples were placed on the APES (3-Amino Propyl Triethoxy Saline) slide having already injection sterile water. The slide along with sample was placed on hot plate (100^0C). On hot plate slide was left for the water to evaporate. When all the water has evaporated, drops of toluidine blue (1%) in PBS (Phosphate Buffer Sodium) borax for epoxy resin was transferred on the slide. This step was done in order to stain the sample. Then slide again placed on the hot plate till the time golden crust forms appear. The slide was washed with distilled water once the golden crust has formed. DPX was placed on 3-Amino Propyl Triethoxy Saline slide and covered with slide cove r(22 x 50mm, No. 1.5).The samples was thoroughly examined under microscope in order to analyses any cutting problem. White patches should not be present.

3.6.8 Preparation of Ultra-Thin Slides for Light Microscopy:

Ultra-thin sections of size (70-90nm) were cut with the use of semi-thin sections as a guide. The ultra-microtone was put to cut between the size range of 80-90nm.When sections were cut they looked gold-silver in color. Any purple or multi-colored sections were thrown away. Water for injection was used for the collection of samples. Sections were set apart with the use of paintbrush. The sections were flattened using a hot pen. Sections were placed on the copper grids (Agar Scientific (02410C). Hexagon (100 mesh) and copper (3.05) are parts of copper grids. Copper rids made up of segments were placed in labeled petri dish. Petri dish then stored for staining.

3.7 Electron Microscopy Imagining:

TEM was used for analyses of sample obtained through histological process. With the use of high-power magnification (43,000x) images of samples were obtained. Random selection of sections containing caveolae was selected and images were compiled and saved into a folder. Firstly, low power magnification was used to identify the tunica media. Tunica media is a part where caveolae can be present. The magnification was gradually increased to 43,0000x. 17 random images were taken using hexagon. For volume fraction investigation these pictures were reserved (Dubes et al., 2003).

3.7.1 Fractional Volume of caveolae within VSMC (TEM):

The cytoplasm of an average smooth muscle cells contains number of caveolae which are examined by two different methods after the treatment with two different potencies of methylβCD (0.75mM and 3mM). The small and cup shaped invaginations are present of

caveolae in the membrane of vascular smooth muscle cells. Caveolae has small size and the structure of caveolae can be visualized by transmission electron microscopy (TEM) (Richter et al., 2008).

The images of structural estimation of membrane caveolae obtained from TEM are quantitatively analysed by stereological study. Stereology is three-dimensional determination of two-dimensional images of vascular smooth muscle cells obtained from TEM.

There are two most used analysis method when quantifying caveolae. The following measurements were taken from Electron Microscopy Images (43,000x) magnification:

- Points on caveolae
- points on smooth muscle cytoplasm

The following Measurements were taken from Light Microscopy images taken at 50 x magnification.

- Mean Nuclear volume by measuring the width and length.

Mean Volume Nucleus

A picture was viewed as an image and a lattice was placed over the picture. The number of points fall on nucleus was counted (Vn) and the number of points on smooth muscle cytoplasm was counted (Vcell), which was summed over all electron microscopy images from each animal.

$$Nuclear\ Volume\ Fraction = \frac{Vn}{Vcell}$$

Mean Volume Fraction

To estimate mean volume of cytoplasm, this was done using the images from the light electron microscopy. Image was viewed and lattice was placed on a computer screen, point falling on nuclei and points falling on smooth muscle cytoplasm (SMCs) were counted. This information was collected over 10 images,so the fractional volume of SMC occupied by nuclei was calculated.

$$Mean\ Fractional\ Volume\ of\ SMC = \frac{Vnuc}{Vsmc}$$

Mean Volume Cell

Image J was used to view images collected using light electron microscopy. 20 random length and diameter of nucleus from control (10 samples) 3mM (10 samples) and 0.75mM MβCD (10 samples) were measured. The average of each 10 samples was collected and the mean apparent diameters were corrected for the effect of sectioning.

$$Estimated\ Nuclear\ Volume = (L\ x\ \pi\ x\ D\ x\ D)/4$$

Mean Nuclear Volume

To work out the mean nuclear volume of cell, the equation of cylinder was used because the nucleus was considered as sausage shaped. The corrected diameter was used to calculate radius.

$$Mean\ Nuclear\ Volume = \pi\ x\ r2\ x\ h$$

The value obtained from the estimated nuclear volume and mean nuclear volumes were used to convert fractional volumes into total volumes (μm^3) of the average caveolae within.

$$Mean\ SMC\ Volume = Mean\ volume\ cell\ x\ 1\ /mean\ volume\ fraction$$

Mean Volume Cytoplasm

Mean volume cytoplasm was calculated by taking away mean smc volume from mean nuclear volume.

$SMC\ Cytoplasm = mean\ nuclear\ colume -$ mean smc volume

Caveolar Volume Fraction

To work out the volume of caveolae per cell, this was done by using images collected from transmission electron microscopy (43000 magnification). A total of 17 images from each sample was collected. Each image was viewed on a computer screen, a lattice was placed and points

falling on caveolae ($Vcav$) and points falling outside the smooth muscle cytoplasm ($Vsmc$) were counted.

$$Estimated\ Caveolae\ Volume = \frac{Vcav}{\quad} Vsmc$$

Volume Per Cell of Caveolae and Mean Diameter of Caveolae

The volume of caveolae per cell was calculated using the following equation:

$$Volume\ of\ Caveolae\ per\ Cell = smc\ cytoplasm\ x\ estimated\ caveolae\ volume$$

The mean diameter of caveolae was calculated by changing the unit in within the FIJI (Image J) software. 10 random caveolae from control and treated segments were measured.

3.8 Drugs

In our study, the drugs involved in determination of contractile response of Mβ-CD treated vascular smooth muscle cells of porcine isolated splenic artery includes KCl, U46619, Noradrenaline, Phenylephrine, carbachol, isoprenaline, 5-hydroxytryptamine and stable prostaglandin endoperoxide. All these drugs are vasoconstrictor in nature acting at different receptors.

3.8.1 Pharmacological activity of vasoconstrictor agonists on the ơ-adeno receptors: Selective agonist that includes phenylephrine and noradrenaline that mediate contractile response in strips of splenic artery from the pig. No-adrenaline is ơ-1 selective agonist and phenylephrine induces contractions in splenic artery preparations at different potencies. The GPCRs are membrane bound protein receptors involved in signal transduction pathways of different extracellular signals or drugs. Nor-adrenaline receptors are part of GPCRs that are associated with caveolae.

3.8.2 Pharmacological activity of vasoconstrictor agonist on 5-HT_2A receptor: Serotonin acting on 5-HT_2A receptor produce contraction after the washout period of the first contractile response. 5-HT receptors belong to family of G-protein coupled receptors. The KCl vasoconstrictor effect on Mβ-CD treated splenic artery remain unaltered. Incubation of vascular smooth muscle cells of porcine isolated splenic artery with Mβ-CD results in altered contractile response of agonist acting on receptors other than 5-HT receptors (Keshavarz et al., 2017). The pre-constricted porcine isolated splenic arteries with KCl show normal contractile response to 5-hydroxytryptamine. The effect of thromboxane mimetic U44619 on 5-HT receptor was

examined which had maximum effect on 5-HT_1A induced contractile response of porcine isolated splenic artery (MacLennan & Martin, 1992).

3.9 Statistical analysis

Statistically significant difference in contractile response of porcine isolated splenic artery previously treated with MβCD to different drugs was determined by one-way analysis of variance (ANOVA) with post hoc Dunnett's test and student's t-test. Post hoc Dunnett's test was used to compare the treatment group with the control group. Statistically significant results were obtained when probability value of <0.05. ANOVA with post hoc Dunnett's test expressed the difference of vasoconstrictor agonist action on three different groups i.e. Control group, 3mM Mβ-CD and 0.75mM Mβ-CD. Contractile responses of Mβ-CD treated porcine isolated splenic artery was expressed as $PD_2 \pm SEM$.

3.10 Results

Table 2: The effect of cholesterol depletion using MβCD on contraction-induced by different G-coupled receptor agonists. Cells were incubated overnight treated with MβCD at 37°C and then contracted with agonist using the organ bath.

	Agonist (pD₂ ±SEM)						
Treatment	KCl	U44619	Noradrenaline	Phenylephrine	Carbachol	5-HT	Isoprenaline
Control	1±0.027	-7±0.063	-6 ±0.087	-5 ±0.074	-5±0.053	-5±0.104	-4 ±0.522
3mM MβCD	1±0.039	-7.2 ±0.092	-6.5±0.116	-7 ±0.092	-6 ±0.045	-4±0.211	-4 ±0.621
0.75mM MβCD	1±0.080	-7 ±0.085	-5 ±0.109	-6±0.121			

3.10.1 Pre-constriction of porcine isolated splenic artery incubated overnight:

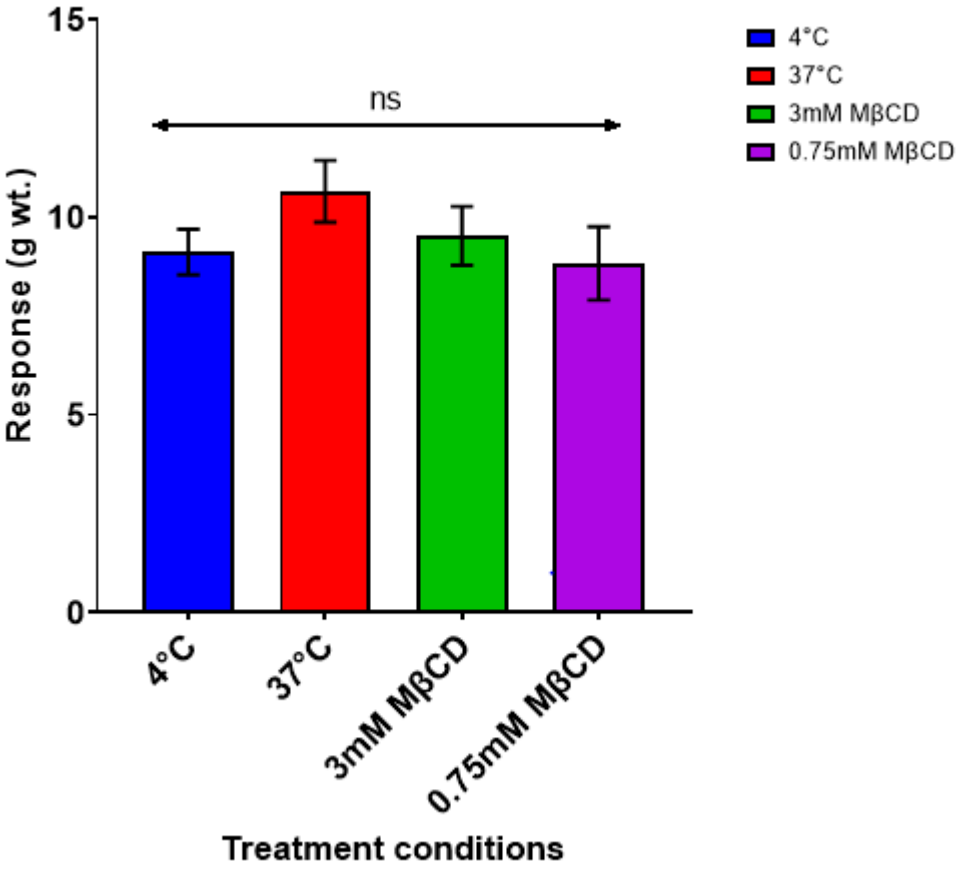


Figure 10. The effect of storage overnight in 4˚C and 37˚C in Krebs-Henseleit solution (with antibiotics) on contractions of the porcine isolated splenic artery elicited by 60mM KCl (n=36) (lower). The effect of overnight storage in Krebs-Henseleit solution with and without 3nM and 0.75mM MβCD on contraction of porcine of porcine isolated splenic artery elicited by 60mM KCl (n=36). ns= $p<0.05$, no significant difference.

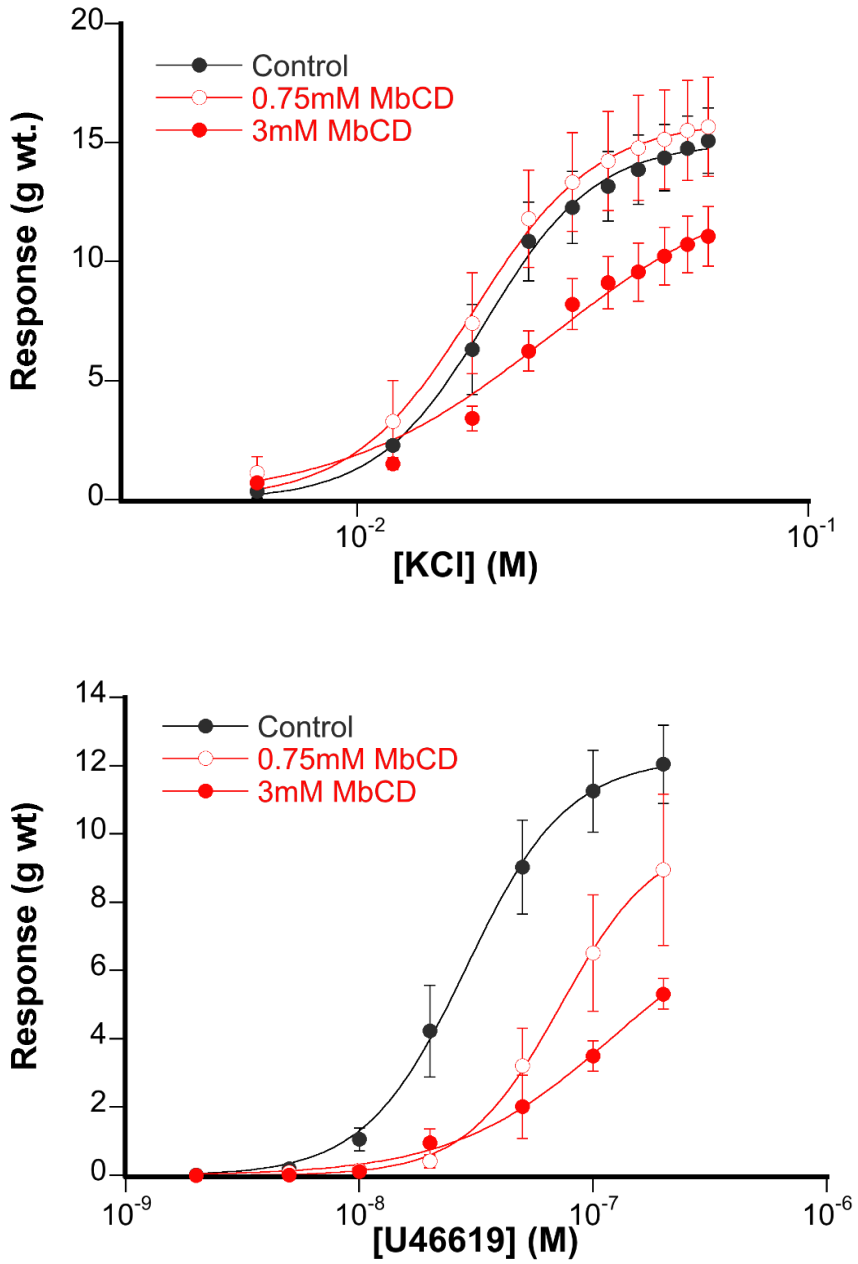


Figure 11. The effect of overnight storage with 0.75mM and 3mM MβCD on contractions of the porcine isolated splenic artery elicited by KCl (n=6). (Below) The effect of overnight storage with 0.75mM and 3mM MβCD on contractions of the porcine isolated splenic artery elicited by U46619 (n=6-9). Error bars represents SEM.

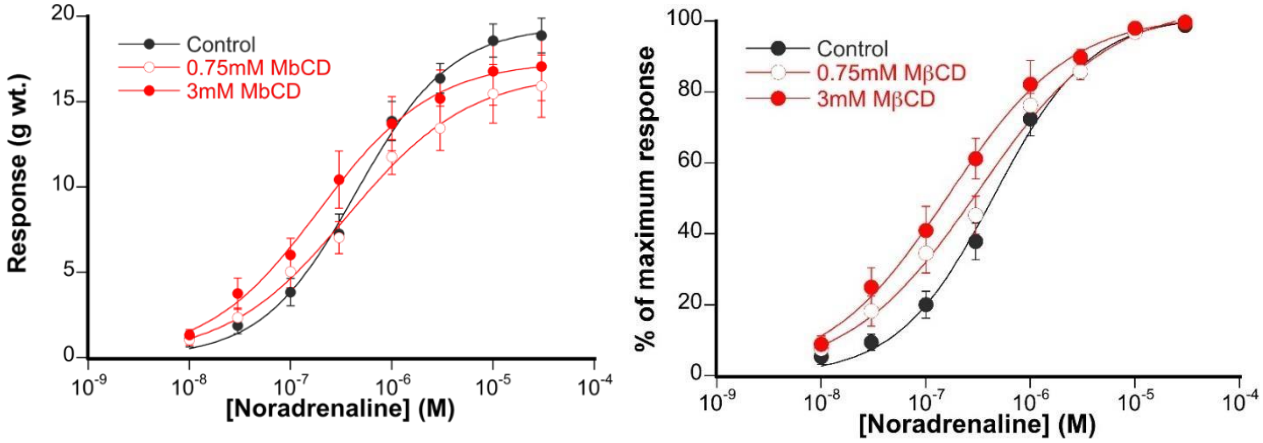


Figure 12. The effect of overnight storage with 0.75mM and 3mM MβCD on contraction of the porcine isolated splenic artery elicited by noradrenaline (n=12). Response expressed as force g.wt (left) and as a percentage of the maximum response (right).

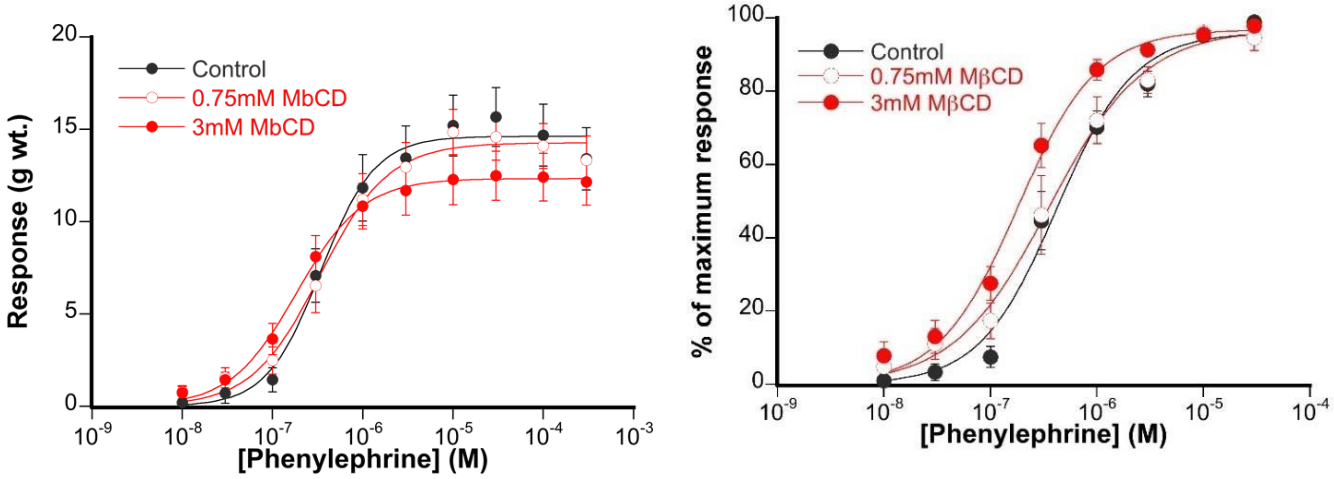


Figure 13. The effect of overnight storage with 0.75mM and 3mM MβCD on contractions of the porcine isolated splenic artery elicited by phenylephrine (n=12). Response expressed as force g.wt (left) and as a percentage of the maximum response (right).

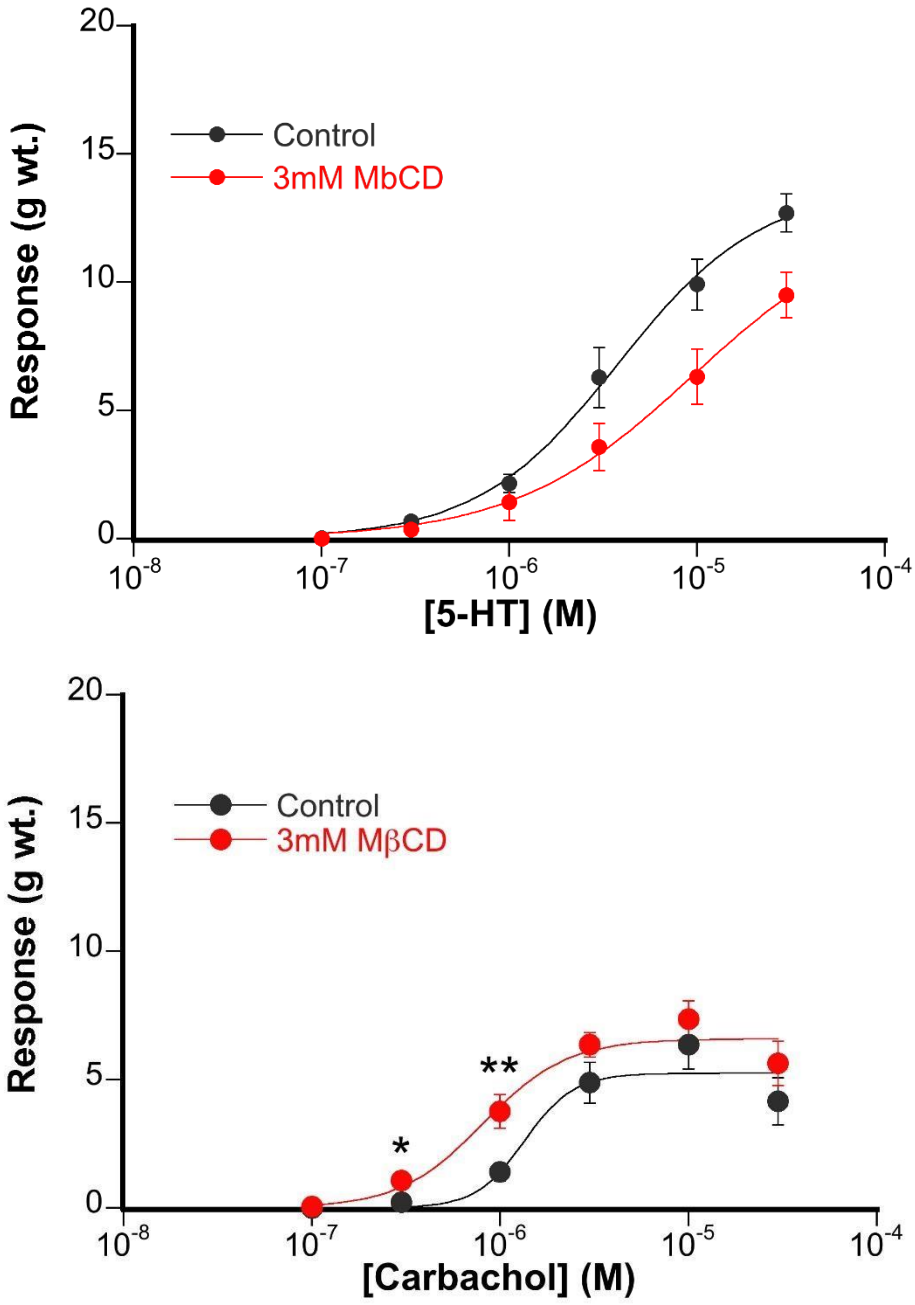


Figure 14. (Above) The effect of overnight storage with 3mM MβCD on contractions of the porcine isolated splenic artery elicited by 5-HT (n=10). (Below) The effect of overnight storage with 3mM MβCD on contraction of the porcine isolated splenic artery elicited by carbochol (n-11) * ($p<0.01$) donates a statistically significant difference (paired student's t-test) in response between control and treated group.

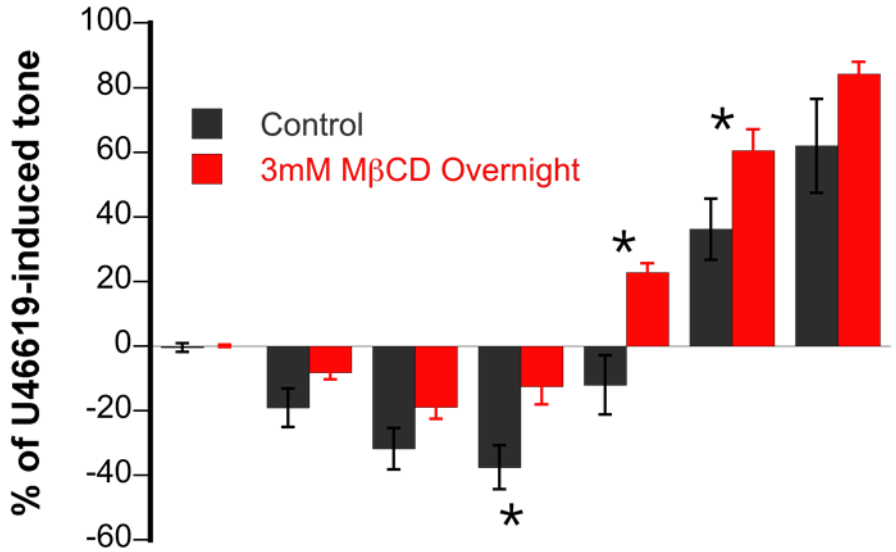


Figure 15.Samples stored overnight with 3mM MβCD , the effect on relaxation and contractions of the isolated porcine splenic artery elicited by isoprenaline against U46619-induced contractions (n=1)* ($p<0.05$) denotes a statistically significant difference (paired student's t-test $p<0.05$). Responses compared between control and treated group. Error bars represent SEM.

3.10.2 Chart Recording representation of contractile responses of different agonists by software:

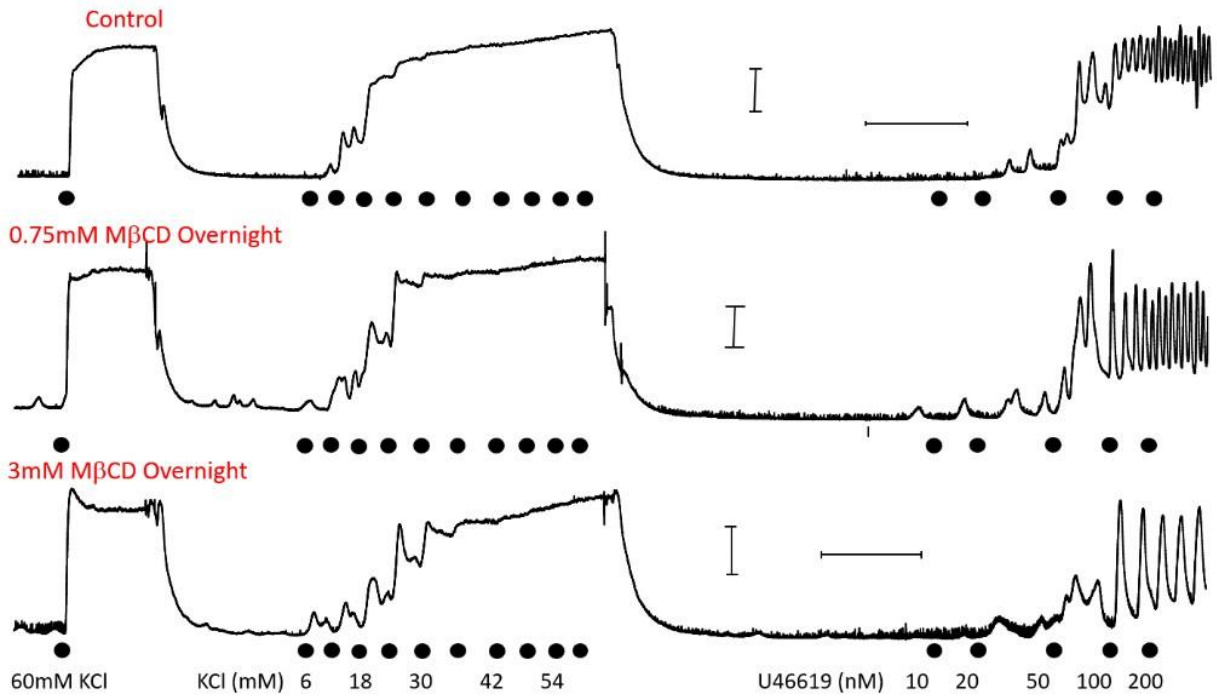


Figure 16. Chart recording of experimental traces increasing concentrations of U46619 in the isolated porcine splenic artery. Responses were recorded using chart software and dots represent increasing concentration(1-200nM)

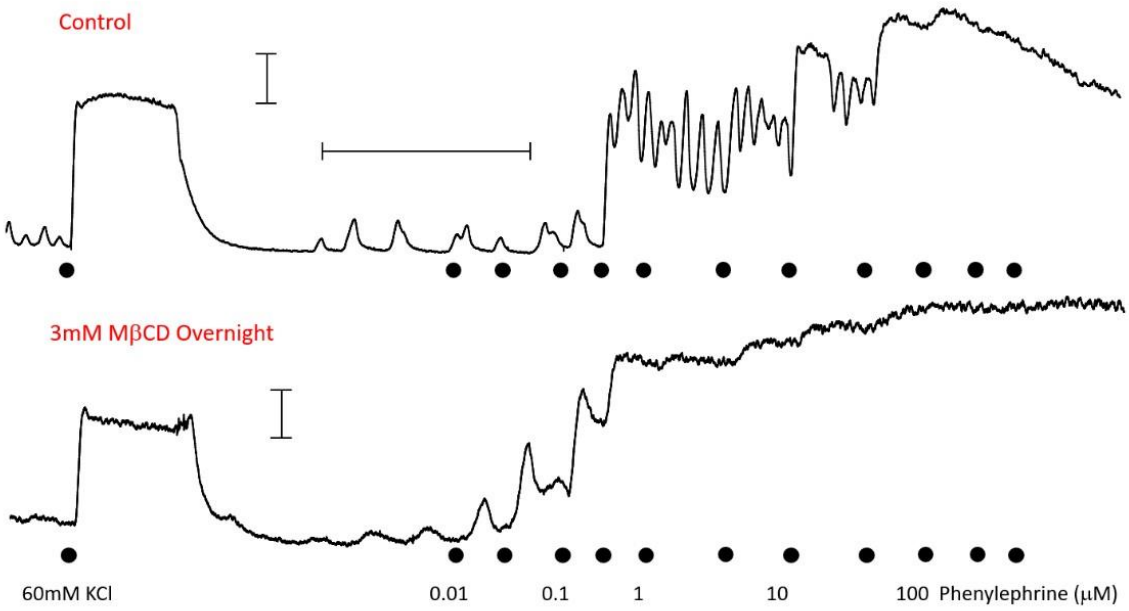


Figure 17. A recorded representative responses trace to increasing concentration of phenylephrine in isolated porcine splenic artery (overnight storage). Control shows the addition of phenylephrine at increasing concentration and declining of traces after the addition of 60mM KCl.

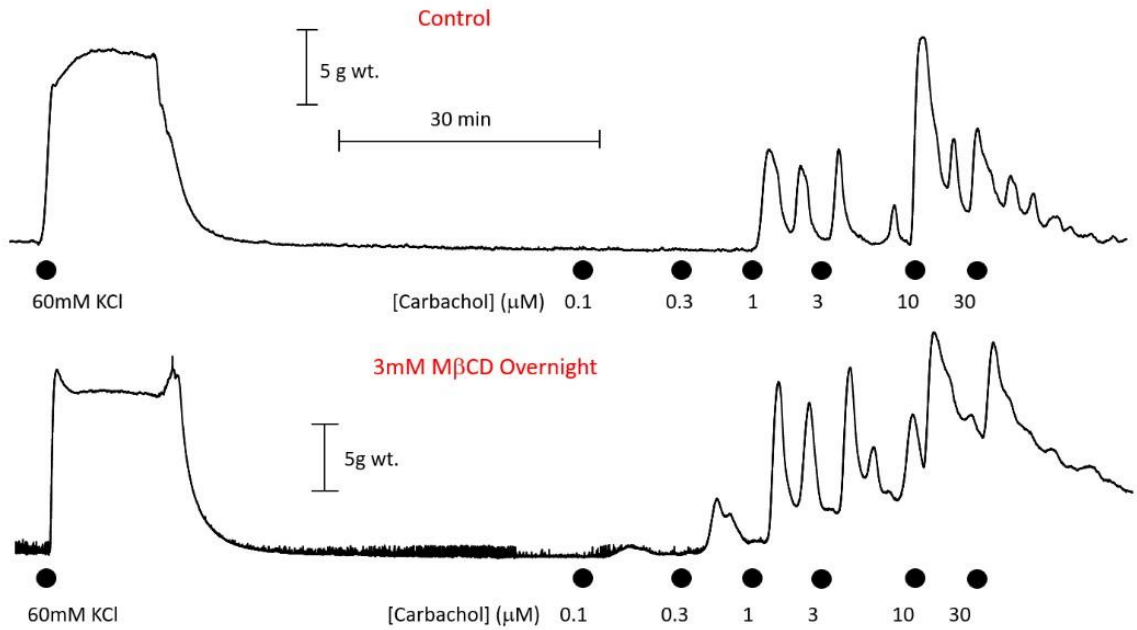


Figure 18. Representative experimental traces of carbochol concentration induced in isolated porcine artery control and treated 3mM MβCD overnight at 37°C. Dots indicate increasing concentration of carbochol 0.1 to 30mM.

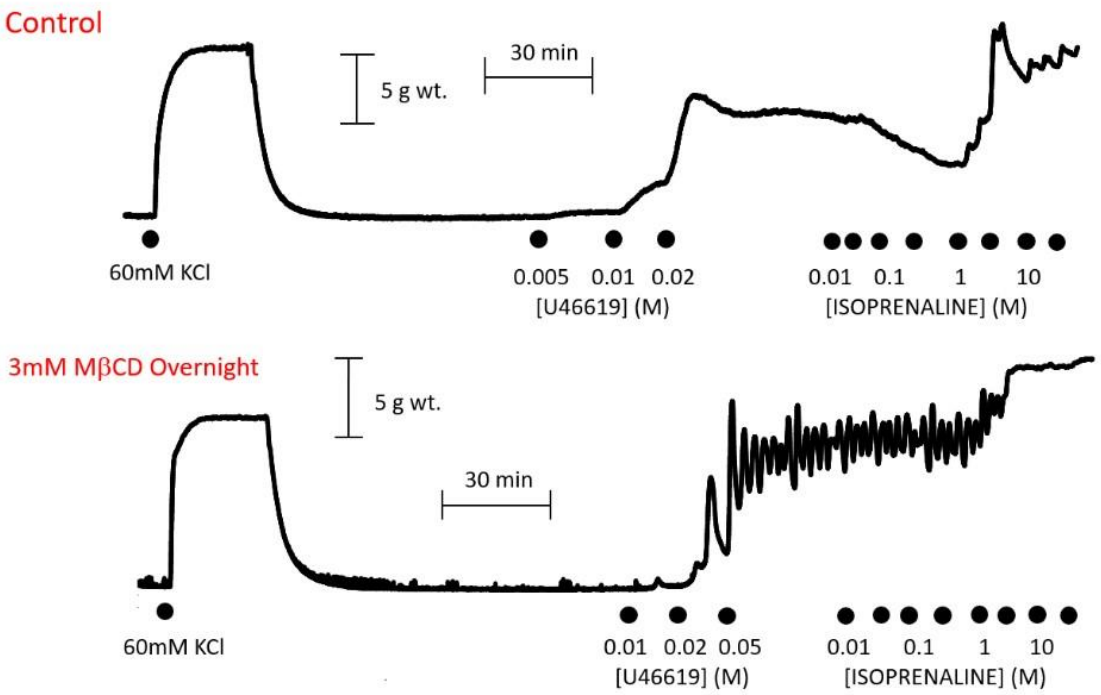


Figure 19. A representative record of the responses obtained from experimental trace showing contractile response to isoprenaline in the presence of U46619 in isolated porcine artery after overnight storage at 37°C. Dots represent concentration added to organ bath in Molar concentration.

3.10.3 Electron microscopy images of porcine isolated splenic artery

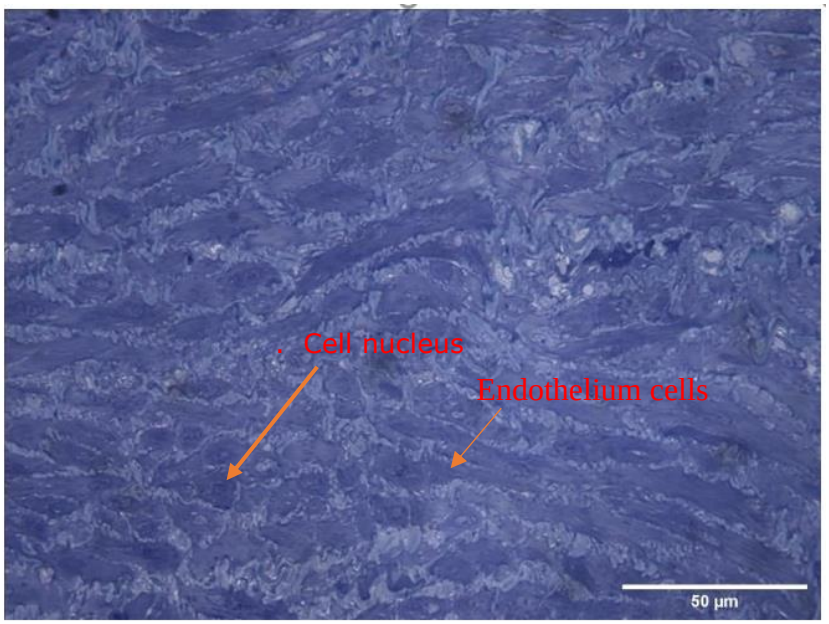


Figure 20. Light electron microscopy of porcine splenic artery (2D image) from control sample at 50x magnification, 50µm scale bar.

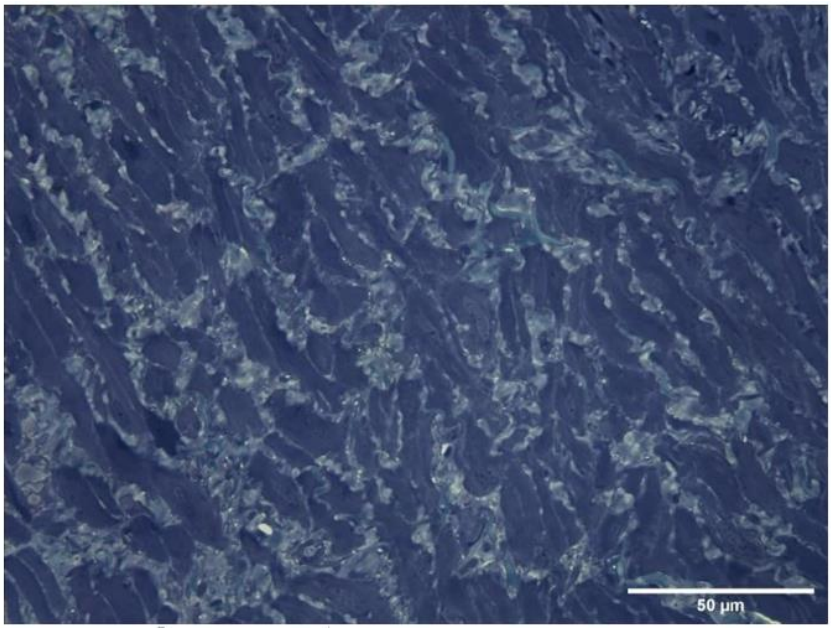


Figure 21. Light electron microscopy of porcine splenic artery (2D image) from control sample (upper) and from treated sample 3mM MβCD (lower). 50x magnification, 50µm scale bar.

Endothelium cells

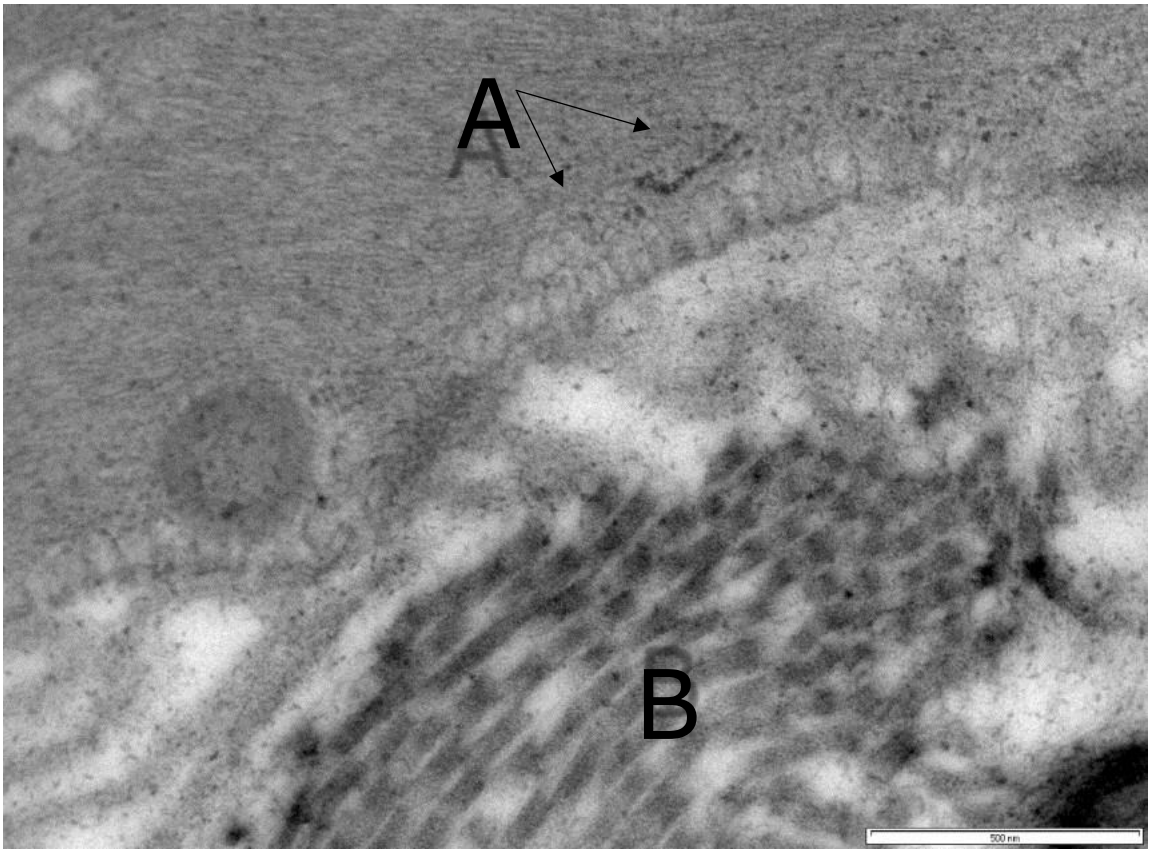


Figure 22. Transmission electron microscopy cross section of a porcine splenic artery in control (a) shows visible caveolae arranged at the border of the plasma membrane (b) collagens. Images were taken at 43000x magnification (n=10)

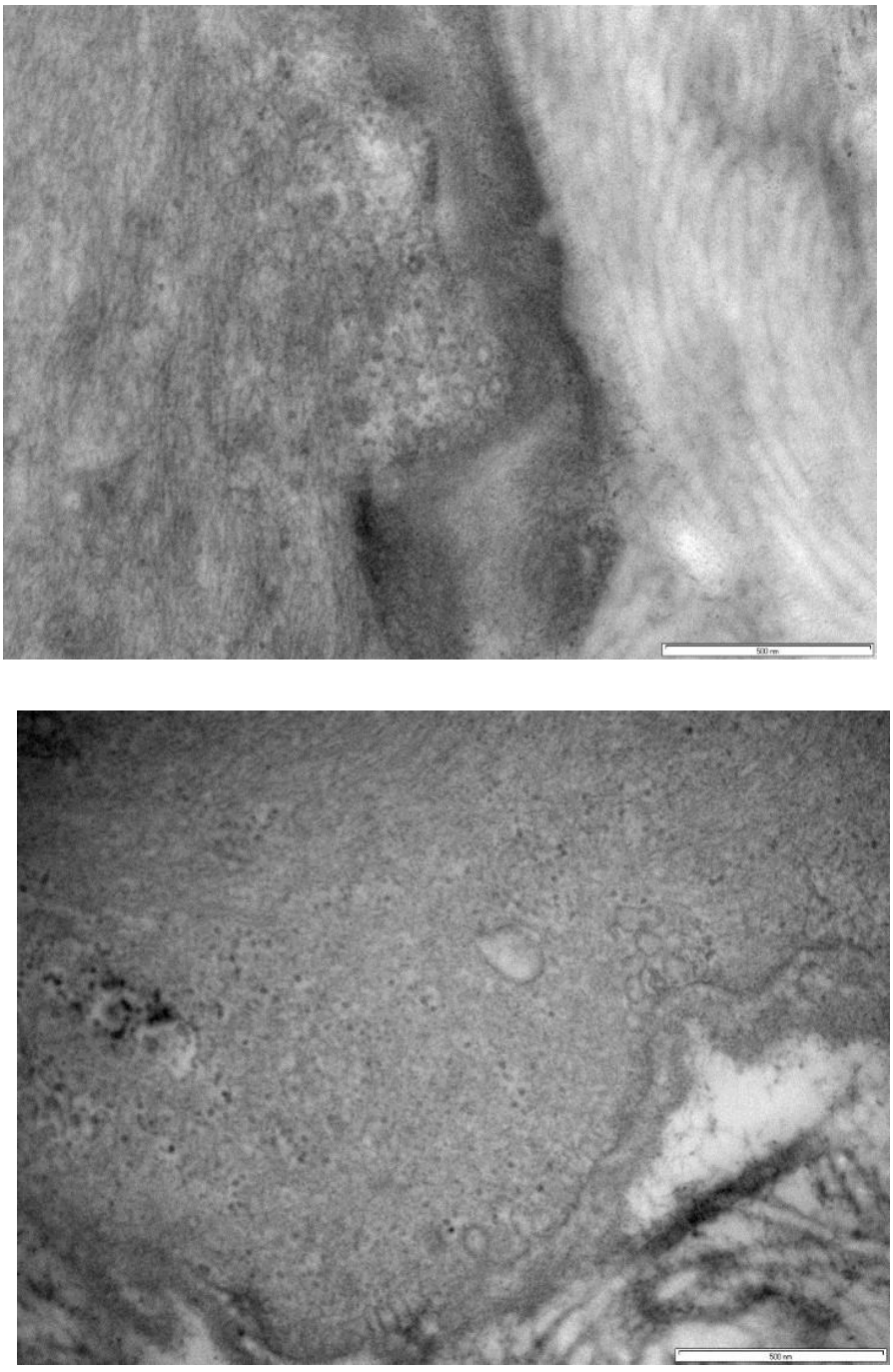


Figure 23. Transmission electron microscopy of treated isolated porcine artery. Isolated porcine splenic artery treatment with 3mM MβCD (upper) and treated isolated porcine artery with 0.75mM MβCD (lower). Images taken at 43000x magnification (n=10 for each treated group). (A) shows visible caveolae at the border of the plasma membrane

3.10.4 Stereological data of caveolae in porcine isolated splenic artery treated with Mβ-CD:

Table 3 Quantification of caveolae from stereological data (n= number of animals used)

Relative Volumes (%), Total Volumes (μm³) and Caveolar Diameters (nm)	**Control n=10**	**0.75 mM MβCD n=10**	**3 mM MβCD n=10**
Mean Nucleus Volume	129.1 ±3.454	109.2 ±3.028	114.3 ±2.943
Nuclear Fraction Volume	9.67% ±2.357	7.59% ±2.030	7.26% ±2.109
Mean Cell Volume	1335 ±4.690	1438 ±4.293	1574 ±4.002
Mean Cytoplasm Volume	1205 ±4.293	1328 ±4.091	1459 ±4.112
Fraction Caveolar Volume	3.04% ±0.938	1.92% ±0.837	0.90% ±0.818
Volume of Caveolae per Cell	36.1 ±1.909	25.6 ±1.834	13.1 ±2.938
Mean Diameter of Caveolae	69±3.147	70±3.019	70±2.877

3.10.5 Response to KCl in porcine isolated splenic artery:

The maximal concentration of KCl produced maximum contraction whether it is highly cholesterol depleted vascular smooth muscle cells of porcine isolated splenic artery or not. However, the pre-contracted porcine isolated splenic artery showed maximum response of contraction. The probability value of less than 0.01 showed statistically significant results. The number of samples of porcine isolated splenic artery used was n=6 which KCl was applied with prior treatment with control, 3mM Mβ-CD and 0.75mM MβCD treatment.

3.10.6 Responses to U46619 in porcine isolated splenic artery:

The maximal concentration of U46619 produced minimum contraction in highly cholesterol depleted vascular smooth muscle cells of porcine isolated splenic artery. However, the precontracted porcine isolated splenic artery showed maximum response of contraction. The probability value of less than 0.01 showed statistically significant results. The number of samples of porcine isolated splenic artery used was 6-9 to which U46619 was applied with prior treatment with control, 3mM Mβ-CD and 0.75mM Mβ-CD diet.

3.10.7 Response to phenylephrine and Noradrenaline:

Both vasoconstrictor agonist act on the ơ1-adenoreceptor and produce vasoconstriction. The contractions reactions towards phenylephrine and adrenaline were extended following disturbance of caveolae.

3.10.8 Response to 5-HT on porcine isolated splenic artery:

The inadequate upstream of Ca^{2+}channel initiation after removal of cholesterol undermine 5HT– instigate upgrading of Ca^{2+}. It leads to decreased depolarisation of membrane after triggering of agonist. Hence, cholesterol depletion with 3mM MβCD results in decreased contraction in porcine isolated splenic artery due to application of maximal concentration of 5HT in 10 number of samples. The probability value of less than 0.05 showed statistically significant result.

3.10.9 Response to Carbachol in porcine isolated splenic artery:

The maximal concentration of KCl produced maximum contraction whether it is highly cholesterol depleted vascular smooth muscle cells of porcine isolated splenic artery or not. However, the pre-contracted porcine isolated splenic artery showed maximum response of contraction. The probability value of less than 0.01 showed statistically significant results. The number of samples of porcine isolated splenic artery used was 11 to which carbachol was applied with prior treatment with control and 3mM MβCD.

Chapter 4

Discussion

In this study, we have investigated the effect of cholesterol depletion and overnight exposure of porcine splenic artery (a peripheral blood vessel) to Methyl-β-Cyclodextrin at different concentrations. Segments of isolated porcine splenic artery was treated under three different conditions: control containing krebs Henseleit solution, 2% ficoll and modified pen/strep, 3mM and 0.75mM Methyl-β-cyclodextrin, containing 2% ficoll and modified pen/strep. Contractile study was carried out to investigate the effect of MβCD on muscarinic receptors, serotonergic H2 receptors, β-adrenoceptors and other G protein-coupled receptors. The segments were mounted to organ bath and contracted to different agonist; KCL, U46619, Noradrenaline, Phenylephrine, 5HT, carbochol, electing a pharmacological response. The effect of cholesterol depletion on cells physiology has been examined through stereological examination. Exposed segments of the porcine splenic artery where examined through transmission electron microscopy. Currently knowledge enabled us to identify the location of caveolae, which was then used to quantify the volume of caveolae in control and treated cells.

In recent studies, cholesterol-extracted tissues were observed, due to depletion of cholesterol: an increase in conductance of K^+ and a reduction in sarcolemma's hyperpolarisation was observed. Variations in the amount of cholesterol found within the plasma membrane can affect a variety of important properties of membrane such as ion transport channels and other proteins associated with the membrane. These variations are basically due to either fixed alteration of interaction of protein and cholesterol or due to huge alterations in fluidity of membrane. Hence, it has been proven that cholesterol is not irregularly disperse in plasma membrane. Cholesterol has been found to be present in concentrated form in spatially compacted rafts or in micro domains. As a result, the physio-chemical properties of a distinct micro domain instead of vastness membrane may particularly affect the tasks of proteins associated with membrane. Remarkable advancement has been assembled in the biochemical and biophysical enactment of Raft structure and in the molecular configuration and dynamics. The physiological significance of Raft structure is poorly recognised. When high concentration of cholesterol was removed using MβCD from the smooth muscle, destabilisation of membrane rafts occurs. This results in the loss of micro domain segregation in the membrane. It also results in disruption of caveolae and reduced contractility. The plasma membrane consist of lipid rafts which consist of cholesterol embedded in-between the lipid bilayer. They may also be defined as sphingolipid-rich regions of membrane. Highly compacted cholesterol-rich lipid rafts has manifest that there iare different types types of micro domains that differ in lipid and protein composition (Krause and Regen, 2014). The visible subtype of lipid rafts is the caveolae. Caveolae are membrane invaginations. These are described as existence of caveolins. With the

help of caveolins a lot of proteins are attached with caveolae. For example, the ATP-sensitive potassium channel pore-forming subunit precipitates with caveolin in porcine isolated arterial smooth muscle.

G-protein coupled receptors (GPCRs) link various body processes including signalling with neurotransmitters and hormones. This protein has three subunits named α (Alpha), β (Beta), γ (Gamma) and is bound to the cytosolic surface of the plasma membrane in an inactive form. When a ligand binds with the receptor, it changes the structure of the receptor that allows it to bind with the alpha-subunit of G protein. This binding breaks down GDP from protein and converting it into an active form by GTP formation that causes α-subunit to remove inhibitory gamma and beta subunits. This G protein activation is multistep. It has been observed, GPCRs response to wide variety of ligands, it facilitates the initial stages of receptor signalling. Some models recommended that receptors are moving in and out of caveolae while others recommended that they remain in the caveolae. Upon activation, the G protein alpha subunit leaves the caveolae. Many studies have been conducting to verify the role of caveolae in the activation of the protein (Hanlon and Andrew, 2015), (Prabhu and Tarapore , 2017).

Removal of cholesterol using MβCD leads to the re-distribution of receptors which occur along the membrane. Re-distribution is associated with disturbance of the lipid rafts. Indistinguishable gesticulation has been showed for a variety of ion channels and raft associated proteins. Along with disruption of lipid rafts when cholesterol is depleted by MβCD treatment, reduced receptor-mediated responses was also observed. Reduced receptormediated responses were detected due to segregation of proteins from rafts. This results in the inactivation of signalling cascades such as, the Src family of tyrosine kinase which can be located in the lipid rafts. When disturbance of local signalling cascades happened due to cholesterol depletion, it resulted in disturbance of ongoing regulatory mechanisms, which report in reduced reactions. Membrane rafts has reduced effects on β-adrenergic receptor and its responses (Mohammad et al., 2014). Caveolae has many signalling proteins. It also participates in signalling of receptors coupled with G protein. It is described in present study that cholesterol removal undermines the functions of many receptors. In the new study it is showed that the arrangements of caveolae was distinctly and revocable by extraction of cholesterol along with various stages of flattening, showing the disturbance of structure of caveolae.5-HT2A receptor is responsible for contractile reactions towards 5-HT in porcine isolated splenic artery. Caveolin-1–expressing cells did not show morphological localisation of 5-HT_{2A} or V_1receptors. For membrane earmarking of receptors caveolae are not needed. In smooth muscle its function shows that it requires cholesterol corresponding with smooth caveolar structure. Fractions of ą1AAdrenergic receptors were also present (Muhammad and Parymryd, 2015). This has been observed in this study.

It has been investigated, in an untreated samples, the L-type channel initiates the depolarisation of the membrane, however, in the treated samples the inadequate upstream of $ca2^{+}$ depleted the activity of 5-HT. Hence, it leads to decreased depolarization of membrane after triggering of agonist. Besides this undeviating activation of G-protein resulted in normal retaliation after removal of cholesterol. Moreover, down drift signalling from G-protein initiation was not influenced. Agonist mediated Ca^{2+} signal venture, that depends on reoccur inositol triphosphate ($InsP_3$)-activated Ca^{2+}emancipate from SR was particularly ostentatious by removal of cholesterol, due to immense subside for 5-HT. This refers to the production of inositol triphosphate by 5-HT activation, which was accommodated by removal of cholesterol. MβCD based treatment results in enhanced basal Ca^{2+}level and as likely as not as a result enhanced Ca^{2+}stocking of the SR. Moreover, enhanced basal Ca^{2+} and contractile tone was also occurred due to enhanced membrane permeability. When is cholesterol removed from membrane, quantity of receptor protein was not contrived. Caveolae (cholesterol affluent) is an area with presence of receptors that is obligatory for real function (Dreja et al., 2002). Caveolae disturbance has no effect on contractions induced by Carbachol and KCL. But contractions reactions towards phenylephrine were extended following disturbance of caveolae (Babiychuk et al., 2004). In this study, we have investigated that, exposing isolated porcine splenic artery to MβCD shows no change in contractile-response to phenylephrine in isolated porcine splenic artery after removal of cholesterol. Contractions of other adrenergic agonists are also unchanged. Contractile mechanism's involvement is unusual. Response to depolarization by high potassium is unchanged. This findings agrees with the Babiychuk study.

Lipid raft contains high concentration of cholesterol and huge measures of signaling molecules. Non rafts membrane in smooth muscle do not have high level of cholesterol. Lipids rafts are now of huge interest. These contributed in the pathology of human diseases. These diseases are cancer, atherosclerosis, HF, ischemic heart disease. Lipids rafts consist of high cholesterol content and cholesterol contributes to the strength vascular smooth muscle contractility that is mediated by various signaling pathway.

In this study, we have investigate the effect of alpha-adrenoreceptors that conciliate contractions in splenic artery strips of pig. Noradrenaline contraction was observed at 6.5 potency value. Alpha selective agonist phenylephrine brings about the contraction of 7 potency value. When effects of adrenaline and alpha selective agonist phenylephrine were compared, it was observed that phenylephrine induced more effect as compared to noradrenaline and that was 93% .The strips of splenic artery of pig contract in reaction to phenylephrine by the initiation of alpha 1-adrenoreceptors which show the pharmacological behaviour of subtype of alpha 1A .It was as shown that porcine splenic artery has own functional α_2-adrenoceptors.Though revealing the effects needs at first initiation of both mechanisms(constrictor and relaxation).Such as α_2-adrenoceptor contrive effects, the

contractile effects are increased in the existence of U46619 in porcine splenic artery nevertheless in the existence of membrane cholesterol.

The signalling mechanisms based on adynylyl cyclase as second messenger to transmit signals to intracellular effectors has been proven to be significantly important in signal transduction pathways. Signal transduction pathways contributes to vasoconstriction. Study conducted using immunofluorescence microscopy, huge number of non clathrin-coate flask similar to caveolae were absorbed at the plasma membrane. Splendidly outline of caveolar often made big, deviant, and grape like collections that expanded deep into the cytoplasm. Electron microscopies substantiate the discomposure of normal caveolar task. Electron micrographs of whole cell contour was used to quantify the invaginations of plasmalemmal. Electron micrographs of whole cell contour was also used to quantify the surface density of caveolae. Various powers of magnification of electron microscopy was used in order to obtain micrographs. Overlay grid has a property to enumerate nucleus in the cytoplasm for the quantification of aggregate of invaginated plasma membrane. According to the point of view of structural benchmark Caveolae can be described as vesicles of plasmalemmal with diameter in range of 50 and 80 nm without any clear coats of cytoplasm. Ordinary transmission electron cannot immediately sort out Caveolae with cytoplasmic coat. Rapid freezing techniques can immediately sort out Caveolae with cytoplasmic coat. High resolution scanning electron microscopy can also be used instead of Rapid freezing techniques (Henley et al., 1998). They perform various roles. These roles are:

1. Signal transduction

2. Endocytosis

3. Mechanoprotection

Caveolins is an essential proteins (Jonathan 2014) in the formation of caveolae. Caveolin is important for production of caveola. It commands the vital functions of caveolae. Caveolin is suggested to perform like a cantilever in large MW brindled layer that covers the corm of caveolae and balancing it. Caveolin can interrelate deviating and undeviating with huge number of signalling molecules. Caveolin moderate on the endocytosis of molecular consignment with the help of caveolae. Overactivity and non-adaptivity of channels of ion are observed due to alteration in caveolar micro-domain.

A simple model is made that verify the activation and deactivation of signalling protein in normal body functions. Different methods were adopted to identify the interaction of caveolin and caveolae with G protein due to its widely used. Upon different studies, we find fluctuating results. One study tells us that Gq which is a unit of G alpha protein found in caveolae and Gb, Gi, and Gs are not found in it. The result of this study is different from others and tells us that

lipid raft size hinders the larger nanoparticle internalization. While we see that lipid raft has the ability to internalisation many molecules due to its expanding property. The size and strength of caveolae that are measured using nanoparticles suggesting that a method may be used to explain the many structures of these organelles in living cells. Within the plasma membrane, there are varieties of lipids and protein. To understand these components, we easily determined the exchange of material across the plasma membrane. One model that is Fluid Mosaic tells us membrane parts that include lipid bi-layer, extracellular matrix, cytoskeleton, and protein.

This model stated that the signal is transduced through the lipid layer that is called are caveolae. It is complex to identify the exact order and types of lipid raft in the plasma membrane. It has been noticed that lipid rafts are caveolae but not all. Plasma membrane and intracellular organelles have different functions. Caveolae has many sphingolipids connecting each other through head groups and has many cholesterol molecules that interact with sphingolipids. Now caveolae are crucial in signalling and they have been found to have other function that is found outside the caveolae region and is responsible for signalling of pathway (Thomas & Smart, 2008).

Depletion of cholesterol using MβCD on contractile response is implicated in the amount of caveolae in smooth muscle. From this study, we have established that small concertation MβCD treatment reduces the number of caveolae at the border, however maintains cell function. This result suggests that caveolae size restricts internalization of larger nanoparticles. We observed that caveolae were able to internalize multiple particles in single caveolae as measured suggesting that caveolae are compliant structures with an intrinsic ability to expand their size. The size and dynamics of caveolae measured using nanoparticles suggests that it is possible using the approach as described to define specific characteristics of these organelles in living endothelial cells. This finding agrees with research (Yamada, 1995), (Wang et al, 2009) and (Stan, 2005). The arrangement of caveolae was liner arrays over the cell body. This has been observed and agrees with other research (Stand, 2005). By monitoring the apparent diameters of caveolae outlines in control and treated cells we could test whether there is variation between the groups.

Chapter 5

Conclusion

The porcine splenic artery was obtained from local abattoir from porcine spleen. The sample of splenic artery was stored in Krebs Henseleit solution overnight at 37°C and 4°C to observe if there was any effect on the contraction after exposure to 60mM KCl. The vascular smooth muscle cells of porcine splenic artery were targeted to investigate the contractile response of different vasoconstrictor agonist. Methyl-β-cyclodextrin is a cholesterol acceptor that deplete the membrane cholesterol by disrupting cholesterol enriched membrane domains. The receptors responsible for contractile response are present in cholesterol enriched membrane domains. Cholesterol depletion in these membrane microdomains resulted in disruption of signaling pathways involved in G-protein coupled receptors for contractile response. By the application of different vasoconstrictor agonist acting at different receptors in cholesterol depleted microdomains in the membrane of porcine splenic artery, the contractile response to each agonist varied. The cholesterol depletion has disrupted the structure of caveolae of the membrane examined by TEM. Transmission electron microscopy was used to examine the effect of cholesterol depleted membrane in porcine splenic artery to different agonist. The results of control group and treated methyl-β-cyclodextrin treated group were compare by using statistical model ANOVA with post hocs Dunnett's test. Hence, it was concluded that cholesterol depletion had varied effect on contractile response induced by the application of different vasoconstrictor agonist.

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